Heterotropic Effectors Promote a Global Conformational Change in Aspartate Transcarbamoylase†

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ABSTRACT: The sigmoidal dependence of activity on substrate concentration exhibited by the regulatory enzyme aspartate transcarbamoylase (ATCase) of *Escherichia coli* is generally attributed to a ligand-promoted change in the quaternary structure of the enzyme. Although a global conformational change in ATCase upon the binding of ligands to some of the six active sites is well documented, a corresponding alteration in the structure of the wild-type enzyme upon the addition of the inhibitor, CTP, or the activator, ATP, has not been detected. Such evidence is essential for testing whether heterotropic, as well as homotropic, effects can be accounted for quantitatively in terms of coupled equilibria involving a conformational change in the enzyme and preferential binding of ligands to one conformation or the other. This evidence has now been obtained with a mutant form of ATCase in which Lys 143 in the regulatory chain was replaced by Ala, thereby perturbing interactions at the interface between the regulatory and catalytic chains in the enzyme and destabilizing the low-activity, compact (T) conformation relative to the high-activity, swollen (R) state. Difference sedimentation velocity experiments involving measurements of the changes caused by the binding of the bisubstrate analogue *N*-(phosphonacetyl)-L-aspartate demonstrated that the sedimentation coefficient of the mutant enzyme was intermediate between that observed for the T and R states of wild-type ATCase. We interpret the results as indicating that the [T]/[R] ratio in phosphate buffer at pH 7.0 is reduced from about 2×10^2 for the wild-type enzyme to 2.7 for r143Ala ATCase. The addition of CTP to the mutant enzyme led to an increase in [T]/[R] to 34, whereas the ratio was lowered to 0.2 in the presence of ATP. Equilibrium dialysis measurements for the binding of PALA demonstrated that the Hill coefficient, n_H, which was 1.4 in the absence of effectors, was increased to 2.0 in the presence of CTP and decreased to 1.0 by the addition of ATP. Analysis of the equilibrium binding data, in terms of the two-state model of Monod, Wyman, and Changeux, yielded [T]/[R] ratios in accord with those deduced from the sedimentation velocity experiments. These results provide convincing evidence that heterotropic effectors cause an alteration in the average quaternary structure of ATCase and thereby regulate the activity of the enzyme by perturbing the T $\rightleftharpoons$ R equilibrium. Moreover, the observations refute suggestions that only local changes in the structure of the enzyme occur upon the binding of CTP and ATP.

Numerous studies over the past 30 years have demonstrated that the unliganded regulatory enzyme aspartate transcarbamoylase (ATCase, carbamoyl phosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2)[1] from *Escherichia coli* is converted from a relatively compact (T) conformation to a more swollen (R) state upon the binding of substrate analogues (Gerhart & Schachman, 1968; Howlett et al., 1977; Kim et al., 1987; Krause et al., 1987; Kantrowitz & Lipscomb, 1988; Ke et al., 1988; Schachman, 1988). This global change in quaternary structure coupled with the assumption of a higher affinity of the R conformation for substrates is presumed to account for the sigmoidal dependence of enzyme activity on the concentration of the substrate, aspartate, when the other substrate, carbamoyl phosphate, is present in saturating amounts (Gerhart & Pardee, 1962). In very recent studies (Newell et al., 1989), the uncertainty inherent in interpreting steady-state kinetics in terms of affinities for substrates was circumvented by direct equilibrium dialysis measurements of the binding of the bisubstrate analogue, *N*-(phosphonacetyl)-L-aspartate (PALA). The results established that ligand binding was cooperative and that the isotherms were shifted by the presence of the inhibitor, CTP, or the activator, ATP, in exactly the same fashion as enzyme activity was altered by the addition of these effectors. These observations are in direct accord with expectations based on coupled equilibria (Wyman, 1964; Monod et al., 1965). Although there is substantial agreement that the homotropic effect exhibited by ATCase is attributable to the preferential binding of substrate analogues at the active sites of the R conformation (Schachman, 1988), there is controversy over how the heterotropic effectors regulate enzyme activity (Howlett et al., 1977; Thiry & Hervé, 1978; Hensley & Schachman, 1979; Tauc et al., 1982; Hervé et al., 1985; Hsuanyu & Wedler, 1988).

