Aspartate-90 and arginine-269 of hamster aspartate transcarbamylase affect the oligomeric state of a chimaeric protein with an Escherichia coli maltose-binding domain

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Residues Asp-90 and Arg-269 of Escherichia coli aspartate transcarbamylase seem to interact at the interface of adjacent catalytic subunits. Alanine substitutions at the analogous positions in the hamster aspartate transcarbamylase of a chimaeric protein carrying an E. coli maltose-binding domain lead to changes in both the kinetics of the enzyme and the quaternary structure of the protein. The \( V_\text{ox} \) for the Asp-90 \( \rightarrow \) Ala and Arg-269 \( \rightarrow \) Ala substitutions is decreased to 1/21 and 1/50 respectively, the \( [S]_{1/2} \) for aspartate is increased 540-fold and 826-fold respectively, and the \( [S]_{1/2} \) for carbamoyl phosphate is increased 60-fold for both. These substitutions decrease the oligomeric size of the protein. Whereas the native chimaeric protein behaves as a pentamer, the Asp-90 variant is a trimer and the Arg-269 variant is a dimer. The altered enzymes also exhibit marked decreases in thermal stability and are inactivated at much lower concentrations of urea than is the unaltered enzyme. Taken together, these results are consistent with the hypothesis that both Asp-90 and Arg-269 have a role in the enzymic function and structural integrity of hamster aspartate transcarbamylase.

INTRODUCTION

The aspartate transcarbamylase (ATCase) (EC 2.1.3.2) of Escherichia coli is an independent, monoenzymic protein composed of two catalytic trimers held together through interactions with three regulatory dimers (2C\(_2\)3R\(_2\)) [1]. In higher eukaryotes the ATCase exists as one domain, corresponding to the catalytic subunit only of the E. coli enzyme, in a multienzymic protein called CAD, the other two enzymic domains being carbamoyl phosphate synthetase II (EC 2.7.2.9) and dihydro-orotase (DHOase) (EC 3.5.2.3). Native CAD is found as multimers of identical subunits, mostly hexamers [2]. X-ray crystallographic analyses [1] and site-directed mutagenesis studies [3] have demonstrated that the active site of the sequence-related E. coli enzyme is formed at the interface of adjacent catalytic subunits. Several residues might interact to hold the catalytic subunits together; one such pair seems to be aspartate-90 and arginine-269. Asp-90 from the C1 subunit interacts with the side chain of Arg-269 from the C2 subunit [4]. An Arg-269 \( \rightarrow \) Ala substitution in the E. coli ATCase led to decreased substrate affinity and disruption in the structural stability of the E. coli enzyme [5]. Substitutions at the Asp-90 position have not yet been reported in the E. coli enzyme. The Arg-269 and Asp-90 residues occur in the sequences of both E. coli [6] and hamster ATCases [7] (For simplicity, the E. coli numbering system is being used for the hamster sequence. Asp-90 and Arg-269 refer to hamster CAD residues Asp-2009 and Arg-2187 respectively from the start codon.) Moreover Asp-90 and Arg-269 are also conserved in the ATCases from more than 20 other species [8], including Serratia marcescens [9], Bacillus subtilis [10], Saccharomyces cerevisiae [11], Pismum sativum [12], Drosophila melanogaster [13], Dictyostelium discoideum [14] and human [15]. We hypothesized that these residues might prove equally important in the structural integrity and enzymic function of the hamster ATCase, even though it is part of a multienzymic protein.

To obtain purified hamster ATCase, the enzyme was over-expressed in E. coli as a chimaeric protein with an E. coli maltose-binding domain and purified by affinity chromatography. A similar system has recently been used to express the catalytic chain of E. coli ATCase [16].

