Replacement of Asp-162 by Ala prevents the cooperative transition by the substrates while enhancing the effect of the allosteric activator ATP on *E. coli* aspartate transcarbamoylase

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Abstract

The available crystal structures of *Escherichia coli* aspartate transcarbamoylase (ATCase) show that the conserved residue Asp-162 from the catalytic chain interacts with essentially the same residues in both the T- and R-states. To study the role of Asp-162 in the regulatory properties of the enzyme, this residue has been replaced by alanine. The mutant D162A shows a 7700-fold reduction in the maximal observed specific activity, a twofold decrease in the affinity for aspartate, a loss of homotropic cooperativity, and decreased activation by the nucleotide effector adenosine triphosphate (ATP) compared with the wild-type enzyme. Small-angle X-ray scattering (SAXS) measurements reveal that the unliganded mutant enzyme adopts the T-quaternary structure of the wild-type enzyme. Most strikingly, the bisubstrate analog *N*-phosphonacetyl-L-aspartate (PALA) is unable to induce the T to R quaternary structural transition, causing only a small alteration of the scattering pattern. In contrast, addition of the activator ATP in the presence of PALA causes a significant increase in the scattering amplitude, indicating a large quaternary structural change, although the mutant does not entirely convert to the wild-type R structure. Attempts at modeling this new conformation using rigid body movements of the catalytic trimers and regulatory dimers did not yield a satisfactory solution. This indicates that intra- and/or interchain rearrangements resulting from the mutation bring about domain movements not accounted for in the simple model. Therefore, Asp-162 appears to play a crucial role in the cooperative structural transition and the heterotropic regulatory properties of ATCase.

Keywords: Aspartate transcarbamoylase; small-angle X-ray scattering; allostery; cooperativity; quaternary structural changes

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Abbreviations: ATCase, aspartate transcarbamoylase (carbamoyl phosphate: L-aspartate transferase) from *E. coli* (EC 2.1.3.2.); $[Asp]_{0.5}$, the aspartate concentration at half the maximal observed specific activity; PALA, *N*-(phosphonacetyl)-L-aspartate; 160's loop, loop region in the catalytic chain corresponding to residues 160–166; 240's loop, loop region encompassing residues 230–245 in the catalytic chain; SAXS, small-angle X-ray scattering; holoenzyme, the entire molecule consisting of six catalytic chains and six regulatory chains; c followed by a number, e.g., c1 or c4, refers to a particular catalytic chain.

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The enzyme aspartate transcarbamoylase (ATCase) from *Escherichia coli,* a canonical model system of allosteric regulation (for reviews see Hervé 1989; Kantrowitz and Lipscomb 1990; Lipscomb 1994), catalyzes the first step of the de novo pyrimidine biosynthetic pathway, the condensation of L-aspartate with carbamoyl phosphate to form *N*-carbamoyl-L-aspartate and inorganic phosphate (Jones et al. 1955; Reichard and Hanshoff 1956). ATCase is a highly regulated enzyme with positive homotropic cooperativity for the binding of the substrate L-aspartate, heterotropic activation by adenosine triphosphate (ATP), and inhibition by cytidine triphosphate (CTP), and by uridine triphosphate (UTP) in the presence of CTP (Gerhart and Pardee 1962; Bethell et al. 1968; Wild et al. 1989).