Is there a dynamic equilibrium between the different quaternary structures? Do the heterotropic effectors regulate enzyme activity by shifting the equilibrium between the low-activity T state and the high-activity R conformation? Or do CTP and ATP control enzyme activity by promoting only local structural changes that are communicated to the active sites by another mechanism? These questions are difficult to address for unliganded, wild-type ATCase because the T con-

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

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formation is the predominant form. On the basis of the two-state model (Monod et al., 1965) along with appropriate physical chemical data, the [T]/[R] ratio has been estimated as 2×10^2 in phosphate buffer at pH 7.0 (Howlett et al., 1977; Schachman, 1988; Newell et al., 1989). Hence, it has not been possible to detect the presence of R-state molecules in unliganded ATCase or the dynamic equilibrium between the two conformations. Moreover, any shift in the putative T $\rightleftharpoons$ R equilibrium caused by the addition of CTP or ATP to the wild-type enzyme is undetectable. These difficulties have now been surmounted by experiments with a mutant form of ATCase that behaves as a mixture containing nearly equal amounts of both the T and R conformations. In this mutant the interactions at the interface between the catalytic (c) and regulatory (r) chains were perturbed by substituting Ala for Lys 143 in the r chain, thereby destabilizing the T state relative to the R conformation. Sedimentation velocity experiments and equilibrium dialysis measurements of PALA binding to the mutant enzyme showed that the relative fraction of molecules in the T and R states was shifted markedly by the addition of nucleotides and by changes in pH (Markby et al., 1990). The data presented here demonstrate that heterotropic effectors promote significant shifts in the equilibrium between the global T and R conformational states of ATCase, and the findings support fully the predictions of the two-state model of Monod et al. (1965) that heterotropic effectors alter the equilibrium by preferential binding to the T or R state.

Experimental Rationale

Multiple interchain interactions are implicated in stabilizing the oligomeric structure of ATCase (Kim et al., 1987; Ke et al., 1988), and these interactions are altered when the enzyme is converted from the T state to the R conformation (Krause et al., 1987). Previous studies in this laboratory (Eisenstein et al., 1989) have shown that different replacements of amino acids in the r chains at the contact regions with c chains have marked effects on the allosteric properties of the enzyme as well as its stability. For the present investigation, we required an enzyme in which the T state was destabilized relative to the R conformation so that approximately equal amounts of both forms would be present. With such a mutant enzyme, even small shifts in the equilibrium toward the T state by the slight preferential binding of CTP to that conformation or toward the R state in the presence of ATP might be detectable both by physical–chemical measurements sensitive to the quaternary structure and by equilibrium dialysis experiments that indicate changes in the cooperativity of PALA binding.[2]

A mutant form of ATCase with the desired properties was obtained by replacing Lys 143 in the r chains by Ala. This substitution was made because Lys 143 in the r chains is located at the c1–r4 interface, a region that has been indicated by crystallographic studies (Kim et al., 1987; Krause et al., 1987; Kantrowitz & Lipscomb, 1988; Ke et al., 1988) to undergo considerable rearrangement when wild-type ATCase is converted from the T to the R conformation. Because contacts at the c1–r4 interface are eliminated in the R state, a perturbation in this region might be expected to destabilize the T state relative to the R state sufficiently to yield an enzyme amenable for measuring directly the effects of CTP and ATP.

The relative amounts of the mutant enzyme in the T and R states were determined by difference sedimentation velocity experiments. With wild-type ATCase, which is largely in the T state when unliganded, the binding of PALA promotes a 3.5% *decrease* in the sedimentation coefficient (Howlett & Schachman, 1977). This decrease is attributable to an increase in the frictional coefficient corresponding to about a 10% increase in the hydrodynamic volume of the enzyme. In contrast, a mutant form of ATCase, r111Ala ATCase, which is devoid of cooperativity and is predominantly in the R state, exhibits no change in sedimentation coefficient upon the binding of PALA (Eisenstein et al., 1989). Moreover, this mutant enzyme has the same sedimentation coefficient as PALA-liganded wild-type ATCase. Mixtures containing equal amounts of protein molecules in the T and R states have an average sedimentation coefficient which is 1.8% less than that of unliganded, wild-type enzyme (Werner & Schachman, 1989). Thus, sedimentation velocity measurements can provide reliable estimates for the fraction of protein molecules in the T and R conformations.

