An Invariant Threonine Is Involved in Self-catalyzed Cleavage of the Precursor Protein for Ornithine Acetyltransferase*

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In Bacillus steaerothermophilus ornithine acetyltransferase is a bifunctional enzyme, catalyzing the first and the fifth steps of arginine biosynthesis; it follows a ping-pong kinetic mechanism. A single chain precursor protein is cleaved between the alanine and threonine residues in a highly conserved ATML sequence leading to the formation of α and β subunits that assemble into a heterotetrameric 2α2β molecule. The β subunit has been shown to form an acetylated intermediate in the course of the transacytelylation reaction. The present data show that the precursor protein synthesized in vitro or in vivo undergoes a self-catalyzed cleavage involving an invariant threonine (Thr-197). Using site-directed mutagenesis T197G, T197S, and T197C derivatives have been generated. The T197G substitution abolishes both precursor protein cleavage and catalytic activity, whereas T197S and T197C substitutions reduce precursor cleavage and catalytic activity in the order Thr-197 (wild type) → Ser-197 → Cys-197. A mechanism is proposed in which Thr-197 plays a crucial role in the autoproteolytic cleavage of ornithine acetyltransferase.

Ornithine acetyltransferase (OATase, 1 N2-acetyl-l-ornithine: l-glutamate l-acetyltransferase; EC 2.3.1.35, encoded by the argJ gene in procaryotes) participates in arginine biosynthesis in microorganisms (for a review see Ref. 1). The OATase-mediated transacytelylation follows a ping-pong kinetic mechanism in which the enzyme is acetylated as an intermediate protein from which the acetyl group is subsequently transferred to glutamate (2). In the thermophilic bacteria Bacillus steaerothermophilus and Thermotoga neapolitana, in the thermophilic archaeon Methanococcus jannaschii (2), and in Saccharomyces cerevisiae (3), OATase has been shown to be synthesized as a precursor protein that undergoes proteolytic cleavage, leading to the formation of α and β subunits that assemble into heterodimeric or heterotetrameric molecules in eucaryotes or procaryotes, respectively. The N-terminal amino acid residue of the β subunit of mature OATase has been shown to be a threonine and cleavage shown to occur between the alanine and threonine residues in a conserved ATML sequence of the precursor proteins (2–4). Cleavage of the yeast OATase precursor has been suggested to be self-catalyzed rather than protease-assisted (5).

Posttranslational autoproteolysis has been described for several proteins from eucaryotes and procaryotes (6). In glycosylasparaginase from Flavobacterium, structural and biochemical studies have shown that cleavage results from a nucleophilic threonine attack on the preceding aspartate residue (7, 8). In addition to threonine the deprotonated side chains of cysteine and serine residues, are also able to attack a preceding carbonyl group resulting in N → O or N → S shift (8, 9). The peptide amide bond is therefore replaced with a more reactive (thio)ester bond, and a subsequent attack by a second nucleophile, which can be an activated water molecule, breaks the ester bond thereby exposing Thr, Cys, or Ser at the N terminus of the downstream peptide (10).

Thus the question arises as to how the cleavage of the OATase precursor protein is accomplished in procaryotes. To answer this question we have investigated the thermolstable OATase from B. steaerothermophilus. Our data indicate that precursor protein cleavage is necessary for enzyme activation and that the invariant threonine Thr-197 is responsible for the self-catalyzed cleavage reaction.

EXPERIMENTAL PROCEDURES

Strains and Materials—The argJ gene was cloned from the B. steaerothermophilus NCIB 8224 strain (11). OATase from B. steaerothermophilus is able to accept acetyl donors both from acetyl-CoA and N-acetyl-l-ornithine and therefore catalyzes the first (argA) and fifth (argJ) steps in arginine biosynthesis (12). Escherichia coli K12 strains XA4 (F− argA nadA λ− l kbdR) and XSI2DR (F− Δ[pi]-argE) nadA rpoB λ hsdR recA) were used in complementation tests to check the functionality of B. steaerothermophilus argJ derivatives in E. coli cells as described previously (13). The E. coli BLR(DE3) was used to overexpress the B. steaerothermophilus mutant argJ gene. The separate expression of α and β subunits of OATase was carried out from the strong PorgC promoter of B. steaerothermophilus (14) in E. coli K802 (F− metB).

