Pancreatic ribonucleases
Welling, Gjalt Wietze

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1976

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
CHAPTER 1

Introduction

Ribonuclease from cow pancreas was the first enzyme of which the amino acid sequence was determined (1). It consists of a single polypeptide chain with 4 disulfide bridges and has a molecular weight of 13,680. There has been a considerable interest in many aspects of this enzyme. The tertiary structure was determined (2,3,4) and to date it still is one of the most important model proteins for a large number of chemical and other studies (5).

Comparative studies were started by Anfinsen et al. (6) by comparing semi-quantitative sequence data of sheep ribonuclease with the amino acid sequence of the cow enzyme. In our laboratory, Beintema started a project to elucidate the evolutionary history of ribonucleases, the first step being the determination of the amino acid sequence of rat ribonuclease (7,8). Comparative studies of proteins may give not only valuable information on their phylogeny, but also on their structural properties. Several aspects of these comparative studies are dealt with in this thesis.

i) In chapter 2, the determination of the amino acid sequence of Arabian camel (dromedary) ribonuclease is described and its position in the evolutionary history of ribonucleases is discussed.

ii) In chapter 3, the amino acid sequence determination of bactrian camel ribonuclease is described and a genetic problem encountered in chapter 2 is solved.

iii) Native cow ribonuclease can be cleaved by subtilisin in an external loop of the polypeptide chain between residues 20 and 21 (9,10). The resulting product, ribonuclease S is still 100% active and can be separated in two inactive components, S-peptide (residues 1-20) and S-protein (residues 21-124). Upon remixing, a fully active product, ribonuclease S', is regained. It seemed of interest to determine whether ribonucleases with a different amino acid sequence in the external loop, were susceptible to cleavage by subtilisin. This comparative study is presented in chapter 4.

iv) S-peptides with different amino acid sequence, obtained either by chemical synthesis or by subtilisin cleavage,
can be used to prepare hybrid ribonucleases. This presents an intriguing opportunity to study peptide-protein interaction and biological activity. In chapter 5, the activity and antigenicity of such hybrid enzymes are described.

v) Comparative studies of homologous proteins may give information on their evolutionary relationship. In chapter 6, the N-terminal amino acid sequences of pancreatic ribonucleases from 23 species are compared and the reasons for different degrees of variability at particular positions are discussed.

vi) One of the objectives of immunochemistry is to understand the exact chemical nature of an antigenic determinant and how it reacts with its counterpart, the antibody combining site.

In the history of immunochemistry, determination of the antigenic structure of proteins is a relatively underdeveloped area of investigation. One factor is the structural complexity of protein antigens compared with for example polysaccharide antigens. Another important factor is that it was not before early in the 1960s that primary and tertiary structures of proteins became available. Interpretation of differences in antigenic reactivity require knowledge of both the amino acid sequence and the conformation of the protein antigen. Recently, the first immunochemical anatomy of a protein was described by Atassi (11). This protein, sperm whale myoglobin, contains 5 independent antigenic regions. Delineation of this antigenic structure relied on the following experimental approaches.

a) The antigenicity of synthetic peptide fragments and fragments obtained by chemical or proteolytic cleavage of myoglobin was determined.

b) The antigenicity of myoglobin or myoglobin fragments modified by chemical reagents was determined.

c) The influence of conformational changes on the antigenicity was studied.

It seems reasonable to assume that proteins obtained by modification of a few residues, or homologous proteins which have become molecular evolutionarily similar conformational role of a common difference in structure.

Special features are:

a) The reactive site consist of 6-8 residues.

b) From the structure reactive role with the antigen nature. In the altered by homologous regions were.

c) The determinants from the antigenic structure were.

The aforementioned antigenic structure of lysozyme and lysozyme antigenic structure years ago. Initial experiments are compared with antibody antiseraum differences in the immunodiffusion, complement fixation test to assign different molecular residue by comparison, conception of antigenic made with lysozyme.

vii) In chapter 8, closely related to the inhibition of the conjugate (15,16) by utilizing discriminative fixation technique. Nuclease A is a conjugate can be
which have been changed at particular positions during molecular evolution, represent groups of proteins of very similar conformation, but one cannot entirely exclude the role of a conformational change being responsible for a difference in antigenicity.

Special features of the antigenic structure of myoglobin are:

a) The reactive regions possess sharp boundaries and consist of 6-7 amino acid residues.
b) From the surface location of the amino acids in the reactive regions it was concluded that the interaction with the antibody combining site is primarily polar in nature. In addition, stabilizing effects are contributed by hydroxy and non-polar amino acids.
c) With 8 antisera studied, always the same 5 reactive regions were found.

The aforementioned features may be expanded when antigenic structures of more native proteins are determined. At the moment, Atassi's group is working on hen egg-white lysozyme and in our laboratory, the determination of the antigenic structure of cow ribonuclease was started a few years ago. In chapter 7, nine pancreatic ribonucleases are compared concerning their antigenic reactivity with antiserum directed against four ribonucleases. By using the immunodiffusion technique (12,13) and the micro-complement fixation technique (13,14) an attempt is made to assign differences in antigenic reactivity to particular residues or a set of residues. Besides this, a comparison, concerning the correlation between differences in antigenic reactivity and in amino acid sequence is made with lysozymes and azurins.

In chapter 8, the antigenic reactivity of a group of closely related artiodactyl ribonucleases was determined by utilizing a less frequently used type of assay, inhibition of the inactivation by antiserum of a phage conjugate (15,16). This technique showed itself to be more discriminative than the immunodiffusion and complement fixation technique. In this particular case, cow ribonuclease A is coupled to bacteriophage T4 (17). This conjugate can be inactivated i.e. is no longer able to act
as a phage (to penetrate its host bacterium) if antiserum
directed against cow ribonuclease A is added. This in-
activation can be inhibited by prior incubation of a
suitable dilution of the antiserum with increasing amounts
of homologous ribonucleases, modified ribonucleases
or ribonuclease fragments.

viii) In chapter 9 attention is paid to the current research
on the antigenicity of chemically modified ribonucleases
and ribonuclease fragments. Preliminary results are
given and the results of chapters 7, 8 and 9 are dis-
cussed.

References

Chem. 238, 227-234.
862-865.
3) Carlisle, C.H., Palmer, R.A., Mazumdar, S.K., Gorinsky,
4) Wyckoff, H.W., Tsernoglou, D., Hanson, A.W., Knox, J.R.,
Lee, B., and Richards, F.M. (1970) J.Biol.Chem. 245,
305-328.
5) Richards, F.M., and Wyckoff, H.W. (1971) in The Enzymes,
6) Anfinsen, C.B., Åqvist, S.E.G., Cooke, J.P., and Jönsson,
Acta, 147, 612-614.
Acta, 310, 161-173.
9) Richards, F.M. (1955) Compt rend.trav.lab.Carlsberg,
29, 329-343.
234, 1459-1465.
Press, New York.
13) Roitt, I. (1974) in Essential Immunology, 2nd ed., 99-


The Amino Acid Sequence of Dromedary Pancreatic Ribonuclease

B. GIJALT W. WELLING, GERDA GROEN and JAAP J. BEINTEMA
Bioc hemisch Laboratorium, Zernikeplein, Rijksuniversiteit,
Groningen, The Netherlands

(Received 13 November 1974)

A dromedary (Camelus dromedarius) RNAase (ribonuclease) was isolated from pancreatic juice by affinity chromatography. Peptides obtained by digestion with different proteolytic enzymes and CNBr were isolated by gel filtration, preparative high-voltage paper electrophoresis and paper chromatography. Peptides were sequenced by the dansylrhodamine method. All peptide bonds were overlapped by one or more peptides. The polypeptide chain consists of 123 amino acids. A deletion (position 39) was observed in an internal loop of the polypeptide chain (residues 35-40), as was found earlier in horse RNAase (Scheffer & Beintema, 1974). A heterogeneity was found at position 103 (glutamine and isoleucine). Dromedary RNAase differs at 23-32% of the positions from all other RNAases sequenced to date. In evolutionary terms this indicates that dromedary RNAase has evolved independently during the larger part of the evolution of the RNAases. Detailed evidence for the sequence has been deposited as Supplementary Publication SUP 50046 (14 pages) at the British Library (Lending Division), Boston Spa, Wetherby, W. Yorks. LS23 7BQ, U.K., from whom copies may be obtained on the terms given in J. Biochem. J. (1975) 145, 5.
Trehfluoroacetic acid was 'sequanal-grade' from Pierce or from Merck A.G. (Darmstadt, Germany). It was twice-distilled before use. Silica-gel thin layers with fluorescence indicator were from M. Woelm (Eschwege, Germany). Phenanthrenequinone was from Schuchardt (München, Germany).

Methods

Isolation. Dromedary RNAase was isolated from the combined pancreatic tissue of six animals by affinity chromatography as described by Wierenga et al. (1973) by acetone precipitation. Carbohydrate was detected by the orcinol-H$_2$SO$_4$ method (Winnler, 1955).

Electrophoresis in polyacrylamide gels was performed with 10% gels in 35mM-ficoll buffer, pH 4.5. Gels were stained in 0.5% Amido Black in 7% Na$_2$CO$_3$ acid, followed by destaining in 7% acetic acid. Duplicate gels were incubated with yeast RNA (BDH, Poole, Dorset, U.K.) and stained with 0.2% Toluidine Blue as described by Wilson (1969).

Amino acid analysis. Amino acid analyses were performed with a Beckman 120-C or Technicon TSM-4 amino acid analyser. Samples were hydrolysed in 0.4ml of 6M-HCl at about 110°C. In evacuated [13.3Pa (0.1mmHg)] sealed glass tubes for 18-96h. For peptides usually 18-20h was used. The amounts of cysteic acid, homoserine, homoserine lactone and aminocrotonic acid were not determined. The recovery of aminohexane was often low.

Hydrolysis with carboxypeptidases A and B and with aminopeptidase M. Dromedary RNAase (0.7mg) in 0.2m-N-ethylmorpholine acetate buffer, pH 6.5, containing 0.1% sodium dodecyl sulphate was incubated at 37°C with 0.05mg (1 al) of carboxypeptidase A suspension for 4h. Free amino acids were detected with the TSM-4 amino acid analyser.

Aminopeptidase M digestion of peptides was performed in 0.2m-N-ethylmorpholine acetate buffer, pH 8.0, at 37°C. Digestion of peptides with a mixture of carboxypeptidases A and B and with aminopeptidase M was performed as described by Saigo & Devényi (1972). The amino acids generated by carboxypeptidase digestion were analysed on ionex-25 SA layers (Dêvényi et al., 1972). The overall recovery of aminoethylcysteine was often low.

Aminopeptidase M digestion of peptides was performed with subtilisin Carlsberg (Welling et al., 1974a). The fragment obtained from this digest is denoted by the letter S. Dromedary RNAase (4mg) was digested with pepsin (Ambler, 1963). The larger peptides were incubated with 0.7mg of chymotrypsin in 0.2m-N-ethylmorpholine acetate, pH 8.0, for 4h at 37°C. The smaller peptides were digested with trypsin (Ambler, 1963). The fragment obtained from this digest is also prefixed by the letter G. Dromedary RNAase (4mg) was digested with pepsin (Ambler, 1963). The larger peptides were incubated with 0.7mg of chymotrypsin in 0.2m-N-ethylmorpholine acetate, pH 8.0, for 3h at 37°C. The fragment obtained from this digest is denoted CN1CH. Dromedary RNAase (4mg) was digested with subtilisin Carlsberg (Welling et al., 1974a). The fragment obtained from this digest is denoted by the letter S.

Peptides have been numbered according to their position in the polypeptide chain.

Gel filtration of peptides. After cleavage of dromedary RNAase the first purification step was gel filtration on Sephadex G-25 or G-50. Generally peptides were fractionated on a column (0.8cm x 190cm) of Sephadex G-25 (superfine-grade) with 0.1M-acetic acid at a flow rate of 7ml/h, 1.5ml fractions being collected.

The thermolysin and pepsin digests were fractionated on a column (1cm x 100cm) of Sephadex G-25 (fine grade) with 5% formic acid and 0.1M-NH$_3$, respectively, at a flow rate of 20ml/h, 1 ml fractions being collected. The 3-5-8-9-10-11 peptides were isolated by gel filtration on a column (1.0cm x 100cm) of Sephadex G-50 (superfine-grade) with 0.05M-HCl at a flow rate of 15ml/h, 1.5ml fractions being collected.

Determination of peptide positions in column eluates. The eluates of the column fractionations were monitored for $E_{280}$ by using an LKB Uvicord II instrument or by measuring either the $E_{280}$ or $E_{340}$ or both, in a Zeiss PMQ II spectrophotometer. Peptide 'maps' were obtained by subjecting a suitable portion of each fraction appearing after the void volume to high-voltage paper electrophoresis.
...and any other content that was previously extracted for this document as if you were reading it naturally.
Fig. 1. Amino acid sequence of dromedary pancreatic RNAase. The numbering of bovine RNAase was used. The sequence was determined by dansyl-Edman degradation (---). Additional information was obtained by carboxypeptidase digestion (-----). Dashed lines indicate results which were not quite certain. Attention is also drawn to the part of the Discussion section, "Reliability of the sequence determination". Those amino acids differing from bovine RNAase are indicated in blocks. For peptide nomenclature, see under 'Methods'.
Dansyl-Edman results were also more difficult to interpret because we had to use peptides with amino acid analyses which were not always satisfactory.

In Tables 2-5 of Supplementary Publication SUP 50046 a distinction has been made between very pure peptides, peptides with small impurities and rather impure peptides. In Table 6 of the supplement the peptides used for the amino acid sequence are shown. Six peptides have been used containing small impurities. These impurities, however, did not interfere with the dansyl-Edman technique.

The amino acid composition of the impure peptide H3 (residues 19-42) contains more lysine residues than suggested by the sequence. Compared with the other pancreatic RNAses sequenced to date, a lysine residue could be inserted between residues 33 and 34 or residues 40 and 41. However, because the recovery of free lysine in the tryptic digest was rather low and could be accounted for by the lysine residues at position 66 and position 104, we think this insertion to be less probable.

To obtain an overlap we also used the rather impure peptide T13 (Table 3 of Supplementary Publication SUP 50046). Obviously this is a peptide mixture, but it apparently connects the sequence His-Gln-Ser-Thr-Thr-Met (73-79) with His-Ile-Thr-Asp-Cys (80-84). Additional evidence for this rather difficult part of the sequence was provided by the large peptide PC3, which connects the sequence 56–72 with 73–79. Peptides T5 (residues 27–31; Asn-Gln-Met-Met-Lys) and T10 (residues 62–65; Ser-Val-Thr-Cys) were isolated as a mixture in relative amounts of 42 and 58% respectively. They showed the same mobility on paper electrophoresis and after paper chromatography. However, in the latter case, the relative amounts were changed in favour of peptide T10 (see Table 3 of the supplement), probably because of oxidation of the two methionine residues in peptide T5. This mixture was sequenced by the dansyl-Edman technique, during which peptide T5 only interfered with the Edman degradation by giving a weak spot of dansyl-glutamic acid after one step.

From the amino acid composition of the mixture and the sequence results, the composition of peptide T5 could be deduced. One peptide, CN3, showed a rather peculiar amino acid composition: Asp (4), Ser (7), Glu (1), Tyr (2) and Hse (1). The sequence determination proved to be fairly easy, because the peptide contained the invariant residue tyrosine-25. After 11 Edman steps had been carried out on peptide CN3, dansylation of the remaining peptide showed bis-Dns-Tyr. The sequence was supported by the dansyl-Edman degradation of the cyanogen bromide peptide T4, which partially overlaps CN3 and contains tyrosine-25.
Table I. Difference matrix of pancreatic RNAases

Data were derived from publications mentioned in the introduction. A deletion was treated as an amino acid. Compared with horse and rat RNAase, the relative differences were calculated on the basis of 126 and 127 amino acid residues respectively. Latin names for the species investigated are as follows: cow (Bos taurus), sheep (Ovis aries), goat (Capra hircus), giraffe (Giraffa camelopardalis), reindeer (Rangifer tarandus), dromedary (Camelus dromedarius), pig (Sus scrofa), horse (Equus caballus) and rat (Rattus rattus).

