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Amino acid substitutions which stabilize aspartate transcarbamoylase in the R state disrupt both homotropic and heterotropic effects

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We have used site-specific amino acid substitutions to investigate the linkage between the allosteric properties of aspartate transcarbamoylase and the global conformational transition exhibited by the enzyme upon binding active-site ligands. Two mutationally altered enzymes in which an amino acid substitution had been introduced at a single position in the catalytic polypeptide chain (Lys-164 → Glu and Glu-239 → Lys) and a third species harboring both of these substitutions (Lys-164 : Glu-239 → Glu : Lys) were constructed. Sedimentation velocity difference studies were performed in order to assess the effects of the amino acid substitutions on the quaternary structure of the holoenzyme in the absence and presence of various active-site ligands, including the bisubstrate analog, *N*-(phosphonacetyl)-L-aspartate (PALA), which has been shown previously to promote the allosteric transition. *In the absence of ligand*, two of the mutationally altered enzymes, Lys-164 → Glu and Lys-164 : Glu-239 → Glu : Lys, existed in the R conformation, isomorphous with that of the PALA-liganded wild-type holoenzyme. These enzymes exhibited no conformational change upon binding PALA. The unliganded Glu-239 → Lys enzyme had an average sedimentation coefficient intermediate between that of the unliganded and PALA-liganded states of the wild-type enzyme which could be accounted for in terms of a mixture of T- and R-state molecules. This mutant enzyme was converted to the fully swollen conformation upon binding PALA, phosphate or carbamoyl phosphate. The allosteric properties of the mutationally altered species were investigated by PALA-binding studies and by steady-state enzyme kinetics. In each case, the mutationally altered enzymes were devoid of both homotropic and heterotropic effects, supporting the premise that the allosteric properties of the wild-type enzyme are linked to a ligand-promoted change in quaternary structure.

1. Introduction

Allosteric interactions in oligomeric proteins are ubiquitous and often play crucial roles in the regulation of metabolism. The molecular mechanisms by which these interactions occur are therefore of considerable interest and have been the focus of intensive experimental and theoretical investigation. Although it is accepted that allosteric linkage generally arises from ligand-promoted conformational changes in the macromolecule, in most systems considerable uncertainty remains about the molecular details of the process. One particularly well-characterized allosteric enzyme is ATCase from *Escherichia coli* (ATCase, carbamoylphosphate : L-aspartate carbamoylphosphate transferase, EC 2.1.3.2) which catalyzes the condensation of aspartate and carbamoyl phosphate to yield carbamoyl aspartate and inorganic phosphate, the first committed step in pyrimidine biosynthesis. ATCase displays both homotropic properties – cooperatively with respect to the concentrations of both substrates [1,2] – and hetero-

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Abbreviations: ATCase, aspartate transcarbamoylase; C, catalytic subunit; R, regulatory subunit; PALA, *N*-(phosphonacetyl)-L-aspartate; Mops, morpholinopropanesulfonic acid. Amino acid substitutions are designated by the original amino acid (standard three-letter abbreviation) and the position in the amino acid sequence [46] followed by an arrow and the substituted amino acid.

tropic properties – activation by ATP and inhibition by CTP [1]. Analogous allosteric effects have been demonstrated in the binding of the bisubstrate analog *N*-(phosphonacetyl)-L-aspartate (PALA) to ATCase [3]. The holoenzyme is composed of two trimeric catalytic (C) and three dimeric regulatory subunits (R) [4–8]. Isolated C subunits are catalytically active but display neither homotropic nor heterotropic effects whereas the R subunits bind the nucleotide effectors but exhibit no catalytic activity [4,7].

ATCase undergoes a global conformational change upon the binding of substrates and/or substrate analogs. This increase in hydrodynamic volume was demonstrated initially by the ligand-promoted decrease in the sedimentation coefficient [9] and subsequently confirmed by a variety of techniques, including X-ray crystallography [10] and low-angle X-ray scattering [11]. Significant effort has been directed at investigating the relationship between this observable conformational transition and the homotropic and heterotropic properties of the enzyme. The results of these studies generally support the premise that this global conformational isomerization serves as the mechanism by which the allosteric effects are transmitted. Nevertheless, some crucial predictions of this proposition have remained refractory to direct experimental verification. In particular, a direct and convincing demonstration that the observed structural alteration is *required* for the enzyme to exhibit allosteric behavior has been lacking. Toward this end, we have used techniques for introducing site-specific amino acid substitutions into proteins in order to construct mutant forms of ATCase unable to undergo the allosteric transition. Studies on these mutationally altered enzymes demonstrate that they are devoid of the allosteric properties characteristic of the wild-type enzyme.

2. Rationale for amino acid substitutions

One may conceive of several different mechanisms by which the introduction of site-specific amino acid substitutions could disrupt the capacity of the enzyme to exhibit the characteristic conformational isomerization upon binding ligand. In this study, we used amino acid replacements in order to promote the isomerization of the unliganded enzyme into the more swollen conformation adopted by the liganded wild-type enzyme (referred to as the R conformation). Such an enzyme, existing in the R conformation in the absence of ligand, would be expected to exhibit no global change in quaternary structure upon binding ligands which promote the allosteric transition of the wild-type enzyme. If the transmission of allosteric effects in ATCase is mediated by the global conformational isomerization, the altered enzyme would not display the allosteric properties characteristic of the wild-type enzyme.

Since the objective for introducing amino acid substitutions into the enzyme was to alter the structure of the enzyme from the compact T state adopted by the wild-type enzyme in the absence of ligand to the more swollen R conformation, the principal consideration in the choice of amino acid replacements was that they be more easily accommodated in the R conformation than in the T. The choice of substitutions was based on structural information from X-ray crystallographic studies of the unliganded T and PALA-liganded R states of ATCase [10,12,13] *. Gross features of these two forms of the enzyme are illustrated in fig. 1. In the absence of PALA, the assembly of the holoenzyme from two trimeric C subunits and the three dimeric R subunits involves three classes of subunit interactions: two sets of contacts between C and R subunits, the c1-r1 and c1-r4 interfaces, and one set of contacts between apposing C subunits, the c1-c4 interface. The transition to the PALA-liganded R state involves a separation of the two apposing C subunits by approx. 12 Å along the three-fold axis of symmetry and is associated with significant alterations of the interactions between subunits. Although the extensive

* The atomic coordinates upon which descriptions of these structures are based are available only for the T state of the enzyme. Coordinates for the PALA-ligated R state were not available through the Brookhaven protein data bank at the time this manuscript was submitted and our descriptions of this structure therefore rely instead on the numerous published accounts [10,13,42,47–49].