The holoenzyme (306 kD) is composed of two catalytic trimers (102 kD) and three regulatory dimers (34 kD) assembled with approximate D_3 symmetry. Each catalytic chain contains two domains, the aspartate domain and the carbamoyl phosphate domain, the active site being located between the two domains (Monaco et al. 1978; Robey and Schachman 1985; Krause et al. 1987; Wente and Schachman 1987). Two domains are also present in the regulatory chains: the allosteric domain, which contains the nucleotide binding site located some 60 Å away from the nearest active site, and the zinc domain, which is in contact with the catalytic chain. The cooperative and regulatory properties of ATCase have been related to ligand-promoted conformational changes (Gerhart and Schachman, 1968; Monaco et al. 1978; Moody et al. 1979), interpreted for the most part within the framework of the concerted Monod, Wyman, and Changeux (1965) model with two quaternary structures, T and R (Monod et al. 1965). Crystallographic studies (Honzatko et al. 1982; Ke et al. 1984; Krause et al. 1987; Ke et al.1988) revealed that the cooperative transition after binding of various substrate analogs results in an expansion of 10.8 Å along the threefold axis, with a 12° rotation of the catalytic trimers relative to one another around this axis, coupled to a 15° rotation of each of the three regulatory dimers around the twofold axes (Lipscomb 1994). Small-angle X-ray scattering (SAXS) studies showed that the scattering pattern of the holoenzyme, characteristic of the quaternary structure of the enzyme, changes dramatically on substrate binding (Moody et al. 1979; Hervé et al. 1985). Comparison of the experimental SAXS profiles with calculated curves from atomic coordinates showed the existence of large differences between the solution and crystal quaternary structures in the R state (Svergun et al. 1997; Fetler and Vachette 2001). The elongation along the molecular threefold axis is about 2.8 Å larger in solution than in the crystal, accompanied by increased amplitudes of rotation. These large quaternary changes are accompanied by various alterations in the tertiary structure of both the catalytic and regulatory subunits, as observed by X-ray crystallography (Krause et al. 1987; Ke et al. 1988; Jin et al. 1999). More specifically, the cleft between the carbamoyl phosphate and the aspartate domains of the catalytic chains closes and the 240's loop, encompassing residues 230–245, at the c1-c4 interface undergoes a major reorientation. Numerous studies with site-specific mutants have pointed out the importance of this loop in the homotropic cooperative and allosteric properties of the enzyme (Middleton and Kantrowitz 1986; Ladjimi et al. 1988; Middleton et al. 1989; Newton and Kantrowitz 1990; Stebbins et al. 1990), and other studies have shown that its position is directly related to substrate binding in the corresponding active site (Fetler et al. 1995). Close to the 240's loop and the active site, another loop region (161–166) plays a potential role in the internal architecture of the enzyme and its regulatory properties (Newton et al. 1992). Asp-162 interacts with nitrogen atoms of the peptide backbone of Lys-164 and Tyr-165, two residues that have been shown to stabilize the c1-c4 interface through intertrimer interactions with Glu-239 in the T-state and intratrimer interactions with Glu-239 in the R-state (Fig. 1) (Kantrowitz and Lipscomb 1988). The side chain of Asp-162 makes another interaction, in both T- and R-states, with Gln-231, a residue involved in the binding of aspartate. To investigate the functional role of Asp-162, a phylogenetically conserved residue that has no direct catalytic role, it was replaced by an alanine residue (Newton et al. 1992). This D162A mutant shows a major reduction in the maximal observed specific activity, a loss of homotropic cooperativity, an absence of inhibition by CTP and, at variance with former results (Newton et al. 1992), a twofold decrease for aspartate affinity and a twofold reduced activation by ATP. In the absence of additional structural data, earlier results were interpreted assuming the mutant enzyme was in the R-functional state. The low activity, atypical of the R-state, was attributed to a repositioning of the side chains in the immediate vicinity of Asp-162, which could destabilize crucial interactions involving neighboring residues Lys-164, Tyr-165, and Gln-231. In the absence of high resolution atomic coordinates for the D162A mutant, SAXS patterns were recorded for this mutant in the presence of different substrates and/or nucleotide effectors to determine whether the postulated R-functional state in this mutant correlates with an R-structure.

Results and Discussion

X-ray scattering pattern of the unliganded enzyme

The X-ray scattering curves of the wild-type and the D162A enzymes recorded in the absence of ligand are virtually identical, except for a minor difference at the first subsidiary maximum (Fig. 2). Furthermore, the radius of gyration of both enzymes, as derived from a Guinier analysis of small-angle scattering profiles obtained with dilute solutions of