<table>
<thead>
<tr>
<th></th>
<th>Cow</th>
<th>Sheep/goat</th>
<th>Giraffe</th>
<th>Reindeer</th>
<th>Dromedary</th>
<th>Pig</th>
<th>Horse</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow</td>
<td>3%</td>
<td>11%</td>
<td>23%</td>
<td>21%</td>
<td>28%</td>
<td>35%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sheep/goat</td>
<td>4</td>
<td>7</td>
<td>14</td>
<td>32</td>
<td>24%</td>
<td>36%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Giraffe</td>
<td>11%</td>
<td>7</td>
<td>14</td>
<td>32</td>
<td>24%</td>
<td>36%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reindeer</td>
<td>14</td>
<td>10</td>
<td>14</td>
<td>32</td>
<td>24%</td>
<td>36%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dromedary</td>
<td>28</td>
<td>28</td>
<td>33</td>
<td>32</td>
<td>24%</td>
<td>36%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pig</td>
<td>36</td>
<td>23</td>
<td>28</td>
<td>25</td>
<td>28%</td>
<td>24%</td>
<td>36%</td>
<td></td>
</tr>
<tr>
<td>Horse</td>
<td>35</td>
<td>31</td>
<td>34</td>
<td>33</td>
<td>33</td>
<td>30</td>
<td></td>
<td>33%</td>
</tr>
<tr>
<td>Rat</td>
<td>44</td>
<td>42</td>
<td>48</td>
<td>45</td>
<td>41</td>
<td>46</td>
<td>45</td>
<td></td>
</tr>
</tbody>
</table>

Heterogeneity

One unmistakable heterogeneity (Lys, Gln) was found at position 103. Several peptides in this region have been isolated.

T18 Ala-Ser-Asn-Leu-Lys
T19 Ala-Ser-Asn-Leu-Gln-Lys
H14 Leu-Gln-Lys-His
PC6 Lys-Lys-His-Ile

The thermolysin peptide H14 showed a mobility of 0.80 relative to lysine at pH 6.5. This could explain why the very basic thermolysin peptide Leu-Lys-Lys-His was lost (see Fig. 2 of the supplement). Whether this heterogeneity in dromedary RNAase reflects polymorphism in the population or the existence of two different structural genes needs to be investigated on individual pancreatic tissue. Despite the fact that the heterogeneity involves a difference in charge, only one band was observed on gel electrophoresis. This would suggest that there should be another heterogeneity elsewhere in the protein, which compensates for the difference at position 103. No such heterogeneity has been found, however.

Camel RNAase (Camelus bactrianus) was isolated from one pancreas. A sample was aminoethylated and digested with trypsin. In this case only glutamine was observed at position 103.

Comparison of sequences

In Table I a difference matrix of pancreatic RNAases is shown. One outstanding feature that can be deduced from this matrix is that dromedary RNAase differs to the extent of between 23 and 32% of its residues from all other pancreatic RNAases sequences thus far. This suggests that dromedary RNAase has evolved independently during the larger part of the evolution of the mammals. This agrees with the classical taxonomy (Romer, 1962), in which the dromedary is an early offshoot in the phylogenetic tree of the artiodactyls.

Another interesting point is that dromedary RNAase exhibits a deletion at position 39 compared with all other pancreatic RNAases sequenced to date, except for horse RNAase which has the same deletion (Scheffer & Beintema, 1974).

Comparison with the tertiary structure of bovine RNAase

To compare the amino acid sequence of dromedary RNAase with the three-dimensional structure of bovine RNAase, we used a model of RNAase-S (Wyckoff et al., 1970). Some 28 differences with the bovine enzyme were observed. Of the substitutions 80% were found clustered in three parts of the molecule: residues 62, 64 and 73; a large cluster with residues 16, 17, 19, 20, 22, 76, 77, 80, 99, 100, 101, 102 and 103; and a loop (34-39) with substitutions of residues 34, 35, 37, 38 and 39 (Fig. 2).

Seven of the replacements involve hydrophobic residues, of which two are completely internal Val (47) → Ile and Val (108) → Ile. The side chains of the two isoleucine residues can easily be fitted in the largest of three internal cavities termed cavity B by Lee & Richards (1971) without altering the polypeptide backbone.

Introduction of the other substitutions also did not bring about alterations in the polypeptide chain of any significance, except for the deletion at position 39. This replacement introduces a radical change in the polypeptide backbone. It shortens an external loop in the structure of the molecule. In having this deletion, dromedary RNAase differs from all other RNAases except for the horse enzyme, in which the same important mutation has occurred in parallel.

The loop in the RNAases of these two species differs only at position 37 (asparagine in the dromedary and
The phylogenetic relationship of the dromedary (Camelus dromedarius) compared to the domesticated species (Camelus domesticus), horse (Equus caballus), and rat (Rattus norvegicus) is shown in the phylogenetic tree. The amino acid residues compared are from the following species:

- Horse (Equus caballus)
- Rat (Rattus norvegicus)
- Dromedary (Camelus dromedarius)

The substitutions are as follows:

- Horse: 35%
- Rat: 33%
- Dromedary: 39%
- Domesticated species: 35%
- Horse: 32%
- Rat: 36%

The structure of RNase A is also shown, with the active site shown in black. The structure of the enzyme is a dimer, with each subunit containing two domains: the N-terminal and C-terminal domains. The active site is located at the interface between the two domains, with the enzyme catalyzing the hydrolysis of RNA.

References

The Amino Acid Sequence of Dromedary Pancreatic Ribonuclease
By Gjalt W. Welling, Gerda Groen & Jaap J. Beintema

Table 1  Amino acid composition of dromedary ribonuclease

<table>
<thead>
<tr>
<th></th>
<th>Beckman</th>
<th>TSM-1</th>
<th>sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>24h</td>
<td>96h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asp</td>
<td>13.6</td>
<td>13.7</td>
<td>14.4</td>
</tr>
<tr>
<td>Thr</td>
<td>2.5</td>
<td>3.4</td>
<td>8.5</td>
</tr>
<tr>
<td>Ser</td>
<td>10.0</td>
<td>12.9</td>
<td>17.9</td>
</tr>
<tr>
<td>Glu</td>
<td>14.4</td>
<td>14.5</td>
<td>14.1</td>
</tr>
<tr>
<td>Pro</td>
<td>3.8</td>
<td>4.1</td>
<td>4.0</td>
</tr>
<tr>
<td>Gly</td>
<td>3.8</td>
<td>4.2</td>
<td>3.6</td>
</tr>
<tr>
<td>Ala</td>
<td>6.8</td>
<td>6.9</td>
<td>6.9</td>
</tr>
<tr>
<td>Cys</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Val</td>
<td>6.4</td>
<td>5.5</td>
<td>6.5</td>
</tr>
<tr>
<td>Met</td>
<td>4.8</td>
<td>5.2</td>
<td>3.8</td>
</tr>
<tr>
<td>Ile</td>
<td>3.3</td>
<td>4.8</td>
<td>4.8</td>
</tr>
<tr>
<td>Leu</td>
<td>2.0</td>
<td>2.2</td>
<td>1.6</td>
</tr>
<tr>
<td>Tyr</td>
<td>5.2</td>
<td>4.9</td>
<td>5.4</td>
</tr>
<tr>
<td>Phe</td>
<td>2.8</td>
<td>3.0</td>
<td>2.8</td>
</tr>
<tr>
<td>Lys</td>
<td>10.8</td>
<td>10.9</td>
<td>9.0</td>
</tr>
<tr>
<td>His</td>
<td>5.2</td>
<td>5.7</td>
<td>5.8</td>
</tr>
<tr>
<td>Arg</td>
<td>3.7</td>
<td>3.8</td>
<td>5.9</td>
</tr>
</tbody>
</table>

Amino acid analysis of dromedary ribonuclease
Dromedary ribonuclease was hydrolysed for 24 or 96 h and analysed as described under 'Methods'.

The 24 h values for serine and threonine are corrected by extrapolation to zero hydrolysis time (underlined). For valine and isoleucine 96 h values are taken (underlined). The values in the second and third columns were obtained with a Beckman 120C amino acid analyser (kindly performed by Dr. R.P. Ambler, University of Edinburgh). Those in the fourth column were determined with a TSM-1 (Technicon) amino acid analyser by comparison with the data for an identically treated sample of bovine ribonuclease.
The composition of peptides derived from the amino acid sequence is shown in parentheses. Peptides were purified by paper electrophoresis at pH 6.5. In some cases additional purification was achieved by paper electrophoresis and/or paper chromatography. Cysteic acid was not determined. Serine and threonine values were not corrected for degradation losses.

| Position | 1-3 | 4-7 | 14-42 | 43-45 | 46-50 | 51-53 | 54-56 | 57-62 | 63-70 | 70-80 | 81-95 | 96-98 | 99-101 | 102- | 103- | 105- | 107- | 109- | 110- |
|----------|-----|-----|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|------|------|------|------|------|------|
| Arg      | Arg | Arg | Arg   | Arg   | Arg   | Arg   | Arg   | Arg   | Arg   | Arg   | Arg   | Arg   | Arg   | Arg   | Arg   | Arg   | Arg   | Arg   | Arg   | Arg   |
| Asp      | Asp | Asp | Asp   | Asp   | Asp   | Asp   | Asp   | Asp   | Asp   | Asp   | Asp   | Asp   | Asp   | Asp   | Asp   | Asp   | Asp   | Asp   | Asp   | Asp   |
| Glu      | Glu | Glu | Glu   | Glu   | Glu   | Glu   | Glu   | Glu   | Glu   | Glu   | Glu   | Glu   | Glu   | Glu   | Glu   | Glu   | Glu   | Glu   | Glu   | Glu   |
| Pro      | Pro | Pro | Pro   | Pro   | Pro   | Pro   | Pro   | Pro   | Pro   | Pro   | Pro   | Pro   | Pro   | Pro   | Pro   | Pro   | Pro   | Pro   | Pro   | Pro   |
| Ala      | Ala | Ala | Ala   | Ala   | Ala   | Ala   | Ala   | Ala   | Ala   | Ala   | Ala   | Ala   | Ala   | Ala   | Ala   | Ala   | Ala   | Ala   | Ala   | Ala   |
| Ile      | Ile | Ile | Ile   | Ile   | Ile   | Ile   | Ile   | Ile   | Ile   | Ile   | Ile   | Ile   | Ile   | Ile   | Ile   | Ile   | Ile   | Ile   | Ile   | Ile   |
| Leu      | Leu | Leu | Leu   | Leu   | Leu   | Leu   | Leu   | Leu   | Leu   | Leu   | Leu   | Leu   | Leu   | Leu   | Leu   | Leu   | Leu   | Leu   | Leu   | Leu   |
| Tyr      | Tyr | Tyr | Tyr   | Tyr   | Tyr   | Tyr   | Tyr   | Tyr   | Tyr   | Tyr   | Tyr   | Tyr   | Tyr   | Tyr   | Tyr   | Tyr   | Tyr   | Tyr   | Tyr   | Tyr   |
| Phe      | Phe | Phe | Phe   | Phe   | Phe   | Phe   | Phe   | Phe   | Phe   | Phe   | Phe   | Phe   | Phe   | Phe   | Phe   | Phe   | Phe   | Phe   | Phe   | Phe   |
| His      | His | His | His   | His   | His   | His   | His   | His   | His   | His   | His   | His   | His   | His   | His   | His   | His   | His   | His   | His   |
| Arg      | Arg | Arg | Arg   | Arg   | Arg   | Arg   | Arg   | Arg   | Arg   | Arg   | Arg   | Arg   | Arg   | Arg   | Arg   | Arg   | Arg   | Arg   | Arg   | Arg   |

Amino acid compositions of thrombolytic peptides. The composition of peptides derived from the amino acid sequence is shown in parentheses. Peptides were purified by paper electrophoresis at pH 6.5. In some cases additional purification was achieved by paper electrophoresis and/or paper chromatography. Cysteic acid was not determined. Serine and threonine values were not corrected for degradation losses.

* Amino acid composition was not quite satisfactory. Generally, however, no problems were encountered during dansyl-Edman degradation.
<table>
<thead>
<tr>
<th>Peptide</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>T5</th>
<th>T6</th>
<th>T7</th>
<th>T8</th>
<th>T9</th>
<th>T10</th>
<th>T11</th>
<th>T12</th>
<th>T13</th>
<th>T14</th>
<th>T15</th>
<th>T16</th>
<th>T17</th>
<th>T18</th>
<th>T19</th>
<th>T20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>1.4(1)</td>
<td>2.0(2)</td>
<td>1.1(1)</td>
<td>1.5(1)</td>
<td>2.0(2)</td>
<td>0.3</td>
<td>1.3(2)</td>
<td>1.1(1)</td>
<td>1.2(1)</td>
<td>0.9(1)</td>
<td>0.8(1)</td>
<td>2.0(2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thr</td>
<td>1.1(1)</td>
<td>1.1(1)</td>
<td>0.9(1)</td>
<td>0.7(1)</td>
<td>1.0(1)</td>
<td>2.0(2)</td>
<td>1.0(1)</td>
<td>0.6</td>
<td>0.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ser</td>
<td>0.9(1)</td>
<td>1.1(1)</td>
<td>5.5(6)</td>
<td>1.8(1)</td>
<td>1.2(1)</td>
<td>0.7(1)</td>
<td>1.6(3)</td>
<td>2.2(2)</td>
<td>0.8</td>
<td>1.1(1)</td>
<td>1.4(1)</td>
<td>2.8(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu</td>
<td>2.0(2)</td>
<td>1.1(1)</td>
<td>0.6(1)</td>
<td>1.0(1)</td>
<td>0.9(1)</td>
<td>3.7(3)</td>
<td>0.9(1)</td>
<td>0.3</td>
<td>0.9(1)</td>
<td>1.1(1)</td>
<td>0.7(1)</td>
<td>1.4(1)</td>
<td>1.1(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td>0.7(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.9(2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gly</td>
<td>1.3(1)</td>
<td>0.5</td>
<td>0.4</td>
<td>0.9(1)</td>
<td>0.7</td>
<td>1.1(1)</td>
<td>0.7</td>
<td>0.5</td>
<td>2.1(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>2.0(2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.7(3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>2.7(3)</td>
<td>0.9(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Met</td>
<td>0.9(1)</td>
<td>1.7(2)</td>
<td>0.8(1)</td>
<td>0.3</td>
<td>0.5(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.7(3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ile</td>
<td>0.9(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.6(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>1.2(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.1(1)</td>
<td>0.6(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyr</td>
<td>0.6(1)</td>
<td>1.0(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.9(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phe</td>
<td>0.9(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.0(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td></td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>0.2</td>
<td>1.0(1)</td>
<td>1.4</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>0.9(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>His</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td></td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td></td>
<td>1.9(2)</td>
<td></td>
<td>1.0(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg</td>
<td>1.1(1)</td>
<td>2.0(2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.4</td>
<td>1.0(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Amino acid compositions of tryptic peptides.** The composition of peptides derived from the amino-acid sequence is shown in parentheses. Peptides were purified by paper electrophoresis at pH 3.5. In some cases additional purification was achieved by paper electrophoresis at pH 9.8 and/or paper chromatography. Aminohexyloxylysiene recoveries were often low. Serine and threonine values were not corrected for degradation losses.

*See Table 2.*

† Relatively impure peptides.

‡ The amino acid composition of peptide T5 was derived from the composition of a mixture of peptides T5 and T10 (see 'Reliability of the sequence determination').
Amino acid compositions of tryptic peptides. The composition of peptides derived from the amino-acid sequence is shown in parentheses. Peptides were purified by paper electrophoresis at pH 3.5. In some cases additional purification was achieved by paper electrophoresis at pH 3.5 and/or paper chromatography. See Table 2.

+ Relatively impure peptides.

The amino acid composition of peptide 95 was derived from the composition of a mixture of peptides 75 and 110 (see Reliability of the sequence determination)
Table 5

<table>
<thead>
<tr>
<th>Peptide</th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
<th>G5</th>
<th>G6</th>
<th>CN1</th>
<th>CN2</th>
<th>CN3</th>
<th>CN4</th>
<th>CN5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>1.0(1)</td>
<td>1.4(1)</td>
<td>1.0(1)</td>
<td></td>
<td></td>
<td></td>
<td>3.7(1)</td>
<td>1.0(1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thr</td>
<td>1.1(1)</td>
<td>0.9(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.0(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ser</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>2.1(2)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>7.2(7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu</td>
<td>1.1(1)</td>
<td>2.1(2)</td>
<td>1.0(1)</td>
<td></td>
<td></td>
<td></td>
<td>3.0(3)</td>
<td>1.0(1)</td>
<td>1.2(1)</td>
<td>1.0(1)</td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.0(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gly</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.1(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>0.9(1)</td>
<td></td>
<td></td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.9(2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>1.6(2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.3(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ile</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.8(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>1.0(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyr</td>
<td></td>
<td>1.6(2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.9(2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phe</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.0(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>1.1(1)</td>
<td></td>
<td></td>
<td>1.0(1)</td>
<td>1.3(1)</td>
<td>0.9(1)</td>
<td>0.9(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>His</td>
<td>1.2(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.0(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg</td>
<td></td>
<td>0.8(1)</td>
<td></td>
<td></td>
<td>1.0(1)</td>
<td>0.5</td>
<td>2.0(2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hae (lactone)</td>
<td>+ (1)</td>
<td>+ (1)</td>
<td>+ (1)</td>
<td>+ (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Position: 50-52 53-61 60-86 87-91 92-98 120-124 1-10 11-13 14-29 26-29 30 31-33

Amino acid compositions of peptides obtained on digestion with the staphylococcal proteinase and trypsin (G) and of the CIIBr peptides (CB)

The composition of peptides derived from the amino acid sequence is shown in parentheses. Peptides were purified by paper electrophoresis at pH 3.5. Homoserine (lactone) was not determined quantitatively. Serine and threonine values were not corrected for degradation losses.