EXPERIMENTAL

Materials

The site-directed mutagenesis kit was purchased from Clontech Laboratories. The protein fusion and purification system was obtained from New England Biolabs. Restriction endonucleases, T4 DNA kinase and T4 DNA ligase were obtained from Gibco BRL. The plasmid purification kit was purchased from Qiangen Inc. The mutagenic oligonucleotides and sequencing primers were made on a 394 Applied Biosystems oligonucleotide synthesizer. 5'-Aspartate, N-carbamoyl-L-aspartate, carbamoyl phosphate, ampicillin, uracil, antipyrimidine, diacetyl monoxime, agarose, Tris, glycerol, isopropyl \( \beta \)-D-thiogalactoside and \(^{14}\)C]Aspartate were obtained from Sigma. Casamino acids, yeast extract and tryptone were obtained from Difco. Electrophoresis-grade acrylamide was purchased from Amresco. Dye terminator cycle sequencing with AmpliTag DNA polymerase was performed on a 373A Applied Biosystems DNA Sequencer (Foster City, CA, U.S.A.). The E. coli pycB strain AT2535 (F\(^{−}\), pycB59, hisG1, purF1, argH1, thi-1, ara-13, lacY1, malA1, mtl-2, rpsL8, 9 or 14, tonA2 or ton-14, tsx-25, \( \lambda^{\lambda}\) λ\(^{−}\), supE44\(^{+}\)) (A. L. Taylor strain obtained from B. Bachman, CGSC4517) was used for complementation.

Abbreviations used: ATCase, aspartate transcarbamylase; DHOase, dihydro-orotase; CAD, tri-enzymic protein with carbamoyl phosphate synthetase II, ATCase and DHOase activities; MBP, Escherichia coli maltose-binding protein; C, catalytic polypeptide chain of E. coli ATCase; R, regulatory polypeptide chain of E. coli ATCase; MBP–ATCase, chimaeric protein containing the E. coli maltose-binding protein and hamster aspartate transcarbamylase.

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Site-directed mutagenesis was performed with pATC21, a plasmid encoding the complete hamster ATCase domain of CAD [17]. The method [18] of mutagenesis was based on simultaneously annealing two oligonucleotide primers to one strand of the denatured double-stranded pATC21. One oligonucleotide primer was for the purpose of selection (loss of a unique CfrI site on the vector sequence with the gain of a unique XhoI site). Another one, the mutagenic primer, encoded the desired mutation. The sequences of the mutagenic primers were:

- 5'-GCC GAA TCC CTT GCC GCC TCT GTG CAG-3' for Asp-90→Ala;
- 5'-CGG ATG CCC GCA GTC AAT GAG-3' for Arg-269→Ala (changes from wild-type sequence are shown in bold). The mutations were confirmed by DNA sequencing with the dyeoxy chain-termination method with the Sequenase 2.0 kit (USB). Genetic complementation was tested by growing the transformed pyrB (ATCase-deficient) E. coli as described previously [19]. The EcoR1–XbaI DNA fragments, which corresponded to the complete hamster ATCase sequence from pATC21 and from the pATC21 derivatives carrying the Asp-90→Ala and Arg-269→Ala mutations, were subcloned into pMAL-c2 [20] downstream and in-frame with the malE gene, which encodes the E. coli maltose-binding protein (MBP). The resulting constructs thus encoded a chimaeric protein with an N-terminal MBP domain followed by a C-terminal ATCase domain.

**Purification of hamster ATCase**

The recombinant plasmids, pMAL-c2-ATCase, pMAL-c2-D90A and pMAL-c2-R269A, were expressed in E. coli strain BL21 (F ompT Rho-) (Novagen). Cells were grown at 37°C overnight in 500 ml minimal M9 medium supplemented with 200 µg/ml ampicillin and 100 µg/ml uracil. The chimaeric protein was induced by adding an additional 500 ml of M9 medium (with 0.3 mM isopropyl β-D-thiogalactoside) at mid-exponential phase. After induction for 3 h, cells were harvested and lysed with a French press. The protease inhibitor 1 mM PMSF was added to all of the buffers for purification. The MBP moiety of the MBP–ATCase chimaera allowed purification to near-homogeneity by amylose-column affinity chromatography, in accordance with the kit instruction manual (New England Biolabs). The one-step purification procedure yielded approx. 4 mg of fusion protein that was approx. 95% pure.

**Determination of protein concentration**

Concentrations of protein were determined by the method of Bradford [21], with the Bio-Rad protein assay dye.

**ATCase assay**

ATCase activity was measured in vivo by either a radioactive [22] or a colorimetric [23,24] method. The conversion of [14C]aspartate to [14C]carbamoyl aspartate was performed in 50 mM Hepes buffer, pH 8.3, at 37°C for 30 min. Aspartate and carbamoyl phosphate concentrations were 1.6 and 2.5 mM respectively. The reaction was quenched by adding 50 µl of 95% (v/v) cold ethanol to the 100 µl reaction; 20 µl of the reaction was separated by cellulose TLC with butanol/acetate/water (60:20:20, by vol.) as solvent. The TLC was exposed to Kodak X-Omat film to locate the radioactivity. The product, [14C]carbamoyl aspartate, was quantified with a scintillation counter. Colorimetric assays were performed in duplicate in 50 mM Tris/acetate buffer, pH 8.3, at 25°C, and the data points in the figures represent the average values.