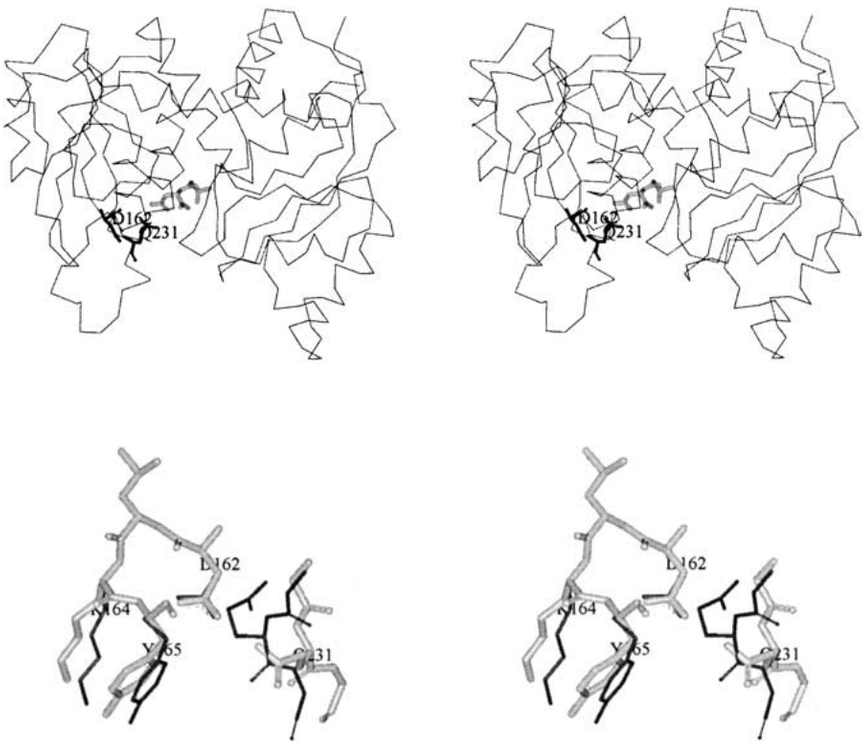

Fig. 1. (*Top*) Stereoview of the α-carbon trace of one catalytic chain in the R-state oriented approximately along a 2-fold axis (from the PALA-bound structure 1D09; PALA is shown in the active site). (*Bottom*) Close-up stereoview of the superimposed Asp-162 region in the T-state (unliganded structure 6AT1; thick gray line) and the R-state (1D09; thin black line). Asp-162 is located in a loop region near the active site and the 240's loop, and it interacts with backbone nitrogens of Lys-164, and Tyr-165, and with the side chain of Gln-231. This figure was drawn with WebLab ViewerLite from Molecular Simulations Inc.

the enzymes, are identical within experimental error. This shows that the unliganded mutant enzyme adopts a quaternary structure very similar, if not identical, to the T-structure of the wild-type enzyme. This result excludes the earlier hypothesis of a mutant enzyme locked in the R-state (Newton et al. 1992). The rationale for this hypothesis relied on the loss of cooperativity, the absence of activation by *N*-(phosphonacetyl)-L-aspartate (PALA), and a high affinity for aspartate. In mutant enzymes that do not show PALA activation, the R-state may be unattainable or the enzyme may already be in the R-state as postulated in the former work. To clarify the apparent discrepancy between the interpretation of the activity measurements and the structural data, activity measurements were repeated. Some differences with the previously published results were observed, especially regarding the $[Asp]_{0.5}$ value that is about 60% larger in the noncooperative mutant than in the wild-type enzyme. This result is consistent with the observation of a T-state conformation for the unliganded mutant enzyme.

X-ray scattering pattern of the PALA-bound enzyme

When present at substoichiometric concentrations, the bisubstrate analog PALA has been shown to activate the wild-type holoenzyme by stabilizing the R-state of the enzyme (Collins and Stark 1971). PALA has a very high affinity for the active site of the wild-type enzyme ($K_D = 27$ nM) (Collins and Stark 1971; Jacobson and Stark 1973). Indeed, the concentration of active sites used in our SAXS experiments is about 2000-fold higher than the dissociation constant of PALA for the wild-type enzyme, assumed to be comparable to that of the mutant enzyme. Therefore, virtually no PALA is left free in solution. It has been shown by sedimentation velocity measurements and SAXS titration studies that the entire enzyme population is in the R-structure when, on average, four out of the six active sites are occupied by PALA (Howlett and Schachman 1977; Fetler et al. 1995). SAXS showed that the half structural transition point corresponds to an average of two of six active sites occupied by PALA (i.e., 33%) (Fetler et al. 1995). In contrast, the presence of a 0.33 molar equivalent concentration of PALA per active site barely alters the SAXS pattern of the mutant enzyme (data not shown). The addition of a twofold molar excess of PALA per active site modifies the X-ray scattering profile of the mutant enzyme as compared with the unliganded enzyme, but to a much smaller extent than the full T to R transition observed with the wild-type enzyme (Fig. 2). Furthermore, the radius of gyration of the mutant