See legend of Table 3.
<table>
<thead>
<tr>
<th>Amide assignments</th>
<th>comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>Au, El</td>
</tr>
<tr>
<td>Serine</td>
<td>Au, El</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>El</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>Au</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>CF</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>El</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>El</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>El</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>El</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>El</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>El</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>El</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>El</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>El</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>El</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>El</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>El</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>El</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>El</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>El</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>El</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>G, El</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>El</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>El</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>El</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>El</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>Am</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>Am</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>El</td>
</tr>
</tbody>
</table>

Amide residues

Reagents used: Au, automatic Edman degradation (identification as amino acid "amidohydantoins"). El, high-voltage paper electrophoresis at pH 6.5. The mobility of basic peptides was measured relative to that of lysine, indicated by a positive sign, and acidic peptides relative to that of aspartic acid, indicated by a negative sign; G, specificity of Glu-enzyme. CF, Carboxypeptidase digestion. Am, amide.

The peptide is a rather basic peptide (mobility +0.26) and contains two cysteic acid, one serine and one histidine residues and is possibly four residues (Thr, Ser, Met, Ile) longer (see composition in Table 2). This suggests that the two Asx and Glx residues are amidated.
Fig. 1. Elution pattern of the fractionation on a Sephadex G-25 (fine grade) column (1 x 100 cm) of a thermolysin digest of dromedary ribonuclease. Elution was carried out with 5% formic acid at a flow rate of 20 ml/h, 1 ml fractions being collected.

Fig. 2. Analysis by paper electrophoresis at pH 6.5 of the thermolysin digest of dromedary ribonuclease fractionated on Sephadex G-25 (see Fig. 1). For details see the text.
Fig. 3. Gel filtration of CNBr-treated drosophila ribonuclease on a Sephadex G-25 superfine grade column (0.8 x 100 cm). Elution was carried out with 0.1 M acetic acid at a flow rate of 7 ml/hr; 1.5 ml fractions being collected. The fractions indicated by a bar were oxidized with performic acid. The other peaks were analysed by paper electrophoresis.

Fig. 5. Gel filtration on the column used in Fig. 3, of the void-volume peak of Fig. 3 after digestion with the diastase. The fractions indicated by bars were pooled and digested with trypsin, except for the fourth peak, which was analysed by paper electrophoresis. The tryptic peptides were fractionated by preparative paper electrophoresis, which resulted in peptides G2, G3, G4, and G5.
Allelic Polymorphism in Arabian Camel Ribonuclease and the Amino Acid Sequence of Bactrian Camel Ribonuclease

G. W. Welling, H. Mulder, and J. J. Beintema

Received 23 Sept. 1975—Final 13 Oct. 1975

Pancreatic ribonucleases from several species (whitetail deer, roe deer, guinea pig, and arabian camel) exhibit more than one amino acid at particular positions in their amino acid sequences. Since these enzymes were isolated from pooled pancreas, the origin of this heterogeneity is not clear. The pancreatic ribonucleases from 11 individual arabian camels (Camelus dromedarius) have been investigated with respect to the lysine-glutamine heterogeneity at position 103 (Welling et al., 1975). Six ribonucleases showed only one basic band and five showed two bands after polyacrylamide gel electrophoresis, suggesting a gene frequency of about 0.75 for the Lys gene and about 0.25 for the Gln gene. The amino acid sequence of bactrian camel (Camelus bactrianus) ribonuclease isolated from individual pancreatic tissue was determined and compared with that of arabian camel ribonuclease. The only difference was observed at position 103. In the ribonucleases from two unrelated bactrian camels, only glutamine was observed at that position.

KEY WORDS: ribonuclease; amino acid sequence; polymorphism; Camelidae, pancreas.

INTRODUCTION

The amino acid sequences of many pancreatic ribonucleases have been determined (Welling et al., 1975). In a number of cases, heterogeneity in the...
structure was encountered. In whitetail deer (Odocoileus virginianus) serine and threonine were found at position 3 (Barnard et al., 1973). Ribonuclease from bactrian camel (Camelus bactrianus) exhibits heterogeneity with alanine and isoleucine at position 64 (Zwiebs et al., 1973). This is also heterogeneous in guinea pig (Cavia porcellus) B ribonuclease showing a proline or a leucine residue (Van den Berg and Beintema, 1975). The position 32 in chinchilla (Chinchilla breviceps) is occupied either by an alanine or isoleucine (Van den Berg and Beintema, 1975), and in camel (Camelus dromedarius) ribonuclease glutamine or lysine was found in position 103 (Welling et al., 1975). However, the sequence determination of these heterogeneous ribonucleases were carried out with pooled tissue. In this study, the Arabian camel ribonuclease from the glandular tissue were investigated with respect to the heterogeneity at position. The amino acid sequence of bactrian camel ribonuclease isolated from individual pancreatic tissue was determined and compared with that of Arabian camel ribonuclease.

MATERIALS AND METHODS

Methods were as described by Welling et al. (1975) unless stated. Bactrian camel ribonuclease was isolated by affinity chromatography. Peptides were numbered according to their position in the chain. Peptides from a trypsin digest of reduced and amino-ribonuclease were prefixed by the letter T. Peptides obtained from a digest of oxidized ribonuclease were prefixed by the letter H. Digestion of 400 nmole of a trypsin peptide (residues 111–124) was carried out by digestion with 10 μl of a chymotrypsin solution (1 mg/ml) in 0.1M acetate, pH 8.0, at 37°C for 2 hr. The peptides digested were prefixed by the letters TC. Peptides were isolated by preparative paper electrophoresis. Additional purification was carried out by paper chromatography.

RESULTS AND DISCUSSION

Pancreas (115 g), 28 mg of purified bactrian camel ribonuclease was isolated. The gland pancreas (102 g), 14 mg of pure ribonuclease was obtained.

Pancreas (115 g), 28 mg of purified bactrian camel ribonuclease was isolated. The gland pancreas (102 g), 14 mg of pure ribonuclease was obtained.

Pancreas (115 g), 28 mg of purified bactrian camel ribonuclease was isolated. The gland pancreas (102 g), 14 mg of pure ribonuclease was obtained.
<table>
<thead>
<tr>
<th>Peptide</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>T5</th>
<th>T6</th>
<th>T7</th>
<th>T8</th>
<th>T9</th>
<th>T10</th>
<th>T11</th>
<th>T12</th>
<th>T13</th>
<th>T14</th>
<th>T15</th>
<th>T16</th>
<th>T17</th>
<th>T18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg</td>
<td>0.7</td>
<td>1.3</td>
<td>1.6</td>
<td>1.4</td>
<td>1.5</td>
<td>0.1</td>
<td>0.9</td>
<td>1.2</td>
<td>2.1</td>
<td>1.0</td>
<td>0.4</td>
<td>0.6</td>
<td>1.0</td>
<td>0.9</td>
<td>1.2</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Thr</td>
<td>0.9</td>
<td>0.6</td>
<td>1.0</td>
<td>2.6</td>
<td>1.0</td>
<td>0.8</td>
<td>0.8</td>
<td>1.2</td>
<td>2.0</td>
<td>1.2</td>
<td>0.8</td>
<td>0.8</td>
<td>1.7</td>
<td>1.0</td>
<td>0.8</td>
<td>1.5</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Ser</td>
<td>2.0</td>
<td>2.2</td>
<td>3.2</td>
<td>2.4</td>
<td>2.4</td>
<td>0.8</td>
<td>0.6</td>
<td>1.0</td>
<td>0.8</td>
<td>0.8</td>
<td>1.0</td>
<td>0.8</td>
<td>1.7</td>
<td>1.0</td>
<td>0.8</td>
<td>1.5</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Glu</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.8</td>
<td>0.8</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.8</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Pro</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Ala</td>
<td>2.1</td>
<td>2.4</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Val</td>
<td>0.9</td>
<td>1.8</td>
<td>1.3</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Met</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>He</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Leu</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Phe</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Lys</td>
<td>0.9</td>
<td>0.6</td>
<td>0.6</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Asp</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>His</td>
<td>1.0</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Arg</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Position in sequence


---

*The number of residues derived from the amino acid sequence is given in parentheses.

*Peptides eluted from paper were sometimes contaminated with glycine and serine.

*Not determined quantitatively.

*Tyrosine and methionine values were sometimes low, probably because of oxidation.
Table II. Amino Acid Composition of Thermolysin Peptides (H) and Chymotrypsin Peptides (TC) of Salt-reconstituted Retinase

<table>
<thead>
<tr>
<th>Peptide</th>
<th>H1</th>
<th>H2</th>
<th>H3</th>
<th>H4</th>
<th>H5</th>
<th>H6</th>
<th>H7</th>
<th>H8</th>
<th>H9</th>
<th>TC1</th>
<th>TC2</th>
<th>TC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CySO₃H</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.9</td>
<td>1.3</td>
<td>0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asp</td>
<td>1.1</td>
<td>2.1</td>
<td>2.2</td>
<td>1.7</td>
<td>1.9</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Met(O)₂</td>
<td>1.2</td>
<td>3.4</td>
<td>2.3</td>
<td>1.9</td>
<td>1.8</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thr</td>
<td>2.3</td>
<td>3.4</td>
<td>2.3</td>
<td>1.9</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ser</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gly</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ile</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyr</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phe</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>His</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Position in sequence</td>
<td>47-50</td>
<td>51-56</td>
<td>57-62</td>
<td>63-76</td>
<td>63-80</td>
<td>61-95</td>
<td>81-98</td>
<td>102-105</td>
<td>106-117</td>
<td>111-115</td>
<td>116-120</td>
<td>121-124</td>
</tr>
</tbody>
</table>

* The number of residues derived from the amino acid sequence is given in parentheses.
* n.d., Not determined.
* Ile-Ile bonds are not hydrolyzed completely in 20 hr.
* +, Not determined quantitatively.
* Tyrosine values were sometimes low, probably because of oxidation.
Fig. 1. Gel filtration of a tryptic digest of bactrian camel ribonuclease on a Sephadex G-25 (fine grade) column (0.9 by 200 cm). Elution was carried out with 0.1 M acetic acid at a flow rate of 9.3 ml/hr, 2.3-ml fractions being collected. Each fraction eluting after the void volume was electrophoresed at pH 3.5. Part of the resulting "fingerprint" (of the fractions indicated by a bar) is shown in Fig. 2.

to pool fractions for further purification. The resulting "fingerprint" was very similar to the one obtained for the tryptic peptides of arabian camel ribonuclease. An essential part of both "fingerprints" is compared in Fig. 2. Because of this resemblance, peptides from bactrian camel ribonuclease were positioned by homology with the completely sequenced arabian camel ribonuclease. The alignment of the peptides and the sequence results are shown in Fig. 3. The presence or absence of amide groups on each aspartic or glutamic acid residue in bactrian camel ribonuclease was assumed to be the same as in arabian camel ribonuclease and was verified only for those amino acids which differed from most other ribonucleases: Asn 22, Asn 24, Glu 34, Asn 37, Asn 101, and Glu 103. Peptides were not completely sequenced except for peptides T8 (residues 34–40) and T17 (residues 99–104), differing from all other ribonucleases sequenced to date with the exception of arabian camel ribonuclease, and peptide T14 (residues 86–91) which showed a glycine-serine inversion in horse (Scheffler and Beintema, 1974) and in guinea pig ribonuclease A (Van den Berg and Beintema, 1975) compared with equine ribonuclease (Smyth et al., 1963).

Only one difference was found between bactrian and arabian cam...
A "fingerprint" was prepared in Fig. 2. Results are shown for those amino acids differing of the arabian camel enzyme isolated from unrelated single individuals only glutamine was observed. studies on arabian camel ribonuclease were carried out on several combined pancreas from six individuals, giving rise to conflicting with respect to number of bands on polyacrylamide gel electrophoresis.
Graph of an essential part (indicated by a bar in Fig. 1) of the tryptic digest obtained after electrophoresis of an aliquot of each of 100 fractions from 55-78 of a tryptic digest of Arabian camel (A) and B ribonuclease. From left to right, fractions 55-78.

- Ala-Tyr (92-95),
- Phe-Glu-Arg (8-10),
- Ala-Tyr-Gln-Lys (56-61),
- Ala-Ser-Asn-Leu-Lys (99-103),
- Ser-Glu-Lys (99-103),
- Ser-Asn-Leu-Gln-Lys (99-104).

In the latter, position 103 is occupied by either glutamine or lysine. Preparations of the bactrian camel enzyme isolated from several single individuals only glutamine was observed. Arabian camel ribonuclease were carried out on several pancreases from six individuals, giving rise to conflicting numbers of bands on polyacrylamide gel electrophoresis.
and heterogeneity in amino acid sequence (Welling et al., 1975). Recently, however, new Arabian camel pancreatic tissue became available. Homogenates of individual pancreatic tissue were made and after sulfuric acid extraction and acetone precipitation the resulting protein mixtures were

![Fig. 3. Amino acid sequence of bactrian camel pancreatic ribonuclease. The sequence was determined with Arabian camel ribonuclease. The numbering of bovine ribonuclease was used. For peptide nomenclature, see Materials and Methods.](image-url)
Polyacrylamide gel electrophoresis in 10% gels in β-alanine pH 4.5. Subsequent incubation with yeast RNA and staining with toluidine blue (Wilson, 1969) revealed that in pancreatic tissue of dromedaries only one enzymically active band (see Fig. 4). The remaining ones showed two active bands (see Fig. 4). Ribonuclease, also isolated from individual pancreatic tissue, reacted with the upper (less basic) band of the two. With the gel electrophoresis, we could much more easily interpret Fig. 4 of Fig. 2.

Camel ribonuclease, position 103 is occupied by glutamine after digestion of the aminoethylated protein, Ala-Ser-Asn- residues 99–104) could be isolated (spot No. 7 in Fig. 2B). Arabian camel pancreatic tissue mainly consisted of ribonuclease at position 103 and to a lesser degree consisted of ribonuclease at that position, resulting in a main spot after trypic digestion of Leu-Lys (residues 99–103, spot No. 6 in Fig. 2A) and a faint

Fig. 4. Polyacrylamide gel electrophoresis of individual dromedary pancreatic tissue. Pancreatic tissue was homogenized and extracted with 0.25 M H₂SO₄. After acetone precipitation and subsequent centrifugation, the resulting protein mixtures were electrophoresed. Gels were incubated with yeast RNA and stained with toluidine blue. In each case, two active bands (--) or only the lower band (--) was observed. In most cases, the more basic rat ribonuclease was used as a reference. The thin fast-running band consists of nontransparent material migrating with the front of the β-alanine buffer and is not found on electrophoresis of pure ribonucleases.
spot of Ala-Ser-Asn-Leu-Gln-Lys (residues 99-104, spot No. 7 in Fig. 2A). The main spot was absent in the tryptic digest of bactrian camel ribonuclease (see Fig. 2B).

The results of the polyacrylamide gel electrophoresis of Arabian camel ribonuclease can be explained by allelic polymorphism. Application of the Hardy-Weinberg law suggests a frequency of about 0.75 for the Lys gene and about 0.25 for the Gln gene. Thus the only difference between bactrian and Arabian camel ribonuclease is a very slight transition caused by what probably might be called a neutral mutation from glutamine to lysine at position 103, since the transient phase, with both amino acids present, is observed.

ACKNOWLEDGMENTS

We thank Dr. P. Zwart and Mr. E. de Graaf, Institute for Diseases in Specific Animals, Veterinary Department of the University of Utrecht, for the camel pancreatic tissue. We thank Dr. B. A. Peleg, Kimron Veterinary Institute, Bet Dagan (Israel), for the dromedary pancreatic tissue. We also thank Ms. Gerda Groen for her excellent technical assistance.

