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Effect of amino acid substitutions on the catalytic and regulatory properties of aspartate transcarbamoylase

(site-directed mutagenesis/active-site residues/conformational change)

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ABSTRACT **Although intensive investigations have been conducted on the allosteric enzyme, aspartate transcarbamoylase, which catalyzes the first committed reaction in the biosynthesis of pyrimidines in *Escherichia coli*, little is known about the role of individual amino acid residues in catalysis or regulation. Two inactive enzymes produced by random mutagenesis have been characterized previously but the loss of activity is probably attributable to changes in the folding of the chains stemming from the introduction of charged and bulky residues (Asp for Gly-128 and Phe for Ser-52). Site-directed mutagenesis of *pyrB*, which encodes the catalytic chains of the enzyme, was used to probe the functional roles of several amino acids by making more conservative substitutions. Replacement of Lys-84 by either Gln or Arg leads to virtually inactive enzymes, confirming chemical studies indicating that Lys-84 is essential for catalysis. In contrast, substitution of Gln for Lys-83 has only a slight effect on enzyme activity, whereas chemical modification causes considerable inactivation. Gln-133, which has been shown by x-ray crystallography to reside near the contact region between the catalytic and regulatory chains, was replaced by Ala. This substitution has little effect on catalytic activity but leads to a marked increase in cooperativity. The Gln-83 mutant, in contrast, exhibits much less cooperativity. Since a histidine residue may be involved in catalysis and His-134 has been shown by x-ray diffraction studies to be in close proximity to the site of binding of a bisubstrate analog, His-134 was replaced by Ala, yielding a mutant with only 5% wild-type activity, considerable cooperativity, and lower affinity for aspartate and carbamoylphosphate. All of the mutants, unlike those in which charged or bulky residues replaced small side chains, bind the bisubstrate analog, which promotes the characteristic "swelling" of the enzymes indicative of the allosteric transition.**

Studies of mutationally altered proteins have contributed greatly to our understanding of the effect of amino acid substitutions on function. The most notable example is hemoglobin, for which more than 200 naturally occurring mutant forms have been characterized in terms of variations in oxygen affinity, cooperativity, association–dissociation equilibrium, and the Bohr effect (1–4). Recently, the development of oligonucleotide-directed mutagenesis techniques (5–7) has permitted the construction of mutants of many proteins in which individual amino acid residues of potential interest have been modified. This approach has been applied to the study of a variety of enzymes, including tyrosyl-tRNA synthetase, dihydrofolate reductase, β-lactamase, triose phosphate isomerase, carboxypeptidase, trypsin, aspartate aminotransferase, and aspartate transcarbamoylase. In many studies, the modifications were of value in identifying amino acids at active sites and in elucidating details of enzyme–substrate interactions and catalytic mechanisms. With the regulatory enzyme aspartate transcarbamoylase (ATCase; aspartate carbamoyltransferase; carbamoyl-phosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2) from *Escherichia coli*, site-directed mutagenesis (8) can be used also to study the allosteric transition whereby the enzyme is converted from the T state with low affinity for substrates to the more active R conformation (9). We describe here the characterization of five mutants in which amino acid replacements in the catalytic chains of ATCase cause a marked decrease in enzyme activity or cause large changes in cooperativity without significant decreases in catalytic activity.

ATCase catalyzes the formation of carbamoylaspartate from carbamoylphosphate and aspartate, which in *E. coli* is the first committed reaction in the biosynthesis of pyrimidines. The enzyme exhibits a sigmoidal dependence of activity on the concentration of both substrates, and it is allosterically regulated by CTP and ATP, which, at intermediate substrate concentrations, inhibit and activate, respectively, the catalytic reaction. Physical chemical studies of ATCase in the absence and presence of substrate analogs have demonstrated ligand-promoted global conformational changes in the enzyme that have been interpreted in terms of the T→R transition (9, 10). A variety of inactive mutant forms produced by random mutagenesis have been described (11–13) and active mutants with altered regulatory properties have been produced by suppression of nonsense mutations or by selecting revertants of inactive derivatives (14–17). In one active mutant (18), the alteration occurs in the *pyrI* gene encoding the regulatory chain of ATCase, whereas all other mutants described thus far involve the *pyrB* gene, which encodes the catalytic chain of the enzyme. The application of site-directed mutagenesis of *pyrB* has made possible the investigation of the effect of specific amino acid replacements on the catalytic and regulatory properties of ATCase, and we report here the results of various substitutions in the catalytic polypeptide chain.

