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An approach to prevent aggregation during the purification and crystallization of wild type acyl coenzyme A: Isopenicillin N acyltransferase from *Penicillium chrysogenum*

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Abstract

Acyl coenzyme A: isopenicillin N acyltransferase (AT) from *Penicillium chrysogenum* is an enzyme of interest for the biosynthesis of β-lactam antibiotics. Severe aggregation problems with wild type AT have, however, prevented significant progress in the structure–function analysis of this enzyme for a decade. In this study, we show an approach to solve this aggregation problem by using dynamic light scattering (DLS) analysis to probe the aggregation state of the protein in the presence of various additives. After a one-step purification of recombinant wild type AT with a C-terminal His-tag using Ni2+ affinity chelate chromatography, addition of a combination of 5 mM DTT, 250 mM NaCl, and 5 mM EDTA to the purified AT effectively prevented aggregation. In the presence of these additives, the DLS profile of AT shows a narrow size distribution indicative of a homogeneous protein solution and the absence of aggregation. The purity and mono-dispersity of wild type AT was sufficient for the growth of high quality crystals diffracting to 1.64 Å resolution.

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Keywords: Acyl coenzyme A: Isopenicillin N acyltransferase; Dynamic light scattering; Prevention of aggregation; Purification

Acyl coenzyme A: isopenicillin N acyltransferase (AT) from *Penicillium chrysogenum* catalyzes the conversion of isopenicillin N (IPN) into penicillin G, which is the last step in the in vivo biosynthesis of penicillin G (Fig. 1). This in vivo process is exploited for the commercial production of penicillins [1,2]. Starting from penicillin G, obtained from fermentation of *P. chrysogenum*, several other β-lactam antibiotics can be made via semi-synthesis [3,4]. In this production process, the side chain is first removed from the β-lactam core, after which the β-lactam nucleus is acylated with the side chain of interest in an enzymatic or non-enzymatic process. Since AT catalyzes the direct exchange of the side chains, the enzyme may greatly help to improve the conventional semi-synthetic penicillin production process by allowing a one-pot synthesis process [5,6].

To understand how AT catalyzes the exchange of side chains and to identify the atomic details of how the
enzyme recognizes its substrates, knowledge of the three-dimensional structure of the enzyme is an indispensable requirement. Also, insight into the active site from the three-dimensional structure of AT with its substrates brings the possibility of the in vivo production of novel antibiotics closer. Considering that AT shows a relatively wide substrate specificity, this structural knowledge may give clues how to design AT enzymes with altered specificity allowing alternative transfer reactions.

AT is produced as a 40 kDa pro-enzyme in \textit{P. chrysogenum}. This pro-enzyme is processed post-translationally by cleavage of the peptide bond between Gly102 and Cys103, resulting in \(\text{afii9825}\)- and \(\text{afii9826}\)-subunits of 11 and 29 kDa, respectively [7,8]. Co-expression of plasmids encoding the \(\text{afii9825}\)- and \(\text{afii9826}\)-subunits in recombinant \textit{Escherichia coli} results in correctly folded active enzyme. In contrast, the \(\text{afii9825}\)- and \(\text{afii9826}\)-subunits produced separately are insoluble and inactive, and refolding them separately and mixing them afterwards does not lead to AT activity. Only refolding the two subunits together in the presence of urea yields active enzyme [9]. These in vitro refolding and co-expression experiments thus indicate that both the \(\text{afii9825}\)- and \(\text{afii9826}\)-subunits are necessary for proper folding and activity [9].

Site-directed mutagenesis studies on AT have revealed several interesting mutants that were impaired in post-translational peptide bond cleavage or/and enzymatic activity [10,11]. In particular, mutants of Cys103, which is one of the residues in the cleavage site, were devoid of AT activity and remained in the 40 kDa precursor form [11].

In a previous study, the crystallization and preliminary X-ray diffraction of one of these mutants, Cys103Ala, were reported [12]. However, mature wild type AT could not be crystallized, since it aggregates easily. Recently, we succeeded in solving the aggregation problem, making it possible to crystallize wild type AT. Here, we present the cloning, expression, purification, and successful crystallization of AT as well as our approach to preventing its aggregation.