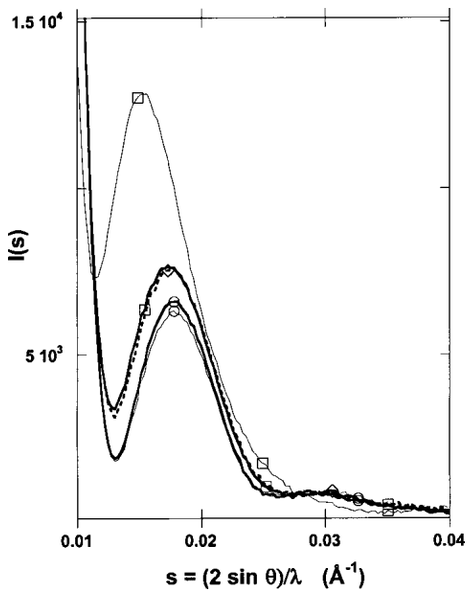

Fig. 2. Solution X-ray scattering spectra of the wild-type and the D162A enzymes. (○, thin line) unliganded wild-type enzyme; (□, thin line) wild-type enzyme in the presence of a twofold molar excess of PALA; (○, heavy line) unliganded D162A enzyme; (□, heavy line) D162A enzyme in the presence of a twofold molar excess of PALA; (◇, heavy discontinuous line) D162A enzyme in the presence of a 10-fold molar excess of PALA.

enzyme increases by only 0.7 Å, as compared with the approximately 3 Å increase observed for the wild-type enzyme under the same experimental conditions (data not shown). To ensure that this result is not caused by a large decrease in the affinity of the mutant enzyme for PALA, the scattering pattern was recorded in the presence of a 10-fold molar excess of the bisubstrate analog. As can be seen in Figure 2, even such a large amount of PALA is unable to convert the D162A mutant to the R-structural state and the corresponding scattering pattern is identical to that obtained in the presence of a twofold molar excess of PALA. These data show that the presence of a very high concentration of PALA, most likely sufficient to saturate all active sites, is unable to convert the entire population of ATCase molecules into the quaternary R-structure as observed for the wild-type enzyme. With the exception of the R105A mutant, which does not bind PALA to the active site (Macol et al. 2001), the D162A enzyme is the first mutant ATCase studied so far to show this behavior (Cherfils et al. 1987; Cherfils et al. 1989; Tauc et al. 1990; Tauc et al. 1994; Baker et al. 1995; Fetler et al. 1995; Baker et al. 1996; Macol et al. 1999). In activity measurements, the bisubstrate analog PALA was unable to activate the D162A mutant (Newton et al. 1992), in contrast with the wild-type enzyme. This result can be explained by the necessity for PALA to occupy all the mutant active sites to promote the limited change in the SAXS pattern, therefore leaving none unoccupied and available for catalysis. Consequently, any further substrate binding is blocked and PALA cannot activate the enzyme. As expected from the failure of PALA to trigger the T to R transition, the addition of other substrates or analogs, such as carbamoyl phosphate and succinate at their respective saturating concentrations of 5 mM and 20 mM (known to convert the wild-type enzyme to the R-structure [Gerhart and Schachman 1968; Fetler et al. 1997]), does not significantly modify the X-ray scattering pattern of the D162A mutant (Fig. 3). More specifically, whereas the binding of the first substrate carbamoyl phosphate causes a small but detectable change very similar to that reported with the wild-type enzyme (Fetler et al. 1997), further addition of succinate appears to have no additional effect.

Is the change observed on PALA binding indicative of a new quaternary structure of the mutant enzyme or of an equilibrium between the T and R quaternary structures of the wild-type enzyme? In the latter case, the scattering pattern of the mutant enzyme in the presence of PALA should be a linear combination of the T and R wild-type patterns (Materials and Methods). However, no satisfactory fit to the experimental pattern can be obtained in this way. Thus, the PALA-bound mutant either adopts a conformation different from both known T- and R-structures or it is in an equilibrium between the T-structure and a new quaternary structure, a possibility that will be discussed in the next section.