Site-directed Mutagenesis and Recombinant DNA Constructions—Mutations were introduced in the B. steaerothermophilus argJ gene by polymerase chain reaction as previously described for the argR gene (15). In pargJ-Bs/N plasmid the argJ gene was fused to an N-terminal His tag encoding sequence (2), and the ACG codon (threonine at a 197-position) was mutated to either AGT (serine), TGT (cysteine) or GGT (glycine). Sequences of mutagenic oligonucleotide primers are available on request. The mutated argJ sequences were cloned in...
pCR1.4 TOPO vectors (Invitrogen). The pargf-Bs/N and the pargf-Bs/C plasmid (2) were also used to construct shortened argJ coding only for the α or β subunit fused to terminal His tag (Fig. 1). To evaluate the role of Thr-197 as an N-terminal residue, two versions of the β subunit were designed: (i) an N-terminal methionine was added upstream of Thr-197 (argJ-β1), or this Thr-197 was replaced by a methionine (argJ-β2; see Fig. 1A). argJ sequences encoding a or β subunit were fused to the PgcP promoter by the “overlapping extension” method (16) and cloned in the EcoRI site of pBR322.

We also constructed an argJ variant with a translation stop codon for the α subunit encoding sequence immediately followed by a ribosome-binding site preceding either β1 or β2 versions (see above). This preserved the single mRNA transcription but resulted in independent translation of the α and β subunits (see Fig. 1B). All mutations were confirmed by DNA sequencing.

**Molecular Mass Determination of Mutant OATases**—Recombinant *E. coli* XS1D2 cells carrying corresponding plasmids were cultivated in arginine-less minimal medium, and His-tagged proteins were then purified using Ni²⁺-affinity column. Molecular masses of purified proteins were estimated by gel permeation chromatography using a TSK-3000SW column (Merck) directly on-line with the HPLC system (Merck) using Tris buffer 0.01 M (pH 7.5 with NaCl 0.15 M) as the mobile phase at a flow rate of 1 ml/min. Detection of proteins was carried out after 280 nm, and molecular masses were calculated using standard protein markers (Amersham Pharmacia Biotech): aldolase (158 kDa), bovine serum albumin (67 kDa), chicken ovalbumin (44 kDa), chymotrypsinogen (25 kDa), and ribonuclease (13.7 kDa). SDS-PAGE of proteins was carried out as described by Ausubel et al. (17). Gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad). Protein concentration was determined by the Bradford method (18) using bovine serum albumin as standard.

**Enzymatic Assays**—A previously developed HPLC-based method was used to evaluate quantitatively OATase-catalyzed formation of N-acetyl-l-glutamate (2). Enzyme activity was measured in a mixed buffer (MES 0.1 M, PIPES 0.1 M, Tris 0.1 M, and K₂HPO₄ 0.1 M, pH 7.0) containing l-glutamate (20 mM) and N²-acetyl-l-ornithine (20 mM). After 2 min of preincubation at 60 °C the reaction was initiated by adding the purified enzyme. The reactions were continued from 5 min to 24 h at 60 °C and then stopped by adding 500 mM phosphoric acid. One enzyme unit is defined as the amount of enzyme producing 1 mol of acetyl CoA, and 5 mol of acetyl-L-glutamate/min.

**Detection of Acetylated Proteins**—Purified His-tagged OATases were incubated with [¹⁴C]labeled N-acetyl-l-glutamic acid as acetyl donor, but in the absence of acetyl acceptor (semi-reaction) in the above mentioned mixed buffer at 37 °C for 30 min or at 65 °C for 1 h, reaction products were then separated on SDS-PAGE (a 12% gel). After coloration with Coomassie Brilliant Blue, gels were treated with an amplifier solution (Amersham Pharmacia Biotech), and radioactive bands were visualized by autoradiography using Hyperfilm TM (Amersham Pharmacia Biotech).

**Measurement of CoA Formation**—In a previous study we noticed that a rate of acetylated intermediates both for *B. stearothermophilus* and *B. neapolitana* OATases was lower as compared with the consumption of [¹⁴C]acetyl-CoA or [¹⁴C]acetyl-L-glutamate substrates in a semi-reaction (2). To find out the matter we performed semi-reaction by quantifying the deacetylated donor. The reaction was carried out in a 200-µl incubation mixture containing the above-mentioned mixed buffer and 100 µM of acetyl CoA, and 5 µM of glutamate. The reaction was initiated by the addition of a purified His-tagged OATase. After 20 min of incubation at 60 °C the reaction was stopped by cooling on ice, and the samples were immediately filtered on Microcon 10 (Amicon) to remove the enzyme. Quantitative determination of CoA was carried out on 100 µl of filtrate by reverse phase HPLC analysis using an Apex ODS 3 µ column (250 × 4.6 mm, from Jones Chromatography, Inc.) as described in (19).