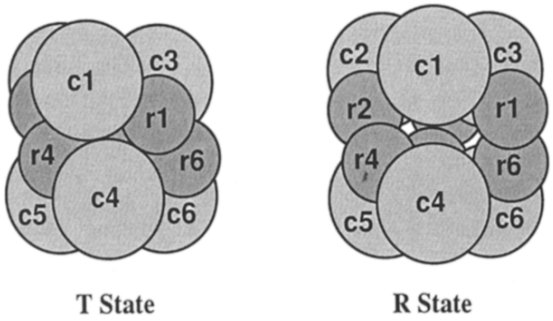

Fig. 1. A schematic view of the subunit structure of wild-type ATCase in the T and R conformations. The relatively compact T conformation is on the left with the more swollen R conformation showing the separation of the two C trimers on the right. Although the folding of the polypeptide chains leads to identifiable domains, they are depicted here simply by spheres so as to illustrate the interface regions.

c1-r1 interface is largely preserved in the transition to the swollen conformation, the c1-c4 and c1-r4 contacts are virtually abolished [13,14]. It therefore seems reasonable to implicate the c1-c4 and c1-r4 interfaces in stabilizing the compact T conformation relative to the R structure. Among the contacts at the c1-c4 interface of the wild-type T state enzyme is an apparent salt-bridge between Lys-164 in one C subunit and Glu-239 in the apposing C subunit (fig. 2). This salt-bridge between c1 and c4 is absent in the PALA-liganded R state structure; instead, these residues form a salt-bridge within the same polypeptide chain. Thus, the salt-bridge is converted from an inter- to an intrasubunit interaction in the transition of

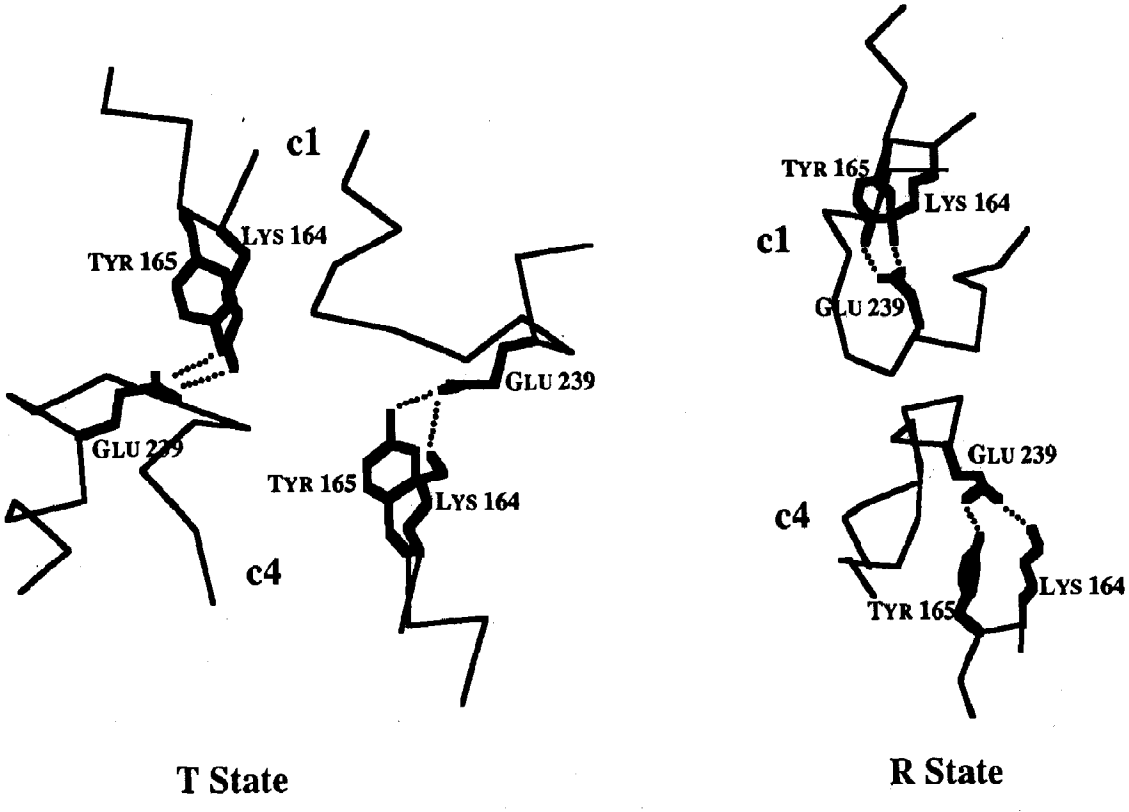

Fig. 2. Rearrangements at the c1-c4 interface accompanying the allosteric transition. Portions of the α-carbon backbone are represented by the lighter segments and the positions of the non-hydrogen atoms of the side chains of the three designated amino acid residues are connected by the darker segments (adapted from ref. 40).

ATCase from the T to the R state. Replacement of Lys-164 or Glu-239 with an amino acid of opposite charge would preclude the formation of this salt-bridge in either state, and hence presumably destabilize both states of the enzyme. However, the salt-bridge appears to be more accessible to solvent in the R state which might allow for some solvation of the unpaired charges. Hence, it is possible that such amino acid substitutions might preferentially destabilize the T state of the enzyme, thereby promoting its conversion to the more-swollen R conformation. We therefore constructed one altered enzyme in which Lys-164 was replaced by Glu (Lys-164 → Glu), and a second in which Glu-239 was replaced by Lys (Glu-239 → Lys). We also constructed a double mutant form of the enzyme harboring both of these amino acid substitutions (Lys-164 : Glu-239 → Glu : Lys).