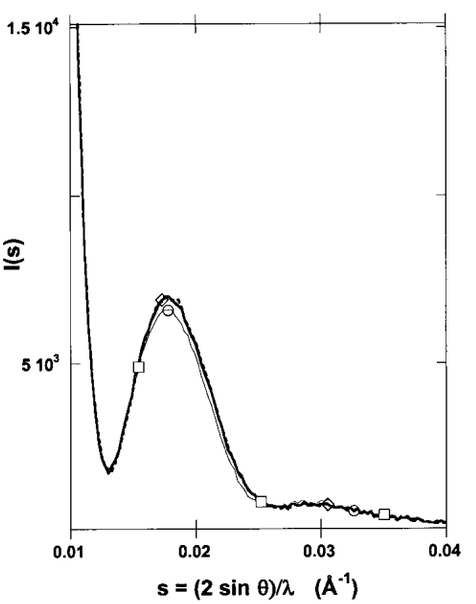

Fig. 3. Solution X-ray scattering curves the D162A enzyme. (○, thin line) unliganded; (◇, heavy line) in the presence of a 5 mM carbamoyl phosphate; (□, heavy discontinuous line) in the presence of a 5 mM carbamoyl phosphate and 20 mM succinate.

Effect of nucleotide effectors on the quaternary structure

SAXS measurements showed that the nucleotide activator ATP only induces a small increase of the first subsidiary minimum of the scattering profile of the wild-type enzyme, which has recently been shown to be a direct contribution of bound ATP to the scattering intensity with no conformational change of the enzyme (Fetler and Vachette 2001). Crystallographic studies revealed an apparent 0.5 Å separation of the wild-type catalytic trimers along the C3 axis of symmetry (Stevens et al. 1990) at the detection limit of SAXS measurements. In the presence of ATP, a significant intensity increase in the first subsidiary minimum and maximum of the scattering profile of the D162A enzyme can be observed (Fig. 4), which is at variance with the results obtained with the wild-type enzyme and the various mutants studied so far. Indeed, the scattering curves of the D162A enzyme in the presence of ATP alone or a twofold molar excess of PALA per active site are quite similar (Fig. 5). This observation must be related to the recent activity measurement data, showing that ATP can activate the mutant enzyme to some extent (Table 1). Hence, this newly observed structure, although different from the R-structure, must have some improved affinity and/or activity for the substrates. Because the mutant does not show any cooperativity for aspartate as judged from activity measurements, this conformation is likely to remain inaccessible even in the presence of the natural substrates carbamoyl phosphate and aspartate, as observed with carbamoyl phosphate and succinate. This could be determined experimentally but because of the consumption of substrates by the enzyme, it would require time-resolved SAXS measurements in which the enzyme is rapidly mixed with the substrates in the presence of ATP and the transient steady-state conformation is recorded (Tsuruta et al. 1998). However, such experiments require a higher flux than that available with the instrument used here.

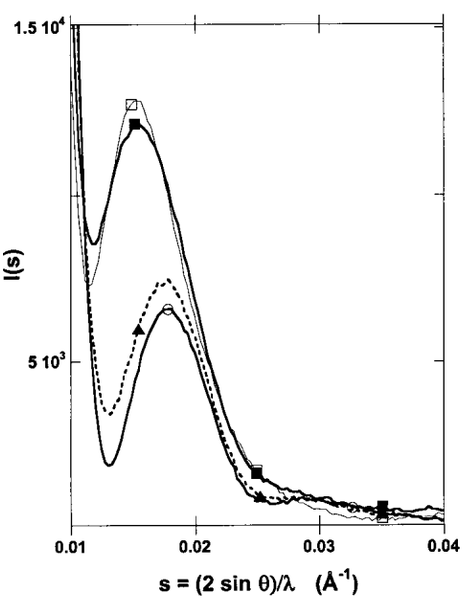

Fig. 4. Solution X-ray scattering spectra of the wild type and the D162A enzymes. (□, thin line) wild-type enzyme in the presence of a twofold molar excess of PALA; (○, heavy line) unligated D162A enzyme; (▲, discontinuous heavy line) D162A enzyme in the presence of 5 mM ATP; (■, heavy line) D162A enzyme in the presence of a twofold molar excess of PALA and 5 mM ATP.

