Effects of Replacement of Active Site Residue Glutamine 231 on Activity and Allosteric Properties of Aspartate Transcarbamoylase†

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ABSTRACT: Since crystallographic studies on *Escherichia coli* aspartate transcarbamoylase (ATCase) indicate that Gln 231 is in the active site of the enzyme and participates in the binding of the substrate, aspartate, it seemed of interest to examine mutant enzymes in which Gln 231 was replaced by Asn or Ile. The two mutant forms containing amino acid substitutions were characterized by a combination of steady-state kinetics, hydrodynamic measurements, and equilibrium ligand binding techniques. Both mutant forms exhibited a dramatic reduction in the affinity of the protein for substrates and substrate analogues as well as a very large decrease in catalytic activity. Moreover, the amino acid substitutions introduced within the active site of the enzyme led to unusual allosteric properties in the mutant enzymes. Although the bisubstrate analogue *N*-(phosphonoacetyl)-L-aspartate promotes the characteristic global conformational change in the mutant forms that is observed with the wild-type enzyme, the combination of substrate and substrate analogue does not. Cooperativity with respect to substrate binding is largely reduced compared to wild-type ATCase. Also, the effector molecules ATP and CTP which bind to the regulatory chains have dramatic effects on the activity of the mutant enzymes containing replacements for Gln 231 in the catalytic chains. In stark contrast to the wild-type enzyme, in which effects of nucleotides are manifested primarily by changes in the $K_{0.5}$ of the enzyme, ATP and CTP have large effects on the V_{max} of the mutant enzymes. Analysis of equilibrium binding of the analogue *N*-(phosphonoacetyl)-L-aspartate to the mutant enzymes based on the two-state model of Monod, Wyman, and Changeux provided insight into the unusual allosteric properties of the mutant forms of ATCase.

As the first committed enzyme in the pyrimidine biosynthetic pathway, aspartate transcarbamoylase (ATCase,[1] carbamoyl phosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2) from *Escherichia coli* catalyzes the condensation of the substrates, carbamoyl phosphate and aspartate, to yield the product, carbamoyl aspartate. The allosteric response of the enzyme to various metabolites allows for modulation of enzyme activity in response to intracellular pools of purines and pyrimidines. Thus, high levels of ATP serve to activate the enzyme, whereas accumulation of CTP acts conversely to inhibit the enzyme. In addition to this heterotropic response to the nucleotide effectors, the enzyme exhibits cooperativity with respect to its two substrates, which is apparent from the sigmoidal shape of the saturation curve for either aspartate or carbamoyl phosphate (Bethell et al., 1968; Gerhart & Pardee, 1962).

The allosteric properties of ATCase can be accounted for satisfactorily by the two-state model of Monod et al. (1965), which assumes that the enzyme molecules exist in a dynamic equilibrium between two global conformational states and that the allosteric transition between these two states results from preferential binding of ligands to one or the other conformation. Abundant evidence has been accumulated showing that binding of substrate analogues to ATCase converts the enzyme from a relatively compact, low-activity, "taut" (T) conformation to a swollen, more active, "relaxed" (R) form (Dubin & Cannell, 1975; Gerhart & Schachman, 1968; Ke et al., 1984; Krause et al., 1987; Moody et al., 1979). The ligand-promoted change in overall quarternary structure from the T form to the R conformation has been demonstrated to account quantitatively for the sigmoidal dependence of enzyme activity on substrate concentration (Howlett et al., 1977) and the cooperativity in binding the bisubstrate analogue, *N*-(phosphonoacetyl)-L-aspartate (PALA) (Newell et al., 1989).

Preferential binding of CTP to the T state and ATP to the R conformation have been invoked to account for their heterotropic effects in altering the affinity of the enzyme for substrates as observed by shifts in the concentration of substrate corresponding to half-maximal velocity [*K* effects, in the terminology of Monod et al. (1965)]. Equilibrium dialysis experiments have demonstrated that the two global conformational states differ in affinity for PALA, but it has not been shown whether the T and R conformations differ in catalytic turnover rate [a *V* component according to Monod et al. (1965)]. With the wild-type enzyme, it has been difficult to measure directly effects of the nucleotides on the global conformational state of the wild-type enzyme because the equilibrium lies far in favor of the T state in the absence of added ligands. However, experiments performed recently on a mutant form of the enzyme in which the allosteric equilibrium constant of the unliganded enzyme is close to unity have

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[1] Abbreviations: ATCase, aspartate transcarbamoylase; PALA, *N*-(phosphonoacetyl)-L-aspartate; C, catalytic trimer; R, regulatory dimer; c, catalytic polypeptide chain; r, regulatory polypeptide chain; n_H, Hill coefficient; MOPS, morpholinopropanesulfonic acid. Mutants with amino acid substitutions in the catalytic chain are denoted in the standard one-letter code by the wild-type residue and numbered position within the sequence, followed by the amino acid substitution. Thus the replacement of Gln at position 231 by Asn is referred to as Q231N.

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demonstrated directly changes in the average conformation of the enzyme molecules and their affinity for PALA as a result of CTP or ATP binding (Eisenstein et al., 1990). Hence, the two-state model provides a unifying framework with which to interpret the allosteric properties of ATCase. It should be noted, however, that some workers have invoked other indirect effects of CTP and ATP in altering the activity of the wild-type enzyme (Hervé et al., 1985; Hervé, 1988, 1989; Tauc et al., 1982; Xi et al., 1991).

This study describes changes in activity and allosteric properties resulting from the substitution of Asn or Ile for Gln 231, which is located within the aspartate binding region of the active site of ATCase. Comparison of the crystallographic structure of ATCase containing PALA (Krause et al., 1987) with that of the enzyme liganded with both the substrate carbamoyl phosphate and the aspartate analogue succinate (Gouaux & Lipscomb, 1988) indicates somewhat different contacts made by the side chain of Gln 231 in the two structures. Gln 231 appears to interact with the β-carboxylate of PALA in the structure of the enzyme ligated with the bisubstrate analogue and with both carboxylates of succinate in the complex of ATCase with carbamoyl phosphate and succinate. In selecting amino acids for replacement of Gln 231, two different considerations were applied. On the one hand, substituting Asn for Gln retains the polar nature of the side chain, although shortening the chain by one methylene group. In contrast, by using Ile as a nonpolar replacement, we maintained some of the steric features of the Gln side chain. The two mutant forms of ATCase characterized in this study can be compared with the work of Stebbins et al. (1990), who replaced Gln 231 by Leu.