Rationale for Amino Acid Replacements

The selection of amino acid residues for replacement was based on structural information obtained from crystallography and changes in the activity and regulatory properties of the enzyme caused by chemical modification. X-ray diffraction studies of ATCase in the T state have yielded a structure to a resolution of 2.6 Å and the location of the binding site for phosphate ligands, which are competitive inhibitors of the substrate carbamoylphosphate (19). In addition (20), the struc-

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Abbreviations: ATCase, aspartate transcarbamoylase; PALA, *N*-(phosphonacetyl)-L-aspartate.

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ture of the R-state enzyme complexed with the bisubstrate analog, *N*-(phosphonacetyl)-L-aspartate (PALA) has been solved to 2.9 Å. Although the phosphonate of PALA is located at the previously identified phosphate binding site, there are large changes in the tertiary structure of the catalytic chains when ATCase is converted from the T state to the R conformation. Some amino acids within contact distance of the inhibitor have been identified (20). It seems likely that PALA binds to the active site of crystalline R-state enzyme, but it cannot be taken for granted that binding of PALA resembles that of substrates in the catalytically productive mode.

Chemical modification studies on the catalytic subunit show a loss in enzyme activity when Lys-84 is pyridoxylated (21, 22) and when both Lys-83 and Lys-84 are modified with bromosuccinate (23) or with trinitrobenzene sulfonate (24). The functional groups of these residues are several Å from the phosphate binding site in the T-state enzyme, but in R-state ATCase, Lys-84 is close to the phosphonate of PALA. Since modification of these lysine residues involved introduction of bulky and charged groups, it is not known whether the resulting inactivation is attributable to indirect effects or the direct consequence of altering active site residues. Hence, site-directed mutagenesis was used to replace Lys-84 by both Gln and Arg. In this way, the role of the position and geometry of the charged group could be assessed. Gln was also substituted for Lys-83 to determine the effect of removing the charged residue.

His-134 is near the phosphate binding site of the T-state enzyme and is close to the carbonyl oxygen of PALA in R-state ATCase. ^{13}C NMR studies led to the suggestion that the carbonyl oxygen of carbamoylphosphate becomes protonated when succinate, an aspartate analog, binds to the enzyme–carbamoylphosphate complex (25). If protonation of the carbonyl oxygen of carbamoylphosphate by the enzyme occurs, His-134 is a likely candidate as the proton donor. In addition, because the amino group of aspartate loses two protons in the conversion to carbamoylaspartate, it has been suggested that base catalysis may be part of the catalytic mechanism (26). Since His-134 is in close proximity to PALA in R-state enzyme (20), it could be a proton acceptor. Therefore, there are at least two ways that His-134 could be involved in catalysis (27). Two unidentified histidine residues are modified by photooxidation of the catalytic subunit–pyridoxal 5′-phosphate complex with a concomitant loss of enzyme activity (21). To determine whether His-134 is critical for catalysis, we studied the effect of removing the functional group in His-134 by substituting Ala in its place.

The intersubunit contacts between catalytic and regulatory subunits are thought to be of importance for mediating the homotropic and heterotropic effects exhibited by ATCase. Since crystallographic data for T-state ATCase show that Gln-133 of a catalytic chain is close to the neighboring regulatory chain, it seemed of interest to determine the effect of an amino acid change in that region. Accordingly, the effect of a change in this intersubunit contact on the allosteric properties was investigated by substituting Ala for Gln-133 in the catalytic subunit.