Materials and methods

Cloning, expression, and purification of wild type AT

Recombinant \textit{E. coli} JM109 containing a plasmid, pHAR11 encoding the \textit{penDE} gene with a 6-His tag at the C-terminus, was used in this study. The plasmid pHAR11 was constructed from pMAT4 [8] (Fig. 2). The \textit{E. coli malE} gene from pMAL-c2 (New England Biolabs, USA) was amplified by PCR using a forward primer designed with a \textit{Bam}HI site (underlined) [5\text/H11032-tcatcatcatggatccaaaatcgaagaaggtaaa-3\text/H11032] and a reverse primer including a \textit{HindIII} site (underlined) after the \textit{malE} gene stop codon [5\text/H11032-gtcgtcgtcgtcatagctctggtcgtctttcag-3\text/H11032]. The obtained fragment was inserted between the \textit{Bam}HI and \textit{HindIII} sites of pHAR6 (constructed by changing the stop codon sequence of \textit{penDE} in pMAT4 into an \textit{Xba}I site by Kunkel mutagenesis [13]), resulting in pHAR9 which encodes an out-of-frame \textit{penDE malE} fusion. To create the correct reading frame of the \textit{penDE malE} fusion, linker oligonucleotides (sense sequence including part of an \textit{Xba}I site and a \textit{Sac}I site (both underlined) [5\text/H11032-ctaga tcgagctcgaacaacaacaaagataacaataacaacag-3\text/H11032] and anti-sense sequence including part of a \textit{Bam}HI site (underlined) [5\text/H11032-gatcttcgatgttattgttattgttattgttattgttattgttattgttattggttacctgagctgat-3\text/H11032]) were inserted between the \textit{Xba}I and \textit{Bam}HI sites of pHAR9, resulting in pHAR10 which is able to express AT-maltose binding protein fusion protein. Additionally, an oligo cassette comprising six histidine codons (sense sequence including part of an \textit{Xba}I site and a \textit{Sac}I site (both underlined)
[5'-ctagtcgagctcagccacccacacattgaggatcca-3'] and anti-sense sequence including part of a HindIII site and an overhanging BamHI site (underlined) [5' -agcttgga tctcaatgatggtgatggtggtgcgagctcgat-3'] was inserted between the XbaI and HindIII sites of pHAR9, resulting in pHAR11. Cells transformed with pHAR11 were grown at 28 °C in 2× YT medium containing 30 μg/ml chloramphenicol, and after 2.5 h of cultivation, 0.1 mM D-isopropylthiogalactopyranoside (IPTG) was added. The cells were harvested after overnight growth, and then resuspended and sonicated in the lysis buffer (50 mM Tris–HCl, pH 7.5, 8.7%(v/v) glycerol, and 0.1%(v/v) Triton X-100). The sonicated sample was centrifuged (27,138 g, 20 min, 4 °C), and the resultant cell-free extract was applied to an affinity column (HiTrap Chelating HP 5 ml, Amersham Biosciences) which had been loaded with NiSO₄ solution and equilibrated with 50 mM Tris–HCl, pH 8.0. After washing away the unbound protein with equilibration buffer, the protein was eluted by a step gradient of 50 mM Tris–HCl, pH 8.0, 500 mM NaCl, with increasing imidazole concentration (0, 20, 50, 150, 250, and 500 mM). The purified protein, which was eluted in 50 mM Tris–HCl, pH 8.0, 500 mM NaCl, 250 mM imidazole, was confirmed by SDS–PAGE, showing two bands of 11 and 29 kDa, respectively.

Dynamic light scattering analysis

The effect of additives on the aggregation of wild type AT was investigated by Dynamic light scattering (DLS) (DynaPro MSTC161, Protein Solutions, USA). For DLS analysis, purified AT, eluted from the Ni²⁺ affinity chelate column, was concentrated and its buffer exchanged with 50 mM Tris–HCl, pH 8.0, 150 mM NaCl using a Centricon YM-10 concentrator (Millipore, USA) to a final protein concentration of 3.30 mg/ml. As additives, we used 5 or 10% glycerol, 1 or 5 mM DTT, 1 or 5 mM EDTA, 5 or 10% ethylene glycol, 1 or 5 mM Tris(2-carboxyethyl)phosphine (TCEP)–HCl, and 250 or 500 mM NaCl. These additives, alone and in combination, were added to eluted protein solution, obtained by the described purification scheme, and incubated at 4 °C for 4, 12, and 33 days. The aggregation state of each sample was evaluated by DLS. Since DLS measures the size distribution of the protein molecules in the solution, it is sensitive to variations in particle size and interactions of
protein molecules in solution [14]. Thus, protein aggregation is recognizable by an increase in the hydrodynamic radius (Rh).