The two mutant forms of ATCase were compared to the wild-type enzyme with regard to catalytic activity, substrate and analogue binding, and allosteric properties. What effects do these substitutions for Gln 231 in the active site of the enzyme have on catalysis? Does removal of a methylene group from the side chain allow for carbamoylation of bulkier amino acids? Is aspartate binding affected in the mutant forms of ATCase? Do the various enzymes differ in their binding of substrates and the bisubstrate analogue PALA? Are allosteric properties of the enzymes altered? Can changes in homotropic or heterotropic behavior of the mutant forms of the enzyme be understood in terms of the two-state model of Monod, Wyman, and Changeux? As shown here, replacements at position 231 in the catalytic (c) chain of ATCase resulted in mutant forms exhibiting striking differences in substrate binding and allosteric properties compared to the wild-type enzyme. The approach which included kinetics, hydrodynamic measurements, and equilibrium ligand binding provided a basis for an internally consistent interpretation of the unusual properties of these mutant forms of ATCase.

MATERIALS AND METHODS

Protein Preparations. Amino acid replacements at position 231 in the c chain were introduced by oligonucleotide-directed mutagenesis as previously described (Eisenstein et al., 1989), using mutagenic primers (21-mers) synthesized on an Applied Biosystems 380B synthesizer by the Microchemical Facility of the University of California at Berkeley. Likewise, the site-directed mutagenesis approach was used to generate a silent mutation changing the codon for Gly 130 from GGC to GGA, which introduced a unique *Bam*HI restriction site at that position. Also, oligonucleotide-directed mutagenesis was used to introduce a unique *Xho*I site in the intercistronic region. Plasmid pCP6 was generated upon introduction of the *Bam*HI and *Xho*I sites into plasmid pPYRB11 (Eisenstein et al., 1989), which contains both the *pyrB* and *pyrI* genes, for expression of intact ATCase. In all cases, mutants were identified by sequence determination using the dideoxynucleotide method (Sanger et al., 1977). The 550-bp *Bam*HI/*Xho*I fragment from identified mutants was subcloned into the 5.6-kb fragment of plasmid pCP6 cut with the same restriction enzymes for expression of variant forms of ATCase with amino acid substitutions at position 231. The DNA from the *Bam*HI to *Xho*I sites was subjected to sequence determination by the dideoxy method (Sanger et al., 1977) to verify the absence of secondary sites of mutation. Wild-type and mutant forms of ATCase were overproduced in *E. coli* strain EK1104 grown on minimal medium with the supplementations described by Nowlan and Kantrowitz (1985). Holoenzymes were purified from the cell extract using the procedures described by Wall et al. (1979). Mercurial reagents were used to dissociate the holoenzymes into C and R subunits, and free subunits were isolated by ion-exchange chromatography as described by Yang et al. (1978). Purity of samples was assessed by polyacrylamide gel electrophoresis using nondenaturing conditions according to Jovin et al. (1964). Activity stains on samples in gels were performed as previously described (Bothwell, 1975).

Enzyme Activity Assays. Measurements of enzyme activity were performed using the method of Davies et al. (1970) utilizing ^{14}C-labeled carbamoyl phosphate. Activity was measured at 30 °C in a 0.1 M potassium MOPS buffer, pH 7.0, containing 0.2 mM EDTA and 0.4 mM zinc acetate. Data for holoenzymes were analyzed on an Apple Macintosh SE microcomputer according to the Hill equation using the nonlinear least squares feature of the program Kaleidagraph, a product of Synergy Software. Kinetic data for mutant and wild-type C subunits were analyzed using the Michaelis–Menten equation.

In addition to an analysis of substrate saturation kinetics, the allosteric nature of Q231I ATCase was assessed by measuring the ability of the bisubstrate analogue PALA to activate the enzyme either at subsaturating or near-saturating substrate concentrations. For these experiments, the aspartate concentration was fixed and small volume aliquots from a stock solution of PALA in water were added to the reaction mixture in the MOPS assay buffer. PALA (lot no. MK 45-89-1) used for these experiments was kindly provided by Dr. Robert R. Engle of the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute.

The affinity of the mutant C subunit for the substrate analogue succinate was determined from the inhibition constant, K_i, measured by Dixon plot analysis (Dixon, 1953) using succinate as a competitive inhibitor. Assay conditions were adjusted such that kinetics were measured using saturating amounts of carbamoyl phosphate, subsaturating concentrations of aspartate, and varying levels of the competitor, succinate. The data were fitted to the following equation using the program ENZFITTER by R. J. Leatherbarrow:

$$v = \frac{V_{app}}{1 + K_{i,app}}$$

to give an apparent inhibition constant, $K_{i,app}$, and the apparent

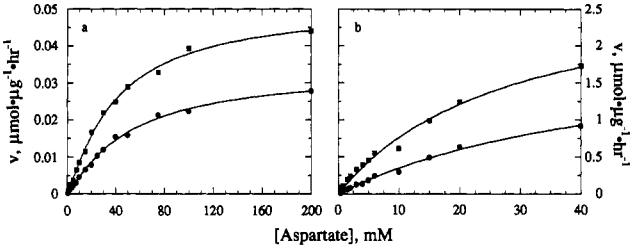

FIGURE 1: Steady-state kinetics for mutant forms of ATCase with replacements at Gln 231. Aspartate saturation plots are shown for holoenzymes in (a) and for C subunits in (b). In both panels, data for Q231N are plotted as (■) and data for Q231I as (●). In measurements on the holoenzymes, excess free R subunits were added to the assay mixture as described in Materials and Methods.

V_{max} (V_{app}). The true K_i was determined according to the relationship:

$$K_i = \frac{K_{i,app}}{1 + A/K_a}$$

where A is the concentration of aspartate and K_a equals the K_m determined for the substrate.

