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Changes in stability and allosteric properties of aspartate transcarbamoylase resulting from amino acid substitutions in the zinc-binding domain of the regulatory chains

(site-directed mutagenesis/subunit exchange/ligand-promoted conformational changes)

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ABSTRACT **Changes in subunit interaction energies linked to the allosteric transition of the regulatory enzyme aspartate transcarbamoylase (ATCase; EC 2.1.3.2) from *Escherichia coli* are localized in part at interfaces between the six catalytic (c) and six regulatory (r) polypeptide chains. Site-directed mutagenesis has been used to construct enzymes with amino acid substitutions in a limited region of the zinc-binding domain of the r chains. Substitution of Ser or His for r114 Cys, one of four cysteines binding the structural zinc ion in the regulatory chain, leads to incorrectly folded chains as shown by the inability to detect stable assembled holoenzyme in cell extracts. Replacement of r111 Asn by Ala at the interface between an r chain and a c chain in the apposing catalytic trimer causes a complete loss of the homotropic and heterotropic effects characteristic of wild-type ATCase. Moreover, sedimentation velocity experiments demonstrated that this mutant enzyme exists in the R ("relaxed") conformation in the absence of active site ligands due to preferential destabilization of the T ("taut") conformation relative to the R state. In contrast, replacement of r113 Asn by Ala at the interface between adjacent r and c chains leads to an increase in the cooperativity of the enzyme. When r139 Lys is replaced by Met, V_{max} is reduced by 50% compared to wild-type ATCase, whereas it is increased about 2-fold when r142 Glu is replaced by Asp. Amino acid substitutions in this domain significantly affect subunit interaction energy as measured by rate of subunit exchange when holoenzymes are incubated with isolated catalytic subunits, thus permitting measurements of the effect of the bisubstrate analog *N*-(phosphonacetyl)-L-aspartate in weakening intersubunit interactions. Subunit exchange increased about 9-fold for the r142 Glu → Asp mutant and almost 20-fold for the r142 Glu → Ala mutant in the presence of the ligand.**

It is generally accepted that ligand or substrate binding at one site in an oligomeric allosteric protein promotes global conformational changes affecting the relative free energy of interaction between subunits and the affinity of ligand binding at other sites. As a consequence, it is of interest not only to study structural alterations resulting from ligand binding but also to investigate changes in intersubunit interactions in order to obtain estimates of the energetic differences between the various quaternary states of the proteins. The regulatory enzyme aspartate transcarbamoylase (ATCase; aspartate carbamoyltransferase, carbamoyl-phosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2), which catalyzes the first committed reaction in the biosynthesis of pyrimidines in *Escherichia coli*, is known to undergo pronounced conformational changes upon binding substrate analogs (1–6) that promote the allosteric transition of the enzyme from the low-activity T ("taut") state to the more active R ("relaxed") state (7). Despite intensive effort, quantitative information on the energetics of the various interchain interactions has been difficult to obtain because of the strength and multiplicity of the subunit contacts stabilizing the quaternary structure of the wild-type enzyme. This difficulty has been circumvented in part by using site-directed mutagenesis to replace various amino acids located in the zinc-binding domain of the regulatory chains of ATCase. In this way we have been able to study mutant forms of ATCase having markedly altered stability, catalytic activity, and regulatory properties.

ATCase is composed of two catalytic trimers (C) and three regulatory dimers (R) linked noncovalently into a stable complex of 12 polypeptide chains having D_3 symmetry (3–5). Although isolated C subunits, produced by dissociation of ATCase upon treatment with mercurials (8), exhibit Michaelis–Menten kinetics, the holoenzyme shows a sigmoidal dependence of activity on the concentration of each of its substrates, carbamoyl phosphate and aspartate (9, 10). Moreover, at subsaturating concentrations of substrates, the enzyme is inhibited by CTP, the end product of the pyrimidine pathway, and activated by ATP, a product of the purine pathway (9).

