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Glucose transport in the extremely thermoacidophilic *Sulfolobus solfataricus* involves a high-affinity membrane-integrated binding protein

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Summary

The archaeon *Sulfolobus solfataricus* grows optimally at a temperature of 80°C and a pH of 2.5-3.5 on carbon sources such as yeast extracts, trypton and various sugars. Cells rapidly accumulate glucose. This transport activity involves a membrane-bound glucose-binding protein that interacts with its substrate with very high affinity (K_d of 0.43 µM) and retains high glucose affinity at very low pH values (up to pH 0.6). The binding protein was extracted with detergent, and purified to homogeneity as a 65-kDa glycoprotein. The gene coding for the binding protein was identified in the *S. solfataricus* P2 genome by means of the amino-terminal amino acid sequence of the purified protein. Sequence analysis suggests the protein to be anchored to the membrane via an amino terminal transmembrane segment. Neighbouring genes encode two membrane proteins and an ATP-binding subunit that are transcribed in the reverse direction, whereas a homologous gene cluster in *Pyrococcus horikoshii* OT3 was found to be organised in an operon. These data indicate that *S. solfataricus* utilizes a binding-protein-dependent ATP-binding cassette (ABC) transporter for the uptake of glucose.

Introduction

Glucose is one of the most important carbon sources for many living organisms. It is mostly metabolized via a mechanism that involves catabolite repression. Such a “glucose effect” has also been described for the extreme thermoacidophilic archaeon *Sulfolobus solfataricus* (Haseltine et al., 1996). The pathways of glucose metabolism in this archaeon have been well-characterised (Schönheit and Schäfer, 1995). However, little is known about the mechanism by which glucose is taken up by *S. solfataricus*. In bacteria, glucose transport occurs often via the phosphoenolpyruvate-dependent sugar phosphotransferase transport system (PTS). In Archaea, a PTS most likely is absent, since the available archaeal genome sequences do not give any indications for the presence of such systems (Sensen et al., 1998). An alternative way for uptake of glucose is ion-symport. In bacteria mostly H^+/glucose symporters are found, whereas in eukaryotes Na^+/glucose symporters are more common. Most of the bacterial sugar symporters belong to a well-characterised family, the major facilitator superfamily (MFS) (Pao et al., 1998). A third mechanism described for sugar uptake is via a binding-protein dependent ATP-binding cassette (ABC) transporter. A well-studied ABC transporter is the maltose transport system of *E. coli* (Boos and Shuman, 1998). It comprises a binding protein in the periplasm, two inner membrane components and two identical domains, which catalyze the hydrolysis of ATP, the energy source for this transport system.
A binding-protein-dependent ABC-transporter for trehalose/maltose has been described for the archaeon *Thermococcus litoralis* (Horlacher et al., 1998). This system is equipped with a binding protein that has an exceptionally high substrate binding affinity (Xavier et al., 1996). We have analysed the mechanism of glucose transport in the extremely thermoacidophilic *S. solfataricus*. Our data indicate that glucose is taken up via a high affinity binding-protein-dependent ABC-transporter. The binding protein is a secreted glycoprotein that is anchored to the cytoplasmic membrane by a membrane-spanning domain and that shows a very low pH optimum for glucose binding.

**Results**

**Sugar transport in *S. solfataricus* cells**

*Sulfolobus solfataricus* can grow on various sugars as sole carbon and energy source (Grogan, 1989). Uptake of glucose, galactose, fructose, sucrose and maltose occurs at a high rate. Glucose is transported most rapidly and was therefore studied in greater detail. The rate of glucose uptake is steeply dependent on the external pH (Fig. 1). The uptake rate is highest at pH 3.0, strongly decreased above pH 4.0, and is no longer detectable at pH values of 5.0 and above. At higher pH values, the ΔpH, and also the internal pool of ATP, rapidly diminishes (Moll and Schäfer, 1988) causing a transient glucose uptake or no uptake at all. The apparent Kₘ for glucose uptake was found to be 1.9 µM at pH 3.0 and 60°C with a Vₘₐₓ of 0.9 nmol min⁻¹ (mg protein)⁻¹. A 10-fold excess of galactose and mannose significantly inhibits uptake of glucose, while 2-deoxyglucose only marginally affected uptake (Fig. 2A). A 100-fold excess of 2-deoxyglucose decreased the glucose uptake only by 50%. Fructose, sucrose and maltose had no effect on glucose uptake (data not shown). These data suggest that *S. solfataricus* cells take up glucose and most likely also galactose and mannose in an energy-dependent manner by means the same high affinity transport system.