Analytical Ultracentrifugation. Difference sedimentation velocity experiments were performed with a Beckman-Spinco Model E ultracentrifuge and a rotor with two single-sector cells (Howlett et al., 1977). Boundary positions, determined as a function of time using schlieren optics, were analyzed to give the difference in sedimentation coefficient, $\Delta s/s$, between the samples in the two cells. Measurements at about 20 °C were made with protein solutions at a concentration of 3 mg·mL^{-1} in 50 mM MOPS buffer, pH 7.0, containing 0.2 mM EDTA and 2 mM β-mercaptoethanol. The effect of active site ligands on the sedimentation coefficient was determined by addition of a small volume of ligand to the sample cell, with an equal volume of buffer or control ligand to the reference cell. Values of $\Delta s/s$ were corrected for the added weight and density of the ligand.

Equilibrium Dialysis. PALA binding to Q231I ATCase was measured by equilibrium dialysis using ^{3}H-labeled PALA as described by Newell et al. (1989). Buffer for the experiments was 0.1 M MOPS, pH 7.0, containing 2 mM β-mercaptoethanol. Samples were dialyzed for 16 h at 15 °C. Bound ligand and free ligand at each PALA concentration were determined directly by measurement of radioactivity in a Beckman LS-7500 counter after addition of scintillation cocktail. The binding isotherm was analyzed by nonlinear least squares fitting to the Hill equation. Data were also analyzed by a nonlinear least squares method in terms of the two-state model of Monod et al. (1965). The allosteric equilibrium constant, L ([T]/[R]), was determined by this method of analysis along with values of the parameter K_R, the dissociation constant of PALA from the complex in the R form, and c, the ratio of K_R/K_T, in which K_T is the dissociation constant for PALA binding to the T form of the enzyme.

RESULTS

Replacement of Gln 231 by Asn or Ile Causes a Large Reduction in Enzyme Activity. Substitution of either Asn or Ile for Gln at position 231 in the active site of ATCase causes a large decrease in the catalytic activity of the holoenzyme and isolated C trimers. Aspartate saturation kinetics for the two mutant holoenzymes are shown in Figure 1a, and for the mutant C subunits, in Figure 1b. As seen in Table I, V_{max} for the Q231N and Q231I holoenzymes is less than 1% that of wild-type ATCase. In contrast, the value of V_{max} for the Q231N C subunit is approximately 8% of the wild-type C subunit, and V_{max} for Q231I C subunit is about 5% that of the wild-type subunit. Although the isolated C subunits from the mutant holoenzymes are substantially less active than wild-type C trimer, they are significantly more active as free subunits than when incorporated into holoenzymes. For Q231I ATCase, the C subunit is approximately 35 times more active than the holoenzyme when activity is related to the number of catalytic sites. Similarly, the C subunit from Q231N ATCase is about 35 times more active than the holoenzyme on a per site basis. In contrast, the activity of wild-type C subunit is only about 2-fold higher per active site compared to that of the intact wild-type enzyme. Table I also shows that binding of aspartate by the mutant forms, as inferred from the values of K_m for the C subunits, is significantly weaker than observed with the wild-type enzyme.

Table I: Kinetic Characterization of Mutant Forms of ATCase with Replacements for Gln 231[a]

		holoenzyme				C subunit		
ATCase	nucleotide	n_H	$K_{0.5}$, mM	V_{max}, mmol·(mg·h)$^{-1}$	activity, %[b]	K_m, mM	V_{max}, mmol·(mg·h)$^{-1}$	activity, %[b]
wild-type		1.5	5.6	16.8	100	5.6	36.6	100
Q231N		1.2	40.1	0.05	0.3	25.0	2.8	7.6
	ATP[c]	1.1	82.1	0.40				
	CTP[d]	1.2	21.7	0.04				
Q231I		1.1	52.9	0.03	0.2	42.2	1.9	5.2
	ATP[c]	1.2	52.7	0.46				
	CTP[d]	1.0	26.2	0.01				

[a] Assays were conducted in 0.1 M MOPS buffer, pH 7.0, containing 0.2 mM EDTA and 0.4 mM zinc acetate as described under Materials and Methods. Concentrations of holoenzyme were 1.6 nM for wild-type ATCase and from 64 to 130 nM for mutant forms. Isolated R subunits were added to the holoenzyme to a final concentration of 600 nM. C subunit concentrations were 2 nM for wild-type protein and 30–50 nM for the mutants. [b] Percent activity is expressed relative to that measured for the wild-type enzyme. [c] Magnesium ATP concentration was 2 mM. [d] CTP concentration was 0.5 mM.

Both Q231I ATCase and Q231N ATCase are substantially less stable than the wild-type enzyme; as a consequence, all preparations of the two mutant holoenzymes contained free C subunits resulting from dissociation of the intact enzymes. Since the activity of the isolated C subunits is so much greater than that of the intact holoenzyme, even a small amount of dissociation complicates the interpretation of enzyme assays of the mutant holoenzymes. Thus, in all the experiments on the holoenzymes illustrated by Figure 1a and Table I, isolated R subunits were added in an amount sufficient for reassembly of any C subunits into intact enzyme. The required amount of R subunits was determined by electrophoretic analysis of polyacrylamide gels stained for enzyme activity. Assays of the decrease in activity upon the addition of R subunits indicated that preparations of Q231I ATCase contained about 5% free mutant C trimers whereas about 2% of Q231N ATCase dissociated under conditions of the assay.

In one of the mutant forms of ATCase used in this study, Gln at position 231 was replaced by Asn, effectively shortening the amino acid side chain of this active site residue by one methylene group. This was done in part to determine whether removal of this group from the side chain would facilitate binding of the alternative, bulkier substrate, glutamate, at the active site. However, attempts to detect carbamoylation of glutamate by the Q231N enzyme were unsuccessful.