3. Experimental

3.1. Chemicals and buffers

Dilithium carbamoyl phosphate was from Boehringer Mannheim (F.R.G.) and dilithium [^{14}C]carbamoyl phosphate was from New England Nuclear. *N*-(Phosphonacetyl)-L-aspartate (PALA) was a generous gift from Dr Jefferson Foote. ATP and CTP were obtained as the potassium salts from Sigma, Mops from Serva, and neohydrin from K&K Labs. Assays were performed in either Mops buffer (50 mM Mops, 0.2 mM EDTA, 2 mM 2-mercaptoethanol, pH adjusted to 7.0 with KOH) or phosphate buffer (40 mM potassium phosphate, 0.2 mM EDTA, 2 mM 2-mercaptoethanol, pH 7.0).

3.2. Site-directed mutagenesis

Oligonucleotides used in the construction of the altered enzymes were as follows (with mismatched nucleotides in bold-face type): Lys-164 → Glu, 5′-CCATATTCCAGATCACC-3′; Glu-239 → Lys, 5′-CGTACTTGGACGGATCCAG-3′. In addition to providing for the desired amino acid substitutions, the oligonucleotides were designed to generate altered restriction site patterns, the removal of a *Bst*EII site in the first case and the introduction of a *Bam*HI site in the second. The template plasmid M13mp8 PYRB2 [15] was isolated from *E. coli* strain RZ1032 in the single-strand form as described [16]. The mutagenesis reactions were performed according to the procedure of Zoller and Smith [17] and the reaction mixtures were transformed into the *E. coli* strain JM103 [18]. Colonies were screened for the desired sequence alterations by the dideoxynucleotide method [19]. Fragments of the *pyr*B gene harboring the desired alterations were subcloned into the expression vector pPYRB11 [20] to create plasmids pJN1 (Lys-164 → Glu) and pJN2 (Glu-239 → Lys), which were subsequently transformed into *E. coli* strain HB101. A plasmid containing the sequence alterations introduced by both mutagenic oligonucleotides, pJN3 (Lys-164 : Glu-239 → Glu : Lys), was constructed by joining the appropriate regions of pJN1 and pJN2. The plasmids pJN1-3 were subsequently transformed into *Salmonella typhimurium* strain TR 4574 from which the chromosomal *pyrBI* genes are deleted [21] and which harbors the pyrH700 allele, causing enhanced expression from the plasmid *pyrBI* promoter [22,23].

3.3. Purification of wild-type and mutationally altered holoenzymes and C subunits

The wild-type and mutationally altered versions of ATCase were purified from *S. typhimurium* strain TR 4574 as described by Gerhart and Holoubek [24] and Wall et al. [25], respectively. The catalytic subunits were isolated as described by Yang et al. [26].

3.4. Experimental procedures

Enzyme activities were assayed at 30 °C by the method of Davies et al. [27] in Mops buffer. PALA-binding isotherms were determined by equilibrium dialysis at 23 °C as described by Newell et al. [3] in standard phosphate buffer.

Sedimentation velocity difference experiments were performed with a Beckman-Spinco model E ultracentrifuge at 20 ± 2 °C as described by Howlett and Schachman [28]. The experiments were

performed at 3.0 mg/ml enzyme in either Mops or phosphate buffer. In all of the sedimentation velocity experiments, the observed difference between the sedimentation coefficients of the unliganded and liganded enzymes ($\Delta s/s$) were corrected to compensate for the effects of the ligand on the mass and density of the sedimenting species [28]. The corrected values of $\Delta s/s$ therefore reflect only alterations in the frictional properties of the enzyme resulting from ligand binding.

3.5. Data analysis

PALA-binding data sets for the mutationally altered holoenzymes and C subunits were analyzed by nonlinear least-squares methods in terms of the equation describing binding to an unknown number of identical and noninteracting sites in order to determine dissociation constants (K_d) and the numbers of binding sites per enzyme molecule (n). Data sets from the steady-state kinetic assays of the mutationally altered holoenzymes and for the wild-type and altered C subunits were analyzed in an analogous manner to derive values of the Michaelis constants (K_m) and maximal velocities (V_{max}). The steady-state kinetic saturation curves for the cooperative wild-type holoenzyme were analyzed by standard graphical methods to derive the substrate concentrations at half-maximal velocity ($K_{0.5}$), the Hill coefficients (n_H), and the maximal velocities (V_{max}) [29].

4. Results

4.1. Amino acid substitutions preferentially stabilize the R conformation even in the absence of active-site ligands

The effects of the amino acid substitutions on the quaternary structure of the mutant enzymes were investigated by difference sedimentation velocity experiments. As shown previously [28], there is a substantial *decrease* in the sedimentation coefficient of wild-type ATCase upon binding PALA. This decrease, represented by a value of -3.8% for $\Delta s/s$, corresponds to the conversion of the enzyme from the compact T state to the more swollen R conformation. Titration curves showing $\Delta s/s$ as a function of PALA/ATCase are shown in fig. 3 for wild-type ATCase and the three mutant holoenzymes *. The conformational swelling exhibited by the wild-type enzyme upon binding PALA was either substantially reduced or abolished in the altered species. In fact, only Glu-239 → Lys exhibited any significant PALA-promoted conformational change; the decrease in the sedimentation coefficient at 10 PALA/enzyme was -1.3% (table 1), approximately one-third of that exhibited by the wild-type holoenzyme. In contrast, the values of $\Delta s/s$ at 10 PALA/enzyme determined for the enzymes Lys-164 → Glu and Lys-164 : Glu-239 → Glu : Lys were insignificant, $+0.1$ and -0.4%, respectively.