Materials and Methods

Oligonucleotide-directed mutagenesis was performed by the method of Zoller and Smith (6, 8). The template DNA, M13 mp8 PYRB2, consisted of the 1.65-kilobase *Eco*RI/*Hin*cII restriction fragment of pPYRB3 (28) cloned into M13 mp8 at the *Eco*RI and *Sma* I sites by blunt-end ligation. Oligonucleotides were synthesized on an Applied Biosystems (Foster City, CA) automated DNA synthesizer by using phosphotriester chemistry (29). Fig. 1 shows the relevant mutagenic oligonucleotides for production of the five mutants. Asterisks denote mismatches used to produce desired amino acid substitutions. In addition, some mismatches were introduced to eliminate or introduce restriction sites without altering the amino acid sequence in order to simplify identification of the desired mutations. Mutations were confirmed by DNA sequence determinations by the procedure of Sanger *et al.* (30). Mutant proteins were expressed in *Salmonella typhimurium* strain TR 4574, which has a large deletion, *pyrB655*, removing *pyrB* and *pyrI* (31). In addition, it contains the *pyrH700* allele causing high expression from the *pyrBI* promoter (32).

The mutant forms of ATCase and their catalytic subunit were purified by the procedures described by Wall *et al.* (11). Enzyme assays were performed by the method of Davies *et al.* (33). For the very inactive mutants in which Lys-84 was replaced by Gln or Arg, the more sensitive assay of Prescott and Jones (34) was used as modified by Pastra-Landis *et al.* (35). Kinetic parameters at various aspartate concentrations were determined at saturating carbamoylphosphate (4 mM for assay with [^{14}C]carbamoylphosphate and 1–4.8 mM for the colorimetric assay). Values of V_{max} for holoenzymes were

		80	81	82	83	84	85	86	87	
WILD-TYPE SEQUENCE		SER	LEU	GLY	LYS	LYS	GLY	GLU	THR	
TEMPLATE		--TCA	CTG	GGT	AAA	AAG	GGC	GAA	ACG--	
LYS 83 → GLN				*	*					
OLIGONUCLEOTIDE	3′	AGT	GAC	CCG	GTT	TTC	CC–			5′
LYS 84 → GLN						*	*			
OLIGONUCLEOTIDE	3′			CCA	TTT	GTC	CCA	CTT	TG–	5′
LYS 84 → ARG						*				
OLIGONUCLEOTIDE	3′			CCA	TTT	TCC	CCG	CTT		5′

		130	131	132	133	134	135	136	137	
WILD-TYPE SEQUENCE		GLY	SER	ASN	GLN	HIS	PRO	THR	GLN	
TEMPLATE		--GGC	TCC	AAC	CAA	CAT	CCG	ACG	CAA--	
HIS 134 → ALA						**				
OLIGONUCLEOTIDE	3′		–GG	TTG	GTT	CGA	GGC	TGC	G—	5′
GLN 133 → ALA					**					
OLIGONUCLEOTIDE	3′	—G	AGG	TTG	CGT	GTA	GGC	T—		5′

FIG. 1. Mutagenic oligonucleotides and wild-type template. The wild-type amino acid sequence from residues 80–87 and 130–137 is shown above the template nucleotide sequence. The nucleotide sequences of the different mutagenic oligonucleotides are presented for the various amino acid substitutions, with asterisks designating the mismatches. Some of the oligonucleotides had additional mismatches that led to silent mutations and changed the sensitivity to various restriction enzymes.

evaluated from plots of v/[aspartate] vs. v, and $K_{0.5}$ and the Hill coefficients, n_H, were obtained from plots of log [$v/(V_{max} - v)$] vs. log [aspartate]. Nucleotide effects on enzyme activity were measured at 0.5 mM CTP and 2.0 mM ATP. Results of assays with isolated catalytic subunits were fit to the Michaelis–Menten equation. For catalytic subunit in which Lys-83 was replaced by Gln, a term for linear substrate inhibition was included in fitting data with the Michaelis–Menten equation (36).

Spectral studies were performed with a Cary Model 118 double-beam spectrophotometer and effects of substrate analogs on the absorption spectra of the various proteins were measured directly as difference spectra (37). Sedimentation velocity studies were performed with a Beckman-Spinco Model E ultracentrifuge and changes in the sedimentation coefficients ($\Delta s/s$) of the enzymes due to PALA binding were made directly in experiments with two ultracentrifuge cells so that patterns for the unliganded and liganded enzymes were recorded simultaneously (38).