Active Site Ligands Are Bound Weakly to the Mutant Forms of ATCase. Since the high values of K_m for the mutant C subunits are indicative of a decreased affinity for the substrate, aspartate, it seemed of interest to examine directly

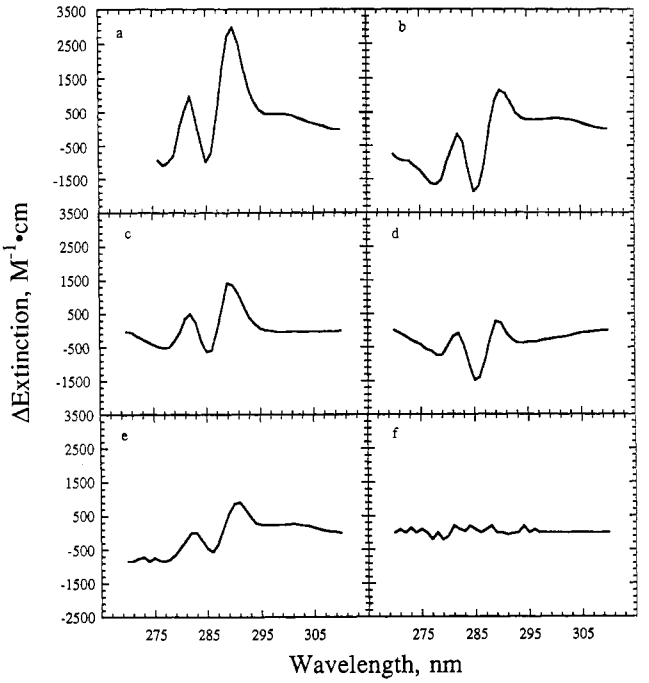

FIGURE 2: Ultraviolet difference spectra upon ligand binding to wild-type and Q231I ATCase. Changes in spectra in the ultraviolet region were recorded as difference measurements on a Cary 118 spectrophotometer using protein samples in both sample and reference cells. Protein concentrations for the experiments were 3 mg·mL^{-1}. Ligand was added to the sample cell, with equivalent volumes of buffer added to the reference cell. Difference spectra observed for the wild-type protein are shown in the panels on the left of the figure and for Q231I ATCase on the right. The uppermost two panels show spectra observed upon addition of saturating amounts of PALA (20 PALA per ATCase) to wild-type (a) or Q231I (b) enzyme. Panels c and d show difference spectra generated upon addition of 2 mM carbamoyl phosphate to wild-type and Q231I ATCase, respectively. The bottom two panels show *subsequent* spectral changes which occur upon addition of 12 mM succinate to samples of wild-type enzyme (e) or Q231I ATCase (f) which have already been saturated with carbamoyl phosphate.

the binding of other active site ligands. Figure 2 shows the difference spectra obtained upon the addition of PALA, carbamoyl phosphate, and succinate (Collins & Stark, 1969). Qualitatively, the difference spectrum produced upon PALA binding to Q231I ATCase (Figure 2b) is similar to that observed for the wild-type enzyme (Figure 2a). A significant decrease in the amplitude at 289 nm is observed with the mutant enzyme. Similar difference spectra for the wild-type and mutant enzyme are obtained upon the addition of carbamoyl phosphate (Figure 2, panels c and d, respectively). Again the magnitudes of the changes are decreased for Q231I ATCase. In contrast to these relatively small differences between wild-type and Q231I ATCase for the binding of PALA and carbamoyl phosphate, a large effect of the amino acid substitution is seen upon the addition of succinate. Whereas succinate causes a significant perturbation of the spectrum of wild-type ATCase in the presence of carbamoyl phosphate (Figure 2e), there is virtually no effect of succinate (12 mM) on the spectrum of the complex of Q231I ATCase and carbamoyl phosphate (Figure 2f).

Although the absence of a succinate-promoted spectral change in Q231I ATCase can be attributed to a greatly reduced affinity for the ligand, it is also possible that binding to the mutant enzyme does not cause the characteristic conformational change leading to an altered environment of the aromatic amino acids which is observed with wild-type ATCase (Collins & Stark, 1969). Hence, the binding of succinate as competitive

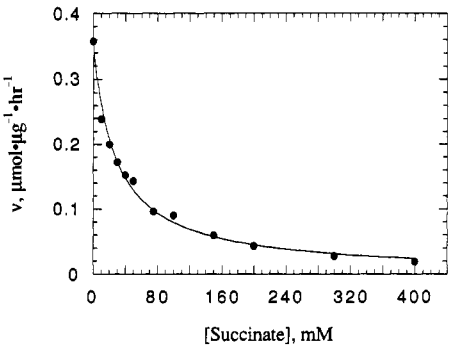

FIGURE 3: Dixon plot analysis of succinate as a competitive inhibitor in assays on the C subunits of Q231I ATCase. Inhibition in kinetic measurements was measured at varying levels of succinate and constant concentrations of 4 mM carbamoyl phosphate and 19 mM aspartate. The curve shown in the figure is the nonlinear least squares fit to the data as described under Materials and Methods.

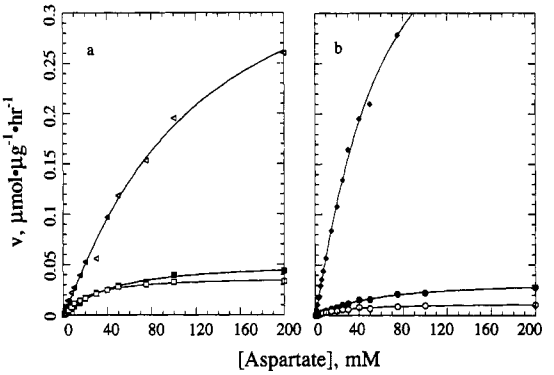

FIGURE 4: Effects of nucleotides on enzyme activity of mutant forms of ATCase containing replacements of Gln 231. Aspartate saturation plots in the presence and absence of nucleotide effectors are shown for Q231N ATCase (a) and for Q231I ATCase (b). For these assays, ATP concentration was 2 mM, and CTP concentration was 0.5 mM. In panel a are plotted results of steady-state kinetic measurements on Q231N ATCase in the presence of ATP (△) or CTP (□) or in the absence of added nucleotides (■). Panel b shows saturation kinetics for Q231I ATCase in the presence of ATP (◆), CTP (○), or no nucleotides (●). As described in Materials and Methods, R subunits were added for all assays of these holoenzymes.

inhibitor of aspartate was measured directly by enzyme kinetics. These experiments were performed with the C subunit in order to eliminate complications resulting from the allosteric transition with the holoenzyme. The inhibition of the enzyme-catalyzed reaction as a function of the succinate concentration is shown in Figure 3. Analysis of the data as described under Materials and Methods yields a value of 20 mM succinate for K_i. In contrast, the wild-type C subunit under comparable conditions exhibits a K_i of about 0.5 mM (J. Turnbull and H. K. Schachman, unpublished). Hence the affinity of the enzyme for the analogue is drastically weakened as a result of the amino acid substitution.