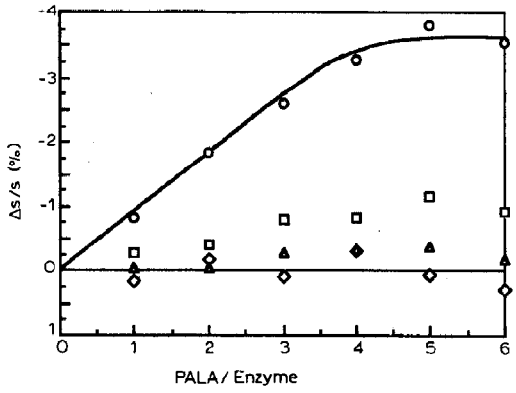

Fig. 3. Effects of PALA on the sedimentation coefficients of the wild-type and mutationally altered holoenzymes. Experiments were performed in Mops buffer as described (section 3.4) and the results are presented as the percentage change in the sedimentation coefficient (adjusted to offset the effects of bound ligand on the mass and density of the enzyme as described [28]) vs the molar ratio of PALA/enzyme. (○) Wild-type ATCase, (△) Lys-164 → Glu, (□) Glu-239 → Lys and (◇) Lys-164 : Glu-239 → Glu : Lys.

Although the results in fig. 3 indicate that PALA has little or no effect on the sedimentation coefficient of the mutant forms of ATCase, they do not in themselves indicate whether the enzymes

* The concentration of binding sites in these sedimentation experiments is at least 75-fold greater than the dissociation constants determined for each holoenzyme. Therefore, under these conditions, PALA is bound to the active sites virtually stoichiometrically, and the abscissa in fig. 3 represents the average number of bound PALA molecules per enzyme.

Table 1

Effect of active-site ligands on the sedimentation coefficient of wild-type and mutationally altered holoenzymes

Sedimentation velocity difference experiments were performed and parameters were determined as described in section 3.4. Each value of $\Delta s/s$ is the average of three independent determinations. The average standard deviation among triplicates was 0.1%.

	$(\Delta s/s)^{a}_{corr}$ (%)			$(\Delta s/s)_{corr}$ (%)
	Mops	Mops + CbmP[b]	Phosphate	Mutant + PALA vs wild-type + PALA
Wild-type	−3.8	−3.0	−3.3	
Lys-164 → Glu	+0.1	−0.1	+0.6	+0.1
Glu-239 → Lys	−1.3	−0.2	−0.1	−0.1
Lys-164 : Glu-239 → Glu : Lys	−0.4	−0.1	−0.2	−0.1

[a] Values of $\Delta s/s$ in % correspond to the change caused by the addition of 10 [PALA]/[enzyme] to one sample compared to the unliganded enzyme.

[b] Experiments were performed with 1 mM carbamoyl phosphate present in each cell.

are in the T or R conformations. It is possible that the enzymes are in the R conformation even when unliganded, or alternatively, they are in the T state and the binding of PALA is insufficient to promote their conversion to the R conformation. To differentiate between these two alternatives we compared directly the sedimentation coefficient of each PALA-liganded mutant enzyme with that of liganded wild-type ATCase which is known to be in the R state. As seen in table 1, the sedimentation coefficients of the wild-type and mutationally altered enzymes were virtually indistinguishable at 10 PALA/enzyme ($\Delta s/s$ approx. ± 0.1%). Hence, the amino acid substitutions have no significant effect on the quaternary structure of the PALA-liganded enzyme. Therefore, the sedimentation velocity experiments for both the Lys-164 → Glu and the Lys-164 : Glu-239 → Glu : Lys enzymes indicate that these *unliganded* mutant enzymes have quaternary structures virtually identical to that of *liganded* wild-type ATCase. In contrast, the *average* quaternary structure of the unliganded Glu-239 → Lys enzyme is intermediate between those of the T and R conformations of wild-type ATCase.

Since the objective of these studies was to correlate the effects of the amino acid substitutions on the structural and allosteric properties of ATCase, it was important that the experimental conditions for the various studies of the mutationally altered enzymes be comparable. However, the PALA-binding studies were performed in the presence of phosphate, a product of the enzymatic reaction, and the kinetic studies were performed in the presence of the substrate carbamoyl phosphate. Given that both phosphate and carbamoyl phosphate bind to ATCase, it is possible that they could alter the quaternary structure of the mutationally altered enzymes. We therefore measured the effects of these ligands on the magnitude of the $\Delta s/s$ values caused by the addition of 10 PALA/enzyme in the presence of either phosphate or carbamoyl phosphate. Due to its extremely tight binding to ATCase, PALA will displace phosphate or carbamoyl phosphate from the active sites. Therefore, differences in the magnitude of the PALA-promoted $\Delta s/s$ in the presence and absence of either phosphate or carbamoyl phosphate would be attributable to effects of bound phosphate or carbamoyl phosphate on the conformation of the enzyme. The results of these experiments are given in table 1. The two mutationally altered species which exhibited no apparent PALA-promoted conformational change in the absence of any additional ligand (Lys-164 → Glu and Lys-164 : Glu-239 → Glu : Lys) also exhibited no significant $\Delta s/s$ upon the addition of PALA in the presence of phosphate or carbamoylphosphate. Thus, the global conformations of these two enzymes were not discernably influenced by the presence of carbamoyl phosphate, phosphate or PALA. The holoenzyme Glu-239 → Lys, which exhibited a significant conformational change upon binding PALA in the ab-

sence of any other ligand, displayed no detectable PALA-promoted $\Delta s/s$ in the presence of phosphate or carbamoyl phosphate. Thus, the addition of carbamoyl phosphate, phosphate, or PALA was sufficient to convert this enzyme to a conformation isomorphous with that of the PALA-liganded wild-type enzyme.