REFERENCES


LOCATION AND PRIMARY STRUCTURE OF S-PEPTIDES FROM DIFFERENT PANCREATIC RIBONUCLEASES

A. WELLING, G. GROEN, D. GABEL*, W. GAASTRA, J.J. BEINTEMA
Biochemisch Laboratorium, Rijksuniversiteit,
Zernikelaan, Groningen, The Netherlands
Received 14 December 1973

Described the isolation of protein during the digestion of S-peptide. The characterisation of enzymatically linked components later [2]. Ribonuclease activity and the same constituent from S-peptide fragments of S-peptide residues linked to S-protein and in the reconstituted RNase S has been studied in an external rat and snapping turtle study. We present the successful reconstitution of RNase S, ribonuclease obtained from RNase S; S-peptide obtained from RNase S; S-peptide obtained from RNase S; S-peptide and S-protein.

The S-peptides from reindeer, kangaroo and dromedary RNase could be obtained easily, using a digestion time of 90 min. The elution patterns were more or less identical (fig. 1A). Digestion times had to be 1 hr for goat and gnu RNases and 2 hr for giraffe.

Miles-Seravac Ltd. (Maidenhead). All other ribonucleases used in this study (goat, giraffe, gnu, reindeer, dromedary, kangaroo, lesser rorqual, pig, and horse) were isolated according to Wierenga et al. [7] and rat RNase, according to Beintema et al. [8]. Subtilopeptidase A (Subtilisin Carlsberg) was a gift from Novo Industri (Copenhagen). Sephadex G-50 (fine) was purchased from Pharmacia (Uppsala). All other reagents were analytical grade products from Merck AG (Darmstadt).

Amino acid analysis, high-voltage paper electrophoresis, dansylation, and dansyl-Edman degradation were performed as described earlier [7, 9].

2.1. Preparation of S-peptides

Four mg of ribonuclease in 200 µl 0.1 M Tris-HCl pH 8.0 was treated with 0.04 mg (0.16% solution in buffer) of subtilopeptidase A at 0°C. After 60–120 min of digestion, 250 µl of 0.5 N HCl was added. The mixture was applied to a Sephadex G-50 (fine) column (1 X 100 cm) and eluted with 0.05 N HCl. The absorbance of the effluent was measured at 280 and 220 nm. The S-peptide peak was lyophilised and further purified by preparative paper electrophoresis at pH 3.5.

3. Results and discussion

The S-peptides from reindeer, kangaroo and dromedary RNase could be obtained easily, using a digestion time of 90 min. The elution patterns were more or less identical (fig. 1A). Digestion times had to be 1 hr for goat and gnu RNases and 2 hr for giraffe.
Fig. 1. Gel filtration on a Sephadex G-50 (fine) of pancreatic ribonuclease from dromedary (B) and lesser torquail (C) digested with 0.05 N HCl. Flow rate 15–20 ml/min. Fractions (•••••••) A220. S-peptide peak positions.

Table 1

Amino acid compositions of S-peptides obtained after cleavage of ribonucleases with subtilisin and subsequent gel filtration on a Sephadex G-50 (fine). Nearest integers are given in brackets.

<table>
<thead>
<tr>
<th></th>
<th>Goat</th>
<th>Cow</th>
<th>Gnu</th>
<th>Giraffe</th>
<th>Reindeer</th>
<th>Dromedar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>2.0</td>
<td>2.1</td>
<td>1.8</td>
<td>2.1</td>
<td>2.0</td>
<td>1.1</td>
</tr>
<tr>
<td>His</td>
<td>0.9</td>
<td>1.0</td>
<td>0.9</td>
<td>1.1</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td>Arg</td>
<td>0.8</td>
<td>1.0</td>
<td>1.2</td>
<td>0.8</td>
<td>1.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Asp*</td>
<td>1.4</td>
<td>1.0</td>
<td>1.5</td>
<td>1.3</td>
<td>1.2</td>
<td>1.4</td>
</tr>
<tr>
<td>Thr</td>
<td>1.0</td>
<td>1.9</td>
<td>1.0</td>
<td>0.8</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Ser</td>
<td>5.5</td>
<td>2.8</td>
<td>5.0</td>
<td>4.2</td>
<td>3.8</td>
<td>4.9</td>
</tr>
<tr>
<td>Glu</td>
<td>2.8</td>
<td>2.8</td>
<td>3.1</td>
<td>3.1</td>
<td>2.9</td>
<td>4.0</td>
</tr>
<tr>
<td>Pro</td>
<td></td>
<td></td>
<td></td>
<td>2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gly**</td>
<td>0.6</td>
<td>0.3</td>
<td>0.3</td>
<td>0.7</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Ala</td>
<td>4.0</td>
<td>4.6</td>
<td>4.0</td>
<td>2.9</td>
<td>4.0</td>
<td>2.2</td>
</tr>
<tr>
<td>Val</td>
<td></td>
<td>0.2</td>
<td></td>
<td>0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Met</td>
<td>0.9</td>
<td>0.6</td>
<td>0.7</td>
<td></td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Leu</td>
<td></td>
<td></td>
<td></td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyr</td>
<td></td>
<td></td>
<td></td>
<td>0.2</td>
<td>0.4</td>
<td>1.0</td>
</tr>
<tr>
<td>Phe</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Total residues: 20 20 20 18 20 19

* Some values are too high because of baseline shift and, sometimes, traces of methionine sulfoxide.
** Glycine values were sometimes too high because of contamination from the paper electropherogram.
Fig. 2 Primary structures of the N-terminal part of ribonucleases from cow[10], horse[11], pig[12,7], rat[13], turtle[14], and kangaroo [14]. The rest of the sequences is determined in our laboratory and will be published elsewhere. In the ribonucleases that are susceptible to cleavage by subtilisin the cleavage points are indicated by arrows. O = carbohydrate chain.
RNase. These elution patterns were comparable to that of giraffe RNase that is shown in fig. 1B. Lesser rorqual, rat, pig, and horse ribonuclease resisted a 120 min subtilisin treatment. The elution pattern obtained after subtilisin digestion of lesser rorqual RNase is shown in fig. 1C.

From the amino acid compositions of the S-peptides (table 1) and sequence information from primary structure studies on the whole ribonuclease molecules — which will be described elsewhere — we derived the primary structure of the isolated S-peptides (fig. 2). This figure also shows the points of cleavage by subtilisin in the RNases attacked. The presence of proline, valine, tyrosine, or glutamic acid in the S-peptide loop does not interfere with the susceptibility to cleavage by subtilisin. However, different sequences are attacked at different positions not easily rationalized from the sequence data. Thus, the change of an alanine in position 20 of goat RNase to a valine in giraffe RNase shifts the cleavage point two residues towards the N-terminus.

The sequences resistant to cleavage by subtilisin are also shown in fig. 2. The reasons for this resistance may be: i) the presence of amino acids incompatible with the substrate binding site of subtilisin; ii) prevention of binding to or hydrolysis by subtilisin due to a different conformation of this part of the RNase, or iii) the presence of carbohydrate attached to Asn 21 in the pig enzyme [15], but not in the horse enzyme [11]. In the latter enzyme, only partial glycosidation is observed.

Empirical methods to predict β-bends using a tetramer [16] or a nonamer [17] correlation have been applied to the sequences known on both sides of the potential cleavage region. The results of the nonamer model (fig. 3) for the rat and turtle enzymes are similar to those for the horse enzyme, whereas the profiles for the cow and reindeer enzymes resemble that of goat RNase. The dromedary profile differs from all others, having a higher-than-average bend probability for all but three of the residues in the region 17–25 (independent of the choice of Asx). The sequences split show a trough in the bend probability around the point of cleavage, whereas bends are predicted in the resistant RNases, with the exception of the pig enzyme. Here, the carbohydrate chain attached to Asn 21 may be important. The shift in the giraffe enzyme seems to be correlated with conformational properties of valine, increased bend probability of residues 11–17 (fig. 3). The tetramer prediction algorithm gives a similar result (not shown) with the exception of fig. 2.

We suggest that, in the S-peptide, there exist minor differences in the three-dimensional structure of the different RNases due to different conformational preferences of different β-bends. Such changes may interfere with the subtilisin.

Acknowledgements

Part of this work has been carried out with the financial support of the Netherlands Foundation for Research (S.O.N.) and with financial assistance from the Netherlands Organisation for the Advancement of Pure Research (Z.W.O.).
ACTIVITY AND ANTIGENICITY OF RIBONUCLEASE HYBRIDS

Gjalt W. WELLING, Johannes A. LENSTRA and Jaap J. BEINTEMA
Biochemisch Laboratorium, Rijkuniversiteit, Zernikelaan, Groningen, The Netherlands

Received 29 October 1975
Revised version received 19 December 1975

1. Introduction

Since the preparation of RNase-S* by Richards [1] there has been a considerable interest in its two components, S-peptide and S-protein. The S-peptide (residues 1–20) has attracted most attention, because it has been possible to synthesize analogs of it. By mixing it with S-protein, the influence of amino acid modifications, replacements or removal on activity and binding, could be assayed. It has turned out that there are residues which are important for binding (Glu-2, Phe-8, Arg-10, Gin-11, Met-13, Asp-14) and for enzymic activity (His-12) [2,3]. Except for Met-13, which is replaced by Val or Ile in a few ribonucleases, all these residues are invariant in pancreatic ribonucleases of 23 animal species [4]. A number of these ribonucleases, too, were capable of cleavage by subtilisin and the S-peptides were isolated [5]. It seemed of interest to determine what effect the substitutions which have occurred during molecular evolution of a certain ribonuclease have on enzymic activity and binding. Natural S-peptides from cow, dromedary and kangaroo ribonuclease were used. Rat ribonuclease cannot be cleaved by subtilisin [5,6], so synthetic rat S-peptides (13 and 17 residues long) were used instead. S-peptides were combined with a constant amount of S-protein from dromedary or bovine,ribonuclease and the activity was measured with cytidine 2’3’-cyclic phosphate as a substrate.

Immunologic techniques have proved to be sensitive probes of the surface structure of proteins in solution [7]. RNase-S, S-peptide and S-protein have been subject of such studies [8,9]. In this study, the surface structure of ribonuclease hybrids consisting of cow, dromedary, kangaroo and rat S-peptides combined with bovine S-protein is scanned by utilising antiserum directed against RNase-S.

2. Materials and methods

Bovine pancreatic ribonuclease was obtained from Miles-Seravac Ltd. (Maidenhead). Kangaroo and dromedary ribonucleases were isolated by affinity chromatography as described by Wierenga et al. [10]. Rat ribonuclease was isolated as described by Beintema et al. [11]. Bovine S-protein was from Sigma Chemical Company (St Louis) or prepared from RNase-S [5], which was purified on SE-Sephadex C-25 (Pharmacia, Uppsala) as described by Dunn et al. [12]. Bovine RNase S and cytidine 2’3’-cyclic phosphate (sodium salt) were purchased from Sigma Chemical Comp. (St Louis). Dromedary S-protein and S-peptides were isolated as described [5]. Rat S-peptides were synthesized by M. Voskuyl-Holtkamp and others (M. Voskuyl-Holtkamp, C. Schattenkerk and E. Hovinga; to be published). Agarose was a product from BDH (Poole, Dorset). All other reagents were analytical grade products from Merck AG (Darmstadt).

Amino acid analysis, high-voltage paper electrophoresis, dansylation and dansyl-Edman degradation were performed as described [10,13]. Concentrations of S-peptide and S-protein solutions were determined by amino acid analysis.

*Abbreviations. RNase-S, native ribonuclease cleaved by subtilisin. The resulting active complex can be separated into its two inactive components: S-peptide and S-protein. Upon remixing equimolar amounts of S-peptide and S-protein, active RNase-S’ is formed.
2.1. Enzyme measurements

The production of cytidine 2',3'-cyclic phosphate was measured with a Zeiss PMQ II spectrophotometer equipped with a recorder (absorbance scale 0 to 0.05) at 25 ± 1°C as described by Crook et al. with some modifications. A first course antiserum (S1) and a second course antiserum (S2) were used for the experiments.

Double immunodiffusion was performed in 2% agarose gel with antigens at a concentration of about 25 nmol/ml in 0.1 M Tris-Cl pH 7.13.

Quantitative precipitation was performed by mixing equal volumes (200 or 50 µl) of antiserum and antigen in 0.1 M Tris-Cl pH 7.13 and subsequent incubation at 37°C for 1 h. After 4 days at 4°C and careful mixing each day, the solutions were centrifuged at 2000 g (1 h) and washed twice with 500 or 50 µl of cold buffer. The precipitate was dissolved in 1 ml or 250 µl of 0.5 M NaOH and the absorption was read at 280 nm using 1 cm or 1 mm quartz cuvettes for the macro and micro procedure, respectively.

3. Results and discussion

Bovine and dromedary S-proteins were titrated with cow, dromedary, kangaroo and rat S-peptides, of which the amino acid sequences are shown in fig.1. The activity was determined with cytidine 2',3'-cyclic phosphate as a substrate and the profiles of these titrations are shown in figs.2 and 3.

![Image](image-url)

![Image](image-url)

The data were used to calculate an apparent dissociation constant. The following scheme was assumed:

\[
S + P \rightleftharpoons E + P
\]

in which E denotes RNase-S, S the substrate, and P the product. The dissociation constant \( K_d \) is equal to:

\[
K_d = \frac{[S][P]}{[E]}
\]
The initial velocity is proportional to the concentration of the ES complex, so if $V_{\text{max}}$ is the maximal velocity (which is reached if no free enzyme or S-protein is left),

$$a = \frac{[\text{ES}]}{[\text{ES}]_{\text{max}}} \times \frac{V_{\text{max}}}{V_{\text{max}}}$$

$$a = \frac{[\text{ES}]}{[\text{ES}] + [E] + [\text{protein}]} \times V_{\text{max}}.$$  

Combining (4), (5) and (6), at a constant concentration of S-protein, $1/a$ is then $K_d$ following from the intercept of the abscissa line.

in which $K_d$ is the apparent dissociation constant for the free enzyme-S-protein complex and $a$ is a constant.

The activity of different S-peptide-bovine S-protein complexes using cytidine 2',3'-cyclic phosphate as substrate is shown in Fig. 2. Rat S-peptide (17 residues) and dromedary S-peptide (17 residues) showed a marked influence, whereas the other two S-peptides did not have a marked influence. Rat S-peptide (17 residues) and dromedary S-peptide (17 residues) were about 60% of the activity of the bovine enzyme.

Fig. 2. Activity of different S-peptide-bovine S-protein complexes using cytidine 2',3'-cyclic phosphate as substrate. (o) Cow S-peptide; (r) kangaroo S-peptide; (l) dromedary S-peptide; (d) rat S-peptide (17 residues); (o) rat S-peptide (13 residues).

The behavior of the dromedary and rat S-peptide complexes was observed in Fig. 3. Rat S-peptide (17 residues) and dromedary S-peptide (17 residues) showed a marked influence.

Fig. 3. Activity of different S-peptide-dromedary S-protein complexes using cytidine 2',3'-cyclic phosphate as substrate. (c) Cow S-peptide; (l) dromedary S-peptide; (o) rat S-peptide (17 residues); (d) rat S-peptide (13 residues).

Fig. 5. Quantitative reaction of bovine S-peptide (17 residues) and dromedary S-peptide (17 residues) with cytidine 2',3'-cyclic phosphate.
combining (4), (5) and (6) and taking the reciprocal results in:

\[ \frac{1}{V} - \frac{1}{V_{\text{max}}} = \frac{1}{V_{\text{max}}} \left(1 + \frac{K_M + K_d \times [\text{peptide}]}{[S]} \right) \]

At a constant concentration of substrate and added S-protein, \( V / V_{\text{max}} \) is plotted against \( 1 / [\text{peptide}] \) at an intercept of the abscissa:

\[ [\text{peptide}] \left( \frac{1}{V_{\text{max}}} \right)^2 - \frac{K_d}{K_M} = -K_d \frac{1}{[S]} \]

in which \( K_d \) is the apparent dissociation constant for a given substrate concentration. From equation (8) we conclude that the substrate concentration may have a marked influence on \( V_{\text{max}} \). However, if \([S] \ll K_M\), \( S/K_M \approx 1 \) and \( K_d' \approx K_d \). To find the right substrate concentration, \( K_M \) values were determined. \( K_M \) values for the five ribonuclease hybrids containing bovine S-protein were about equal, 1.3 to 2.0 mM. A similar behaviour was observed for the four hybrids containing dromedary S-protein, which showed values

Fig. 4. Immuno-diffusion study in which undiluted antiserum directed against bovine RNase-S (S2) was developed against bovine S-protein (wells 1 and 4) and against complexes of S-peptides with bovine S-protein. S-peptides used: dromedary (2), rat 13 residues (3), kangaroo (5) and cow (6). Antigen concentration was 0.5 mg/ml.