Homotropic and Heterotropic Properties of the Mutant Enzymes with Replacements at Position 231 Are Unusual. Fitting of the kinetic data for the holoenzymes in Figure 1 to the Hill equation indicates that homotropic cooperativity of the mutant enzymes is much less than that observed with the wild-type ATCase (Table I). This slight, but detectable, degree of cooperativity was verified using a more sensitive Eadie plot (Hensley et al., 1981). The heterotropic effectors, ATP and CTP, had a marked influence on the kinetics of the mutant holoenzymes containing amino acid substitutions at position 231 as shown in Figure 4. In contrast to the wild-type enzyme, which exhibits only alterations in the $K_{0.5}$ of the enzyme in response to the nucleotide effectors, these mutant

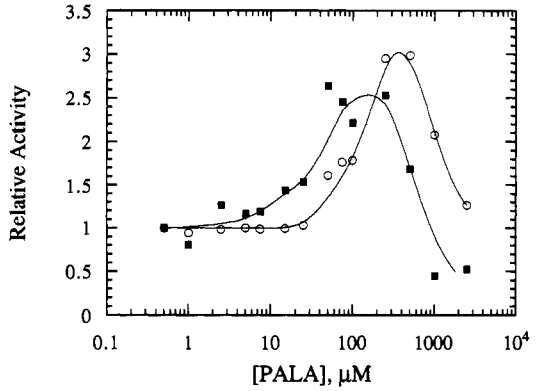

FIGURE 5: PALA activation in kinetic measurements on Q231I ATCase. Varying amounts of PALA were added to assay mixtures containing a fixed level of 4 mM carbamoyl phosphate and either 7.5 mM (■) or 100 mM (○) aspartate. Activity is expressed relative to the activity of the enzyme in the absence of added ligand. Free R subunits were added in excess over the holoenzyme in the assay, as described in Materials and Methods.

forms of ATCase showed large changes in V_{max} upon the addition of ATP or CTP (Table I).

High Concentrations of PALA Are Required To Activate Q231I ATCase. The unusual heterotropic effects for these mutant forms of ATCase coupled with the slight cooperativity prompted further examination of the allosteric properties of these enzymes. The ability of the bisubstrate analogue PALA to activate the wild-type enzyme at subsaturating aspartate concentrations has provided evidence for the conversion of the enzyme from a low- to a high-activity conformation (Collins & Stark, 1971). As seen in Figure 5, PALA also activates Q231I ATCase. In stark contrast to the wild-type enzyme, which is maximally activated by micromolar concentrations of PALA (Collins & Stark, 1971), Q231I ATCase requires almost *millimolar* concentrations of ligand for activation.

Another striking result with the Q231I enzyme is the ability of PALA to activate the mutant form of ATCase at near-saturating concentrations of aspartate. At an aspartate concentration approximately 2 times $K_{0.5}$, the enzyme is activated to an *even greater extent* than at subsaturating substrate concentrations. Activation at the higher substrate concentration requires a higher concentration of PALA. In the case of wild-type ATCase, PALA activation is achieved at an aspartate concentration significantly less than $K_{0.5}$, and no activation by the bisubstrate analogue is observed at near-saturating levels of aspartate.

Allosteric Properties of Q231I ATCase Differ from Those of Wild-Type Enzyme. The ability of PALA to activate the Q231I enzyme at near-saturating levels of substrates suggests that PALA promotes the allosteric transition that cannot be accomplished by binding of substrates alone. Sedimentation velocity measurements were employed to assess the ability of active site ligands to promote the global conformational change associated with the allosteric transition of ATCase. As shown in Table II, binding of PALA to both Q231N ATCase and Q231I ATCase causes the same decrease in sedimentation coefficient ($\Delta s/s$) as that observed with the wild-type enzyme. The value of −4.2% for $\Delta s/s$ exhibited by all three holoenzymes indicates that they undergo the same "swelling" upon the conversion from the T to the R state (Howlett et al., 1977). Addition of the substrate, carbamoyl phosphate, to Q231I ATCase leads to a decrease of 1.1% in the sedimentation coefficient of the enzyme. However, no further change in the sedimentation coefficient is observed upon the subsequent addition of succinate even to a concentration of 90 mM, which

Table II: Effects of Ligands on the Sedimentation Coefficient of Wild-Type and Mutant Forms of ATCase[a]

	ligands		
ATCase	sample	reference	$(\Delta s/s)_{corr}$, %
wild-type	PALA[b]	none	−4.2
Q231N	PALA[b]	none	−4.2
Q231I	PALA[b]	none	−4.2
Q231I	carbamoyl phosphate[c]	phosphate[c]	−1.1
Q231I	carbamoyl phosphate[c] + succinate[d]	phosphate[c] + glutarate[e]	−1.1
G231I	PALA[b] + carbamoyl phosphate[c] + succinate[d]	carbamoyl phosphate[c] + succinate[d]	−3.1

[a] Experiments were performed at 3 mg·mL^{-1} and 20 ± 2 °C as described under Materials and Methods. Buffer was 50 mM MOPS, pH 7.0, containing 0.2 mM EDTA and 2 mM β-mercaptoethanol. [b] PALA was added to the sample from a concentrated stock in water to give a molar ratio of 90 PALA per ATCase. An equal volume of water was added to the reference. [c] Carbamoyl phosphate concentration was 10 mM. Phosphate was added in an equal concentration to the reference cell. [d] Succinate concentration was 90 mM. [e] Glutarate was added at a concentration of 90 mM to the reference to mimic the density and viscosity of free succinate. A correction factor for $\Delta s/s$ due to succinate was, therefore, not applied. Uncertainty as to the validity of using glutarate to compensate for effects of succinate at such high ligand concentrations may lead to a small error in the value of $\Delta s/s$.