4.2. PALA binding to the mutationally altered enzymes

The PALA-binding isotherms of the mutationally altered holoenzymes were determined in phosphate buffer both in the presence and absence of the nucleotide effectors. In contrast to the results obtained with the wild-type holoenzyme (described by Newell et al. [3] and included in fig. 4 and table 2 for comparison), the mutationally altered holoenzymes exhibited neither any apparent cooperativity nor any sensitivity to the nucleotide effectors (fig. 4). Scatchard plots of the binding data provide very sensitive graphical indicators of cooperativity, since even a very slight degree of positive cooperativity in binding results in a concave curvature of the data with respect to the abscissa [30]. In contrast to the Scatchard plot of the binding data for the wild-type holoenzyme which exhibits obvious curvature in both the absence (inset in fig. 4a) and presence (not shown) of nucleotides, no cooperativity was apparent in

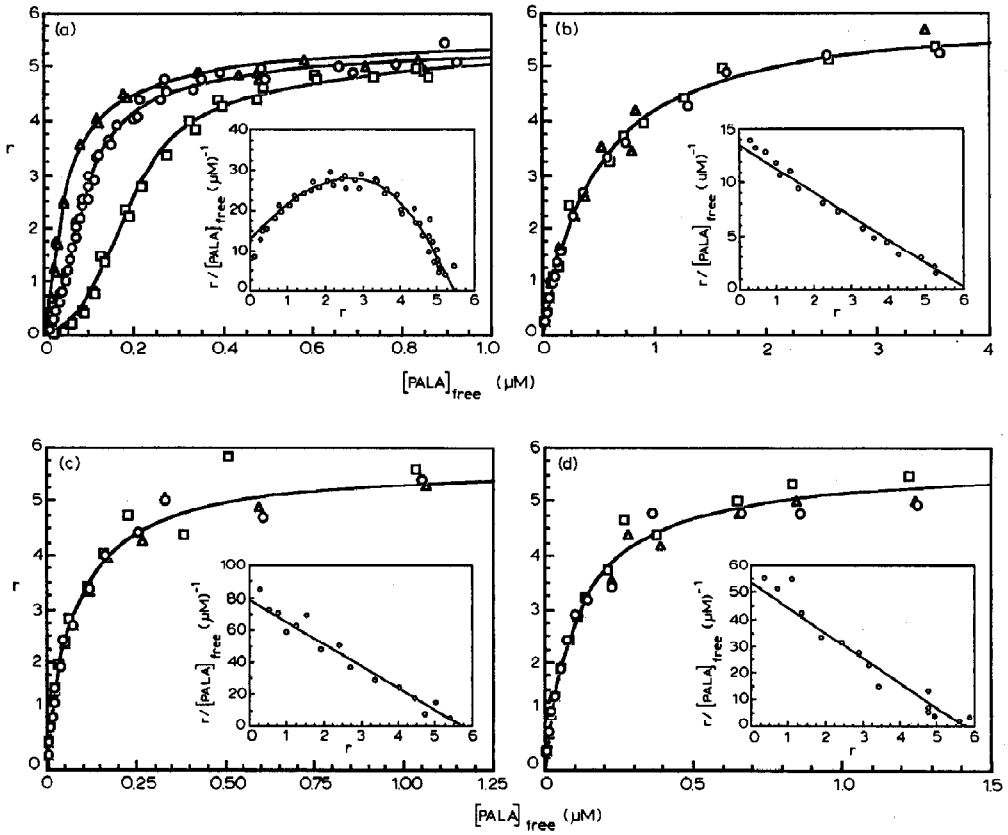

Fig. 4. PALA binding to wild-type and mutationally altered holoenzymes. Experiments were performed in standard phosphate buffer as described (section 3.4) in the absence of nucleotides (○), in the presence of 2 mM ATP (△), and in the presence of 0.5 mM CTP (□). r equals $[PALA]_{bound}/[Enzyme]_{total}$. (Inset) Scatchard plots of the data in the absence of nucleotides. Lines correspond to the parameters given in table 2. (a) Wild-type ATCase (data from ref. 3), (b) Lys-164 → Glu, (c) Glu-239 → Lys, (d) Lys-164 : Glu-239 → Glu : Lys.