Previous investigations have demonstrated the striking stability of the native enzyme by the lack of exchange of subunits in the holoenzyme upon incubation with free C or R subunits (11); consequently, studies of the effects of ligands on the strength of intersubunit interactions in ATCase have been limited to the disproportionation of less stable enzyme-like molecules lacking one R subunit (11, 12) or temperature-induced changes in enzyme activity (9, 13, 14). Here we report the effects of amino acid substitutions located at interfaces between C and R subunits; the alterations were produced by site-directed mutagenesis of *pyrI*, which encodes the regulatory (r) polypeptide chain of ATCase. These mutant enzymes have been used to study the regulatory properties of ATCase and to determine the effects of ligands on the dissociation of the holoenzymes.

RATIONALE FOR AMINO ACID REPLACEMENTS

In the unliganded T state of ATCase, three classes of intersubunit interactions have been implicated in stabilizing the enzyme (3). There is a contact region between adjacent catalytic (c) and r chains, designated the c1–r1 interface, another between apposing C trimers, designated the c1–c4 interface, and finally, a region between an r chain and an apposing c chain, designated the c1–r4 interface. Studies on solutions of ATCase (1, 2) and x-ray diffraction of crystals (4)

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Abbreviations: ATCase, aspartate transcarbamoylase; PALA, *N*-(phosphonacetyl)-L-aspartate; C, catalytic trimer; R, regulatory dimer; c chain, catalytic polypeptide chain; r chain, regulatory polypeptide chain.

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have demonstrated that the enzyme is converted to a swollen conformation upon the binding of substrates or the bisubstrate analog *N*-(phosphonacetyl)-L-aspartate (PALA). This global structural alteration, representing the allosteric transition from the T state to the R state, consists of a 12-Å expansion along the threefold symmetry axis and occurs at the expense of the interactions at the c1–c4 and c1–r4 interfaces, which are virtually eliminated in the liganded R state. In contrast to the extensive contacts which stabilize the T state of the enzyme, the c1–r1 interface provides the only significant intersubunit interaction in the R state.

Despite the obvious importance of domains in the C subunit in the allosteric transition of the enzyme (15, 16), we wanted to avoid alterations in catalytic activity as a consequence of amino acid substitutions in c chains. Hence, replacements were confined solely to r chains to determine the effect of amino acid substitutions at subunit interfaces in perturbing the allosteric equilibrium and to permit an evaluation of the contribution of various interactions to the relative stability of the T and R states. Amino acid residues in the zinc-binding domain (about residue 100 to C-terminal residue 153) of the r chain located at intersubunit contact regions were chosen for replacement based on structural information obtained from x-ray diffraction studies of ATCase (3). Because interactions at the c1–r4 interface are present only in the T state, the impact of perturbations at this interface was evaluated by removing functional groups of amino acid side chains. Thus r111 Asn, which is in contact with residues in both a c1 chain and a c4 chain, was replaced by Ala. Since the c1–r1 interface plays a major role in stabilizing both the T and the R state, the effects of amino acid replacements at r142 Glu, r139 Lys, and r113 Asn were evaluated in terms of the stability of the mutant enzymes and their catalytic and regulatory properties. Four Cys residues in the r chain coordinate tetrahedrally a structural zinc ion required for the assembly of the holoenzyme, and therefore we sought to evaluate their contribution to the stability of ATCase by altering r114 Cys.