**Isolated membranes of *S. solfataricus* bind glucose with high affinity**

To further characterise glucose transport in *S. solfataricus*, membrane vesicles are the preferred model system for uptake studies as they are devoid of substrate metabolising activities and the energy-supply for transport across the membranes of these vesicles can be well controlled. However, attempts to construct closed membrane vesicles from *S. solfataricus* have so far been unsuccessful, partly due to the difficulty to remove the membrane-anchored S-layer. Membranes derived by French Press treatment of *Sulfolobus* cells form vesicle-like structures, which are, however, leaky for protons and small ions and thus are unable to maintain a proton-motive force. These membranes show a distinct glucose-binding activity, which was strongly pH dependent (Fig. 3A) with an optimum at pH 1.5. At this pH value, binding occurred most rapidly.

Glucose binding in membranes could not be inhibited by the addition of the ionophores valinomycin and nigericin or was it affected by the presence of the detergent Triton X-100. Moreover, in a total membrane protein extract...
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obtained after detergent solubilization, the same pH-dependency of glucose binding was observed as for the intact membranes (data not shown). Therefore, it is concluded that the *S. solfataricus* membranes harbour a glucose-binding activity.

**Glucose binding is mediated by a membrane-bound 65-kDa glycoprotein**

To identify the glucose-binding protein, the protein was purified to homogeneity from *S. solfataricus* membranes using the binding activity to monitor the purification. The activity appeared to be tightly associated with the membranes as it resists treatment of the membranes with chaotropes like urea or Na₂CO₃, pH 10. Extraction of the protein from the membrane required a high concentration of the detergent Triton X-100 (2 %). Initially, the protein was further purified by FPLC Mono Q chromatography using the glucose-binding assay to monitor the activity. Activity appeared to be related to a 65-kDa polypeptide in SDS-PAGE (Fig. 4A). A glycoprotein specific stain indicated that this binding protein is glycosylated (Fig. 4B). This enabled a larger scale purification by means of Con A sepharose affinity chromatography.

Con A sepharose binds specifically the α-glucosyl- and α-mannosyl side chains of glycoproteins. By increasing the concentration of methyl-α-D-mannopyranoside, 5 to 7 glycoproteins could be eluted from the Con A sepharose column after loading with a total Triton X-100 extract of *S. solfataricus* membranes. The protein was subsequently purified to homogeneity with FPLC Mono Q. The purified 65-kDa protein retained the ability to bind [¹⁴C]glucose on a native PAGE-gel (Fig. 4C). Moreover, the same activity-staining technique reveals the presence of a single band in the total membrane extracts that corresponds to the purified glucose-binding protein (Fig. 4C).

Fig. 2. Glucose uptake by *S. solfataricus* cells (A) and binding of glucose to purified GBP (B) in the presence of competing substrates. Uptake and binding assays were performed at 60°C and 1 µM [¹⁴C]glucose in the presence of the indicated concentration of mannose (black), 2-deoxyglucose (grey), and galactose (white). Cells were preincubated 30 s with non-labelled sugars in medium pH 2.5 without carbon source and uptake was stopped after 10 s. GBP was preincubated for 1 min with non-labelled sugars in buffer pH 2 and the binding reaction was stopped after 2 min.

![Fig. 2](image)

Fig. 3. pH-dependence of glucose binding to *S. solfataricus* membranes (A) and purified GBP (B). Binding assays were performed at 60°C in the presence of 1 µM [¹⁴C]glucose. The buffers used for the different pH values were 250 mM HCl (pH 0.6) (○[only in panel B]), 50 mM HCl (pH 1.5) (●), 50 mM glycine HCl (pH 2 [□] and 3 [■]), and 50 mM citric acid NaOH (pH 4 [▲] and 5 [▲]).