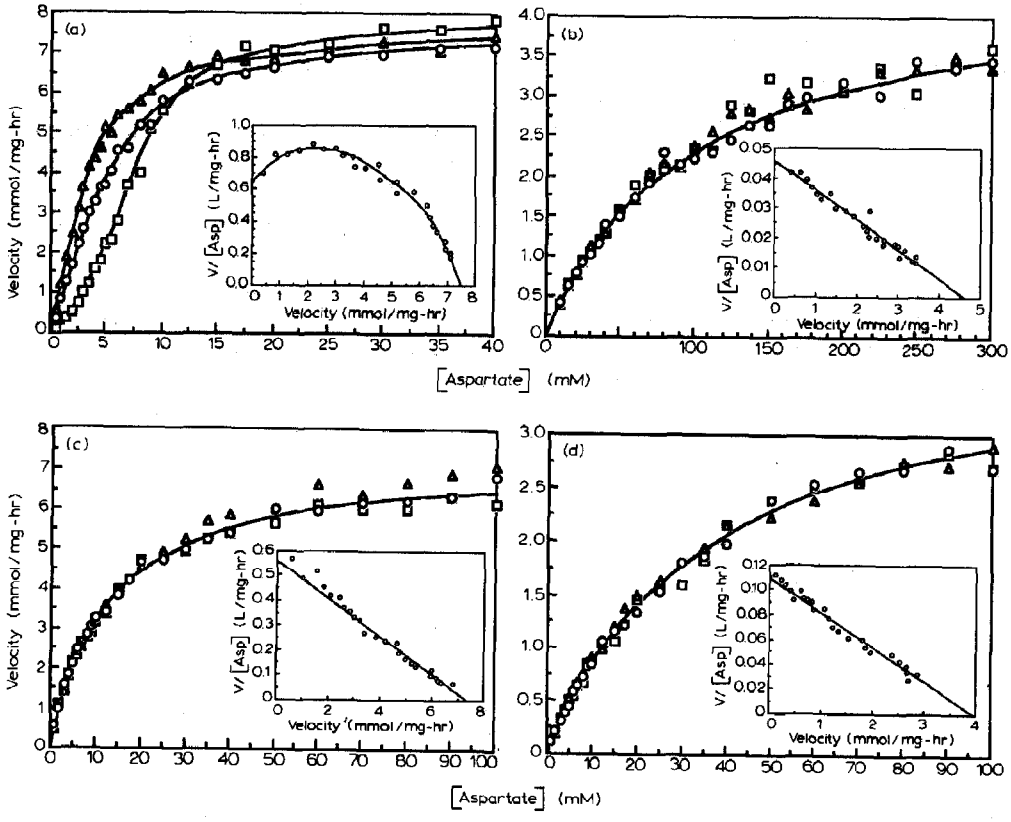

Fig. 5. Dependence of reaction velocity on aspartate concentration for wild-type and mutationally altered holoenzymes. Experiments were performed in standard Mops buffer as described (section 3.4), in the absence of nucleotides (○), in the presence of 2 mM ATP (△), and in the presence of 0.5 mM CTP (□). (Inset) Eadie plots of the data in the absence of nucleotides. Lines corresponding to the best fit parameters of the data in the absence of nucleotides for the mutationally altered holoenzymes given in table 3 are included on graphs b–d. The lines on graph a, data from the wild-type holoenzyme, were drawn by eye. (a) Wild-type ATCase, (b) Lys-164 → Glu, (c) Glu-239 → Lys, (d) Lys-164 : Glu-239 → Glu : Lys.

Scatchard graphs of the binding data from the mutationally altered enzymes, in either the absence (inset in fig. 4b–d) or presence (not shown) of nucleotides. Furthermore, the PALA-binding isotherms of the mutationally altered holoenzymes did not exhibit the sensitivity to the allosteric effectors ATP and CTP characteristic of the wild-type holoenzyme (fig. 4). The dissociation constants of PALA determined for the mutationally altered holoenzymes in the presence and absence of nucleotides are listed in table 2, along with analogous values for the cooperative wild-type holoenzyme.

The PALA-binding isotherms of the isolated C subunits of each of the mutationally altered enzymes were also determined. Scatchard plots of the PALA-binding isotherms were examined and in no case was any cooperativity detected (data not shown). The dissociation constants of PALA from the altered C subunits are included in table 2. In contrast to the wild-type enzyme for which the K_{av} of the holoenzyme and the K_d of the C subunit are similar, the affinities of the mutationally altered C subunits for PALA were substantially less than those of the corresponding holoenzymes.

Table 2

Parameters for PALA binding to wild-type and mutationally altered holoenzymes and catalytic (C) subunits

Experiments were performed as described in section 3.4. Data for the wild-type enzyme are from ref. 3 and are included for comparison.

	Effector	K_d or K_{av} (nM) [a]	n_H [b]
Wild-type			
holoenzyme		110	1.95
	ATP	65	1.35
	CTP	266	2.27
C		95	[c]
Lys-164-Glu			
holoenzyme		460	[c]
	ATP	470	[c]
	CTP	460	[c]
C		24000	[c]
Glu-239 → Lys			
holoenzyme		73	[c]
	ATP	76	[c]
	CTP	77	[c]
C		370	[c]
Lys-164 : Glu-239 → Glu : Lys			
holoenzyme		108	[c]
	ATP	104	[c]
	CTP	107	[c]
C		3700	[c]

[a] For the wild-type holoenzyme, which exhibits cooperativity, the average dissociation constant (K_{av}) is given (see ref. 3), whereas isotherms for preparations devoid of cooperativity are characterized by the dissociation constant (K_d).

[b] The Hill coefficient at half-saturation n_H. The method by which the coefficients for the wild-type enzyme were determined is described by Newell et al. [3].

[c] No cooperativity detected in Scatchard plot.

4.3. Catalytic properties of the mutationally altered enzymes

The rates of the initial steady-state reactions catalyzed by each of the mutationally altered holoenzymes were determined at varying concentrations of aspartate in the presence of 4 mM carbamoyl phosphate. The results are shown in fig. 5, along with data from the wild-type holoenzyme for comparison. Eadie plots of the data in the absence of nucleotides (a sensitive graphical method of detecting cooperativity analogous to the Scatchard plot of binding data) are inset on each graph. In contrast to the marked cooperativity exhibited by wild-type ATCase, no cooperativity was detected for the mutationally altered enzymes. Furthermore, the presence of the nucleotide effectors had no discernible effect on the catalytic properties of the mutationally altered enzymes; the K_m and V_{max} in the presence and absence of nucleotides were in each case identical within the limits of experimental error (table 3).

The catalytic properties of the isolated mutationally altered C subunits were also examined,

Table 3

Kinetic parameters for wild-type and mutationally altered holoenzymes and catalytic (C) subunits

Experiments were performed and parameters were determined as described in section 3.4.

	Effector	K_m or $K_{0.5}$ [a]	V_{max}	n_H
Wild-type				
holoenzyme		4.8	7.5	1.5
	ATP	3.2	7.8	1.3
	CTP	7.4	8.1	2.3
C		5.3	24	[c]
Lys-164 → Glu				
holoenzyme		100	4.6	[c]
	ATP	97	4.7	[c]
	CTP	97	4.7	[c]
C		> 300 [b]	4.0 [b]	[c]
Glu-239 → Lys				
holoenzyme		13	7.3	[c]
	ATP	15	8.0	[c]
	CTP	12	7.1	[c]
C		11	24	[c]
Lys-164 : Glu-239 → Glu : Lys				
holoenzyme		36	3.9	[c]
	ATP	32	3.8	[c]
	CTP	36	3.9	[c]
C		260	21	[c]

[a] The cooperative kinetic saturation curves of the wild-type holoenzyme are characterized by ($K_{0.5}$), and the noncooperative saturation curves of the C subunits and the mutationally altered holoenzymes by K_m.

[b] The K_m of the Lys-164 → Glu C subunit for aspartate appeared to be dramatically increased and no reliable value could be established. At the highest aspartate concentration examined there is no evidence of a decrease in the concentration of free active sites, so it is assumed that the concentration of aspartate at half-saturation is higher than this value and that V_{max} is higher than the observed maximal velocity.