Materials and Methods

Site-directed mutagenesis, as described by Eisenstein et al. (1989), was used to replace Lys 143 in the r chain by Ala, yielding r143Ala ATCase, which was purified as described previously for a variety of mutant forms containing amino acid replacements in the r chains. Assays of enzyme activity were performed at 21 °C by the procedure of Davies et al. (1970) with 4 mM carbamoyl phosphate and varying amounts of aspartate. The same buffer consisting of 0.04 M potassium phosphate at pH 7.0, 2 mM 2-mercaptoethanol, and 50 μM zinc acetate was used for enzyme kinetics, sedimentation velocity, and equilibrium dialysis experiments.

Difference sedimentation velocity experiments were performed as described previously (Howlett & Schachman, 1977; Werner & Schachman, 1989) with a Beckman-Spinco Model E ultracentrifuge and a rotor containing two single-sector cells with graphite-filled epoxy centerpieces and quartz windows. One cell had conventional plane windows, while the other had a lower plane window and a 1° positive wedged window. In this way, the schlieren pattern of the solution in one cell was displaced relative to that for the other cell, permitting simultaneous measurements of the boundary positions of both solutions. From measurements of these boundary positions as a function of time, the difference in sedimentation coefficient, $\Delta s/s$, between the two samples was calculated directly. The effect of PALA on the sedimentation coefficient was determined by adding the ligand in H_2O to one solution and an equal amount of H_2O to the other. Values of $\Delta s/s$ were corrected for the added weight and density due to PALA binding as described by Howlett and Schachman (1977). The ratio, [T]/[R], or the allosteric equilibrium constant, L, was determined from the maximum PALA-promoted $\Delta s/s$ by assuming that the T and R states have $\Delta s/s$ values of −3.5% and 0.0%, respectively, and that the maximum observed $\Delta s/s$ is directly proportional to the fraction of molecules in the T state. Since the precision in the measurement of $\Delta s/s$ is about 0.05%, ratios of [T]/[R] ranging from 0.02 to 70 can be estimated by this method. It should be noted that Howlett et al. (1977) fit the two-state model (Monod et al., 1965) to complete titration curves of $\Delta s/s$ versus PALA/ATCase to obtain values for L. Although this curve-fitting approach

[2] Curve fitting of experimental data for wild-type ATCase to the two-state model (Howlett et al., 1977) indicates that the preferential binding of ATP and CTP to the R and T conformations, respectively, is very slight. The ratio of the dissociation constant for ATP from the R conformation relative to that from the T state was estimated as 0.81. Hence, according to the model, the [T]/[R] ratio for the wild-type enzyme decreases from about 2×10^2 to 70 upon the addition of ATP. This shift is so slight as to be virtually undetectable, and therefore, it was essential for the present investigation to use a mutant enzyme for which the [T]/[R] ratio was close to unity.

expands the range of [T]/[R] ratios that can be estimated, it relies on additional postulates of the two-state model and was not used in this study.

PALA binding to r143Ala ATCase was measured by equilibrium dialysis according to the procedure of Newell et al. (1989), and the data were analyzed by nonlinear least-squares (Johnson & Frasier, 1985) in terms of the two-state model (Monod et al., 1965). This analysis yielded the allosteric equilibrium constant, L, K_R, the dissociation constant for PALA binding to the R conformation, and c, the ratio of K_R/K_T, where K_T is the dissociation constant for PALA binding to the T state. In this way an independent estimate of the [T]/[R] ratio was determined, not subject to the limits in the difference sedimentation velocity technique.

Two different numerical approaches were used to treat the binding data, and both yielded values of the parameters that were identical within experimental errors. The first method consisted of analyzing each independent binding curve to give values of the parameters and confidence limits for L, K_R, and c. In the second approach, essentially an extension of nonlinear least-squares with multiple independent variables, all the binding data in the absence and presence of nucleotides were analyzed to yield unique values for L_0 ([T]/[R] in the absence of effectors), L_{CTP}, L_{ATP}, K_R, and c. This method uses fewer parameters for the total analysis and is based on the requirements of the two-state model that K_R and c are the same in the absence and presence of the nucleotides. Results from this approach are presented below, but it should be noted that the parameters evaluated from both approaches are in excellent agreement.

Hill coefficients, n_H, were determined from the maximum slope of plots of log $(\nu_{max} - \nu)/\nu$ versus log [PALA], where ν is the average number of molecules of PALA bound per ATCase molecule and ν_{max} is the maximum number of bound PALA molecules.