Fig. 3. pH-dependence of glucose binding to *S. solfataricus* membranes (A) and purified GBP (B). Binding assays were performed at 60°C in the presence of 1 µM [¹⁴C]glucose. The buffers used for the different pH values were 250 mM HCl (pH 0.6) (○[only in panel B]), 50 mM HCl (pH 1.5) (●), 50 mM glycine HCl (pH 2 [□] and 3 [■]), and 50 mM citric acid NaOH (pH 4 [▲] and 5 [▲]).
strongly inhibited by galactose and mannose, but, not by 2-deoxyglucose (Fig. 2B). We were unable to detect any significant level of $[^3]$H 2-deoxyglucose binding to the membrane vesicles or purified binding protein (data not shown). Taken together, these data demonstrate that glucose binding by \textit{S. solfataricus} cells is mediated by a membrane-bound glycoprotein with an apparent molecular mass of 65-kDa.

**Genetic characterisation of glucose-binding protein**

To identify the gene coding for the glucose-binding protein, the amino-terminal amino acid sequence of the purified protein was determined. A stretch of 31 amino acid residues could unequivocally be determined (Fig. 5A), and this sequence was used to design two degenerated primers to amplify part of the gene by PCR. The

*Fig. 4.* Purification of the glucose-binding protein from \textit{S. solfataricus} membranes. Coomassie brilliant blue (A) and glycoprotein (B) staining of SDS-PAGE of Triton X-100 solubilized membrane proteins (lane 1), glycoprotein fraction eluting from the ConA Sepharose column (lane 2), and the purified binding protein after FPLC MonoQ (lane 3). Positions of the molecular mass standards are indicated. (C) $[^1]$C glucose staining of native gel electrophoresis of purified glucose binding protein (lane 1) and solubilized membrane proteins (lane 2).

*Fig. 5.* Amino acid sequence of the glucose-binding protein. (A) Sequence of the amino-terminus of the purified protein. Also indicated are the nucleotide sequences of the PCR primers used to clone the gene encoding GBP. An asterisk indicates differences in the nucleotide sequence of \textit{S. solfataricus} P1 and P2. (B) Complete amino acid sequence of the binding protein derived from the nucleotide sequence found in the \textit{S. solfataricus} genomic bank. The sequenced amino-terminal fragment is framed, the positions of the putative transmembrane segments are shadowed grey, and putative glycosylation sites are shaded black.
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primers allowed the PCR of an 80 base-pair DNA fragment when either \( S. solfataricus \) P1 or P2 chromosomal DNA was used as template. The translated nucleotide sequence of the PCR product corresponded to the short amino-terminal sequence of the purified glucose-binding protein (Fig. 5A,B), and allowed the identification of the complete open reading frame (accession code c42_036) in the genomic database of \( S. solfataricus \) P2 (Fig. 6B). This gene codes for a protein of a molecular mass of 61 kDa. The size difference with the purified protein (~65 kDa) is most likely due to the glycosylation of the mature protein. The protein contains eleven possible glycosylation sites (Fig. 5B), while hydrophathy analysis revealed a strong hydrophobic region at the amino-terminus and at the carboxyl-terminus of the protein. Both may form a transmembrane segment that anchors the protein to the cytoplasmic membrane.

The amino-terminal amino acid sequence of the purified glucose-binding protein completely matched the predicted sequence from the DNA database, except that the first 12 amino acids were lacking in the purified protein. One possibility is that the protein is truncated as a result of a proteolytic degradation. The other possibility is that the protein is processed after synthesis and before transport over the cytoplasmic membrane (Chapter 5).

Databank searches revealed that the GBP shares 24 % (40 % similarity) and 19 % (30 % similarity) identical residues with the products of the \( P. horikoshii \) PH1214 (accession no.g3915507) and PH1039 (accession no. g3915490) genes, respectively. It shares 15 % (29 % similarity) with the product of the \( bxlE \) gene of \( Streptomyces lividans \) (accession no. g3941369), that corresponds to a putative sugar-binding protein. Downstream of PH1214 of \( P. horikoshii \), two other ORFs are located in the same transcription direction, PH1215 (accession no. g3915506) and PH1216 (accession no. g3915506) (Fig. 6). These two ORFs code for integral membrane proteins that are homologous to sugar permeases bearing the inner membrane component signature typical for binding-protein-dependent-transport systems. Further analysis of the DNA sequence surrounding the glucose-binding protein gene of \( S. solfataricus \) revealed three upstream genes, i.e., ORF32, ORF34 and ORF35 that are transcribed in the reverse direction (Fig. 6). ORF34 and ORF35 show homology to binding-protein-dependent sugar permeases, while ORF32 is similar to several ATP-binding proteins (Fig. 7). Furthermore, ORF34 and PH1215 (28 % identity, 51 % similarity), and ORF35 and PH1216 (24 % identity, 54 % similarity) are homologous (Fig. 6). This genetic organisation suggests that the GBP is a subunit of an ABC-transporter.