Fig. 5. Quantitative precipitation (micro-method, see 2.2.) in which antiserum directed against bovine RNase-S (S1), was allowed to react with bovine S-protein (*) and complexes of bovine S-protein with different S-peptides from cow (○), dromedary (☆), rat 17 residues (●) and rat 13 residues (△).
ranging from 0.7 to 1.1 mM. Activity measurements were made at a substrate concentration of 0.16 mM, so \( K_d \) should be 1.1 to 1.2 \( K_d' \). In this way, \( K_d' \) values for the hybrids containing bovine S-protein were found to be 1.0–4.5 \( \times 10^{-8} \) M and those for the hybrids containing dromedary S-protein, 0.5–1.3 \( \times 10^{-8} \) M. These values are in reasonably good agreement with those determined by others using cytidine 2',3'-cyclic phosphate as a substrate (1 \( \times 10^{-7} \) [17]; 1 \( \times 10^{-8} \) [18]; 5 \( \times 10^{-7} \) [19]; 3.6 \( \times 10^{-8} \) [20]). The dissociation constants found by using direct binding assays [21] are consistently three orders of magnitude higher, which is most probably due to aggregation of S-protein at the high protein concentrations used [19,22]. However, the value found with agarose-bound S-protein is also rather high (2.5 \( \times 10^{-8} \) M) [19], indicating that in some way agarose may influence the binding.

An immunologic comparison has been made for the ribonuclease hybrids containing bovine S-protein. In Fig. 4 an example is shown of an Ouchterlony immunodiffusion test. For all hybrids, and also for free bovine S-protein, complete identity was observed. Quantitative precipitation (micro-adaption, see Materials and methods), too, failed to reveal any difference between the various hybrid ribonucleases and bovine S-protein (Fig. 5). In this case antiserum S1 was used. To enhance possible differences, the macro-test was performed using antiserum S2 and as antigens, bovine S-protein, and cow and rat S-peptide (17 residues) combined with bovine S-protein. Again no differences were observed (Fig. 6). The reaction of free S-protein indicates that it has a similar structure as the whole complex. However, antibodies fitting to the hybrid molecule may force an S-protein into the correct shape.

In conclusion, the results indicate that despite the many differences in primary structures of the S-peptides which have originated during molecular evolution, there are no important differences between the RNase hybrids with respect to binding constant, maximal activity, Michaelis constant of the catalysed reaction and antigenic properties.

Acknowledgements

We are grateful to J. Schermer, S. K. Oosterloo and D. Hoekstra for carrying out preliminary experiments. We thank R. N. Campagne for helpful comments. Part of this work has been carried out under auspices of the Netherlands Foundation for Chemical Research (S.O.N.) and with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

References

In this case antiserum S has detectable differences in comparison to the antiserum S1. The results indicate that the S-proteins exhibit similar epitopes. It was observed that the antibodies raised against S-protein 5 indicate that denatured structures of S-protein 4 during monomer-dimer conversion differ in the antigenic binding constant of the...
Studies of proteins can provide information for the elucidation of molecular evolution. The best example is cytochrome c. Amino acid sequences have been published of more than 45 species (McDonald and Dayhoff, 1973). From these, a genetic tree was constructed representing the evolutionary history of this protein. Cytochrome c is a rapidly evolving protein (Leijenaar and Gruber, 1967) compared with other proteins (see Table 1). It has been a highly variable enzyme and an excellent candidate for studying molecular evolution within the framework of comparative studies of ribonucleases. The N-terminal amino acid sequences of more than 40 species have been determined: cow (1963), rat (Beintema and Gruber, 1966), sheep (Jackson and Hirs, 1970), Welling et al. (1974), horse (Scheffer and Welling et al., 1974), reindeer (Leijenaar-van den Berg and Beintema, 1974), chinchilla, coyus, guinea pig (Van den Berg and Beintema, unpublished), European elk, fallow deer (Leijenaar-van den Berg and Beintema, unpublished) and muskrat (Van Dijk et al., unpublished).

The N-terminal amino acid sequences up to 30 residues (1/4 of the molecule) are fairly representative parts of the whole molecules as to their variabilities. They contain the external, so-called S-peptide loop, which is highly variable and also internal and active center residues, which are more or less invariant. In this study, the 24 N-terminal amino acid sequences of 23 species are compared to give more insight into the processes of the molecular evolution of ribonucleases. The species of which pancreatic ribonuclease was investigated are listed in Fig. 1 according to classical taxonomy (Romer, 1962; Morris, 1965). The 24 N-terminal amino acid sequences are shown in Fig. 2a. The occurrence of particular amino acids at each position is shown in Fig. 2b. A difference matrix is given Table 1.

### Table 1

<table>
<thead>
<tr>
<th>Protein Family</th>
<th>UEP in MY</th>
<th>Paulings</th>
</tr>
</thead>
<tbody>
<tr>
<td>cytochromes c</td>
<td>20</td>
<td>0.25</td>
</tr>
<tr>
<td>globins</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>fibrinopeptides</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>ribonucleases</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>4 external loops of ribonuclease: residues 1-6, 15-24, 31-39 and 98-104</td>
<td>0.75</td>
<td>7</td>
</tr>
</tbody>
</table>

Evolutionary rates are given in UEP (Unit Evolutionary Period) and Paulings (See Dickerson, 1971).
Fig. 1. Species of which the N-terminal amino acid sequence has been determined, divided into groups according to classical taxonomy (Morris, 1965; Romer, 1962).

### TABLE 2

Difference matrix of ribonucleases. Data are derived from the N-terminal sequences and are given in number of differences between each of the species and as % difference. A chain length of 33 residues was used for rat ribonuclease and 25 for kangaroo ribonuclease. A deletion was treated as an amino acid.

<table>
<thead>
<tr>
<th></th>
<th>cow</th>
<th>goat</th>
<th>sheep</th>
<th>pig</th>
<th>boar</th>
<th>camel</th>
<th>dromedary</th>
<th>camel</th>
<th>horse</th>
<th>reindeer</th>
<th>fallow deer</th>
<th>dromedary</th>
<th>camel</th>
<th>pig</th>
<th>guinea pig</th>
<th>musk rat</th>
<th>rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>cow</td>
<td>0%</td>
<td>7%</td>
<td>17%</td>
<td>10%</td>
<td>23%</td>
<td>27%</td>
<td>31%</td>
<td>40%</td>
<td>28%</td>
<td>40%</td>
<td>27%</td>
<td>34%</td>
<td>37%</td>
<td>27%</td>
<td>17%</td>
<td>34%</td>
<td>28%</td>
</tr>
<tr>
<td>goat</td>
<td>2%</td>
<td>7%</td>
<td>10%</td>
<td>3%</td>
<td>27%</td>
<td>30%</td>
<td>37%</td>
<td>42%</td>
<td>31%</td>
<td>37%</td>
<td>27%</td>
<td>34%</td>
<td>37%</td>
<td>27%</td>
<td>17%</td>
<td>34%</td>
<td>28%</td>
</tr>
<tr>
<td>sheep</td>
<td>2%</td>
<td>4%</td>
<td>17%</td>
<td>23%</td>
<td>25%</td>
<td>27%</td>
<td>31%</td>
<td>40%</td>
<td>25%</td>
<td>37%</td>
<td>27%</td>
<td>30%</td>
<td>37%</td>
<td>27%</td>
<td>17%</td>
<td>34%</td>
<td>28%</td>
</tr>
<tr>
<td>pig</td>
<td>2%</td>
<td>4%</td>
<td>17%</td>
<td>23%</td>
<td>25%</td>
<td>27%</td>
<td>31%</td>
<td>40%</td>
<td>25%</td>
<td>37%</td>
<td>27%</td>
<td>30%</td>
<td>37%</td>
<td>27%</td>
<td>17%</td>
<td>34%</td>
<td>28%</td>
</tr>
<tr>
<td>boar</td>
<td>1%</td>
<td>4%</td>
<td>17%</td>
<td>23%</td>
<td>25%</td>
<td>27%</td>
<td>31%</td>
<td>40%</td>
<td>25%</td>
<td>37%</td>
<td>27%</td>
<td>30%</td>
<td>37%</td>
<td>27%</td>
<td>17%</td>
<td>34%</td>
<td>28%</td>
</tr>
<tr>
<td>camel</td>
<td>7%</td>
<td>8%</td>
<td>10%</td>
<td>8%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>dromedary</td>
<td>7%</td>
<td>8%</td>
<td>8%</td>
<td>8%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>camel</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
</tr>
<tr>
<td>pig</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
</tr>
<tr>
<td>boar</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
</tr>
<tr>
<td>camel</td>
<td>12%</td>
<td>12%</td>
<td>12%</td>
<td>12%</td>
<td>12%</td>
<td>12%</td>
<td>12%</td>
<td>12%</td>
<td>12%</td>
<td>12%</td>
<td>12%</td>
<td>12%</td>
<td>12%</td>
<td>12%</td>
<td>12%</td>
<td>12%</td>
<td>12%</td>
</tr>
<tr>
<td>dromedary</td>
<td>9%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
<td>11%</td>
</tr>
<tr>
<td>camel</td>
<td>8%</td>
<td>8%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
</tr>
<tr>
<td>boar</td>
<td>8%</td>
<td>8%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
<td>9%</td>
</tr>
<tr>
<td>camel</td>
<td>16%</td>
<td>15%</td>
<td>15%</td>
<td>15%</td>
<td>15%</td>
<td>15%</td>
<td>15%</td>
<td>15%</td>
<td>15%</td>
<td>15%</td>
<td>15%</td>
<td>15%</td>
<td>15%</td>
<td>15%</td>
<td>15%</td>
<td>15%</td>
<td>15%</td>
</tr>
</tbody>
</table>
Fig. 2a. N-terminal sequences of ribonucleases. From 18 species the complete primary structure has been determined (see introduction). The remaining N-terminal sequences have been determined in our laboratory. Capybara ribonuclease was sequenced by automatic Edman degradation. Bison, gnu, kangaroo and camel ribonuclease sequences were determined with manual Edman techniques from peptides of different digests. The sequence of lesser rorqual ribonuclease was determined with both methods. The N-terminal sequences of roe deer and red deer ribonuclease are different from the ones published by Zwiers et al. (1973). It was found that upon cyanogen bromide treatment of these ribonucleases an Asp-Pro (14-15) bond was cleaved, next to the expected cleavage of the Met-Asp (13-14) bond. Dansyl-Edman degradation on the mixture of peptides 14-29 and 15-29 resulted in data which were interpreted wrongly. The sequences are being corrected at the moment by sequencing of peptides from proteolytic digests. The N-terminal sequence of kangaroo ribonuclease differs at position 15 from the one determined earlier (Barnard et al., 1972). The bison sequence differs at position 16, 18, 22 and 23 from the one determined by Barnard et al. (1972), but agrees at position 22 and 23 with the tentatively assigned sequence (20-29) of Stewart and Stevenson (1973). † Identity uncertain.

<table>
<thead>
<tr>
<th>Species</th>
<th>N-terminal sequence</th>
</tr>
</thead>
</table>
Fig. 2b. Occurrence of particular amino acids at each position of the N-terminal amino acid sequence, in decreasing order of frequency. Numbering of bovine ribonuclease has been used.

in Table 2, providing the number of differences and the relative differences between each of the species. With this matrix it was possible to construct an approximate phylogenetic tree (Fig. 3), which generally appeared to agree with the common zoological classification. Notable are the positions of 3 of the species. Rat ribonuclease apparently has evolved with an increased evolutionary rate after divergence from the muskrat. Horse ribonuclease appears to differ less from the artiodactyl ribonucleases than from the ribonucleases of the remaining species. Kangaroo ribonuclease differs less from the other ribonucleases than expected from the zoological classification (see Figs. 1 and 3).

13 out of 30 residues in each sequence are invariable. The other amino acids are subject to alteration, though in different ways. This is clearly understood by observing the degrees of variability of some residues: (i) Histidine 12 is an active center residue and therefore unchanged in all mammalian ribonucleases. However, in turtle ribonuclease a tyrosine is observed at this position (Barnard, 1972), indicating that it is not completely invariable. (ii) Position 3 can only be occupied by a serine or a threonine residue. At this position threonine is found in four species which have evolved independently from one another. A serine—threonine replacement is a conservative substitution, suggesting that this position is almost invariable. (iii) Alanine 5 is a special feature of the ruminants, the dromedary, serine 4 is a particular residue for the rodents, whilst proline is found in other species investigated. (iv) Position 25 appears to be rather variable. Alanine, aspartic acid, lysine, glutamic acid, glutamine were found. However, apparently not all amino acids are permitted at this position. From the sequences and the evolutionary relationships between the different species, it can be deduced that quite some lel and back mutations must have occurred.

Fig. 3. Approximate phylogenetic tree derived from the % differences between the N-terminal amino acid sequences of each of the species listed in Fig. 1. The 17 residues sequence of capybara ribonuclease was used as a reference.
one of the reasons for this is the importance of an amino acid of the molecule (Wyc.) Positions 16 and 17 are the S-peptide loop, which is variable parts of the whole. A difference with the bovine position 16, serine, aspartic acid, glutamic acid, arginine, lysine and at position 17 the asparagine, methionine, serine and preserved, while in the ribonuclease residue is invariably preserved that in homologous proteins no differences exist in the conformation of the main chain, except for external loops, where it is not unusual to find differences in conformation. We have found evidence that the latter may not be true for several S-peptide loops in different ribonucleases. Richards (1955, 1959) cleaved bovine ribonuclease with subtilisin and isolated 'a' active intermediate' called ribonuclease S. Predominantly peptide bond 20-21 in an external loop was cleaved (Doscher and Hirs, 1967). A number of ribonucleases with considerable differences in primary structure in this loop showed the same behaviour towards subtilisin (Welling et al., 1974b). In Fig. 5 the
sequences of cow, reindeer, red deer, dromedary, giraffe and kangaroo ribonucleases around the point of cleavage are given. For comparison the sequence of lesser rorqual ribonuclease which is not cleaved, is also shown. We calculated by an empirical method the $\beta$-bend probability in this part of the sequence. (Burgess et al., 1974; Scheraga, 1974). In Fig. 6 the profiles of this prediction for bovine, reindeer and lesser rorqual ribonuclease are shown. Cleavage of the S-peptide loop by subtilisin is correlated with a low bend probability in the region of cleavage. Apparently the conformation of the S-peptide loops of different ribonucleases with different amino acid sequences is not identical. However, in a number of ribonucleases, the ones suscetible to cleavage by subtilisin, comparable bend probabilities are observed and, together with the certainty that the external loop of these ribonucleases must fit in the active site cleft of subtilisin, this suggests that even with different amino acid sequences they possess the same conformation. The presence of this loop with the same conformation in different amino acid sequences in different ribonucleases points to conservation of an ancestral structure or to structural convergence.

The exact reasons for the different degree of conservation of structure are obscure. The speculations can be made: (i) The sequence contains residues belonging to or near a site or site of the chain, for instance the formation of an S-peptide (residue 6), a $\beta$-bend or another special structure. (ii) The sequence consists of residues important for correct folding of the chain. (iii) The sequence contains residues with special properties, others important for accessory function, for instance affecting solubility or carbohydrate attachment. Horse, pig and guinea pig ribonuclease contain a carbohydrate chain attached to asparagine 21. Pig and guinea pig ribonuclease show remarkable homology, primary structure in this region of the molecule (80% identity in the 30 residue terminal sequence). It could be possible that carbohydrate attachment has resulted in a second pressure, which has forced more than the immediate surroundings of asparagine 21 to converge to a similar structure.

These different degrees of selectivity are evident from the information obtain by comparing a set of related amino acid sequences indicate that there are possible reasons for conservation of a particular amino acid during molecular evolution.

Acknowledgements

Part of this work has been carried under auspices of the Netherlands Foundation for Chemical Research (S.O.N.) and financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).


The structure of cytochrome c and molecular evolution, 1971, J. Molec. Evol., 1, 40–45.


A COMPARISON OF PANCREATIC RIBONUCLEASES

E. Welling, Gerda Groen, Jaap J. Beintema, Marchienus Peter Schröder

Double immunodiffusion and micro-complement were used in cross-reactivity studies with nine ribonucleases differing 3-28% in amino acid sequence and rabbit antisera to cow, gnu, reindeer and whale ribonuclease. Generally a correlation was observed between extent of cross-reactivity and amino acid sequence resemblance. The antiserum against whale ribonuclease however, reacted to a larger degree with several antigens than expected from the amino acid sequence difference. Dromedary ribonuclease, which differs at 26% of the positions, showed the least cross-reaction. By comparing the antigenic reactivity and the differences in sequence, an attempt was made to locate antigenically relevant regions.