EXPERIMENTAL PROCEDURES

Site-directed mutagenesis with oligonucleotides (18- to 21-mers), synthesized on an Applied Biosystems 380B synthesizer, was accomplished with a template, M13mp8pyrI4, constructed from the 876 base-pair (bp) *Bgl* II/*Sal* I fragment of pPYRB11 (17) subcloned in the M13mp8 polylinker at the *Bam*HI/*Sal* I sites. Plaques arising from transformation of JM103 were screened by hybridization (18), and potential mutants were screened further by DNA sequence determinations. Replicative form DNA from mutant candidates in M13mp8pyrI4 was cleaved with *Sal* I and either *Hinc*II or *Eco*RV, and the resulting 820- or 670-bp fragments were ligated with a 3.7-kilobase (kb) fragment from similarly digested pPYRB11 treated with calf intestinal phosphatase (19) and used to transform *E. coli* HB101. Mutations were confirmed by determining the nucleotide sequence of the coding region of the subcloned DNA directly from double-stranded, supercoiled plasmid prepared by the boiling Triton lysis method (20, 21).

Mutant forms of ATCase were overproduced in either *Salmonella typhimurium* TR4574 (13, 22) or *E. coli* EK1104 grown on minimal medium supplemented as described by Nowlan and Kantrowitz (23). Purification of the enzymes was performed by the procedure of Wall *et al.* (24) with slight modifications. Because of the lability of several of the mutant proteins to both EDTA and high temperature, the chelating agent and the heat step were omitted in the purification procedure.

Assays of enzyme activity were performed according to Davies *et al.* (25) at 30°C in 0.1 M Mops, adjusted with KOH to pH 7.0, containing 2 mM 2-mercaptoethanol and 0.1 mM zinc acetate. Initial velocities were measured at various concentrations of L-aspartate in the presence of 4.0 mM carbamoyl phosphate in the absence and presence of 2 mM ATP and 0.5 mM CTP. Values of maximal velocities, V_{max}, Hill coefficients, n_H, and aspartate concentrations corresponding to 0.5 V_{max}, $K_{0.5}$, were determined by nonlinear least-squares analysis (26) in terms of the Hill equation.

Alterations in the quaternary structure of the mutants caused by PALA were determined by sedimentation velocity experiments performed with a Beckman–Spinco model E ultracentrifuge, and changes in the sedimentation coefficient, $\Delta s/s$, were obtained directly in difference experiments (2).

The stability of various mutant forms of ATCase was measured by subunit exchange between ^{125}I-labeled holoenzymes and an excess of unlabeled wild-type C subunits at 25°C in 20 mM Tris·HCl at pH 7.15 containing 5 mM 2-mercaptoethanol, 100 μM zinc acetate, and 150 μM potassium phosphate. C and R subunits were prepared by the procedure of Yang *et al.* (27) and iodination of C subunit was performed with chloramine T (28). For the exchange experiments, mutant holoenzymes were reconstituted from ^{125}I-labeled wild-type C trimer and various R dimers. These ^{125}I-labeled mutant enzymes were then incubated with excess unlabeled wild-type C trimer and, at various times, the mixtures were subjected to polyacrylamide gel electrophoresis (29) in a Hoefer Mighty Small II slab gel apparatus. Gels were stained for protein, and zones corresponding to intact holoenzyme and to free C subunit were cut from the gel for direct measurement of the extent of subunit exchange from determination of the amount of radioactivity in the various species.

RESULTS

Stability of Mutant Forms of ATCase. During the purification of the mutant proteins, it became evident that some amino acid replacements in the zinc-binding domain of the r chains caused a marked decrease in stability of the holoenzymes in comparison to wild-type ATCase and various mutants containing substitutions in c chains (24, 30, 31). This decreased stability was manifested by dissociation of the mutant holoenzymes into subunits during the 5-min heat treatment at 65°C. Yields of purified proteins were improved substantially by eliminating the heat step, by omitting EDTA from all buffers, and by adding 100 μM zinc acetate. When the residue r114 Cys, which is involved in the direct coordination of the structural zinc ion in the r chain, was replaced by either His or Ser, no assembled holoenzyme was detected by polyacrylamide gel electrophoresis of cell extracts. Moreover, the absence of any species migrating at the rate of r chains of ATCase in gels containing SDS indicated that the mutant r chains, presumably because of their inability to coordinate zinc and attain a stable conformation, had been degraded within the cell. It is of interest that substitution of Ser for r140 Tyr, which is located very close to the cluster of the four cysteines chelating the zinc ion, yielded a product in which at least 50% of the protein was in the form of free C trimer.