Results

Effect of Mutational Alterations on Catalytic and Regulatory Properties. As shown in Fig. 2, some amino acid substitutions like Gln for Lys-83 and Ala for Gln-133 cause only a slight decrease in enzyme activity with marked alterations in cooperativity. In comparison with wild-type ATCase, the Gln-83 mutant exhibits much less sigmoidality in the dependence of enzyme activity on aspartate concentration, whereas the homotropic effects with the Ala-133 mutant are much more pronounced. Replacement of Lys-84 by either Gln or Arg leads to almost a complete loss of catalytic activity and substitution of Ala for His-134 causes a large decrease in enzyme activity, a much higher $K_{0.5}$, and an increase in cooperativity. For comparison, the saturation curve of the previously described mutant (8) in which Tyr-165 is replaced by Ser is also shown in Fig. 2. Values of V_{max}, $K_{0.5}$, and n_H for the holoenzymes are summarized in Table 1.

The active mutants also exhibit the heterotropic effects characteristic of wild-type ATCase at subsaturating concentrations of aspartate. For Gln-83 ATCase in the presence of 4 mM carbamoylphosphate and 5 mM aspartate, 2 mM ATP causes a 38% increase in activity and 0.5 mM CTP leads to 50% inhibition. Activation by ATP is 50% and inhibition by CTP is 85% for the Ala-133 mutant in the presence of 4 mM carbamoylphosphate and 50 mM aspartate. With Ala-134 ATCase in the presence of 4.8 mM carbamoylphosphate and 80 mM aspartate, the activation by ATP is 21% and inhibition by CTP is 86%.

Table 1. Kinetic parameters and effect of PALA on the sedimentation coefficient of wild-type and mutant holoenzymes and catalytic trimers

	Holoenzyme				Catalytic subunit	
Mutant	V_{max}	$K_{0.5}$	n_H	$\Delta s/s$	V_{max}	K_m
Wild-type	11	7	1.7	−3.1	34	13
Gln-83	8.3	19	*	−3.0	15	19
Ala-133	8.0	48	2.7	−2.8	23	50
Ala-134	0.5	120	2.0	−2.9	2.0	45
Gln-84	0.003	10	†	−3.2	0.003	11
Arg-84	0.01	6	†	—	0.018	4

Assays and sedimentation velocity experiments were performed as described. Values of $\Delta s/s$ are expressed as percent at 3 mg per ml of enzyme and were determined for saturating levels of PALA (200 μM). V_{max} is in μmol·hr^{-1}·μg^{-1} and $K_{0.5}$ and K_m are mM.

*Pronounced substrate inhibition was observed; hence, the estimate of V_{max} is not sufficiently reliable for an accurate determination of n_H. Since the saturation curve exhibits little sigmoidality, n_H is not much larger than 1.0.

†Activities are so low that determinations of n_H are not warranted.

Enzyme assays were also performed on isolated catalytic subunit in an attempt to differentiate between effects of amino acid substitutions on catalytic and regulatory properties. The mutant catalytic subunit exhibits Michaelis–Menten kinetics and values for V_{max} and K_m are summarized in Table 1. When Lys-83 is replaced by Gln there is only a 50% decrease in V_{max} and a slight increase in K_m. Similarly, replacement of Gln-133 by Ala causes only a small decrease in activity, but there is a substantial increase in K_m. The His-134→Ala mutation causes a large decrease in activity and a marked increase in K_m. In agreement with the kinetics for mutant holoenzymes, when Lys-84 is changed to Gln or Arg, there is a dramatic loss in activity of the catalytic subunit with relatively small changes in K_m.