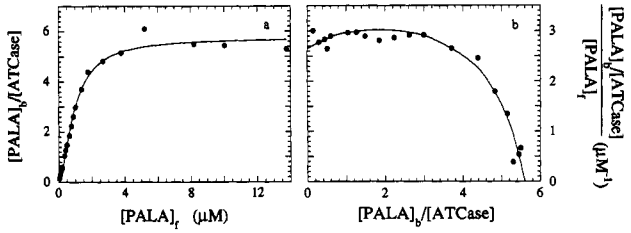

FIGURE 6: Equilibrium binding of PALA by Q231I ATCase. Binding of PALA to the mutant form of ATCase was measured by equilibrium dialysis as described under Materials and Methods. Data are represented as a saturation plot in (a) and as a Scatchard plot in (b). The subscripts b and f represent bound and free ligand, respectively.

corresponds to about 90% saturation of the active sites.[2] An independent demonstration that 90 mM succinate in the presence of carbamoyl phosphate does not promote the T → R transition is shown by the effect of PALA in the presence of these two ligands. As seen in Table II, the addition of PALA to a solution containing carbamoyl phosphate and succinate causes a 3.1% decrease in the sedimentation coefficient of Q231I ATCase. This value, −3.1%, for $\Delta s/s$ is precisely the difference between the measured values, −4.2% and −1.1%, for the effects, respectively, of PALA alone and carbamoyl phosphate alone.

Further insight into the unusual allosteric properties of the Q231I enzyme was gained from measurements of the equilibrium binding of [^{3}H]PALA (Newell et al., 1989). Direct measurements by equilibrium dialysis provide evidence both for the affinity of the mutant enzyme for PALA and for the cooperativity of binding. A fit of the binding data in Figure 6 to the Hill equation yields 1.6 for n_H and a value of 960 nM for the concentration of PALA corresponding to 50% saturation. This estimated value for the affinity of Q231I ATCase for PALA is significantly lower than that for the wild-type

[2] This calculation was based on the measured K_i of 20 mM for succinate at 30 °C. Since the ultracentrifugation experiments are performed at a temperature of 20 °C, which is 10 °C lower than that at which the K_i was measured, a value of 10 mM was used to estimate the fractional saturation of the mutant form of ATCase under conditions of the ultracentrifugation experiment.

enzyme (K_{av} = 110 nM).[3] Fitting the binding data by a nonlinear least squares method for the two-state model (Monod et al., 1965) yielded estimates of the parameters used to characterize allosteric proteins. For the allosteric equilibrium constant, L, a value of 2.2×10^3 was obtained for Q231I ATCase compared to 250 for the wild-type enzyme (Newell et al., 1989).[4] In addition, a value of 0.136 for c (K_R/K_T) was obtained compared to 0.05 for wild-type ATCase, and K_R for Q231I ATCase was 250 nM as contrasted to 50 nM for the wild-type enzyme.

These results from the equilibrium dialysis experiments are in accord with the enzyme kinetics and the ligand-promoted changes in the sedimentation coefficient in showing that the population of molecules in Q231I ATCase is shifted substantially toward the T state in comparison to the wild-type enzyme and that there is less preferential binding of PALA to the R conformation relative to the T state than is observed with wild-type ATCase.

DISCUSSION

Replacement of Gln 231 Has Pronounced Effects on the Activity of the Enzyme and Its Affinity for Active Site Ligands. Crystallographic studies have indicated that Gln 231 contributes to the aspartate-binding portion of the active site, interacting with the β-carboxylate of PALA in the structure of the ligated enzyme (Krause et al., 1987) and with both carboxylates in succinate in the complex of the enzyme ligated with carbamoyl phosphate and succinate (Gouaux & Lipscomb, 1988). It might be expected, therefore, that the affinities of both Q231N and Q231I ATCase for aspartate should be decreased in comparison to that of the wild-type enzyme. Indeed, the large increase in K_m of the C subunit is indicative of a much weaker affinity (10–20%) of the enzyme for the substrate. Moreover, the maximal activity of the C subunit from both mutant holoenzymes is reduced to less than 10% the activity of the wild-type C subunit, and both mutant forms of the holoenzyme exhibit less than 1% the activity of wild-type ATCase. Similarly, the replacement of Gln 231 by Leu (Stebbins et al., 1990) leads to a reduction in V_{max} of the holoenzyme to less than 10^{-3} of wild-type ATCase. The significant disparity between the activity of the C subunits and holoenzymes containing replacements for Gln 231 can be understood, in part, in terms of the alteration in the allosteric properties of the mutant forms of ATCase, as discussed below. The reduced affinity for aspartate and the lowered activity observed for the altered enzymes support a role for the side chain of Gln 231 in binding and appropriately orienting aspartate for carbamoylation, as suggested by crystallography.

Although the substantial increase in K_m for aspartate exhibited by the mutant C trimers is indicative of a decreased affinity for active site ligands, more direct evidence is required in order to avoid the ambiguity inherent in analyzing enzyme kinetics. Measurements by ultraviolet difference spectroscopy as described by Collins and Stark (1969) provide useful information about the binding of carbamoyl phosphate and succinate. As seen in Figure 2, there is no perturbation of the absorption spectrum by the addition of 12 mM succinate to Q231I ATCase in the presence of carbamoyl phosphate. Although this result could be interpreted in terms of succinate binding without a concomitant conformational change affecting the environment of tyrosine and tryptophan residues, it is more likely that there was little binding of succinate at the active sites. The latter interpretation is supported by the inhibition study in Figure 3 showing that K_i for succinate is 20 mM, a value almost 50-fold greater than that observed for wild-type C subunit.