Discussion

In this study we have investigated the mechanism of glucose transport in \( Sulfolobus solfataricus \). Transport of glucose is mediated by a high affinity binding-protein-dependent system that is specific for glucose, galactose, and mannose were unable to detect any 2-deoxyglucose binding to the membrane vesicles and to the purified binding protein. Moreover, 2-deoxyglucose is a very poor
inhibitor of glucose binding and transport. This implies that a hydroxyl group at the C2 position of the sugar is critical for binding of the substrate, but there is no discrimination between C2 and C4-epimers of glucose. Studies of Cusdin et al. (Cusdin et al., 1996) suggest that 2-deoxyglucose is transported in *S. solfataricus* (DSM 1616) by a glucose/galactose/mannose transporter albeit with a 15-fold lower affinity than glucose. In our study, the difference in affinity between 2-deoxyglucose and glucose appears even larger. The exact reason for this discrepancy is not clear, but could relate to differences in the used strains. Nevertheless, both studies show that 2-deoxyglucose is a poor substrate for this transporter.

A transporter with affinity for glucose and galactose has previously been identified in *Brucella abortus* (Essenberg et al., 1997). This system presumably catalyses a sugar/H⁺ symport reaction. Energetically such a mechanism would also be favourable for *S. solfataricus*, since it maintains a very large ∆pH across its membrane (Moll and Schäfer, 1988). Uptake of glucose by *S. solfataricus* could possibly be mediated by a binding protein that is associated with a secondary transport system (Jacobs et al., 1996; Shaw et al., 1991), but the gene encoding the glucose-binding protein is located adjacent to genes encoding two integral membrane proteins and one ATPase subunit that are typical for ABC transporters. Since these three genes are transcribed in the reverse direction relative to the binding protein, a direct link is not immediately obvious. However, homologues of these genes in the genome of *Pyrococcus horikoshii* OT3 (Kawarabayasi et al., 1998) are contained in a single operon-like structure.

Moreover, the two integral membrane proteins, ORF34 and ORF35, are also homologous to many other sugar permeases belonging to an ABC transporter. We therefore conclude that the glucose-binding protein is a subunit of an ABC transport system. The binding-protein-dependent maltose/trehalose transporters of *Thermococcus litoralis* (Horbacher et al., 1998) and *S. shibatae* (Yallop and Charalambous, 1996), and the

**Fig. 7.** Alignment of ORF32 of the ABC operon of *S. solfataricus* with other ATP-binding proteins. (PH0203 of *Pyrococcus horikoshii*, MsiK of *Streptomyces lividans*, and AF084104 of *Bacillus firmus*). The ABC transporters family signature is underlined double and the Walker A (*) and Walker B (+) motif of the nucleotide binding site are indicated. Residues, which are conserved in all four proteins, are shaded black and the ones only found in three of the four aligned proteins are shaded grey.
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glucose transporter of \textit{S. solfataricus}, exhibit very high affinities for their sugar substrates, i.e., in the submicromolar concentration range. The high affinity of the binding protein allows these archaeal cells to efficiently utilise carbon sources in substrate poor environments such as the hydrothermal vents in the deep sea or the hot sulphuric pools.

We noted that a short amino-terminal sequence of 12 amino acids is not present in the purified GBP whereas it is predicted on basis of the nucleotide-sequence. Similar observations have been made for the flagellin proteins in methanogenes (Kalmokoff and Jarrell, 1991). This suggests that these proteins may use a similar mechanism of processing and possibly even secretion (Chapter 5).