For wild-type ATCase, the multiplicity and strength of various interchain interactions are sufficiently large that there is virtually no subunit exchange when the holoenzyme is incubated with an excess of free succinylated C trimers (11). With the mutant enzymes containing replacements in the zinc-binding domain, however, such exchange was detected readily. Hence quantitative studies of the rate of subunit exchange were performed with excess unlabeled wild-type C subunit and holoenzymes reconstituted from ^{125}I-labeled wild-type C trimer and various R subunits. Fig. 1 shows results for two mutant enzymes along with control

experiments on wild-type ATCase. Whereas no exchange could be detected with wild-type ATCase over 50 hr, there was a significant decrease in radioactivity migrating as holoenzyme when r142 Glu was replaced by either Asp (Fig. 1*A*) or Ala (Fig. 1*B*). Moreover, the stability of both mutant enzymes was decreased substantially by the addition of PALA as shown by the increased rate of exchange, about 9-fold for the mutant containing r142 Asp and about 20-fold for the mutant in which Ala was substituted for r142 Glu. In contrast, wild-type ATCase was so stable that the effect of PALA could not be detected.

Catalytic Activity and Allosteric Properties of ATCase Mutants. The various amino acid substitutions in the r chains at the c1–r1 and c1–r4 interface had strikingly different effects on the maximum velocity and allosteric properties of the mutant holoenzymes. As seen in the plot of specific activity vs. aspartate concentration, replacement of r111 Asn by Ala (Fig. 2*A*) completely eliminates the sigmoidal kinetics characteristic of wild-type ATCase. In contrast, the mutant containing Ala substituted for r113 Asn exhibits more pronounced cooperativity than wild-type enzyme. The values of n_H for these two mutants were 1.0 and 2.4, respectively, compared to 2.0 for wild-type ATCase.

Some amino acid substitutions in the r chains also had pronounced effects on the maximum velocity of the mutant holoenzymes (Fig. 2*B*). Replacement of r139 Lys by Met caused almost a 50% reduction in V_{max} (4.1 μmol/hr per μg), whereas substitution of either Ala or Asp for r142 Glu led to an increase in V_{max} to 12.0 or 15.7 compared to 8.4 μmol/hr per μg for wild-type ATCase.

As seen in Table 1, those holoenzymes which exhibit cooperativity are also activated by ATP, indicated by the decrease in $K_{0.5}$ and inhibited by CTP as shown by the increases in $K_{0.5}$. Table 1 also shows that the mutant r111 Asn → Ala, which exhibits no cooperativity, is not affected significantly by ATP or CTP.

Ligand-Promoted Changes in Quaternary Structure of Mutants. With wild-type ATCase and mutants exhibiting cooperativity, the addition of active site ligands causes a *decrease* of about 3% in the sedimentation coefficient of the enzyme (1, 2, 30, 31). This decrease, attributable to a swelling of the enzyme as it is converted from the T state to the R conformation, was observed for all of the mutants described above except for that in which r111 Asn is replaced by Ala. The value of $\Delta s/s$ caused by the addition of PALA is virtually zero for that mutant, as contrasted to values of −2.8% to −3.9% for all the other holoenzymes (Table 1).