[c] No cooperativity was apparent in Eadie plots of the experimental data.

and the results are summarized in table 3. For the wild-type enzyme, the $K_{0.5}$ of the holoenzyme and the K_m of the C subunit for aspartate are similar, as has been found consistently in previous studies. The holoenzyme and C subunit of the enzyme Glu-239 → Lys also exhibited similar K_m values for aspartate. In contrast, for the mutationally altered enzymes Lys-164 → Glu and Lys-164 : Glu-239 → Glu : Lys, the K_m values of the C subunits for aspartate were significantly increased from those of the corresponding holoenzymes. *

5. Discussion

Among allosteric proteins, the regulatory enzyme ATCase has played a central role in the evolution of our current understanding of the structural basis of allosteric linkage. The demonstration by Gerhart and Schachman [9] that ATCase undergoes a large conformational change upon binding the substrate carbamoyl phosphate and the substrate analog succinate was of particular importance, since it was in accord with the fundamental premise of allosteric theory that the interactions between bound ligands are mediated by conformational changes in the macromolecule [31]. Moreover, this conformational transition appeared to be concerted, in the sense that it was complete at substoichiometric concentrations of bound ligand, contradicting any scheme in which the conformation of a subunit is uniquely determined by its state of ligation. Studies of the behavior of ATCase have therefore largely focused on testing the applicability of the concerted model of Monod, Wyman and Changeux [32]. In the simplest formulation of the model, the polyvalent enzyme exists in two conformational states which may differ in their affinities for various ligands. The model is not only consistent with the observation on ATCase that the extent of the conformational change and the fractional saturation of the protein with ligand are not co-linear, but actually predicts that this will in general be the case. Subsequent studies utilizing diverse experimental strategies and techniques have overwhelmingly supported the hypothesis that a two-state allosteric equilibrium underlies the homotropic cooperative behavior of ATCase. Particularly compelling was the demonstration that the binding of one molecule of PALA to ATCase may, under certain conditions, convert all of the unliganded subunits to the high-activity state [33] and the results of sedimentation studies which indicated that, at sub-saturating concentrations of PALA, ATCase exists as a mixture of T and R state molecules rather than as a homogeneous population of molecules in a partially swollen form [34]. While the preponderance of evidence has led to a general acceptance of the two-state concerted model as the mechanistic basis for the observed homotropic cooperativity, the mechanisms by which ATP and CTP exert their heterotropic effects on the enzyme are less certain. Given the strong evidence that the enzyme exists in an equilibrium between two relatively stable conformational states, the most straightforward explanation is that heterotropic effectors simply bind preferentially to one of the two states of the enzyme [32]. From this simple postulate, a quantitative description of the effects of nucleotides on the behavior of ATCase may be derived. Indeed, Howlett et al. [35] have shown that the two-state model may account for a wide spectrum of data pertaining to the heterotropic properties of ATCase with a single set of allosteric parameters. However, the results of other experiments have been reported which are not as readily reconciled with the two-state model of heterotropic effects, and other researchers, focusing on these studies, have advanced more complicated models to account for the allosteric properties of ATCase [36,37]. We feel that the two-state model provides a useful and adequate description of the allosteric behavior of ATCase [38]. However, additional experiments may be useful in resolving some of the existing ambiguities.

* In order to examine the possibility that the mutationally altered catalytic subunits might have been irreversibly damaged during isolation, a sample of each catalytic subunit was reconstituted to the holoenzyme level by the addition of an approx. 2-fold excess of regulatory subunits and the sample was assayed for catalytic activity. In each case, the reconstituted holoenzyme was indistinguishable from the holoenzyme which had not been dissociated (data not shown).

While considerable evidence is available correlating the observable conformational isomerization with the homotropic and heterotropic properties of ATCase, a direct demonstration that this conformational isomerization is *required* for the manifestation of these allosteric properties has remained elusive. Although it is difficult to conceive of an experimental approach to address this issue directly, we sought to focus on a closely related question: will a modified version of ATCase which does not exhibit the characteristic conformational isomerization in response to ligand binding exhibit allosteric properties? To address this question, we attempted to use site-specific amino acid substitutions to perturb the structure of the enzyme such that only the swollen R conformation was significantly populated regardless of the presence or absence of ligands. If the conformational isomerization which is observed when certain ligands bind to the wild-type enzyme forms the structural basis for the transmission of allosteric effects between sites, such an enzyme would be devoid of allosteric properties. If, on the other hand, homotropic and heterotropic effects are mediated by some other mechanism (including a different conformational isomerization which is not observed), there is a priori no reason to expect that the modified enzyme would have lost these properties. We have therefore used site-specific amino acid substitutions in an attempt to create modified versions of ATCase which are isomorphous with the fully swollen (R) conformation of the wild-type enzyme. Three mutationally altered versions of the enzyme were constructed, one in which Lys-164 in the catalytic subunit was replaced by Glu (Lys-164 → Glu), a second in which Glu-239 was replaced by Lys (Glu-239 → Lys), and a third harboring both of these amino acid substitutions (Lys-164 : Glu-239 → Glu : Lys). The rationale for these specific amino acid substitutions is described in section 2.

A series of sedimentation velocity difference experiments were performed in order to assess the effects of these amino acid substitutions on the conformation of the enzyme in the presence and absence of PALA. The results show clearly that the amino acid substitutions led the enzymes to adopt a more R-like conformation. In fact, in the absence of any ligands, two of the altered enzymes (Lys-164 → Glu and Lys-164 : Glu-239 → Glu : Lys) existed in a conformation indistinguishable from that of the PALA-liganded wild-type holoenzyme (table 1). These enzymes exhibited no conformational change in response to the binding of PALA (fig. 3), carbamoyl phosphate or phosphate (table 1). The third mutant enzyme, Glu-239 → Lys, was found to exist in the absence of any ligand in an average conformation intermediate between those of the unliganded and PALA-liganded wild-type enzyme (fig. 3 and table 1); this amino acid substitution alone was apparently not sufficient to convert the enzyme quantitatively to the fully swollen conformation. The addition of PALA, carbamoyl phosphate, or phosphate was sufficient to cause the enzyme to adopt the fully swollen R conformation (table 1). These observations, plus others on wild-type ATCase and additional mutant forms [39a], are readily accounted for if the unliganded Glu-239 → Lys ATCase exists as an equilibrium mixture of the two quaternary forms, T and R, in a ratio of about 1 : 2.