**Gel electrophoresis**

Purified protein was subjected to SDS/PAGE [8–25% (w/v) gel] [25] or non-denaturing PAGE [4–25% (w/v) gradient gel] [25]. In the non-denaturing electrophoresis system used here, 1 mM maltose was always present. The presence of maltose maintains the MBP domain as a monomer [26]. Non-denaturing electrophoresis was followed by either Coomassie Blue staining or ATCase activity staining [27]. The bands on the non-denaturing polyacrylamide gel were also cut out and separated by SDS/PAGE to check for subunit size [25].

**Thermal stability assays**

Purified enzyme aliquots were incubated for 5 min at different temperatures and chilled quickly on ice for 15 min. Colorimetric assays for ATCase activity were then performed. For the unaltered hamster ATCase, the concentrations of aspartic acid and carbamoyl phosphate were held constant at 0.4 and 0.3 mM respectively; for the altered hamster ATCase, concentrations were held constant at 180 mM aspartic acid and 18 mM carbamoyl phosphate respectively.

**Partial purification of native CAD**

Wild-type Chinese hamster ovary cells (CHO-K1) were harvested and lysed by sonication in 0.02 M Hepes (pH 7.4)/0.05 M KCl/4 mM glutamine/1 mM diethiothreitol/5% (v/v) glycerol/30% (v/v) DMSO, then MgCl₂ was added to a final concentration of 25 mM and the solution was centrifuged at 18000 g for 10 min at 4°C. The supernant was loaded on a 8–30% (v/v) glycerol gradient and centrifuged for 18 h at 194000 g. Fractions were collected and assayed for ATCase activity. Fractions with peak ATCase activity were stored at −70°C. Buffer exchange was performed with a Centricron-30 before the colorimetric assay to fit the same condition as used for MBP–ATCase.

**RESULTS**

**Construction of the mutations by site-directed mutagenesis**

The Asp-90→Ala and Arg-269→Ala changes were introduced into the hamster ATCase by site-specific mutagenesis of plasmid pATC21. PyrB (ATCase-deficient) E. coli was transformed with either mutant or wild-type plasmids. The resulting transformants were capable of growth in the absence of uracil, whereas the parent strain required uracil for growth. Therefore both mutant constructs produced an enzymically active ATCase in vivo. For further analysis the mutations were subcloned into the pMAL-c2 expression vector. The construct sequence actually includes the last 12 amino acid residues of the CAD DHOase–ATCase linker region followed by the entire ATCase sequence [17]. The chimaeric protein synthesized has been named MBP–ATCase. The recombinant plasmids, pMAL-c2-ATCase, pMAL-c2-D90A and pMAL-c2-R269A were checked by complete DNA sequencing of one strand and confirmed to be correct, containing no other mutations.

**Enzyme activity and kinetic properties**

Initial assay of enzyme activity was determined with the radioactive method and crude extracts of the wild-type and mutations in pATC21 (i.e. lacking a maltose-binding domain). The results revealed that with 1.6 mM aspartate and 2.5 mM carbamoyl phosphate the activities of the Asp-90→Ala and Arg-269→Ala enzymes were 5–10% and 2-8%, respectively of that of the normal hamster enzyme (results not shown).
Kinetic studies were performed on the affinity-purified MBP–ATCase chimaeric proteins. The aspartate saturation curves of the normal, Asp-90 → Ala and Arg-269 → Ala enzymes are shown in Figure 1 and the kinetic parameters are summarized in Table 1.

The kinetic data of wild-type MBP–ATCase are very similar to those previously obtained with crude extracts of the wild-type hamster ATCase enzyme [5]. Replacements of Asp-90 and Arg-269 by alanine lead to significant decreases in the affinities for both substrates and in the V_max. In addition, whereas the aspartate saturation curve of normal hamster ATCase shows substrate inhibition at aspartate concentrations above 1.5 mM, neither altered enzyme exhibits substrate inhibition even at 480 mM aspartate. Loss of substrate inhibition was also reported in the Arg-269 → Ala-substituted E. coli enzyme [5].