Another unusual aspect of the glucose-binding protein is its extreme acid resistance. The protein exhibits a very low pH optimum, i.e., around pH 1.5, which is comparable to that of pepsin in the stomach (Fox and Whitaker, 1977). In this respect GBP differs from two other extracellular enzymes of \textit{Sulfolobus} that have been analysed, i.e., \(\alpha\)-glucosidase (Rolfsmeier et al., 1998) and an esterase (Huddleston et al., 1995), which exhibit a pH optimum of 4.5 and 6, respectively. The purified GBP in detergent solution appeared somewhat more acid susceptible than the membrane-bound enzyme (compare Fig. 3 A and B).

In Gram-negative bacteria, binding proteins exist in a soluble form in the periplasm. \textit{S. solfataricus} lacks an outer membrane, and is instead surrounded by an S-layer. This hexagonal-paracrystalline-proteinaceous structure contains large pores of 4-5 nm (König, 1988) to allow contact with the external medium. It is thought, that this structure serves as a molecular sieve, but it is not known if it can act as a barrier for proteins that are present in the space between the cytoplasmic membrane and the S-layer. The transmembrane segment(s) of GBP most likely serve as an anchor to the membrane, like the lipid moiety that retains binding proteins at the cytoplasmic membrane of Gram-positive bacteria. Although the exact membrane topology of the protein is not yet known, it is most likely that the major part of the protein is located outside where it is glycosylated and where it can perform its function as a binding protein. Future experiments will address the membrane topology of this protein.

\section*{Materials and Methods}

\subsection*{Organisms and growth conditions}

\textit{Sulfolobus solfataricus} P1 was obtained from W. Zillig (Martinsried, Germany) and \textit{S. solfataricus} P2 (DSM 1617) from the Deutsche Sammlung von Mikroorganismen und Zellkultur GmbH (Braunschweig, Germany). Cells were grown aerobically at 80°C using the mineral base of Allen and Zellkultur (Brock et al., 1972) supplemented with 0.1 % yeast, 0.2 % trypton and 0.5 % sucrose or 0.5 % glucose as a sole carbon source at pH 3.

\subsection*{Uptake experiments}

Cells grown in 50-ml cultures were harvested at an OD \(_{660}\) of 0.5-0.8. The cells were washed twice in medium without carbon source, resuspended at 10 mg protein/ml, and pre-incubated for 15 min at 60°C. Subsequently, 10 \(\mu\)l of this cell suspension was added to 90 \(\mu\)l pre-warmed medium without carbon source containing different concentrations radio-labelled sugars (specific activities: \([14C]\)glucose, 291 mCi/mnmole; \([3H]\)glucose, 13 Ci/mole (Amersham, The Netherlands)). At different time intervals the reaction was stopped by adding 2 ml of ice cold 0.1 M LiCl, and the mixture was rapidly filtered through 0.45 \(\mu\)m nitro-cellulose filters BA 85 (PROTRAN, Schleicher & Schuell, Germany). Filters were washed with 2 ml 0.1 M LiCl and dissolved and counted in 2 ml scintillation fluid (Packard, The Netherlands). For the determination of the kinetic parameters \(K_m\) and \(V_{max}\) of glucose uptake in cells, incubation was stopped after 10 s. Data were analysed with the direct linear plot.

\subsection*{Preparation of membranes of \textit{S. solfataricus}}

Cells were suspended in 20 mM Bis-Tris propane, pH 6.5, containing a small amount of DNase I, and subsequently passed through a French pressure cell at 800 lb/in\(^2\). Unbroken cells were removed by a low spin at 3,000 \(\times\) g for 20 min at 4°C, and membranes were collected from the supernatant by centrifugation (100,000 \(\times\) g for 45 min at 4°C). To remove peripheral membrane proteins, the pellet was resuspended in 20 mM Bis-Tris propane, pH 6.5, and 6 M urea at a protein concentration of 5 mg/ml. After 30 min on ice, the suspension was centrifuged (100,000 \(\times\) g for 45 min at 4°C), and the membrane pellet was resuspended in 20 mM Bis-Tris propane, pH 6.5, and stored in liquid
nitrogen. Alternatively, membranes were extracted with 22 mM Na₂CO₃ for 40 min at 45°C (Grogan, 1996).