Conclusion

Amino acid sequence studies of homologous proteins may yield information on their molecular evolution and on the relationship between protein structure and function. In the case of pancreatic ribonucleases, sequences are manifold as investigated (Welling et al., 1975a) and the evolutionary history of these enzymes has been investigated (Wyckoff et al., 1970). A search for a similar relationship between pancreatic ribonucleases from different species seems of interest. Moreover, the antigenic differences related with the known tertiary structure of cow ribonuclease A (Kartha et al., 1967; Carlisle et al., 1974) and ribonuclease S (Wyckoff et al., 1970), ultimately resulting in knowledge about the location and conformation of anti-
genic determinants.

The interaction of native and oxidized cow ribonuclease with their antibodies was extensively studied by Brown et al. (1959). Native cow ribonuclease did not react with antibody to the oxidized enzyme, indicating that in the native protein only conformational dependent antigenic determinants are present. Changes in the structure by removal of the N-terminal amino acid (Brown, 1960), by using homologous proteins (Ern et al., 1960) or by rupturing the peptide bond between residues 20 and 21 (Singer and Richards, 1959), have been shown to result in less antigenic reactivity with antiserum directed against the native molecule.

In this study an immunologic comparison is made for the following 9 pancreatic ribonucleases: from cow (Smyth et al., 1963), goat (Welling et al., 1974a), giraffe (Gaastera et al., 1974), reindeer (Leijenaar-van den Berg and Beintema, 1975), pig (Jackson and Hirs, 1970; Wierenga et al., 1973), dromedary (Welling et al., 1975a), horse (Scheffer and Beintema, 1975), and pike whale or lesser rorqual (Emmens et al., 1976). Four different antisera against cow, gnu, reindeer and whale ribonuclease respectively, were utilized. Ouchterlony double immuno diffusion and quantitative micro-complement fixation, which has been shown to be very sensitive to small differences in primary structure (Prager and Wilson, 1971a, 1971b) have been used to measure the antigen-antibody reaction.

Experimental procedure

Materials

Cow ribonuclease was a product from Miles (Maidenhead). Cow ribonuclease A (carbohydrate free) was purchased from Schram Mann (Orangeburg). Agarose was from BDH (Poole) and Freund's complete adjuvant was purchased from Difco (Detroit). All other reagents were of analytical grade.

Ribonucleases were isolated by affinity chromatography as described by Wierenga et al. (1973).
cow, gnu, reindeer and whale ribonucleases were produced in rabbits as described by Westen- et al. (1974) for hemoglobins with some modifi-
...rabbit was used for each immunogen. At days 1
...ml of an 0.5% ribonuclease solution in 0.9% NaCl
...of Freund's adjuvant and sub-
...injected in each thigh of a rabbit. At day 22,
...0.25% ribonuclease solution was injected sub-
...followed after 30 min by 0.2 ml intravenously.
...amounts (0.5 - 2.5 ml) of the same solution were
...intravenously at days 24, 26, 29, 31, 33, 35 and 38.
...were taken at day 45. After 3 weeks rest, four
...ections (0.2 - 2 ml) were given at intervals of 2
...cts were bled one week after the last injection.
...limited amount of reindeer ribonuclease was
...this antigen was injected once a week during the
...ration period. The concentration of the cow ribo-
...ution used, was twice that of the other ribonucle-
...al, 80 mg of cow ribonuclease, 40 mg of gnu and
...uclease and 12.5 mg of reindeer ribonuclease were
...munication. Most of the work described here was
...ith second bleeding sera (after three months of

## Methods

...odiffusions were performed in 2% agarose gel with
...centration of 0.5 mg/ml in 0.9% NaCl solution.
...iserum wells were 4 mm and 6 mm in diameter,
...e. The centres of antiserum and antigen wells were
...tore. About 12.5 μl of undiluted antiserum was used. The
...rected to reindeer ribonuclease was 4 fold con-
...with an Amicon S 125 serum concentrator (Amicon

...plement fixation was carried out in disposable
...lates containing 96 wells as described by Casey
...units complement were used. When comparing
very related antigens, the antiserum was diluted by steps of 20%. The titer difference between two proteins was a measure of antigenic difference. The titer in the heterologous system is sometimes equal to but generally lower than in the homologous system. The ratio of the antiserum dilutions used, \( \frac{A}{B} \), is defined as the titer difference.

Antigen concentrations were determined by amino acid analysis with a Technicon TSM-1 amino acid analyzer. Samples were hydrolyzed in 0.4 ml of 6 M HCl at about 110°C in evacuated sealed glass tubes for 18-22 h. Because of differences in molecular weights, quantities of the various ribonucleases were expressed in molar amounts.

**Results**

**Antiserum production**

Cow, gnu, reindeer, and whale ribonucleases were rather immunogenic in rabbits giving antiserum titers of about 4,000-66,000, 1,000, and 1,000, respectively as measured by the micro-complement fixation method.

![Immuno-diffusion](image)

**Fig.1.** Immunodiffusion of antiserum to cow ribonuclease (central well) against different ribonucleases from reindeer (1), pig (2 and 4), gnu (3), giraffe (5) and dromedary (6). Dromedary ribonuclease gave a faint precipitation line which is hardly visible on the photograph.
The relative insensitivity of immunodiffusion to differences in structure as reported by Arnheim and Tjio (1967), spurs were frequently observed (see Fig. 1). Showing the results of the comparison of the 9 ribonucleases using the 4 antisera, are shown in Fig. 2a, b, c, d. With these data, a relative order of antigenic reactions drawn up, as shown in Table 1. Immunodiffusions were carried out with 2 first course antiserum directed to cow ribonuclease and 1 first course antiserum directed to cow ribonuclease A (carbohydrate free). Only minor differences observed compared with the results given in the matrix.
Table 1

A

imm. dif. cow > gnu > reindeer > pig > dromedary > whale
CF cow > gnu > giraffe > reindeer > dromedary > pig > whale > horse
sequence cow > goat > gnu > giraffe > reindeer > pig > dromedary > whale > horse

B

imm. dif. gnu > cow > giraffe > reindeer > pig > dromedary > whale
CF gnu > goat > cow > giraffe > reindeer > pig > dromedary > whale > horse
sequence gnu > goat > cow > giraffe > reindeer > pig > dromedary > whale > horse

C

imm. dif. reindeer > goat > gnu > cow > giraffe > dromedary > whale
CF reindeer > goat > gnu > cow > giraffe > dromedary > pig > whale > horse
sequence reindeer > goat > gnu > cow > giraffe > pig > whale > dromedary

D

imm. dif. whale > dromedary > goat > gnu > reindeer > giraffe
CF whale > dromedary > goat > gnu > cow > reindeer > giraffe > dromedary
sequence whale > pig > gnu > reindeer > horse > dromedary

Table 1. Relative order of antigenic reactivity of different ribonucleases determined with immunodiffusion and micro-complement fixation using antiserum directed against cow ribonuclease (A), gnu ribonuclease (B), reindeer ribonuclease (C) and whale ribonuclease (D). The relative order of resemblance in amino acid sequence is also shown. With immunodiffusion the relative order was determined as follows: with the data from the second row of Fig.2a, a first classification was made: cow, goat, gnu, giraffe > reindeer, pig, dromedary > whale, horse. With the data from the fourth and fifth row, a further classification could be made: cow, goat > gnu, giraffe > reindeer > pig, dromedary > whale, horse. The spur formed by pig over dromedary ribonuclease supports the final classification listed in Table 1.
### Table 3

| Titer differences, optimal antigen concentrations in pmol/ml, and sequence differences in amino acid sequence. Optimal antigen concentrations for gnu ribonuclease, 4 pmol/ml for reindeer ribonuclease and whale ribonuclease. The relative order was deduced from the % difference and after that, a further classification was made by optimal antigen concentration. The more antigen used to obtain complement fixation, the less was the antigenic reactivity. |

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Titer difference</th>
<th>Optimal antigen concentration (pmol/ml)</th>
<th>% difference in amino acid sequence</th>
<th>Reindeer Pro Ala Asp Leu Thr Ser Lys Thr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gnu</td>
<td>81</td>
<td>1.6</td>
<td>2.6</td>
<td>1.3</td>
</tr>
<tr>
<td>Cow</td>
<td>11</td>
<td>1.2</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Giraffe</td>
<td>21</td>
<td>1.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Whale</td>
<td>16</td>
<td>3.3</td>
<td>2.8</td>
<td>2.8</td>
</tr>
<tr>
<td>Horse</td>
<td>23</td>
<td>4.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

### Table 3

| Amino acid sequence differences between reindeer, red deer, fallow deer ribonuclease with titer differences obtained reaction with antisem to reindeer ribonuclease. | O-2-deoxy attached to this residue. |
Micro-complement fixation

In Table 2, the titer differences (see methods) and the amino acid differences are given. From these data, a relative order of antigenic reactivity could be deduced. The results are compared with the relative order obtained from the differences in amino acid sequence (see Table 1). In Table 3, the amino acid sequence differences between the ribonucleases from four deer species, reindeer, moose, fallow deer (Leijenaar-van den Berg and Beintema, 1975) and red deer (Zwiers et al., 1973; Welling et al., 1975b) are given with the titer differences observed by using antiserum directed against reindeer ribonuclease.

Discussion

Sequence-antigenicity correlation

The results from immunodiffusion and micro-complement fixation experiments coincide rather well (see Table 1). Evidently, complement fixation is more sensitive to small differences in primary structure. Employing antisera directed against cow, gnu and reindeer ribonuclease, a rather good correlation with the differences in sequence is obtained.

From the results with antiserum to whale ribonuclease it is obvious that a method which uses only one antiserum does not hold to predict sequence differences between homologous proteins. The ribonucleases, varying 18-26% in amino acid sequence from whale ribonuclease, show titer differences ranging from 4 - 128 (Table 2).

Reciprocal measurements were done for four species (cow, gnu, reindeer and whale). Expressing our data in 'immunological distances' as defined by Prager and Wilson (1971a) made possible a comparison between our results and those of others (Champion et al., 1975). In Fig.3 it is shown that our data fit fairly well to the line drawn by Champion et al. (1975). One point ( ), representing the reaction of dromedary ribonuclease with antiserum to whale ribonuclease, is rather off the straight line; this will be discussed below (see iv).

Fig.3. Dependence among pancreatic ribonucleases. Averages ( ), bacterial azo-synthetases ( ), those of other species ( ) and the straight line which is defined by the averages ( ).
...retation of antigenic differences

... part of the amino acid sequences of the 9 ribonucleases... Residues common to all sequences have been omitted.

...paring the differences in sequence and the titer difference (listed in Table 2), we attempted to localize anti-
...ently relevant regions. It was assumed that most of the

...3. Dependence of immunologic distance on percent sequence difference among pancreatic ribonucleases, bacterial azurins, bird lysozymes and a subunits of bacterial tryptophan synthetases. The expression immunologic distance (Prager and Wilson, 1973a) was used to compare our results with those of others. The immunologic distance between different ribonucleases is defined as 100x log titer difference (Table 2). The data for bacterial azurins, bird lysozymes and a subunits of bacterial tryptophan synthetases (O) are from Champion et al. (1975). Solid points represent averages of reciprocal tests.
Fig. 4. Amino acids at variant positions in the nine ribonucleases studied. One letter code is used: A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; Y, tyrosine. The numbering of cow ribonuclease is used.
acid substitutions fixed during evolution had only a
no effect on the main chain conformation of a series
ambiguous proteins (Champion et al., 1975).
reaction with antiserum to cow ribonuclease.

A difference in antigenic reactivity was found using cow and
ribonuclease, suggesting that Thr 3, Ala 19, Lys 37 and
are not part of an antigenic determinant. Reaction
ribonuclease, differing more from cow ribonuclease
f two positions (residues 50 and 99), results in a
difference of 1.8. This suggests that Ser 50 and/or
are part of antigenic determinants. Using the titer
reactions of giraffe and reindeer ribonuclease (2.6 and 3.2
respectively), it is possible to predict that these differen-
ted result from substitutions at positions 13, 15, 17,
34, 59, 70, 76, 78, 80, 89, 96, 98 and 120. The amino
chains in the 5 antigenic determinants of myoglobin
1975) generally are accessible to solvent molecules
Richards, 1971). If a side chain of an amino acid in
ogenic region is not accessible, then at least the im-
surroundings is accessible. In ribonuclease, the side
residues 13 and 120 together with their surroundings
ly inaccessible to solvent molecules (Lee and Richards,
ating that these residues are unimportant for
icity.
reaction with antiserum to gnu ribonuclease.
iter difference (1.2) was found for goat ribonuclease,
that the amino acids which it differs from gnu ribo-
(residues 50, 99 and 103) are not in an antigenic
cow ribonuclease shows a titer difference of 2.5,
ould be the result of amino acids which it differs
ribonuclease in addition to those it already differs
ribonuclease. This suggests that residues 3, 19 and/
part of an antigenic region. The same procedure can
ribonuclease (titer difference 2.6), indi-
that residues 13, 20, 31, 78, 89, 98 and 120 are in
ogenic reactive region. Residues 13 and 120 are less
be in such a region (see i). Reindeer ribonuclease,
ters only 6% in amino acid sequence from gnu ribo-
shows the relatively high titer difference of 4.0,
ould be caused by one or more of the residues 15, 17,
6, 78, 80 and 96.
iii) reaction with antiserum to reindeer ribonuclease.
The reaction of goat and gnu ribonuclease with antiserum to
reindeer ribonuclease, shows a relatively high difference
(titer difference 1.6 and 3.2, respectively) with regard to
the small differences in primary structure. This suggests
that residues 50, 99 or 103, in which they differ from each
other, may be part of an antigenic determinant. Moose ribo-
nuclease differs like fallow deer and red deer ribonuclease
at 6 positions from reindeer ribonuclease. However, it saw
a titer difference of 2.5 upon reaction with antiserum to
reindeer ribonuclease. This should be the result of amino
acids at positions different from the other three ribonu-
clases. From Table 3 it can be deduced that this concerns re-
dues 20 and 91, suggesting that one of these residues or
both are part of an antigenically important region. Residue
91 is most drastically changed in moose ribonuclease: free
positively charged lysine in the other deer ribonucleases.

iv) reaction with antiserum to whale ribonuclease.
As mentioned before, dromedary ribonuclease differs 26%
amino acid sequence from whale ribonuclease and shows a re-
tively low titer difference after reaction with antiserum to
whale ribonuclease (see Table 2). Also the immunodiffusi-
experiments reveal a remarkable similarity between drome-
ad and whale ribonuclease (see Fig. 2d). These results could
be explained by the presence of identical antigenic structure
in whale and dromedary ribonuclease. We have compared the
amino acid sequences (Fig. 4) to find short stretches of se-
quence which dromedary and whale ribonuclease both have in
common but different from all other ribonucleases: a) the
region containing amino acid residue Asn 22. However, this
region is believed to have a different conformation in the
enzymes. Subtilisin Carlsberg cleaves dromedary ribonuc-
lease in an external loop of the polypeptide chain between res-
e 19 and 20 (Welling et al., 1974b; Welling et al., 1975b
whereas whale ribonuclease is not cleaved. b) the region con-
taining amino acid residue Glu 52. Glutamic acid residue is
accessible to solvent molecules as has been calculated by
Lee and Richards (1971) and is surrounded by amino acid resi-
dues (Ser, Leu, Asp, Val) that have been found earlier as
antigenic reactive regions (Atassi, 1975). Experiments
to determine which part of the surroundings of resin also belongs to an antigenic reactive region.

It is interesting to note that in cow, reindeer and ribonuclease (see i, iii, and iv) apparently the same of the molecule is part of an antigenic reactive region 50 and 52 are rather close to each other, as can be

in a 3-dimensional model of cow ribonuclease S), whereas ribonuclease, serine 50 is substituted by a proline which probably is the reason why this part of gnu ribo

not in an antigenic region (see ii).

ence of carbohydrate chain

of the surface structure comprising antigenic determinants would be rendered inaccessible to antibodies by carbohydrate chains attached to ribonucleases. In seven out of the ribonucleases, carbohydrate is attached to residues at nos 21, 34, 62 or 76. Some of them, giraffe and pig

lease, are completely glycosidated. Nevertheless this marked influence on the relative order of reactivity ascribed with their relative difference in amino acid se-

(see Table 1A, B and C). For example, the completely glycosidated giraffe ribonuclease reacts with antiserum di-

to cow ribonuclease in a manner expected from its relative difference in amino acid sequence. This behaviour has been verified by using antisera to cow ribonuclease (con-
glycosidated and a non-glycosidated component) pro-
m ated rabbits and by using an antiserum to cow ribonuclease A (carbohydrate free). Only minor differences in relative order of reactivity were observed. For instance antiserum to cow ribonuclease A; dromedary

gnu > giraffe > reindeer > pig > horse

a second course antiserum directed to cow ribonuclease:

gnu > giraffe > reindeer > pig > dromedary > whale

clude that the presence of a carbohydrate chain in these ribonucleases is not of major importance for the antigenicity of these proteins.
In conclusion, ribonucleases from different animal species with different amino acid sequences have been used to determine which part of their molecular structure is important for their antigenicity.