Electrophoretic mobility

SDS/PAGE analysis revealed that the chimaeric proteins were purified to near-homogeneity, and the subunit sizes were 76 kDa for each (close to the size expected, 35.6 kDa for hamster ATCase plus 42.9 kDa for maltose-binding domain) (Figure 2). On native polyacrylamide gels the chimaeric proteins were separated according to size not charge. The wild-type MBP–ATCase exhibited an apparent size of approx. 400 kDa (predicting at least a pentamer and perhaps a hexamer), whereas the major band for the Asp-90 → Ala-substituted enzyme ran at approx. 240 kDa (predicting a trimer) and the major band for the Arg-269 → Ala-substituted enzyme ran at approx. 160 kDa (predicting a dimer) (Figure 3A). Each major band demonstrated ATCase activity, as shown by the activity staining in Figure 3(B). To determine the subunit size and composition of the major bands, each was excised from the native gel and subjected to SDS/PAGE. Each major band yielded a single band of 76 kDa (results not shown).

Alterations in thermal stability

If Asp-90 and Arg-269 function in the hamster ATCase analogously to the same residues in the E. coli enzyme, then mutations at these codons should lead to enzymes more sensitive to...
Figure 3 Non-denaturing PAGE [4–25% (w/v) gel] of purified MBP–ATCases

The electrophoresis was performed for 16 h at 4 °C. (A) Gel stained with Coomassie Blue. Arrows indicate the major bands for wild-type and altered ATCases. (B) Polyacrylamide gel specifically stained for ATCase activity by precipitating the enzymically released orthophosphate as the insoluble lead salt, followed by conversion to lead sulphide [27]. Arrows indicate the enzymically active proteins.

Figure 4 Thermal stability curves for the wild-type and mutant MBP–ATCases

Purified enzyme was incubated for 5 min at different temperatures and chilled quickly on ice for 15 min, followed by colorimetric assays for ATCase activity. A sample of each enzyme was kept on ice for 20 min before assay; its activity was set at 100% of the specific activity. Results are shown for the wild-type (●), Asp-90 → Ala (▲) and Arg-269 → Ala (■) enzymes.

Figure 5 Influence of urea on enzyme activities of MBP–ATCases

ATCase activity was measured by the radioactive method with different concentrations of urea included in the reactions. Wild-type (●), Asp-90 → Ala (▲) and Arg-269 → Ala (■) enzyme activities are shown.

Effect of urea on enzyme activity

Disruption of the Asp90–Arg269 interaction by replacing one of the residues with alanine might weaken the remaining interactions that hold the catalytic subunits together [5]. If this hypothesis is correct, the altered MBP–ATCases might also be more sensitive to the denaturing action of urea. Both the Asp-90 → Ala and the Arg-269 → Ala enzymes show marked decreases in activity at urea concentrations at which the unaltered enzyme continues to show substantial activity (Figure 5).

DISCUSSION

Effect of maltose-binding domain on MBP–ATCase

Finding a way to study hamster ATCase structure and function with an E. coli expression system proved more difficult than expected. Initially it was thought that these studies could be performed with a bacterial expression vector that encoded a six-histidine-tagged hamster ATCase domain (Y. Qiu and J. N. Davidson, unpublished work). However, such protein appeared as a large aggregate (15–16-mers) on a native gel, a structure inconsistent with the trimers and hexamers usually observed for hamster CAD [2]. The large aggregate is presumed to result from additional non-specific interactions of unknown origin and has been observed when CAD is treated with trypsin [25].

A six-histidine-tagged ATCase with either Asp-90 → Ala or Arg-269 → Ala alterations showed the same size aggregates on native gel electrophoresis as did the wild-type protein, indicating that the non-specific aggregation was due to sites other than the two altered here.