We investigated the allosteric properties of the mutationally altered holoenzymes in two different functional processes, PALA-binding (in phosphate buffer) and steady-state enzymatic activity (dependence of activity on aspartate concentration in the presence of a constant concentration carbamoyl phosphate). In each of these processes wild-type ATCase exhibits homotropic cooperativity and sensitivity to nucleotide effectors (inhibition by CTP and activation by ATP). If the global swelling characteristic of the wild-type enzyme is inextricably linked to the allosteric properties of the enzyme, the mutationally altered enzymes, which were found to have adopted the fully swollen R conformation in the presence of phosphate or carbamoyl phosphate, would be expected to display neither homotropic nor heterotropic effects. In fact, none of the mutationally altered holoenzymes exhibited any discernible homotropic cooperativity in these assays; for each mutationally altered enzyme, the dependence of fractional saturation of the enzyme with PALA on free PALA concentration (fig. 4) and the dependence of catalytic rate on aspartate concentration (fig. 5) both

followed the rectangular hyperbola characteristic of identical, noninteracting sites. Furthermore, neither the PALA-binding nor catalytic properties of the mutationally altered holoenzymes showed any discernible sensitivity to the presence of the allosteric effectors ATP and CTP (figs 4 and 5). In each case, the mutationally induced isomerization of the holoenzyme into the fully swollen R conformation in the absence of the reference ligand (PALA or aspartate) is associated with a complete loss of the allosteric properties. While alternative explanations for these data may be proposed, we feel these results strongly reinforce the hypothesis that the global conformational transition observed with the wild-type enzyme serves as the structural basis for the transmission of *both* homotropic and heterotropic effects in ATCase.

A number of mutant forms of ATCase harboring site-specific amino acid substitutions have been described [15,20,39–43]. Perhaps not surprisingly, many of these exhibit altered allosteric properties. In many cases, however, there is a complete lack of any direct structural information on the effects of the substitutions with which to correlate these changes, so any structural interpretation of the functional alterations should be viewed with circumspection. Ladjimi and Kantrowitz [40] have reported that the replacement of Glu-239 with Gln yields an enzyme devoid of both homotropic and heterotropic effects in enzyme kinetics. The crystal structure of this mutationally altered enzyme has been described by Gouaux et al. [44] as intermediate between that of the unliganded T state than the PALA-liganded R state. In fact, the gross conformation of this enzyme is very similar to the unliganded T conformation, with a very slight perturbation toward a more swollen structure. Previously published accounts have shown that the transition between the unliganded T to PALA-liganded R states of the wild-type enzyme is accompanied by a 12 Å separation and a 5° relative rotation of the C subunits, of which the Glu-239 → Gln enzyme exhibits 1.5 Å and 0.5°, respectively. Perhaps it would be more appropriate to consider this a slightly perturbed T state enzyme rather than intermediate state. Eisenstein et al. [43] have described a number of other mutant forms of ATCase harboring amino acid substitutions at different positions in the regulatory subunit polypeptide. One of these mutationally altered holoenzymes in which Asn-111 in the regulatory chain was replaced by Ala was shown by sedimentation velocity difference experiments in phosphate buffer to exist in the fully swollen R conformation. This enzyme exhibited neither homotropic nor heterotropic effects in enzyme kinetics, reinforcing the conclusions drawn from the work reported in this paper.

It is presently difficult to predict how multiple amino acid substitutions will interact, i.e., what is the relationship between the net energetic perturbation in a process which arises from multiple amino acid substitutions and the perturbations of the individual substitutions? What evidence is available suggests that the answer to this question will depend in large part on the details of the system, as one might expect, but few relevant data are available. As may be seen in fig. 2, the salt-bridge between the Lys-164 and Glu-239 exists in both the unliganded T and PALA-liganded R states of ATCase. Generation of the mutant enzyme harboring two amino acid substitutions, Lys-164 : Glu-239 → Glu : Lys, may therefore be considered an attempt to reverse the polarity of a salt-bridge, an operation which, without consideration of the other interactions in which the residues are involved in the protein, one might naively expect to have a minimal effect on the energy of the salt-bridge and, therefore, on the structure of the protein. As demonstrated by the sedimentation studies, the reversal of the polarity of this salt-bridge in fact had a dramatic effect on the structure of the enzyme. Hwang and Warshel [45] have argued that the principles underlying the formation of stable salt-bridges in proteins dictate that the reversal of a salt-bridge which in its native configuration contributes to the stability of the protein is energetically unfavorable; factors which make the salt-bridge effective with one polarity will tend to make it ineffective with the other. The bond between Glu-239 and a tyrosine at position 165 illustrated in fig. 2 may be an example of just this type of interaction, although the potential of the hydroxyl group to participate both as a hydrogen bond donor and as an acceptor might also allow it to participate in a favorable interaction

with a lysine substituted at position 239.

Finally, while this discussion has focussed on the allosteric properties of the holoenzyme, previous investigations of the isolated catalytic and regulatory subunits of ATCase have provided insight into the advantages of oligomerization in this system. For this reason, we also studied the isolated mutationally altered C subunits and have made some interesting observations. Unlike the wild-type enzyme, the mutationally altered enzymes exhibited large differences in affinity for PALA at the holoenzyme and C subunit levels. In particular, the enzymes Lys-164 → Glu and Lys-164 : Glu-239 → Glu : Lys exhibited dissociation constants of PALA from the C subunits which were more than 40-fold higher than those of the corresponding holoenzymes. Similar, although less dramatic, observations were made in enzyme kinetics. We hesitate to speculate on the significance of these observations, but do consider this an intriguing subject for further investigation.

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