Binding of Substrate Analogs to the Various ATCase Mutants. As shown by Collins and Stark (37, 39), binding of the

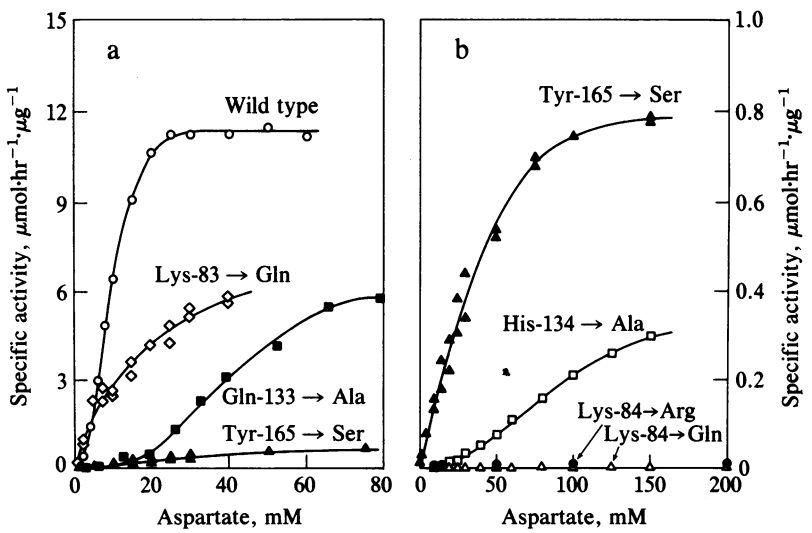

FIG. 2. Dependence of enzyme activity on aspartate concentration for wild-type ATCase and the various mutant holoenzymes. Assays were performed at 30°C in 50 or 200 mM imidazole acetate buffer (pH 7.0) containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA. Specific activities are in units of μmol of carbamoylaspartate formed per hr per μg of protein. The carbamoylphosphate concentration was 4 mM for all the mutants in *a* and for the Tyr-165→Ser and His-134→Ala mutants in *b*; for the mutants involving replacement of Lys-84 by either Gln or Arg, the concentration of carbamoylphosphate was 4.8 mM.

substrate carbamoylphosphate, the aspartate analog succinate, and the bisubstrate analog PALA, can be monitored by the perturbation of the ultraviolet absorption spectrum of the enzyme. With wild-type ATCase, carbamoylphosphate causes a small change in the spectrum, which is readily detected by ultraviolet difference spectroscopy. Addition of succinate to the enzyme–carbamoylphosphate complex causes a further change, whereas succinate alone produces no difference spectrum. Binding of PALA to ATCase yields a difference spectrum very similar to that produced by the combination of carbamoylphosphate and succinate.

For the virtually inactive Gln-84 mutant, carbamoylphosphate causes a change in the spectrum similar to that with wild-type ATCase. However, unlike wild-type enzyme, the subsequent addition of succinate causes no additional change. No perturbation in the spectrum of Ala-134 ATCase results upon adding carbamoylphosphate, but succinate in the presence of carbamoylphosphate produces a difference spectrum resembling that observed with wild-type ATCase. Succinate alone causes no perturbation of the Ala-134 ATCase absorption spectrum. These spectral studies indicate that for Ala-134 ATCase, both carbamoylphosphate and succinate must be present for either to bind.

Fig. 3 shows the PALA-promoted difference spectra for wild-type ATCase and the mutant holoenzymes containing the Gln-133→Ala, His-134→Ala, and Lys-84→Gln substitutions. The general similarity in the difference spectra indicates that PALA binds strongly to the mutant enzymes and causes changes in the local environment of some tyrosyl and tryptophanyl side chains in all of them even though one (Gln-84 ATCase) is virtually inactive and a second (Ala-134 ATCase) has greatly reduced activity and a low affinity for aspartate (cf. Fig. 2). The spectral titration curves in Fig. 3*e* show that Gln-133 ATCase has a high affinity for PALA. Similarly, the inactive mutant, Gln-84 ATCase, binds PALA strongly. In contrast, the His-134→Ala mutant has a reduced affinity for PALA, as indicated by the higher ratio of PALA/ATCase required for the maximal change in the absorption spectrum.