Binding of the bisubstrate analogue, PALA, to ATCase can also be measured by difference spectroscopy (Collins & Stark, 1971), but the concentrations of protein (approximately 10 μM) required for this type of experiment are so much greater than the K_d exhibited by the enzyme for the ligand (generally about 100 nM) that PALA is usually bound stoichiometrically during the course of a titration, precluding determination of affinity from this type of data. However, evidence for weak binding of the bisubstrate analogue PALA was provided in the PALA activation experiments (Figure 5). Whereas wild-type ATCase is activated by PALA at micromolar concentrations, 100–1000 times more PALA is required to activate the Q231I enzyme under the conditions of the kinetic assay. Recently, direct determination of PALA binding by equilibrium dialysis measurements (Newell et al., 1989) using lower concentrations of enzyme has circumvented the problems with the spectral titration approach, as well as the inherent uncertainty in determining substrate affinities from steady-state kinetic measurements. This equilibrium dialysis approach verified weak binding of PALA by the Q231I enzyme, with a calculated affinity that is less than 10% that for wild-type ATCase.

Allosteric Equilibrium of Q231I ATCase Is Shifted toward the T State. Analysis of the equilibrium dialysis data in Figure 6 according to the two-state model of Monod et al. (1965) yielded a value of the allosteric equilibrium constant (L) of 2.2×10^3, about 10 times the value for wild-type ATCase (Newell et al., 1989). This value of L for Q231I ATCase corresponds to 4.4 kcal·mol^{-1} for the free energy difference between the T and R states as contrasted to 3.3 kcal·mol^{-1} for the wild-type enzyme. Despite this enhanced stability of the T state relative to the R conformation, Q231I ATCase is converted to the R state upon the binding of PALA. This conversion is shown by the decrease in sedimentation coefficient of the enzyme upon the addition of PALA. The value of –4.2% of $\Delta s/s$ of Q231I ATCase is the same as that for wild-type enzyme and for Q231N ATCase (Table II).

Experiments on Q231I ATCase in the presence of carbamoyl phosphate result in a change in sedimentation coefficient of –1.1%, indicating that the distribution of T and R forms in the enzyme population has shifted somewhat toward the R form upon substrate binding. This shift in the allosteric equilibrium is comparable to that observed with wild-type ATCase upon binding carbamoyl phosphate. However, addition of high concentrations of succinate to the carbamoyl phosphate–enzyme complex results in no further alteration in the sedimentation coefficient of Q231I ATCase. This result

[3] The value quoted for the dissociation constant for PALA with wild-type ATCase from Newell et al. (1989) was measured in phosphate buffer, rather than in the MOPS buffer used in the present study. Since phosphate is an active site ligand, it actually competes with and effectively *weakens* binding of PALA to the enzyme. The binding of PALA in non-phosphate buffers is estimated to be at least an order of magnitude tighter than the value measured in the presence of phosphate (Markby, 1990). Consistently, K_i measured for PALA at pH 7.0 is approximately 10 nM (J. Turnbull and H. K. Schachman, unpublished).

[4] The allosteric parameters cannot be considered a unique fit of the data to the model of Monod et al. (1965) due to the fitting procedure which involves solution of an equation with multiple interdependent variables. Since L, K_R, and c are not independent variables, alterations in one necessarily affect the other two. The values for these parameters are those which yielded a theoretical curve in satisfactory agreement with the experimental data. All reasonable fits to the data yield values of L greater than 2×10^3, K_R values that are at least 3 times larger than observed with wild-type ATCase, and c values which are significantly elevated compared to those evaluated for the wild-type enzyme.

is in contrast to that found for wild-type enzyme, where the combination of carbamoyl phosphate and succinate yields a value of $\Delta s/s$ similar to that observed with PALA. These data indicate clearly that the bisubstrate analogue PALA binds to the mutant form of ATCase differently than carbamoyl phosphate and the analogue succinate. It appears that binding of PALA is sufficient to overcome the free energy difference of 4.4 kcal·mol^{-1} between the T and R states for Q231I ATCase, whereas binding of carbamoyl phosphate and succinate is not.

In addition to the hydrodynamic data, other results from this study indicate that PALA binding to the Q231I enzyme differs from the binding of the two enzyme substrates. Notably, PALA can still activate the enzyme at near-saturating concentrations of aspartate. Clearly, PALA promotes the full allosteric transition in the enzyme population from predominantly T to predominantly R state, whereas the enzyme substrates alone do not.

Apparent "Decoupling" of Homotropic and Heterotropic Effects in Mutant Forms of ATCase Containing Substitutions for Gln 231. As seen in Figure 1 and Table I, kinetic assays of enzyme activity for both Q231N and Q231I ATCase showed a marked reduction in cooperativity with respect to aspartate when compared to that of the wild-type enzyme. A similar result was observed by Stebbins et al. (1990) for the ATCase mutant in which Gln 231 was replaced by Leu.

In contrast, there is not a corresponding decrease in the heterotropic effects. Indeed, Figure 4 and Table I show that the nucleotide effectors, ATP and CTP, have marked effects on enzyme activity. Although the values of n_H (for aspartate) are not affected significantly by the addition of ATP or CTP, the values of V_{max} are altered markedly. For Q231I ATCase, V_{max} is increased more than 10-fold when ATP is present and conversely V_{max} in the presence of CTP is reduced to about 33% that for the enzyme in the absence of nucleotides. These results are in striking contrast with those observed for wild-type ATCase for which nucleotide effectors cause changes in $K_{0.5}$ and n_H without significant alterations in V_{max}. The heterotropic effects with wild-type ATCase are observed only at subsaturating substrate concentrations, whereas with Q231N and Q231I ATCase the effects of nucleotides are manifested at saturating concentrations of aspartate.

In view of this apparent "decoupling" of the homotropic and heterotropic effects in these mutant forms of ATCase, it is important to determine whether the results can be interpreted in terms of the two-state model or, alternatively, whether they are inconsistent with a model linking the homotropic and heterotropic effects to the same global conformational change.