temperature. Figure 4 shows that the altered enzymic forms lost activity at much lower temperatures than did the normal hamster enzyme as the 5 min heat treatment was increased from 37 to 65 °C. The temperature required for the loss of half the enzyme activity decreased from 62.5 °C for the normal enzyme to 46.2 °C for the Asp-90 → Ala enzyme and to 40.5 °C for the Arg-269 → Ala enzyme, consistent with the hypotheses that these residues have a role in holding together the oligomeric structure of the hamster ATCase and that the active site is formed at the interface of adjacent subunits. Moreover the greater sensitivity of the Arg-269 → Ala enzyme than the Asp-90 → Ala enzyme to temperature might reflect its smaller oligomeric size observed with the native gel (Figure 3).
It was thought that linking the hamster ATCase domain downstream of the MBP domain might mimic the carbamoyl phosphate synthetase II and DHaloase domains found in CAD and prevent the non-specific aggregation. This seems to be true, as the large aggregates seen with the six-histidine tag were not observed. The chimaeric MBP–ATCase protein is at least a pentamer, whereas hamster CAD is often found as a hexamer. Given that the multimer size is based on separation according to size not charge, a technique far from the accuracy of SDS/PAGE, it is possible that the MBP–ATCase protein is actually a hexamer.

A pentamer or hexamer of MBP–ATCase was not expected. Previous work with proteolytic fragments of CAD have found that the cleaved DHaloase can form dimers [25,30] and the cleaved ATCase can form trimers [29,30]. This has led to the general assumption that a hexamer of CAD is the result of a DHaloase-mediated dimer of ATCase-mediated trimers. This has never been confirmed experimentally. Moreover, studies from three laboratories have shown that native CAD can be found as monomers, trimers, tetramers, pentamers, hexamers and higher oligomers [2,25,31], a result that leaves open which assumption that a hexamer of CAD is the result of a DHOase-mediated dimer of ATCase-mediated trimers. This has never been confirmed experimentally. Moreover, studies from three laboratories have shown that native CAD can be found as monomers, dimers, trimers, tetramers, pentamers, hexamers and higher oligomers [2,25,31], a result that leaves open which domains are or are not involved in CAD oligomer formation. Hence the pentameric or hexameric structure of MBP–ATCase might or might not reflect the oligomer size mediated by the ATCase in native CAD.

Although it is possible that the MBP domain affects the kinetics or structure of the hamster ATCase in the chimaeric protein, none of the data presented here support this possibility. Because the two amino acid substitutions studied here were generated in the same chimaeric MBP–ATCase protein background, the decrease in the multimeric size of the altered proteins most probably reflects changes in the ATCase subunit interactions.

Another point can be derived by having expressed the MBP–ATCase vectors in E. coli carrying wild-type levels of bacterial ATCase. As can be seen in Figure 2, none of the bacterial catalytic subunit (33 kDa) of ATCase co-purifies with the MBP–ATCase. Thus, even though the hamster and E. coli enzymes are approx. 60% similar [8] in amino acid sequences, there are sufficient differences for these two enzymes not to form heteromultimers.

Asp-90 and Arg-269 are important for enzyme activity and oligomeric structure of hamster ATCase

Our results for the Arg-269 → Ala substitution in hamster ATCase reveal similar changes in kinetic properties and structural stability to those seen for the same residue in the E. coli ATCase. The altered kinetics and structure of the substituted hamster enzymes described here suggest that the salt link between Asp-90 and Arg-269 seen in E. coli is likely to be present in the hamster ATCase and seems to have an important role in oligomer stabilization. The oligomeric structure might be important to the ATCases of all species, if the catalytic site, like that of the E. coli enzyme, is always formed at the interface of adjacent subunits. It remains possible that altering Asp-90 or Arg-269 somehow grossly affects the conformation of the protein and only indirectly leads to the changes observed here. It should be noted that substitutions at some residues nearby were also made. For example, Ser-80, which has been suggested to form another salt linkage between the C1–C2 interface of E. coli ATCase in the T-state (a less active state of the bacterial enzyme and unlikely to be relevant for the hamster ATCase as there is no allosteric subunit), and Ser-79, which is conserved from yeast to human, have been replaced with alanine in the MBP–ATCase vector (Y. Qiu and J. N. Davidson, unpublished work). Neither of the resulting chimaeric proteins exhibits changes in oligomeric structure, although the altered enzymes are observed to have decreased activities compared with the wild-type MBP–ATCase.

The smaller size of the Arg-269 → Ala chimaeric protein compared with Asp-90 → Ala chimaeric protein (Figure 3) suggests that Arg-269 is involved in additional salt linkages that directly or indirectly affect the stabilization of the C1–C2 interaction. Arg-269 has an additional, intrasubunit salt linkage with Asp-278 in the E. coli ATCase. Because neither substitution leads to a monomeric protein, other residues seem to be involved in holding the subunits together.

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