Differences in sequences from 3-28% affect the antigenic reactivity, which could be used to elucidate the evolutionary relationship between pancreatic ribonucleases. A serious drawback of this approach when used with small proteins like ribonucleases with only a few antigenic regions, is that the extent to which particular amino acid replacements will affect the antigenic reactivity is not known in advance.

Acknowledgements

We thank Dr. F. Westendorp Boerma and Mrs. J. Schröder-Nijboer for their help in preparing antiserum to cow ribonuclease and Mr. J.S. Bouwer for his assistance in preparing the other antisera. We thank Drs. H.G. Seijen, A.C. Wilson and J. Drenth for helpful comments. Part of this work has been carried out under auspices of the Netherlands Foundation for Chemical Research (S.O.N.) and with financial aid from the Netherlands Organisation for the Advancement of Pure Research (Z.W.O.).

References

[References]

CHAPTER 8

ANTIGENIC REACTIVITY OF ARTIODACTYL pancreatic RIBONUCLEASES WITH ANTISERUM TO COW RIBONUCLEASE A

by Gjalt W. Welling & Gerda Groen

The antigenic structure of proteins is of interest if it is to be understood, which amino acids in a particular structure (conformation) elicit an immune response. Such studies were performed with a number of proteins, hemoglobin, cytochrome c, lysozyme and myoglobin (1-4). A prerequisite for such studies is the availability of both the primary and tertiary structure.

To date the primary structures of 18 pancreatic ribonucleases have been determined (5), and the tertiary structures of cow ribonuclease A (6,7) and ribonuclease S (8) are known. To get more insight in the importance of particular amino acids in the reaction of cow ribonuclease A with antibodies directed against it, we selected 6 ribonucleases differing up to 11% in amino acid sequence. It was assumed that

Table 1. STATE OF GLYCOSIDATION OF ARTIODACTYL RIBONUCLEASES (See ref.14)

<table>
<thead>
<tr>
<th>species</th>
<th>carbohydrate free component present</th>
<th>carbohydrate containing component present</th>
</tr>
</thead>
<tbody>
<tr>
<td>cow (Bos taurus)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>eland (Taurotragus oryx)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>gnu (Connochaetes taurinus)</td>
<td>+</td>
<td>n.d.</td>
</tr>
<tr>
<td>topi (Damaliscus korrigum)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>goat (Capra hircus)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>reindeer (Rangifer tarandus)</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

n.d., not determined. Cow ribonuclease A was from Schwarz/Mann (Orangeburg), Lot Y-1815; cow ribonuclease B was from Sigma Chem. Company (St. Louis), Type XII-B, Lot 55C-8253; eland ribonuclease A, topi ribonuclease B and goat ribonuclease A were isolated by ion exchange chromatography after sulfuric acid extraction and ammonium sulfate fractionation; goat, gnu and reindeer ribonuclease were isolated by affinity chromatography (15); goat ribonuclease contains the A and B component in a ratio of 8 : 2.
amino acid substitutions fixed during molecular evolution cannot alter the main chain conformation (9).

Since in an earlier study no difference was found between closely related ribonucleases using the immunodiffusion and complement fixation technique (10), it was decided to use the modified phage technique (11, 12) which showed itself to be more discriminative.

Antibodies to cow ribonuclease A were elicited in rabbits as described by Westendorp Boerma et al. (13) for hemoglobin. The second course antiserum of only one rabbit was used for the experiments. Glycosilated, unglycosilated ribonucleases or a mixture of both from cow, goat, eland, topi, gnu and reindeer (see Table 1) and a synthetic cow ribonuclease fragment (residues 1-14) were used to inhibit the inactivation of bacteriophage T4, to which cow ribonuclease A was coupled, the antiserum (11, 12). Increasing amounts of inhibitory ribonucleases were used. The results with 2 different ribonuclease A-phage preparations are shown in Fig. 1a and b. The antigenic reactivities could be interpreted by using the relevant sequence comparisons listed in Table 2. A small difference in antigenic reactivity was observed using cow ribonuclease B as inhibitor, suggesting that attachment of a carbohydrate chain consisting of at least 5 sugar residues (16) to asparagine 34 influences an antigenic reactive region. Goat ribonuclease A differing from the cow enzyme at positions 3, 19, 37 and 103 is less capable of inhibiting the reaction of antiserum to

Table 2

| Sequence Comparisons of Antiodactyl Ribonucleases |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Cow             | Thr             | Ser             | Ala             | Lys             | Leu             | Ser             | Thr            | Tyr             | Thr             | Ala             | Thr             | Lys             | Thr             | Ref.            |
| Goat            | Ser             | Thr             | Ala             | Lys             | Asn             | Lys             |
| Eland           | Ser             | Ser             | Thr             | Tyr             | Thr             | Ser             | Ala             | Thr             | Thr             | Lys             | Thr             | Lys             |
| Topi            | Ser             | Ser             | Thr             | Tyr             | Thr             | Ser             |
| Gnu             | Ser             | Ser             | Ala             | Pro             | Asn             | Ser             | Ala             | Thr             | Tyr             | Thr             | Thr             | Lys             | Lys             |
| Reindeer        | Ser             | Pro             | Pro             | Thr             | Ser             | Pro             | Asp             | Glu             | Ser             | Ala             | His             | Val             | Glu             |

* H.Kuper and J.J. Beintema, unpublished.
tion did not be- come a diffusion medium to use itself to grow rabbits in rabbits. Proglin used for the generation of antigenicity, by treatment of each ribonuclease A and antigenic fragment of topi ribonuclease A, indicating that at least one of the aforementioned amino acid residues plays a role in antigenicity. The antigenic reactivity of topi ribonuclease B is considerably lower, although it only differs from goat ribonuclease A in the carbohydrate chain at position 34 and a lysine instead of a glutamic acid residue at position 103. This points to a definite role for residue 103 in antigenicity. With eland ribonuclease A, the most indicative results are obtained comparing its reactivity with that of cow and goat ribonuclease. The substitutions of asparagine 34 by aspartic acid and of leucine 34 by methionine are definitely responsible for the decrease in antigenic reactivity. Comparing the amino acid sequence of gnu ribonuclease with that of topi B, it is obvious that the lower antigenic reactivity of gnu ribonuclease results from a substitution at position 50 and/or 99. As can be seen from Table 2, reindeer ribonuclease A differs at too many positions to allow an accurate determination of the residues that cause the relatively small reactivity of this ribonuclease. The synthetic ribonuclease fragment (prepared by J. Raap, K.E.T. Kerling and E. Havinga, Organic Chemistry Dept. Leiden, The Netherlands) comprising the first 14 amino acids of cow ribonuclease did not inhibit the phage inactivation using a molar excess up to $10^4$. This suggests that the first 14 amino acid residues are not part of an antigenic determinant.

From the foregoing results it can be concluded that residues 34, 35, 103, 50 and/or 99 and maybe residues 19 and 37 are part of antigenic reactive regions in pancreatic ribonuclease A. Chemical modification studies of cow ribonuclease are in progress to get information on the importance of other residues for antigenicity.

We thank B.Zantinge for a sample of bacteriophage T4 and S.Bron and E.Luxen for their aid in phage isolation. We are indebted to J.Bouwer for his assistance in preparing the antiserum. We also thank J.J.beintema and J.Drenth for helpful comments. Part of this work has been carried out under auspices of the Netherlands Foundation for Chemical Research (S.O.N.) and with financial aid from the Netherlands Organisation for the Advancement of Pure Research (Z.W.O.).
Fig. 1a and b. The inactivation of a cow ribonuclease A - bacteriophage T4 conjugate by antiserum to cow ribonuclease A. Aliquots of 50 µl of increasing amounts of inhibitors (homologous ribonucleases) were incubated for 2 hr at 37°C with 50 µl of antiserum (4.4 x 10^4 and 1.6 x 10^4 times diluted for phage preparation I and II, respectively). Subsequently, the mixture was incubated for 2 hr at 37°C with 50 µl of a suitable dilution of conjugated phage I (Fig. 1a) or conjugated phage II (Fig. 1b) and plated out using the 'direct plating method' (11). Inhibitor concentrations were determined by amino acid analysis. ▲, cow ribonuclease A; ○, cow ribonuclease B; △, goat ribonuclease A; ◇, gnu ribonuclease A; ■, reindeer ribonuclease A; ▽, goat ribonuclease B; ▼, topi-ribonuclease B.

References


Groen, G. Perlett. 60, 311

Leijenaar-F FEBS Lett.
References


CHAPTER 9

ANTIGENIC STRUCTURE OF COW RIBONUCLEASE A

Present state of investigation

The experimental approaches that can be followed to elucidate features of antigenic determinants in proteins are the following:

1) Chemical modification
   Particular side-chains of amino acids can be modified by using one of the numerous methods available in the literature (1,2).

2) Interspecies cross-reaction
   This approach can be used if a homologous group of proteins with known amino acid sequence is available.

3) Intraspecies cross-reaction
   If there is an amount of genetic variability in a species, for instance in immunoglobulins, this approach can be used.

4) Cleavage of peptide bonds at particular positions and generation of peptide fragments
   Fragments are presumed to exist in conformational equilibrium between various disordered or random conformations (P_r), and the native conformation (P_n) (ref.3),
   \[ P_r \leftrightarrow P_n \]
   For those conformational equilibria for which the equation lies far to the left, \[ P_n \] is only sufficiently high to be measured by reaction with antiserum to the native protein if high concentrations of fragments \[ P_r \] are used.

All of these approaches have been used. Approach 2 has been used in chapter 7 and 8. In this chapter, preliminary evidence is presented utilizing approaches 1) and 4):

a) The antigenicity of native ribonucleases of which particular arginine, lysine and tyrosine are modified, was determined.

* This research was carried out in cooperation with Feitse van Duisterwinkel, Leendert van Geldermalsen, Gerda Groen, Albert Scholma and Ruurd van der Zee.
b) Oxidized cow ribonuclease A was allowed to inhibit the reaction of native cow ribonuclease A with antibodies against it.

c) The antigenicity of cow S-protein (residues 21-124) obtained by cleavage of native ribonuclease with subtilisin (4) was studied.

d) Two random copolymers, poly(Asp, Leu, Ala) and poly(Asp, Leu, Glu) related to residues 51, 52 and 53 in cow and dromedary ribonuclease, respectively, were used to inhibit the phage inactivation.

Finally, preliminary conclusions from the results in chapters 7, 8 and 9 are presented.

Materials and methods

Cow ribonuclease A was from Schwarz/Mann (Orangeburg) Lot Y-1816. Cow ribonuclease (A + B) was a product from Miles (Cape Town) batch 228. Cow ribonuclease oxidized Type XII-AC Lot 114C-8320, cow ribonuclease S-protein, Lot 19B-8170 and human serum albumin, Lot 65C-8320 were obtained from Sigma Chem. Company (St. Louis). Chymotrypsin A and chymotrypsinogen A were from Boehringer (Mannheim). Thermolysin, A grade, Lot 100939 was from Calbiochem (San Diego). Pig trypsin, Lot 8, was from Miles (Stokes Poges). γ-benzyl-L-glutamate-N.C.A. (N-carboxyanhydride) was prepared from γ-benzylglutamate (Merck, Darmstadt) as described by Katchalski and Berger (5). L-alanine-N.C.A., β-benzyl-L-aspartate-N.C.A. and L-leucine-N.C.A. were from Miles-Yeda Ltd. (Rehovot). Tetranitromethane and 1,2-cyclohexanenedione were obtained from Fluka A.G. (Buchs). Hydrogenbromide solution in glacial acetic acid (45% w/v HBr) was a product from BDH (Poole). Ampholines were obtained from LKB (Bromma). Carboxymethylcellulose CM-32 was a product from W.R. Balston Ltd. (Maidstone). CNBr-activated Sepharose-4B was from Pharmacia (Uppsala). All other reagents were analytical grade products from Merck (Darmstadt).

Arginines were modified to N', N''- (1, 2- dihydroxy- cyclohex-1,2-ylene)-L-arginine as described by Patty and Smith (6). Lysine residues were modified by alanylation as described by Anfinsen et al. (7) by reacting 100 mg of cow ribonuclease A with 20 ml 0.05 M phosphate buffer pH 6.8 with 100 mg of L-alanine-N.C.A. in 10 ml dioxane for 72 hr at 4°C. Tyrosine side chains were nitratated with tetranitromethane as described by Sokolin et al. (8).
It was found to inhibit the release with antibodies residues 21-124) release with sub-
not inhibit with sub-
not inhibit with sub-
the results in

arginine modification

Cow ribonuclease was modified to a different extent and modified ribonucleases were purified by ion exchange chromatography on carboxymethylcellulose CM-32 (9,10) by the small scale preparative thin layer isoelectric focusing method of Radola (11). Activity of ribonucleases was determined as described (12,13). Concentrations of ribonucleases were determined by amino acid analysis (14). Preparation of fingerprints (gel filtration and paper electrophoresis), digestion with proteolytic enzymes and performic acid oxidation were carried out as described (14). Random copolymers of poly (Asp, Leu, Ala) and poly (Asp, Leu, Glu) were prepared as described by Sela et al. (15). Antiserum against cow ribonuclease was prepared as described (16,17). Inhibition of the inactivation of bacteriophage T4 to which cow ribonuclease A was coupled was performed as described in chapter 8.

Results

Arginine modification

Cow ribonuclease was allowed to react with 1,2-cyclohexanone (6). The resulting product was subjected to ion exchange chromatography on carboxymethylcellulose. A fraction which did not correspond to the elution position of cow ribonuclease with a relative activity of 67% was isolated. Amino acid analysis and fingerprint analysis after tryptic digestion of the oxidized protein showed that arginine 85 and 39 were modified more than 90%, while arginine 10 was modified for about 50%. No reaction was observed with arginine 33. The results from inhibition of the phage inactivation are shown in Fig.1. Tyrosine modification

Cow ribonuclease that was allowed to react with the N-carboxy-
thymydrate of L-alanine (L-alanine-N.C.A.) was purified on carboxymethylcellulose as described by Taborsky (10). Amino acid analysis of the homogeneous peak, showed that about 28 alanine residues were attached to the protein. From a fingerprint of the performic acid oxidized protein after tryptic digestion, it turned out that lysines 1, 61 and 98 were modified. The results from the inhibition of the phage inactivation are shown in Fig.1. Tyrosine modification

Cow ribonuclease was nitrated with tetranitromethane (8). With
preparative isoelectric focusing (11), one derivative was obtained in pure form. After performic acid oxidation, the modified protein was successively digested with chymotrypsin and thermolysin. After fingerprint analysis and dipping of the paper electropherogram in 10% ammonia in aceton, 2 peptides turned yellow. Analysis of those peptides showed tyrosines 76 and 115 to be nitrated. The results from the inhibition of the phage inactivation are shown in Fig. 1.

![Inhibition of the inactivation of cow ribonuclease A by bacteriophage T4 by antiserum to cow ribonuclease A. As inhibitors were used: ▲, cow ribonuclease A, and modified cow ribonucleases; △, arginines modified; ●, lysines modified; ○, tyrosines modified. The assay was performed as described in chapter 8.](image)

Perfominc acid oxidation

The inhibition of the inactivation of conjugated phage by commercially available oxidized cow ribonuclease A is shown in Fig. 2. To assure that no traces of native protein were left, the protein was oxidized again (14) and the inhibitory capacity of the product was measured (See Fig. 2).

Fragmentation

Commercially available cow ribonuclease S-protein, showing considerable ribonuclease activity, was purified using affinity chromatography on Sepharose 4-B to which S-peptide (residues 1-20) was coupled. The starting buffer was 0.001 M
Inhibitor concentration (nmol/ml)

% inhibition

Fig. 2. Inhibition of the inactivation of cow ribonuclease A - bacteriophage T4 by antiserum to cow ribonuclease A. As inhibitors were used: once, ■ and twice, ○ performic acid oxidized cow ribonuclease A. The assay was performed as described in chapter 8.