Unusual Allosteric Properties of Q231I ATCase Are Attributable to Decreased Preferential Binding of Active Site Ligands to the R State. Curve fitting of the equilibrium dialysis measurements for the binding of [^{3}H]PALA to Q231I ATCase indicated that the affinity of the enzyme for the bisubstrate ligand was significantly lower than that exhibited by wild-type ATCase. Moreover, the value of L was almost 10-fold larger than that for the wild-type enzyme. Although a higher value of L would be expected to result in greater cooperativity, n_H for Q231I ATCase was only 1.6 compared to 1.95 observed for wild-type ATCase (Newell et al., 1989). This decrease in cooperativity for Q231I ATCase relative to the wild-type enzyme can be accounted for by the parameter c, which is a measure of the relative affinities of the T and R conformations for the ligand. Whereas c for wild-type enzyme is about 0.05, the binding data for Q231I ATCase indicate that c is increased almost 3-fold to about 0.14. Hence the R state of Q231I ATCase has only about a 7-fold greater affinity for PALA than the T state in contrast to the ratio of 20 observed for the wild-type enzyme (Newell et al., 1989). Because the preferential binding of PALA to the R state is less, the cooperativity as measured by n_H is significantly lower for Q231I ATCase than for the wild-type enzyme.

This decrease in preferential binding to the R state of Q231I ATCase is apparently even more pronounced for the substrate, aspartate, or the analogue, succinate. Very little cooperativity is observed in the enzyme kinetics. Moreover, the addition of succinate in the presence of carbamoyl phosphate causes no decrease in the sedimentation coefficient, indicating that the mutant enzyme is not converted to the R state. Hence the activity measured even at high aspartate concentrations in indicative of enzyme molecules largely in the T state. Unlike wild-type holoenzyme for which V_{max} corresponds to the R conformation with a value (per active site) comparable to that of free C subunits, Q231I ATCase has a V_{max} representative of the T conformation with a value per site substantially less than that for the mutant C trimer.

The striking changes in enzyme activity of Q231I ATCase upon the addition of ATP or CTP can be accounted for by the two-state model (Monod et al., 1965). Preferential binding of ATP to the R conformation, for example, would shift the population of molecules toward that state and lead to an increase in V_{max}. If V_{max} for the R conformation is 100-fold larger than that for the T state, then only a slight decrease in L upon the addition of ATP could result in a substantial increase in the measured value of V_{max}. Conversely, a shift toward the T state caused by preferential binding of CTP to enzyme molecules having that conformation would lead to a decrease in V_{max} as observed. It should be recognized that the activities of both Q231I and Q231N ATCase are much lower than that of wild-type enzyme under comparable conditions and, therefore, experimental errors for both mutant holoenzymes are almost certainly larger than for wild-type enzyme. Moreover, there is the additional complication of the much more active free C trimers. All of the assays were performed with solutions to which free R subunits were added to minimize this complication. Since the values of $K_{0.5}$ for the holoenzymes differ from those of the isolated C trimers, the results are apparently attributable to the holoenzymes rather than preparations containing varying amounts of contaminating C trimers.

The behavior of Q231I ATCase in the presence of carbamoyl phosphate and aspartate (or succinate) is somewhat analogous to the activity of the wild-type enzyme toward some analogues of aspartate such as cysteine sulfonate (Foote et al., 1985) and L-alanosine (Baillon et al., 1985), for which kinetics exhibit virtually no homotropic cooperativity but still show substantial heterotropic effects. Similar loss of homotropic effects is observed in combination with pronounced heterotropic effects in the reverse of the physiologically catalyzed reaction, i.e., with carbamoyl aspartate and phosphate and substrates (Foote & Lipscomb, 1981; Foote & Schachman, 1985). Although some workers interpret this apparent uncoupling of the homotropic and heterotropic effects as being inconsistent with the model of Monod et al. (1965), the results can be explained simply by the hypothesis that these substrates are not bound preferentially to the R state of the enzyme. This interpretation accounts for the observation that wild-type ATCase shows very little activity toward these substrates, little or no cooperativity, and very substantial activation upon the addition of PALA, which converts it to the much more active R conformation.

These studies with Q231I ATCase are of use in illustrating an important aspect of the model of Monod et al. (1965) which has not been given sufficient attention. The model describes two extreme types of allosteric enzymes. On the one hand, there are the K systems for which the T and R states differ in substrate affinity and not in V_{max}. There could be, on the other hand, a V system including enzymes for which the T and R conformations have the same K_m but very different values of V_{max}. A K system exhibits both homotropic and heterotropic effects whereas the latter, V system, exhibits heterotropic effects but is devoid of cooperativity with respect to substrates. With wild-type ATCase it has not been possible directly to demonstrate that V_{max} for the R state differs from that of the T state because the addition of aspartate in the presence of carbamoyl phosphate promotes the T → R conversion. Hence no value for V_{max} for the T state is available. However, the evidence from experiments on the reverse reaction (Foote & Schachman, 1985) demonstrating almost a 50-fold increase in activity upon the conversion of the wild-type enzyme from the T to the R state suggests that V_{max} for the R state is much larger than V_{max} for the T state. Similar but smaller activation for the wild-type enzyme has been shown for the physiological reaction (Collins & Stark, 1971; Gerhart & Pardee, 1962). Thus wild-type ATCase appears to be a mixed V/K system. For such an enzyme, it is not unexpected that some amino acid substitutions could essentially abolish the K component of allosteric behavior with little or no effect on the V component. If this were to occur, there would be a loss in homotropic effects without an accompanying loss of heterotropic effects. Such an uncoupling appears to have resulted from the amino acid replacements at position 231 in the c chain of ATCase.

Several other mutant forms of ATCase with amino acid substitutions leading to weak binding of aspartate, including E50A ATCase (Newton & Kantrowitz, 1990), E50Q ATCase (Ladjimi et al., 1988), S171A ATCase (Dembowski et al., 1990), and R234S ATCase (Middleton & Kantrowitz, 1988), exhibit similar effects on the homotropic and heterotropic properties as seen with ATCase containing replacements at position 231 in the c chain. It is intriguing that replacement of an active site residue, Gln 231, by Asn, Ile, or Leu alters the allosteric properties of the enzyme by reducing the preferential affinity of the R conformation for aspartate compared to the T state. Crystallographic studies of the mutant forms of ATCase in both the T and R conformations would perhaps provide a structural basis for this interpretation of the experimental data.

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Registry No. Gln, 56-85-9; ATCase, 9012-49-1; Asn, 70-47-3; Ile, 73-32-5; Asp, 56-84-8; ATP, 56-65-5; CTP, 65-47-4; PALA, 51321-79-0; carbamoyl phosphate, 590-55-6; succinic acid, 110-15-6.