**Crystallization of AT**

An initial crystallization condition was found using the Wizard II crystal screen kit (Jena Bioscience, Germany) using the microbatch method under oil [15] and an Oryx 6 crystallography robot (Douglas Instruments, UK). Protein solution (1 μl, 8.28 mg/ml) was mixed with 1 μl of reservoir solution containing 2 M phosphate (0.8 M NaH₂PO₄/1.2 M K₂HPO₄) and 0.1 M Na-acetate, pH 6.7. After optimization of this crystallization condition using the hanging-drop setup [16], crystals appeared within a week at room temperature in a mixture of 1.5 μl protein solution (9.03 mg/ml) and 1.5 μl of reservoir solution containing 1.95 M phosphate (0.78 M NaH₂PO₄/1.17 M K₂HPO₄) and 25 mM Na-acetate, pH 6.9.

**Results and discussion**

**Purification of wild type AT**

Purification steps are summarized in Table 1. Since wild type AT with a C-terminal His-tag binds tightly to the Ni²⁺ affinity chelate column, the protein could be purified in a single step, using an elution buffer with a high concentration of imidazole (250 mM). The total yield of recombinant AT was 115 mg protein/L culture. Unfortunately, in spite of the fast single-step purification, protein precipitation was observed in the eluate within several hours after purification. To solve this severe aggregation problem, we investigated the effect of various additives. We did not test AT activity in this study, since an accurate HPLC AT assay is too time-consuming to combine with rapid AT purification. Instead of checking the enzymatic activity of AT, we confirmed the maturation to wild type, i.e., the presence of post-translationally cleaved AT (11 and 29 kDa subunits) by SDS-PAGE (See Improved purification and storage conditions of wild type AT), since this cleavage has been found previously to be essential for enzyme activity.

**Dynamic light scattering analysis**

Fig. 3 shows the results of Dynamic light scattering (DLS) experiments with the protein after 4 days in the presence of various additives. Compared to the sample without additives (50 mM Tris–HCl, pH 8.0, 150 mM
NaCl), the samples in the presence of 5 mM EDTA and 5 mM TCEP·HCl show reduced aggregation (no signal at Rh higher than 10 nm). With 5 mM DTT, the signal at Rh = 4.0 becomes narrower, which is an indication that the particles become more uniform in size, i.e., there are less interactions between the protein molecules [17].

To optimize the conditions for purification and storage of AT, various combinations of additives were studied. The results of the DLS measurements are summarized in Fig. 4. No aggregation is observed after 33 days in the presence of 1 mM TCEP + 250 mM NaCl + 1 mM EDTA. Although we also tested the effect of each single additive after 33 days, none of them on their own was effective in preventing aggregation during this time period (Fig. 4). Addition of 1 mM TCEP shows broader peaks than the sample without additives (50 mM Tris–HCl, pH 8.0, 150 mM NaCl), but the intensity of the signal from the latter sample indicates that most of protein is aggregated. Addition of 1 mM DTT does not show any effect. Higher concentrations of the single additives might be required for long-term storage.

In the investigation of combinations of additives, the substitution of 1 mM TCEP with 1 mM DTT narrows the peak between 1 and 10 nm (compare graphs 6 and 7 in Fig. 4), although some aggregation remains in another area (>10 nm). Thus, at least a reducing reagent like TCEP or DTT is beneficial for preventing aggregation, but they are not sufficient for long-term storage of protein in isolation. TCEP is generally a more effective reducing reagent than DTT and more stable without metal chelators in the buffer, however, in the presence of a chelating agent, DTT can also be used for long-term storage of proteins [18]. Because DTT gives a narrower size distribution of the protein particles in solution, we prefer the combination of DTT, NaCl, and EDTA as the additive to prevent the severe aggregation of AT.

**Improved purification and storage conditions of wild type AT**

Purified AT, eluted from the Ni²⁺ affinity chelate column according to the procedure described above, was...