Ligand-Promoted Global Conformational Changes in the Various ATCase Mutants. Previous studies on wild-type and several active mutant forms of ATCase, as well as hybrids composed of native and chemically modified polypeptide chains, have shown that those proteins that exhibit homotropic effects also undergo global conformational changes upon addition of active-site ligands (10, 17, 38). It was of interest, therefore, to determine whether similar changes occurred with the mutants described above.

As shown in Table 1, which summarizes the effect of PALA on the sedimentation coefficient of the various holoenzymes, the values of $\Delta s/s$ for the mutants are similar. Even though Gln-84 ATCase is virtually inactive, binding of PALA causes a 3.2% decrease in the sedimentation coefficient, indicative of the swelling of the enzyme as it is converted from the T state (in the absence of PALA) to the liganded R conformation. Similarly, the Ala-134 mutant, which is low in activity and has a lower affinity for PALA as indicated by the spectral titration in Fig. 3, undergoes the characteristic swelling revealed by the −2.9% value of $\Delta s/s$ resulting from a saturating amount of PALA.

Although the maximal values of $\Delta s/s$ for the two active mutants Gln-83 ATCase and Ala-133 ATCase are similar, the two proteins differ in their dependence of $\Delta s/s$ on the molar ratio of PALA/ATCase at subsaturating levels of PALA. The Gln-83 mutant, which exhibits much less cooperativity in measurements of enzyme kinetics (Fig. 2), attains the maximal value of $\Delta s/s$ at a much lower ratio of PALA/ATCase than is required for the more cooperative mutant Ala-133 ATCase.

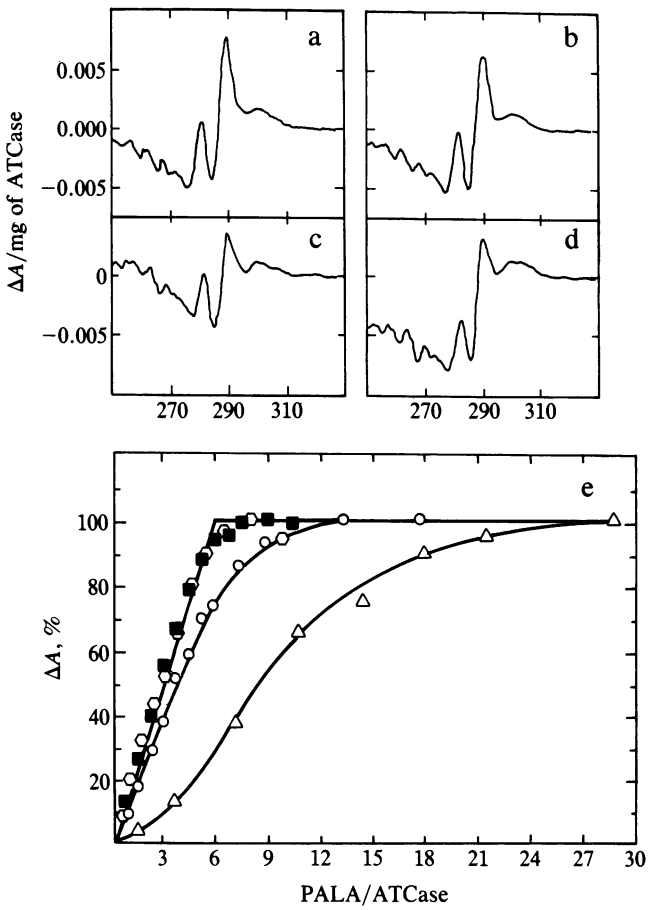

FIG. 3. Ligand-promoted difference spectra of wild-type and mutant holoenzymes. Difference spectra were measured at ≈2.5 mg/ml as described. Absorbance differences ($\Delta A_{290nm} - \Delta A_{286nm}$) were corrected to correspond to a protein concentration of 1 mg/ml; the concentration of PALA (60–215 μM for the various mutants) was sufficient to produce a maximal change in the absorption spectrum for each protein. (*a*) Wild-type enzyme; (*b*) Ala-133 ATCase; (*c*) Ala-134 ATCase; (*d*) Gln-84 ATCase; (*e*) spectral titration for wild-type and mutant enzymes. Data are plotted as percent of maximal change in absorbance ($\Delta A_{290nm} - \Delta A_{286nm}$) vs. molar ratio of PALA/ATCase. ■, Wild-type ATCase; ○, Ala-133 ATCase; ○, Gln-84 ATCase; △, Ala-134 ATCase.