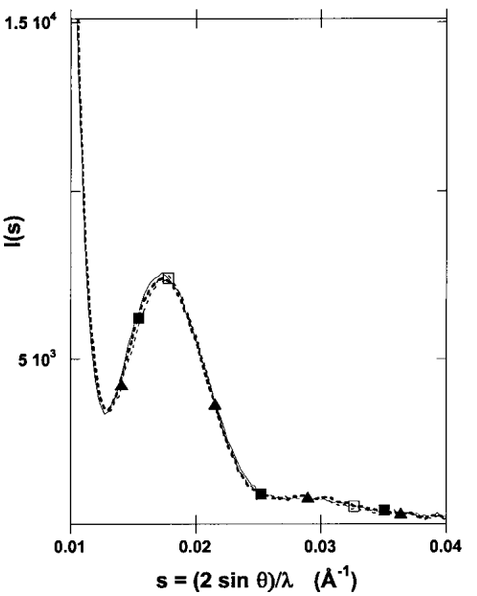

Fig. 5. Solution X-ray scattering spectra of the D162A enzyme. (□, thin line) D162A enzyme in the presence of a twofold molar excess of PALA; (▲, discontinuous thin line) D162A enzyme in the presence of 5 mM ATP; (■, discontinuous heavy line) linear combination of 80% mutant T and 20% mutant R-like (PALA + ATP) scattering patterns.

Table 1. *Kinetic parameters for the wild-type and mutant holoenzymes*

Enzyme	Maximal velocity (mmole/h/mg)	$[Asp]_{0.5}$ (mM)	n_H^{Asp}	% ATP activation[a]	% ATP activation[a] + 2 μM PALA
Wild-type	18.6	10.9	2.3	437	116
D162A	0.0024	19.9	1.0	227	243

Kinetic parameters were derived from the saturation curves for the wild-type and the mutant holoenzymes. Assays were performed at 25°C in 50 mM Tris acetate buffer (pH 8.3). Maximal velocity and Hill coefficients were calculated by a nonlinear least square procedure with a modified Hill equation that incorporates a term for substrate inhibition (Pastra-Landis et al. 1978).

[a] Percent ATP activation is defined as 100 (A_{ATP}/A), where A_{ATP} is the activity in the presence of saturating ATP, and A is the activity in the absence of ATP. (ATP) Adenosine triphosphate; (PALA) *N*-(phosphonacetyl)-L-aspartate.

In contrast with the limited structural changes reported above, the addition of both a twofold molar excess of PALA per active site and ATP significantly modifies the scattering profile (Fig. 4). The corresponding X-ray scattering pattern is much closer, although different, to that of the wild-type PALA-ligated R-structure than the scattering pattern obtained in the presence of PALA or ATP alone. This scattering profile is not a linear combination of the wild-type T and R quaternary structures. However, it is interesting to note that the solution scattering pattern obtained in the presence of ATP or saturating PALA alone can be approximated by a linear combination of the unliganded mutant pattern (80%) and the profile in the presence of both PALA and ATP (20%) (Fig. 5). Therefore, in the presence of a single ligand (ATP or PALA), the mutant enzyme appears to be in an equilibrium between the T quaternary structure and the conformation it adopts when both ligands are bound. Furthermore, the scattering patterns in the presence of ATP and the substrate analogs carbamoyl phosphate, either alone or in the presence of 20 mM succinate, do not show any difference as compared with the scattering pattern of the D162A enzyme in the presence of ATP alone (data not shown).

To complement these structural observations, ATP activation was measured with the wild-type and the D162A enzyme in the presence or absence of PALA. For the wild-type enzyme, the more than fourfold activation by the nucleotide activator ATP vanishes in the presence of 2 μM PALA (Table 1). This is interpreted as indicating that PALA binding was sufficient to convert the enzyme to the active R-state, with no further activation left for ATP to perform. Under the same conditions, the mutant enzyme behaves much differently, because a twofold activation by ATP is observed regardless of the presence of PALA. However, the concentration of PALA used, which had to be kept low enough to leave free active sites for substrates to bind and the reaction to occur, was probably insufficient to alter the scattering pattern. Therefore, the apparent inconsistency between structural and activity observations may be attributable to this experimental limitation.