![Fig. 4. DLS analyses after 12 days (upper) and 33 days (lower) of storage of the samples at 4 °C, showing the effect of single additives and the various combinations of additives [(1) buffer: AT in 50 mM Tris–HCl, pH 8.0, 150 mM NaCl; (2) TCEP: AT in 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 1 mM TCEP·HCl; (3) DTT: AT in 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 1 mM DTT; (4) TCEP and NaCl: AT in 50 mM Tris–HCl, pH 8.0, 250 mM NaCl, and 1 mM TCEP·HCl; (5) TCEP and EDTA: AT in 50 mM Tris–HCl, pH 8.0, 250 mM NaCl, 1 mM TCEP·HCl, and 1 mM EDTA; (6) TCEP, NaCl, and EDTA: AT in 50 mM Tris–HCl, pH 8.0, 250 mM NaCl, 1 mM TCEP·HCl, and 1 mM EDTA; and (7) DTT, NaCl, EDTA: AT in 50 mM Tris–HCl, pH 8.0, 250 mM NaCl, 1 mM DTT, and 1 mM EDTA] in preventing aggregation.](image-url)
immediately mixed with 5 mM DTT and 5 mM EDTA. After concentration of the resulting solution and a change of buffer to 50 mM Tris–HCl, pH 8.0, 250 mM NaCl, 5 mM DTT, and 5 mM EDTA using a Centricon YM-10 concentrator (Millipore, USA), a DLS analysis was performed. The quality of the purified AT was confirmed by SDS–PAGE and by the DLS profile (Fig. 5). No aggregation was observed in the purified sample and the DLS profile remained mono-disperse and unchanged after 8 days of storage at −80 °C. The purified and concentrated proteins were divided into smaller volumes, 40 µl/Eppendorf tube, and stored at −80 °C in order to avoid freeze–thawing of undivided samples.

As a final check of the purity of the enzyme, we set up protein crystallization experiments with the AT material prepared according to the final protocol. Good crystals appeared within a week. They showed excellent diffraction up to 1.64 Å resolution on beamline ID14-1 (ESRF, Grenoble, France). The space group of the crystals is C2, with cell dimensions a = 198.71 Å, b = 68.22 Å, c = 146.96 Å, β = 128.64°. There are four molecules per asymmetric unit. The cell dimensions differ from those of the Cys103Ala mutant AT, thus clearly suggesting a different packing of the molecules in the unit cell. Since the post-translational peptide bond cleavage can be expected to give rise to conformational changes, a different packing of the molecules would be in line with such a conformational difference. A full structure determination of mature wild type AT is currently underway. Preliminary results indicate clear changes around the active site region of AT as compared to the Cys103Ala mutant.

Conclusion

In this study, a successful purification protocol to avoid severe protein aggregation was obtained for AT using DLS analysis to study the effect of various additives on protein stability and aggregation. Recombinant wild type AT with a C-terminal His-tag was purified in a single step using Ni²⁺ affinity chelate chromatography. Incubation of the purified AT with a combination of 5 mM DTT + 250 mM NaCl + 5 mM EDTA showed the effectiveness of these additives in preventing aggregation of AT. The DLS profile remained mono-disperse in the presence of these additives, which indicates no aggregation. The quality of purified wild type AT was confirmed by the successful crystallization of the protein, which yielded crystals diffracting up to 1.64 Å resolution.

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References


Fig. 5. Evaluation of the quality of purified AT with additives. (A) The result of SDS–PAGE after addition of DTT and EDTA to AT purified by Ni²⁺ affinity chelate chromatography (1, LMW (low molecular weight markers); 2, cell free extract; 3, unbound material from Ni²⁺ affinity column; 4–8, consecutive sample fractions in 50 mM Tris–HCl, pH 8.0, 500 mM NaCl, 250 mM imidazole). (B) The stability of purified AT in the presence of 5 mM DTT + 5 mM EDTA + 250 mM NaCl, as analysed by DLS. (a) Purified wild type AT-His (9.03 mg/ml in 50 mM Tris–HCl, pH 8.0, 250 mM NaCl, 5 mM DTT, 5 mM EDTA) (0 day). (b) Purified wild type AT-His after 8 days incubation at −80 °C.
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