Discussion

The five mutationally altered forms of ATCase differed strikingly from wild-type enzyme in their catalytic and regulatory properties. Two of the mutants in which Lys-84 was replaced by either Gln or Arg exhibited very little activity both as holoenzymes and as isolated catalytic trimers.‡ Both the Gln-83 and the Ala-133 mutants had activities comparable to wild-type enzyme, with the former exhibiting much less and the latter exhibiting much more cooperativity. The catalytic subunit from Ala-133 ATCase had a greatly increased K_m, whereas that of Gln-83 ATCase is similar to that of wild-type catalytic subunit. Replacing His-134 by Ala causes a large decrease (≈95%) in the activity of both the holoenzyme and the isolated catalytic subunit. Ala-134 ATCase exhibits more cooperativity than wild-type enzyme with a greatly increased value of $K_{0.5}$ and the mutant catalytic subunit has a much higher K_m than its wild-type counterpart.

‡The activities of the Gln-84 and Arg-84 mutants are so low that one must consider the possibility that the residual activities are attributable to a small amount of contamination with wild-type enzyme. Until this possibility is resolved, the specific activities of these mutants should be considered as upper limits.

Although Lys-83 modification by both bromosuccinate (23) and trinitrobenzene sulfonate (24) causes considerable inactivation, it is clear from the high activity of Gln-83 ATCase that Lys-83 does not play a critical role in catalysis. This conclusion is consistent with the crystallographic studies of the ATCase–PALA complex, which shows that Lys-83 is too far from the inhibitor for direct contact (20).

Lys-84 has been implicated in catalysis because of the inactivation caused by the reaction of the catalytic subunit with pyridoxal 5′-phosphate and $NaBH_4$ (21, 22). The side chain of Lys-84 is within contact distance of the α-carboxylate and phosphonate of PALA in the crystal structure of the complex (20). When Lys-84 is replaced by either Gln or Arg, a dramatic decrease in activity occurs, indicating that the precise geometry as well as the charge of the side chain is important for its function. It is especially significant that the virtually inactive mutant Gln-84 ATCase has a high affinity for PALA and that the binding of the bisubstrate analog promotes the global conformational change characteristic of the wild-type enzyme.

On the basis of photooxidation studies, Greenwell *et al.* (21) suggested that one or two unidentified histidine residues may be involved in catalysis. Because of its location deduced from the x-ray diffraction studies of the ATCase–PALA complex (20), His-134 seemed a likely candidate. As shown above, replacement of His-134 by Ala leads to a substantial reduction in activity and a large increase in K_m. The spectral studies indicate that carbamoylphosphate binding to the mutant enzyme is altered considerably and the affinity of the enzyme for PALA is reduced significantly. Nonetheless, the enzyme exhibits cooperativity with respect to aspartate and undergoes the characteristic T→R transition upon the addition of PALA.

Gln-133 is near the interface between the catalytic and regulatory chains in unliganded ATCase (19), and this contact region may be involved in allosteric regulation. Replacement of Gln-133 by Ala causes a large increase in cooperativity, and the mutant retains the characteristic heterotropic inhibition by CTP and activation by ATP. The increased value of $K_{0.5}$ of the mutant holoenzyme may be attributable in part to the lowered affinity of the active sites for aspartate, as indicated by the increase in K_m of the mutant catalytic subunit.

The mutants produced by site-directed mutagenesis with relatively conservative amino acid substitutions differ strikingly from those described previously (11–13) containing charged or bulky residues such as Asp and Phe in place of the much smaller uncharged residues Gly-128 and Ser-52, respectively. Not only were these two mutants inactive, there was no detectable binding of PALA to either of them, presumably due to steric factors and the possible distortion of the active site.

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