Because the r111 Asn → Ala mutant exhibited no homotropic or heterotropic effects, it was important to determine

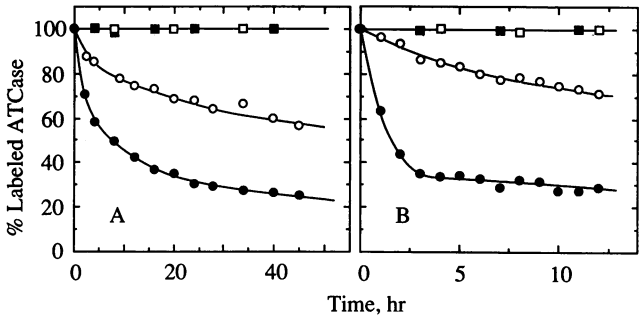

FIG. 1. Effect of PALA on the kinetics of subunit exchange of ^{125}I-labeled mutant holoenzymes with excess unlabeled C subunit. Mutant holoenzymes, assembled from ^{125}I-labeled wild-type C subunits (65 μg, 6.2 × 10^4 cpm/μg) and purified mutant R subunits (35 μg), at a final concentration of 1 mg/ml, were incubated at 25°C with a 20-fold molar excess of unlabeled wild-type C subunit, and aliquots were removed at specific times and analyzed electrophoretically as described in *Experimental Procedures*. The 7.5% polyacrylamide gels were stained with Coomassie brilliant blue G-250 in 12.5% trichloroacetic acid and destained with 5% methanol in 5% acetic acid. Lanes were cut into sections containing stained bands, the amount of radioactivity in these slices was measured in a Packard Multi-Prias γ counter, and the results were plotted as the percent of ^{125}I-labeled holoenzyme remaining vs. time. (*A*) □, Wild-type ATCase; ■, wild-type ATCase + 130 μM PALA; ○, r142 Glu → Asp ATCase; ●, r142 Glu → Asp ATCase + 130 μM PALA. (*B*) □, Wild-type ATCase; ■, wild-type ATCase + 130 μM PALA; ○, r142 Glu → Ala ATCase; ●, r142 Glu → Ala ATCase + 130 μM PALA.

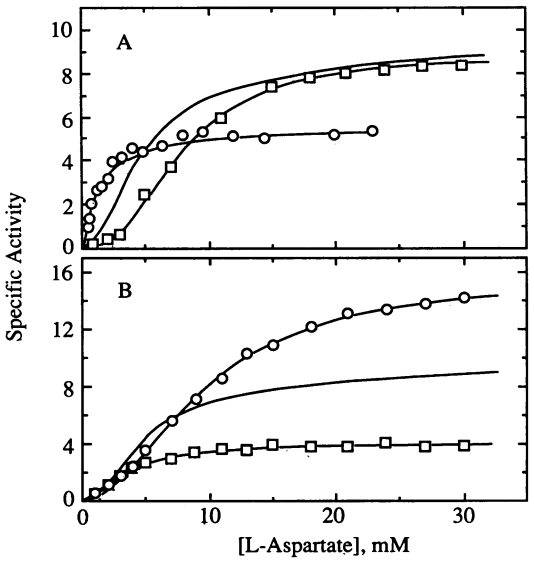

FIG. 2. Enzyme kinetics of various mutant forms of ATCase. The dependence of specific activity, μmol of carbamoyl-L-aspartate formed per hr per μg of protein, is plotted vs. aspartate concentration at 4 mM carbamoyl phosphate. Assays were performed and theoretical curves were determined as described in *Experimental Procedures*. A theoretical curve calculated from the data for wild-type ATCase under identical buffer conditions (data not shown) is presented in each of the two panels. (*A*) ○, r111 Asn → Ala ATCase; □, r113 Asn → Ala ATCase; (*B*) □, r139 Lys → Met ATCase; ○, r142 Glu → Asp ATCase.