**In Vitro Synthesis of OATase**—In vitro synthesis of wild-type or mutant proteins was carried out in S30 extracts of *E. coli* BL21 prepared as described (20) with minor modifications using circular or linear DNA templates. T7 RNA polymerase was purchased from Promega and l-[³⁵S]methionine was from Amersham Pharmacia Biotech. Where indicated a mixture of protease inhibitors (Sigma) against metalloproteases, serine-, cysteine-, and aspartate-proteases, was added to the reaction mixture. The samples were treated at 65 °C for 10 min and then shortly centrifuged, and the supernatant was used for protein separation on SDS-PAGE.

**RESULTS**

Separately Expressed Subunits of OATase Are Not Active—We previously showed that the two subunits of recombinant OATases from three thermophilic microorganisms, always co-purified by affinity chromatography irrespective of the N- or C-terminal His tag, respectively, fused to the α or the β subunit (2). The question arose whether α and β subunits, when synthesized independently, could exhibit enzymatic activity when mixed together in vitro or coexpressed in vivo.

We amplified and cloned separately *B. stearothermophilus* DNA argJ regions corresponding to α and β subunits (two versions, β1 and β2, were obtained for β, differing by their N-terminal sequences, MTMLA and MMLA, respectively; see Fig. 1A). None of the plasmids carrying the shortened argJ gene, namely argJ-α, argJ-β1, or argJ-β2, was able to complement the *E. coli* XS1D2 and XA4 strains deficient for argE and argA genes, respectively. Additionally, none of the purified truncated proteins used either separately or mixed in equimolar concentrations as α and β1 or α and β2 subunits exhibited OATase activity after 24 h of incubation at 37 °C or 50 °C. Moreover, when separately expressed and then mixed together, α and β1 or α and β2 subunits never assembled as judged from PAGE performed in non-denaturant conditions (data not shown).

These data were confirmed by studying plasmid constructions in which the two subunits were independently translated from a common mRNA (see Fig. 1B). Indeed, no complementation was observed in *E. coli* XS1D2 and XA4 mutants, and no OATase activity was detected in cell extracts of the transformants. Thus, the data show that independently synthesized α and β subunits (irrespective of the N-terminal regions created for the latter) do not reconstitute a functional *B. stearothermophilus* OATase in *E. coli* host cells.

Analysis of Thr-197-substituted Mutant Proteins in Vitro—Assuming that the invariant threonine (Thr-197 in *B. stearothermophilus* OATase) in the conserved ATML sequence could cause the deacetylation of the α subunit (2), the question arose whether Thr-197-substituted mutant enzymes in *B. stearothermophilus* S30 extracts. On SDS-PAGE a wild-type *B. stearothermophilus* OATase was detected as two bands corresponding to the α and β subunits, indicating that intramolecular cleavage was a very rapid and efficient process in vitro (Fig. 2A). Added protease inhibitors did not increase the yield of subunit formation; on the contrary, the intensity of the subunit bands decreased and smaller protein appeared. The protease inhibitors mixture was therefore omitted in further experiments.

In contrast to the wild-type argJ gene, the three T197S, T197C, and T197G mutants produced a low migrating band corresponding to the precursor protein (see Fig. 2A). However, in addition, two bands corresponding to the α and β subunits were still present in protein samples synthesized from the T197S and T197C mutant DNA templates; this was not the case for the T197G mutant. In these experiments we noticed that when mutant genes were used as DNA templates, a weak 37-kDa protein band that could be attributed to translation from a potential internal ribosome-binding site in mutant argJ mRNA was present as well.

**Kinetics of Cleavage of Mutant OATase Precursor Proteins**—SDS-PAGE analysis of purified His-tagged wild-type and threonine-substituted mutant proteins synthesized in *E. coli* cells confirmed the results of the in vitro experiments except that the 37-kDa protein was not detected (Fig. 2B). The molecular mass of the native wild-type OATase was determined as 90–
100 kDa by gel permeation as expected for a heterotetrameric structure (2). However, molecular mass determination of purified T197C and T197S mutant proteins gave major protein peaks of 54 kDa and 90–100 kDa corresponding to the precursor and the heterotetrameric molecules, respectively. Only the precursor form of 54 kDa was detected with the T197G mutant. The mutant proteins were diluted in Tris buffer 10 mM, pH 8.0, to a concentration of 1 mg/ml and incubated at 50 °C for 24 h, taking samples regularly to visualize the kinetics of subunits formation by SDS-PAGE (Fig. 3). The T197S mutant precursor underwent the transition to α and β subunits faster than did the T197C mutant. Densitometric analysis of the precursor protein and subunit protein bands formed over a 24-h incubation showed that the kinetics of hydrolysis followed a first order curve and that the apparent half-life of the precursor was 71 min (\( k_1 = 1.1 \times 10^{-2} \text{ min}^{-1} \)) and 35 h (\( k_2 = 2.0 \times 10^{-2} \text{ h}^{-1} \)) for the T197S and the T197C mutant proteins, respectively. The subunit bands did not appear with the T197G mutant confirming its inability to undergo intramolecular cleavage (data not shown). We also found that the ArgJ-T197S mutant precursor was processed throughout the 3.5 to 9.5 pH range and with a maximal rate at 69 °C (Fig. 4).