A modeling procedure using rigid body movements has already been used to propose a model for the quaternary structure of the R-state of the enzyme in solution (Svergun et al. 1997) and, more recently, for the conformation of the ternary complex of the enzyme with PALA and Mg-ATP (Fetler and Vachette 2001). The same approach was applied here to the conformation of the mutant enzyme complexed with PALA and ATP. However, no satisfactory model could be obtained by this simple procedure. The failure of our modeling attempt may be caused by intra- and/or interchain rearrangements following the mutation, which would lead to domain movements not accounted for in our scheme based on the wild-type T to R quaternary structure transition. Previous computer simulations of the energy-minimized D162A enzyme in the T- and R-states showed that the side chain of Gln-231 is able to move into the space once occupied by the side chain of Asp-162 (Newton et al. 1992). This reorientation of Gln-231, preventing its interaction with aspartate, could be responsible for the reduced maximal observed specific activity. The discrepancies between the calculated and the experimental scattering patterns of the mutant enzyme in the presence of ATP and PALA indicate that the perturbations induced by the mutation affects other residues in this critical part of the molecule close to the r1-c1, r1-c4, and c1-c4 interfaces, which are reorganized in the quaternary structure transition. From the scattering pattern of the unliganded mutant enzyme, it appears that the mutation does not affect the T conformation. In contrast, it severely affects the quaternary structure transition because PALA has a very limited effect and even the combined action of PALA and ATP cannot convert the enzyme into the wild-type R conformation. This is most likely attributable to modifications to one or several of the above mentioned interfaces. Only a high-resolution study of the ternary complex of the mutant enzyme with PALA and ATP could give some insight into the mechanism underlying the crucial role played by Asp-162 in the stability of the quaternary structure of ATCase and, most importantly, its central involvement in the transition into the R structure shown by our solution studies.

Materials and methods

Chemicals

Q-Sepharose Fast Flow resin was purchased from Pharmacia. L-aspartate, adenosine triphosphate and cytidine triphosphate (all sodium salts), ampicillin, potassium dihydrogen phosphate, and uracil were purchased from Sigma Chemical Company. Tris(hydroxymethyl)aminomethane (Tris) and magnesium chloride were from Merck. Carbamoyl phosphate dilithium salt, obtained from Sigma, was further purified by precipitation from 50% (v/v) ethanol and was stored dessicated at −20°C. PALA was a generous gift from Drs. V. Narayanan and L. Kedda of the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, NIH, Silver Spring, Maryland.

Enzyme preparation and assay

The wild-type and the D162A mutant ATCase were isolated from the *E. coli* strain EK1104 (F-*ara*, *thi*, Δ*pro-lac*, Δ*pyrB*, *pyrF*±, and *rpsL*) containing the plasmids pEK2 (Smith et al. 1986) and pEK141 (Newton et al. 1992), respectively. The holoenzymes were purified to electrophoretic homogeneity essentially as described by Nowlan and Kantrowitz (Nowlan and Kantrowitz 1985), except that an additional isoelectric precipitation step was used.

The ATCase activity was measured at 25°C by a colorimetric method (Prescott and Jones 1969; Pastra-Landis et al. 1981). Colorimetric assays were performed in 50 mM Tris-acetate buffer, pH 8.3. The nucleotide assays were performed at half the $[Asp]_{0.5}$

value. The analysis of steady-state kinetic data was performed as previously described (Silver et al. 1983). Fitting of the experimental data to theoretical equations was accomplished by nonlinear regression. When substrate inhibition was negligible, data were fit to the Hill equation. When substrate inhibition was significant, data were analyzed using an extension of the Hill equation that included a term for substrate inhibition (Pastra-Landis et al. 1978). The nucleotide saturation curves were fit to a hyperbolic isotherm by nonlinear regression.

Solution X-ray scattering

X-ray scattering curves were recorded on the small-angle scattering instrument D24 using synchrotron radiation at LURE-DCI, Orsay. The instrument, the data acquisition system (Boulin et al. 1986), and the experimental procedures (Fetler et al. 1995) have already been described. The purified enzyme solution was buffered with 50 mM Tris-HCl at pH 8.3 and contained 0.1 mM dithiothreitol, a strong reducing agent. The samples for X-ray scattering experiments were prepared to a final protein concentration of 50 $mg.mL^{-1}$, except for the smallest angle data recorded at 5 $mg.mL^{-1}$. The concentration of protein was determined by absorbance measurements at 280 nm using an extinction coefficient of 0.59 $cm^2.mg^{-1}$ (Gerhart and Holoubek 1967).

The intensity scattered by a noninteracting solution of protein is the sum of the intensities of all individual particles. In the case of a mixture of different particles, the resulting intensity is the sum of the contributions of each particle weighted by their fractional concentrations. In the present work, our samples only contain ATCase, which can adopt different quaternary structures (e.g., the T- and R-state) with different associated scattering patterns. If a solution contains a mixture of two quaternary structures, its scattering pattern will therefore be a linear combination of the individual patterns weighted by the respective fractional concentrations.

The modeling procedure using rigid body movements of the subunits has already been presented (Fetler and Vachette 2001).

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