Table 1. Properties of ATCase r chain mutants

Mutant	Effector	n_H	$K_{0.5}$, mM	V_{max}, μmol/hr per μg	$\Delta s/s$, %
Wild-type	—	2.0	4.5	8.4	−3.5
	ATP	1.6	3.7	8.8	
	CTP	2.6	7.3	8.4	
r111 Asn → Ala	—	1.0	1.4	5.6	−0.2
	ATP	1.0	1.2	5.9	
	CTP	1.0	1.1	5.3	
r113 Asn → Ala	—	2.4	8.0	8.9	−2.8
	ATP	2.4	5.0	7.7	
	CTP	2.4	9.7	8.5	
r139 Lys → Met	—	1.7	3.5	4.1	−2.8
	ATP	1.7	3.1	3.8	
	CTP	2.3	5.1	3.9	
r142 Glu → Ala	—	1.9	31.9	12.0	−3.5
	ATP	2.1	20.0	9.8	
	CTP	1.9	62.1	8.0	
r142 Glu → Asp	—	1.9	9.5	15.7	−3.9
	ATP	1.5	7.4	14.1	
	CTP	2.4	19.3	15.6	

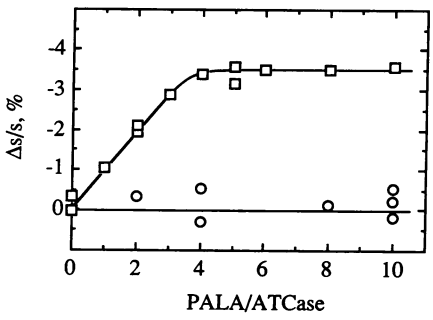

FIG. 3. Effect of PALA on the sedimentation coefficient of wild-type ATCase and a noncooperative mutant holoenzyme, r111 Asn → Ala. Protein concentrations ranged from 2.5 to 3.5 mg/ml in 40 mM potassium phosphate, pH 7.0/2 mM 2-mercaptoethanol/50 μM zinc acetate. The change in the sedimentation coefficient of a sample solution containing PALA was determined relative to a reference solution of unliganded enzyme to which an equal aliquot of water was added. Results are presented as the percent change in sedimentation coefficient, $\Delta s/s$, vs. the molar ratio of PALA to ATCase. □, Wild-type ATCase; ○, r111 Asn → Ala ATCase.

whether its conformation in the absence of ligands corresponded to that of T or R state wild-type enzyme. The titration curves in Fig. 3 show clearly that PALA has no effect on the quaternary structure of the mutant, in contrast to the pronounced effect on wild-type ATCase. When the sedimentation coefficient of PALA-liganded wild-type ATCase was compared to that of the unliganded mutant containing Ala substituted for r111 Asn, the vaue of $\Delta s/s$ was found to be approximately zero. Thus the mutant enzyme is in the R conformation even in the *absence* of active site ligands.

DISCUSSION

Amino acid replacements introduced by site-directed mutagenesis into the zinc-binding domain of the r chains of ATCase have resulted in pronounced and diverse changes in the allosteric properties of the mutant holoenzymes, despite a clustering of the substitutions in a confined surface area (approximately 250 Å^2) at the interface between the c and r chains. Substitutions at the c1–r1 and c1–r4 interfaces yielded enzymes which were more difficult to purify than wild-type ATCase owing to their tendency to dissociate into C and R subunits in the presence of chelating agents at high temperature. A structural zinc ion coordinated tetrahedrally by four cysteines in each r chain is essential for the *in vitro* assembly of ATCase from C and R subunits (32) but may be replaced by cadmium, nickel, or cobalt (33, 34) with little effect on the allosteric properties of the enzymes. Clearly the replacement of r114 Cys by either His or Ser leads to r chains which *in vivo* cannot associate with C trimers to form stable holoenzyme. Since r chains containing these replacements could not be detected in extracts of the cells, we conclude tentatively that the proper folding of the r chains into a stable tertiary structure requires r114 Cys. Determining whether the other three cysteine residues can be replaced by amino acids which chelate metal ions will require additional site-directed mutagenesis experiments.