OATase Activity of Thr-197-substituted Mutant Proteins—Purified His-tagged proteins were tested for OATase activity at 50 °C. A specific activity of 29 μmol of N-acetyl-L-glutamate/min/mg protein was measured for the wild-type enzyme, whereas no activity was detected for the T197G mutant. A very low activity, 0.1 μmol of N-acetyl-L-glutamate/h/mg protein was detected for freshly purified T197S and T197C mutant proteins. Moreover, T197S and T197C mutant proteins exhibited a similar low OATase activity after incubation at 10 °C for a week, although under these conditions the mutant precursor proteins underwent complete cleavage as judged by SDS-PAGE.

In agreement with these data none of the three threonine-substituted argJ mutants supported the growth of E. coli XS1D2 (argE) and XA4 (argA) strains in a medium devoid of arginine. Thus the low level of OATase activity exhibited by T197S or T197C mutants is not sufficient to complement E. coli argE or argA mutant strains.

Acetyl Enzyme Intermediate Formation—We performed a semi-reaction in the presence of only 14C-labeled N-acetyl-L-glutamic acid to determine which subunit is acetylated in OATase. The single band corresponding to the β subunit was detected by autoradiography (Fig. 5) thus showing that the acetyl group is linked to this subunit to form the acetylated intermediate protein. We quantified also the B. stearothermophilus OATase-mediated formation of CoA from non-labeled acetyl-CoA in the absence and presence of L-glutamate as an acetyl group acceptor.
FIG. 2. Synthesis of the wild-type B. stea- rothermophilus OATase and threonine-substituted mutant proteins in vitro (A) and in E. coli cells (B). A, lanes 1 and 3, a wild-type OATase in absence and lane 2, in presence of a protease inhibitors mixture; lanes 4, 5 and 6, mutant proteins T197G, T197S, and T197C, respectively. B, lane M, molecular mass markers; wt, the purified His-tagged wild-type OATase; T197G, T197C, and T197S, the purified His-tagged mutant proteins without further incubation (0 h) and after 21 h of incubation in Tris buffer 10 mM, pH 8.0, at 50 °C.

FIG. 3. Kinetics of intramolecular cleavage of purified mutant precursor proteins at 50 °C. The used buffer was 10 mM Tris, pH 8.0. A, lane 1, molecular mass markers; lanes 2, 3, 4, 5, 6, 7, 8 and 9, the T197S mutant protein sample after 0, 10, 20, 30, 60, 90, 120, and 180 min of incubation; lane 10, after 21 h of incubation. B, lanes 1, 2, 3, 4, 5, 6, 7 and 8, the T197C mutant protein sample after 0, 3, 6, 9, 12, 15, 18, and 21 h of incubation. C and D, densitometric evaluation of a precursor protein band disappearance as a function of the incubation time for T197S and T197C, respectively. The relative efficiency of the cleavage is shown as a log% of the precursor protein band synthesized de novo. Analysis was carried out by a 12% SDS-PAGE.
(thiol ester bond which is then broken to liberate the α and β subunits, exposing alanine at the C terminus of the α subunit and one of the above mentioned amino acids at the N terminus of the β subunit. Therefore, the invariant threonine, being conserved in more than 40 available OATase sequences from bacteria, archaea, and eucaryotes, should play a crucial role in the preOATase protein self-catalyzed cleavage.

Given the crucial role of the invariant threonine in intramolecular cleavage of preOATase, we assume that the processing of a wild-type OATase precursor from B. stearothermophilus is similar to the processing of glycosylasparaginase from Flavobacterium (8, 22). When the de novo synthesized OATase precursor is correctly folded, the hydroxyl group of Thr-197 can be deprotonated and its nucleophilicity become enhanced (Fig. 6). A nucleophilic attack on the α-carbonyl group of Ala-196 can then lead to the formation of a cyclic tetrahydral intermediate with a negatively charged carbonyl oxygen and then to the formation of a reactive ester bond. Hydrolysis by a nucleophile water molecule (or by another nucleophile) can lead to the formation of the next intermediary tetrahydral state from which a breakage reaction liberates two nascent subunits thus exposing the threonine residue at the N terminus of the β subunit.