**Binding assays**

Binding of radiolabeled substrates to membranes or solubilized protein was assayed as described by Richarme and Kepes (1983). Basically, 10 µl of membranes (10 mg/ml) or purified protein (0.3 mg/ml) were added to 90 µl of 50 mM glycine/HCl, pH 2, and preincubated for 5 min at 60°C. [¹⁴C]Glucose was added at various concentrations, and the suspension was incubated at 60°C. At various time intervals, the reaction was stopped by the addition of 2 ml of a chilled 70% saturated ammonium sulphate solution, and the mixture was filtered through 0.2 µm nitro-cellulose filters BA 85 (Schleicher & Schuell, Germany), and washed once with 2 ml of the same solution. Filters were dissolved in 2 ml scintillation fluid and counted. Binding data were analysed according to Scatchard (Scatchard, 1949).

**Purification of glucose-binding protein (GBP) from membranes of S. solfataricus**

Membranes of *S. solfataricus* were solubilized in a buffer containing 20 mM Bis-Tris propane, pH 6.5, and 2 % Triton X-100. The suspension was incubated for 2 h at 37°C, and insoluble material was removed by centrifugation (100,000 x g for 45 min). The supernatant was diluted with 20 mM Bis-Tris propane, pH 6.5, and 0.5 M NaCl to yield a final concentration of 0.05 % Triton X-100. Subsequently, the material was applied to a Con A Sepharose (Pharmacia, The Netherlands) column equilibrated with buffer A (20 mM Bis-Tris propane, pH 6.5, 0.5 M NaCl, 0.05 % Triton X-100). The column was washed with 5-volumes of the same buffer, and bound glycoproteins were eluted with a linear gradient of 0 to 200 mM α-methyl-mannopyranoside in buffer A. Fractions were assayed for glucose-binding activity as described above. α-Methyl-mannopyranoside did not interfere with glucose binding, since the glucose-binding assay was not influenced by the presence of a 100-fold excess of α-methyl-mannopyranoside. Active fractions were pooled and NaCl was removed overnight by dialysis against 1000 volumes of buffer B (20 mM Bis-Tris propane, pH 6.5, 0.05 % Triton X-100). The dialysis buffer was replaced three times. The protein fraction was subsequently applied to a HR5/5 Mono Q column (Pharmacia, Sweden) pre-equilibrated with buffer B. Proteins were eluted with a linear gradient of 0 to 500 mM NaCl. The glucose-binding protein eluted at 120 mM NaCl and was subsequently dialysed against 1000 volumes of 50 mM glycine/HCl, pH 2, 0.05 % Triton X-100 with 10% glycerol. Samples were routinely analysed by SDS-PAGE.

**Detection of binding activity in non-denaturating polyacrylamide gels**

Native PAGE was performed as described by Schägger and Jagow (1991). The glucose-binding activity in these gels was determined as follows: a gel strip was incubated at 80°C for 20 min in 20 mM Bis-Tris propane, pH 6.5, that contained 1 µM of [¹⁴C]glucose (specific activity 291 mCi/mmole). The gel strip was washed for 10 min in water and fixed for 5 min in 50 % (v/v) methanol, and 10 % (v/v) acetic acid. After washing with water for 5 min, the gel was dried and exposed to a high sensitivity X-ray film (Kodak).

**Cloning of the gene of the glucose binding protein**

Two degenerated primers (see Fig. 5A) were designed on the basis of the N-terminal amino acid sequence of the purified glucose-binding protein and used for a PCR reaction with genomic DNA isolated from *S. solfataricus* P1 and P2. The resulting PCR product of 80 bp length was ligated in a pGEM-T-easy vector (Promega, The Netherlands) and subsequently sequenced. The obtained sequences were used to screen the *S. solfataricus* database (http://niji.imb.nrc.ca/sulfolobus/) and genebank (http://www.ncbi.nlm.nih.gov/Entrez/nucleotide.html).

**Other techniques**

For the amino-terminal sequence analysis, purified glucose-binding protein was transferred to a PVDF membrane. DNA and protein sequencing was performed by Eurosequence (Groningen, The Netherlands). Staining of glycoproteins in SDS-PAGE was performed as described by Wardi et al. (Wardi and Michos, 1972). Protein concentrations were determined with the DC Biorad Kit (BIORAD, The Netherlands).

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