Wild-type ATCase and mutant forms of the enzyme that exhibit cooperativity have been shown by sedimentation velocity experiments to be largely in the compact T state in the absence of active-site ligands, and upon addition of such ligands these enzymes are converted to the more swollen R conformation. It was of interest, therefore, to determine the quaternary structure of the r111 Asn → Ala mutant that lacked cooperativity. Is this enzyme unable to undergo the T → R transition? Or is the nonallosteric derivative in the R conformation prior to the addition of any substrates? As seen in Fig. 3, the addition of PALA had no effect on the sedimentation coefficient of the r111 Asn → Ala mutant. Moreover, the unliganded mutant enzyme had the same sedimentation coefficient as wild-type ATCase converted to the R state by the addition of PALA. These results show clearly that the nonallosteric mutant is in the swollen R conformation in the absence of active-site ligands and thereby account for the lack of nucleotide effects on enzyme kinetics. We conclude, therefore, that perturbations of interactions involving r111 Asn at the c1–r4 interface cause a loss in allosteric properties by destabilizing the T state preferentially relative to the R conformation. There is no need to invoke effects of substrate binding in causing the loop movement which has been designated as responsible for the T → R conversion of wild-type ATCase (4, 5, 15, 16).

Amino acid substitutions at the c1–r1 interface, however, resulted in either slight decreases or increases in cooperativity. This is seen in Fig. 2*A* for the mutant containing Ala substituted for r113 Asn; n_H was increased to 2.4 as compared with 2.0 for wild-type ATCase. Recently Xu *et al.* (14) reported a Gly substitution at position r113 Asn for which they too observed an increase in cooperativity. In addition, nucleotides affect the aspartate concentration required to reach half-maximal velocity for mutant holoenzymes altered at the c1–r1 interface; CTP increased $K_{0.5}$, whereas ATP caused a decrease for these enzymes. Thus mutant enzymes with amino acid substitutions at the c1–r1 interface retain substantial cooperativity because they are largely in the T state in the absence of ligands. By contrast, nucleotides have little or no effect on r111 Asn → Ala, a mutant enzyme devoid of cooperativity, as expected for an enzyme predominantly in the R state. These results are in accord with suggestions from crystallography that the c1–r4 and c1–c4 contacts stabilize the T state relative to the R state, whereas the c1–r1 interface contributes to the stability of both states. An interesting but unanticipated result concerns the substantial changes in maximal velocities for several of the mutants. As shown in Fig. 2*B*, r139 Lys → Met exhibits roughly half and r142 Glu → Asp nearly twice the activity of wild-type ATCase. Because these substitutions occur in the r chains of the enzyme and are about 15 Å from the functional groups of active site residues, it seems likely that these changes in activity are attributable to an indirect effect of the mutation.

The cooperative behavior of regulatory enzymes is linked to changes in the energy of interaction between subunits and, therefore, thermodynamic information on the strength of subunit interactions in the presence of various ligands would indeed be valuable in a study of the allosteric transition of ATCase (35). Because the mutational alterations in the r chains resulted in significant changes in the stability of the mutant holoenzymes relative to wild-type ATCase, it was of importance to assess the effects of these amino acid substitutions on the interchain interactions in the holoenzyme. Moreover, the tendency of these mutants to dissociate into C and R subunits afforded an opportunity to measure the relative bond strength between subunits in these enzymes. Subunit exchange experiments with ^{125}I-labeled mutant holoenzymes and wild-type C trimer provided estimates of the effect of PALA on the interchain bond strength of two mutants, r142 Glu → Asp and r142 Glu → Ala, since perturbations at the c1–r1 interface should affect both the T and the R quaternary structures. In the absence of PALA, the half-time for exchange of the Asp derivative is roughly 45 hr, which may be compared to 20 hr for the Ala mutant. Significantly, PALA increases the exchange rate approximately 9-fold for r142 Glu → Asp and almost 20-fold for r142 Glu → Ala. These observations demonstrate that PALA binding to ATCase causes a decrease in the free energy of interaction between C and R subunits. Thus amino acid replacements in the zinc-binding domain of the r chain, which

decrease subunit interactions even in the absence of ligands, provide an opportunity to assess quantitatively the relationship between interchain interactions and allosteric properties of ATCase.

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