The ping-pong kinetic mechanism of OATase indicates that an acetylated intermediate is formed by covalent binding of the acetyl group from a corresponding donor (2). This intermediate appears to be rather unstable because, in the absence of an acetyl group acceptor, some CoA is formed (see Table I) presumably by hydrolysis of the acetylated intermediate. The acetylation reaction itself requires deprotonation of a particular amino acid which should be located in the β subunit as only this subunit has been found to be acetylated in OATase. Our data show that self-catalyzed precursor cleavage is a necessary step to form active OATase, probably by directing appropriate folding and/or topological organization of the active site in the oligomeric molecule. Indeed, replacement of the invariant threonine by glycine completely abolishes enzymatic activity. The two other substitutions, T197S and T197C, slow down precursor protein autocleavage but also cause significant loss of OATase activity. The fact that the T197S and T197C mutant proteins still exhibit low enzymatic activity after complete cleavage of precursors and association of subunits into heterotetrameric molecules indicates that, in addition to autoproteolysis, these mutants are affected in catalysis as well. A probability is that an N-terminally positioned Thr-197 in the β subunit is involved in the enzymatic mechanism; this could be by mediating the transfer of the acetyl group from the acetylated intermediate to the L-glutamate acceptor molecule or by inducing an appropriate conformation at the active site.

It is worth noting that this hypothesis of a crucial function for the invariant threonine in both preprotein cleavage and OATase catalytic mechanism could explain an apparent discrepancy between our results and the conclusions of Abadjieva et al. (5) concerning the maturation of active yeast OATase.
These authors independently proposed OATase autoproteolytic processing and found that the replacement of threonine 215 (referred to us as Thr-197 for *B. stearothermophilus*) by alanine abolishes activity in yeast OATase (5). However, they also observed that a *S. cerevisiae* strain deleted for the OATase gene and carrying two plasmids with separately cloned and expressed yeast α and β subunits (in the latter case a methionine residue had been introduced at the N terminus to initiate translation) still exhibited a weak OATase activity. In contrast, from a plasmid providing concomitant but independent expression of *B. stearothermophilus* OATase α and β subunits from the same mRNA, we have observed no complementation of *E. coli argE* or *argA* mutants and could detect no OATase activity whether the invariant threonine was immediately preceded or replaced by a methionine residue. The fact that OATase activity is only partly restored when using the yeast constructions was attributed by Abadjieva et al. to the presence of the methionine residue introduced at the N terminus of the β subunit (5). However, if we consider that the invariant threonine is essential for the OATase-mediated catalytic mechanism and not only for processing, then the low activity measured in yeast could be due to partial removal of the N-terminal methionine (21); this would expose the threonine residue at the N terminus and thus mimic the result of self-catalyzed processing from a precursor.

Microbial OATases belong to the N-terminal transferases family of enzymes. N-acetyltransferases play important roles in various processes such as the expression of eucaryotic genes, the inactivation or activation of drugs in bacterial and mammalian cells, the morphogenesis of membranes, the formation of nodules during the establishment of symbiotic relationships between bacteria and plants, and the emergence of metabolic pathways for several amino acids. All these enzymes catalyze acetyl group transfer from acetyl-CoA to a primary amino group in different target molecules, including proteins, lipids, amino acids, sugars, and still other compounds (for a review see Ref. 23). Three-dimensional protein structures have been solved for eucaryotic HAT1 histone N-acetyltransferase (24), the GCN5 transcriptional regulator (25), both of which use histones as substrates, as well as for aminoglycoside 3-N-acetyltransferase (26), serotonin N-acetyltransferase (27), and arylamine N-acetyltransferase (28), all of which use non-proteinic substrates. Some N-acetyltransferases share a similar structural core that allows to group them in a common superfamily (29). However, in addition to self-catalyzed intramolecular cleavage OATases exhibit other features not yet detected among the above mentioned N-acetyltransferases; monofunctional OATases are able to accept the acetyl group from a donor other than acetyl-CoA, namely from N-acetyl-l-ornithine, whereas bifunctional OATases are active toward both acetyl-CoA and N-acetyl-l-ornithine. These features as well as the absence of similarity between well characterized N-acetyltransferases and OATases reflect their distant evolutionary relationship (30). Further structural and enzymatic studies of OATases may help to understand the molecular mechanism which couple self-catalyzed cleavage of the precursor to transacylation catalysis and to elucidate the phylogenetic position of these enzymes among other N-acetyltransferases.

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*Autoproteolysis of Ornithine Acetyltransferase Precursor*