Tris-HCl, 0.1 M KCl, pH 7.5. The S-protein was eluted with 0.05 M HCl. The capacity of both preparations to inhibit the enzyme inactivation, is shown in Fig. 3. The specific enzymic activity of purified S-protein was 0.17%.

Fig. 3. Inhibition of the inactivation of cow ribonuclease A - bacteriophage T4 by antiserum to cow ribonuclease A. As inhibitors were used: ▲ commercial cow S-protein; △ commercial S-protein purified by affinity chromatography. The assay was performed as described in chapter 8.
Random copolymers

Random copolymers of poly (Asp, Leu, Ala) in a ratio of 1.0:0.9:1.1 and of poly (Asp, Leu, Glu) in a ratio of 1.0:1.0:0.9 were used as inhibitors of the phage inactivation. Poly (Asp, Leu, Ala) showed 45% inhibition at a concentration of 10 mg/ml, while poly (Asp, Leu, Glu) showed no inhibition at this concentration.

Discussion

From Fig. 1 it can be concluded that only modification of lysines has any effect on the antigenicity of cow ribonuclease. The results indicate that the alanylated ribonuclease resembles the PAR (polyalanylribonuclease) 1 and PAR 2, prepared by Anfinsen et al. (15) despite the fact that we used a relative larger amount of L-alanine-N.C.A. As a result of this the average number of alanines per attached chain is higher, about 9 instead of 4. With ribonucleases alanylated to a higher degree, it was determined that lysines 7, 37 and 41 were resistant to reaction with the L-alanine-N.C.A. (18). Our results are consistent with this finding and it turned out that only lysines 1, 61 and 98 were modified. The decrease in antigenicity should be accounted for by one or more of these residues. Modification of Arg 10, Arg 39, Arg 85, Tyr 76 and Tyr 115 did not affect the antigenicity, which means that they are not in an antigenic reactive region.

Antibodies to globular proteins cross-react very poorly if at all with the denatured protein. The reasons for this are that upon denaturation the polypeptide chain is folded in a different way. Residues which are brought together from distant portions in the native molecule are no longer close to each other. Furthermore, hydrogen bonds, salt bridges and hydrophobic interactions present in the native molecule no longer exist. Oxidation of cow ribonuclease with performic acid results in disruption of the disulfide bridges, conversion of cysteine to cysteic acid and of methionine to methioninesulfone. Brown et al. (19) did not observe any inhibition by oxidized cow ribonuclease of the reaction between native cow ribonuclease and antisera against the native enzyme. In one experiment even a 72,000 fold excess was ineffective. This suggests that also smaller fragments of the oxidized protein would not react with antiserum to the native protein.
protein. In contrast with this, later experiments with the oxidized enzyme and antiserum to native ribonuclease by Brown et al. (20) showed 16% cross-reaction by complement fixation and 1% by precipitation. With the phage inactivation assay, we observed 50% inhibition using a 400,000 fold excess. The small difference in inhibitory capacity between the once and twice oxidized preparations probably means that the former hardly contains any residual native ribonuclease.

The inhibitory capacity of smaller fragments of denatured ribonuclease may be determined now and compared with that of the whole denatured protein.

Several authors have shown that S-protein (residues 21-124) is different from the native enzyme in its antigenic behaviour (21, 22). From these results it was concluded that the S-peptide (residues 1-20) contains at least part of the antigenic determinants (23). In contrast with this, Singer and Richards (21) concluded the opposite from the fact that S-peptide does not inhibit the precipitation of ribonuclease A by antibody. S-protein is difficult to purify and often contains native ribonuclease. Probably the best way to purify S-protein is to utilize its very strong interaction with S-peptide (see chapter 5). An affinity adsorbent of S-peptide coupled to Sepharose was used for purification. The specific activity of the purified S-protein was 0.17% using RNA as substrate. This activity is probably intrinsic. Gutte observed a specific activity of 0.10% with RNA as substrate for purified natural S-protein and for a synthetic 70 residues S-protein (24). With purified S-protein higher concentrations (see Fig. 3) were needed, compared with the commercial product, to inhibit the inactivation of modified phage. Eventually 100% inhibition was reached, suggesting that all antigenic regions were present in the S-protein. The inhibition profile of the two S-protein preparations is different from those of complete ribonucleases. This may be the result of partial denaturation. Detectable thermal denaturation starts at 20-25°C (25, 26). Another reason may be that S-protein tends to polymerize. The sedimentation behaviour of S-protein in the neutral pH range clearly indicates polymerization (27).

One of the sequences present in the random copolymer poly (Asp, Leu, Ala) is Leu-Ala-Asp, also present in cow ribonuclease (residues 51-53). This copolymer showed appreciable inhibitory capacity in phage inactivation, in contrast to the
copolymers that were related to the sequence Leu-Glu-Asp in dromedary ribonuclease (residues 51-53). Fig. 4 shows the relative orientation of residues 50-54 in cow ribonuclease. It is obvious that substitution of alanine 52 by glutamic acid could seriously interfere with the binding to an antibody combining site.

Fig. 4. Computer-drawn stereo picture of a part of cow-ribonuclease S (31) comprising residues 50-53.

From the results in chapters 7, 8 and 9 some preliminary conclusions can be drawn concerning the antigenicity of cow ribonuclease. However, it must be borne in mind that the results concern the antigenic structure of a protein from a particular mammalian species to which antiserum was prepared in another mammalian species. The antigenicity of cow ribonuclease determined with antiserum prepared in other species is probably different.

In chapter 7, antiseras directed against ribonucleases from 4 different species have been used. The antiserum against cow ribonuclease was directed to the mixture of glycosidated and unglycosidated components of which commercial unfractinated cow ribonuclease consists. In immunodiffusion tests, only small differences in the relative order of reactivity were found using either this antiserum or an antiserum directed to cow ribonuclease A. Probably because of the high sensi-
A schematic diagram showing the folding of the cow ribonuclease backbone. The solid black portions are certainly in an antigenic reactive region; these parts of the molecule are less certain to be in an antigenic region (see text); these parts are certainly not in an antigenic region.

A finite difference in antigenicity was found for the reaction of cow ribonuclease A and B with antiserum to cow ribonuclease A (chapter 8). The same holds for residues 3, 19, 37, and 103, which are substituted in goat ribonuclease. No difference in antigenicity was found between goat and cow ribonuclease by immunodiffusion and complement fixation (chapter 8). On the other hand, the antigenic difference in the phage inactivation assay was very obvious.
In chapter 8 and 9, only one antiserum directed against one component (cow ribonuclease A) was used and this time (chapter 8) only very related ribonucleases were compared, allowing a more definite assignment of antigenic important residues. The inhibition of phage inactivation proved to be very sensitive to small differences and from the results presented in chapters 8 and 9, a preliminary picture of the antigenic important regions emerges (see Fig. 5). At least three antigenic regions are present, containing 1) residues 34, 35 and 37; 2) residues 50-53; 3) residues 96, 99 and 103. Besides this, residue 61 could play a role. Residues 34, 35, 51, 52, 53 and 103 are most certain to be in an antigenic reactive region. To get a definite answer, more studies have to be done utilizing fragments prepared either by proteolytic cleavage or by chemical synthesis.

Antigenic determinants of different native proteins may show similar features. The intriguing possibility exists that particular native proteins react with antiserum directed against other proteins. A first attempt has been made using antiserum to whale ribonuclease. Whale ribonuclease probably contains an antigenic region in the immediate surroundings of Glu 52 (amino acid sequence, Leu-Glu-Asp). Two potential inhibitors of the inactivation of whale ribonuclease-phage were tested, human serum albumin containing a sequence Leu-Asp-Glu (28) and chymotrypsinogen containing no such region (29,30). Human serum albumin inhibited the phage inactivation for 45% at a concentration of 10 mg/ml, whereas chymotrypsinogen did not. This suggests that the aforementioned possibility exists.

Acknowledgements

We thank A. Talsma and R.F. Heldeweg (Organic Chem. Dept., Groningen) for preparing the γ-benzylglutamate-N.C.A.; S.K. Oosterloo for preparing the S-peptide-Sepharose column; J. Hofsteenge for performing the activity measurements; D. Mug-Opstelten and H. Hooge for carrying out preliminary nitration experiments; C. Zeestraten for providing us with the computer-drawn stereo picture, and J. Drenth and J.J. Beintema for helpful discussions. Part of this work has been carried out under auspices of the Netherlands Foundation for Chemical Research (S.O.N.) and with financial aid from the Netherlands Organisation for the Advancement of pure Research (Z.W.O.).

References

1) Glazer, A.N. Chemical mod. in biochem. Work, E. eds
3) Sachs, D.H., Richards, F.
4) 234, 1459-14
5) vol.3, 546-554
6) Patty, L., 565-569.
7) Anfinsen, C.
8) Sokolovsky, Biochemistry
9) Movist, S.E
234, 1112-1
10) Taborsky, G
11) Radola, B.J
12) Shapira, R.
13) Campagne, R.
Acta, 55, 3
14) Welling, G.
Biochem.J.
15) Sela, M., 85, 223-223
16) Westendorp, and Huisman
17) Chapter 7.
18) Cooke, J.P.
Chem. 238,
19) Brown, R.K.
(1959) J.B.
20) Brown, R.K.
J. (1967)
21) Singer, S.
References


17) Chapter 7.


SAMENVATTING IN DIT PROEFSCHRIFT

In dit proefschrift is het enzym dat vooral van herkauwers afkomstig is opgenomen. Het enzym dat vooral van herkauwers afkomstig is, heet RNase (RNasen) en is opgebouwd uit een aantal (aminozuren), die met elkaar koppeld zijn. Een eiwit is dan een samengesteld eiwit, waarin de aminozuren gerold zijn tot een doorgaans lineaire betekenis. RNase (RNasen) van koe, wilde en mens vrijwel over het algemeen vrijwel over het algemeen veranderen. Slechts een kameel en een koei blijken voor de RNase (RNasen) van koe, wilde en mens vrijwel over het algemeen vrijwel over het algemeen te verschillen. Een kameel is een kameel en een koei mogelijk als de kameel niet van grond wordt, dan wordt nu zo'n veelrode en daarna worden brokstukken "gehaald" hebben. Die brokstukken worden op grond (b.v. op grond van een zuiver) de stof en een andere stof aan het eind van het onderzoek.
SAMENVATTING IN OMGANGSTAAL

In dit proefschrift worden de eigenschappen behandeld van een enzym dat vooral veel voorkomt in de pancreas (alvleesklier) van herbouwers. Enzymen zijn eiwitten, die een bepaalde werking hebben (ze kunnen b.v. andere eiwitten splitsen) en ze zijn opgebouwd uit van elkaar verschillende bouwstenen (aminozuren), die in een bepaalde volgorde aan elkaar gekoppeld zijn. Er bestaan 20 verschillende aminozuren. Het eiwit is dan een lange keten van aminozuren, die meestal opgerold is tot een soort klouwen (ruimtelijke structuur).

Het eiwit waar het hier om gaat, heet ribonuclease (afgekort: RNase) en is opgebouwd uit ongeveer 124 aminozuren. RNase komt in een aantal verschillende diersoorten voor en het blijkt dat dit eiwit net als andere kenmerken van dieren, kan veranderen tijdens de evolutie. Zo blijkt b.v. dat de RNasen van koe, geit en kameel van elkaar verschillen in de volgorde van hun aminozuren. De RNasen van koe en geit hebben vrijwel op alle plaatsen van de keten dezelfde aminozuren. Slechts op 4 plaatsen hebben ze verschillende aminozuren. Het RNase van kameel verschilt veel meer van de vorige twee. Kamele RNase heeft op 28 plaatsen in de keten een ander aminozuur dan de RNasen van koe en geit. Dit klopt met hetgeen op grond van zoölogische gegevens van deze dieren zou verwacht. Een koe en een geit zijn meer met elkaar verwant dan een kameel en een koe. Een dergelijke vergelijking is alleen mogelijk als de volgorde van de aminozuren in de keten bepaald wordt, dus de volgorde van de ca. 124 bouwstenen. Hoe wordt nu zo'n volgorde bepaald. Het RNase wordt eerst ontrold en daarna met een eiwit-splitsend enzym in een aantal brokstukken "geknipt", waarvan we de grootte niet in de hand hebben. Die brokstukken kunnen we apart in handen krijgen door ze op grond van hun eigenschappen van elkaar te scheiden (bijv. op grond van hun elektrische lading of hun grootte).

Van een zuiver brokstuk wordt nu met een bepaalde techniek steeds van het eind van de keten één aminozuur afgehaald en met een andere techniek wordt dan bepaald welk aminozuur nu aan het eind van de keten zit. Er zijn machines die dit automatisch kunnen doen, ook met een complete keten, maar meestal
niet verder dan 30-40 aminozuren. Zonder het maken van brokstukken is de volgorde van de aminozuren dus niet op te lossen.

We weten nu de volgorde van de aminozuren in de brokstukken, maar nog niet hoe, in welke volgorde, de brokstukken aan elkaar behoren te zitten. Daarom wordt het hele RNase nog eens behandeld met een ander eiwit-splitsend enzym, dat het RNase op andere plaatsen splitst. Van deze brokstukken wordt ook de volgorde van de aminozuren bepaald. Met al deze gegevens over brokstukken, die elkaar in volgorde overlappen (zie tekening) kan nu de volgorde van het hele RNase afgeleid worden.

met eiwit-splitsend enzym 1

\[ 1 2 3 4 \{5 \ 6 \ 7 \} 8 9 10 11 \text{ etc.} \]

met eiwit-splitsend enzym 2

\[ 1 2 \{3 \ 4 \ 5 \ 6 \} 7 8 9 10 11 \text{ etc.} \]

De opheldering van de aminozuurvolgorde van dromedaris- en kamele-RNase is beschreven in hoofdstuk 2 en 3. Factoren, die een rol spelen bij de evolutie van RNases worden besproken in hoofdstuk 6.

Het eiwit is dus een keten van aminozuren, die opgerold is tot een kluit. Sommige delen van die keten steken iets meer naar buiten dan andere en bij RNase uit koe is het mogelijk met een eiwit-splitsend enzym uit een bacterie, de aminozuurketen op één plaats tussen aminozuur 20 en 21 door te "knippen". De eiwitkluit blijft verder één geheel en het RNase werkt nog net als voorheen. Onder bepaalde omstandigheden zijn de 2 in elkaar zittende brokstukken echter van elkaar te scheiden. We hebben dan een stuk van 20 aminozuren en een stuk van 104 aminozuren en geen van beide kan meer als RNase werken. Komen ze weer bij elkaar, dan keert ook de RNase-werking weer terug. In hoofdstuk 4 en 5 is nu beschreven hoe doorknippen op één plaats van een lus in de aminozuurketen van RNases uit verschillende diersoorten. De kleine stukken, die een verschillende aminozuurvolgorde hebben, zijn toegevoegd aan de grote stukken uit koe en dromedaris. Op die manier en onderzocht de aminozuurvolgordes van dromedaris- en koe-RNase.
manier zijn nieuwe RNAsen ontstaan; b.v. kangoeroe-koe en koe-dromedaris. De enzym-werking van deze combinaties is onderzocht en het verbazingwekkende was nu dat de combinatie-RNasen even goed werkten als de oorspronkelijke RNasen.

Omdat we veel van RNase uit koe weten, is het zeer geschikt als model-stof. Het RNase van koe kan als lichaamsvreemde stof dienen bij b.v. een konijn. Tegen een lichaamsvreemde stof kunnen antilichamen gemaakt worden. Deze antilichamen zijn evenals RNasen ook eiwitten en ze passen op de lichaamsvreemde stof. Wat zijn nu de gedeelten van een lichaamsvreemde stof, die veroorzaken dat er antilichamen gemaakt worden en die kunnen reageren met de antilichamen?

We hebben bij konijnen antilichamen opgewekt tegen RNase uit koe en de antilichamen zijn gebruikt voor het onderzoek beschreven in hoofdstuk 7, 8 en 9. We hebben b.v. een bepaald aminozuur in RNase op chemische wijze groter gemaakt. De antilichamen, die op de onveranderde RNase pasten, passen nu misschien niet meer (zie tekening). Als dat zo is, dan zit dit aminozuur op een plaats in de ruimtelijke structuur (kluwen) die belangrijk is voor het opwekken van en reageren met antilichamen.

![Diagram]

Dit onderzoek, wat nog niet is afgerond, kan interessante gegevens opleveren over het hoe en waarom van de reactie lichaamsvreemde stof en antilichamen.