Results

r143Ala ATCase Exhibits No Cooperativity in Enzyme Kinetics but Binds PALA Cooperatively. The characteristic sigmoidal dependence of enzyme activity on the concentration of aspartate (in the presence of saturating carbamoyl phosphate) exhibited by wild-type ATCase is eliminated as a result of the replacement of Lys 143 in the r chains by Ala. This is illustrated by the linearity of the Eadie plot in Figure 1A. In addition, n_H for r143Ala ATCase is 1.0 compared to 1.8 for wild-type enzyme under the same conditions. These results demonstrate that the mutant enzyme obeys Michaelis–Menten kinetics with a K_m of 2.5 mM aspartate and a V_{max} of 2.7 μmol h^{-1} μg^{-1}.

In contrast to the absence of homotropic effects in enzyme kinetics, the mutant exhibits cooperativity in the binding of PALA under identical conditions (buffer and temperature). This cooperativity is illustrated in Figure 1B by the curved Scatchard plot of the data from the equilibrium dialysis experiments. Analysis of the binding data yields 1.4 for n_H, a value significantly less than the 1.9 observed for the wild-type enzyme under similar conditions (Newell et al., 1989).

Carbamoyl Phosphate Alters the Quaternary Structure of r143Ala ATCase. How can an enzyme that exhibits cooperativity in PALA binding not show cooperativity in enzyme kinetics? How can one account for these seemingly contradictory observations? Are some of the r143Ala ATCase molecules in the T conformation and others in the R state? Does the substrate carbamoyl phosphate have a marked effect in shifting the T $\rightleftharpoons$ R equilibrium? These questions were addressed by measuring the effects of PALA on the sedimentation coefficient of the mutant enzyme in the absence and presence of carbamoyl phosphate. As shown in Figure 2, the maximum value of $\Delta s/s$ promoted by PALA binding is −2.55%, less than the value of −3.5% for wild-type ATCase (Howlett & Schachman, 1977; Eisenstein et al., 1989) and greater than 0.0% for ATCase mutants in the R conformation (Eisenstein et al., 1989; Newell and Schachman, unpublished results; Wente and Schachman, unpublished results). In contrast, as seen in Figure 2, PALA has no effect on the sedimentation coefficient of r143Ala ATCase when carbamoyl phosphate is present. Moreover, in independent experiments the effect of carbamoyl phosphate on r143Ala ATCase was determined directly without addition of PALA by comparing the sedimentation coefficients of the mutant enzyme in the presence and absence of 1 mM carbamoyl phosphate. The value of $\Delta s/s$ was −2.67%, in excellent agreement with that determined by the effect of PALA in the absence and presence of carbamoyl phosphate. Thus, the mutant enzyme–carbamoyl phosphate complex is largely in the R conformation. Hence,

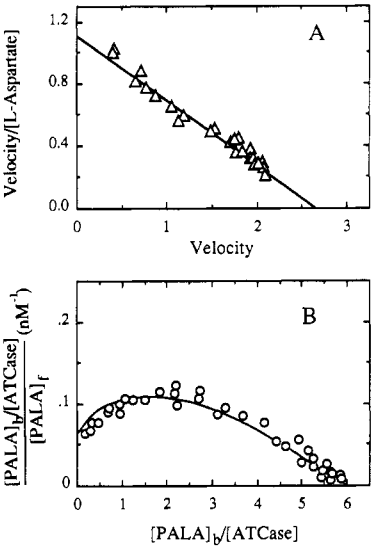

FIGURE 1: (A) Eadie plot of L-aspartate saturation kinetics for r143Ala ATCase. Initial velocity is given in units of μmoles of carbamoyl L-aspartate h^{-1} (μg of enzyme)$^{-1}$ and concentrations of L-aspartate are in millimolar. Steady-state kinetics were performed, as described under Materials and Methods, in phosphate buffer, pH 7.0. The theoretical curve was obtained from fitting the Michaelis–Menten equation to the primary experimental data for the initial velocity versus L-aspartate concentration by using nonlinear least-squares analysis. (B) Scatchard plot for PALA binding to r143Ala ATCase. PALA binding was measured by equilibrium dialysis in phosphate buffer, pH 7.0, using 66.7–167 nM r143Ala ATCase. The theoretical curve was generated by fitting the two-state model to the primary experimental data, $[PALA]_{bound}/ATCase$ versus $[PALA]_{free}$, using nonlinear least-squares analysis as described under Materials and Methods.

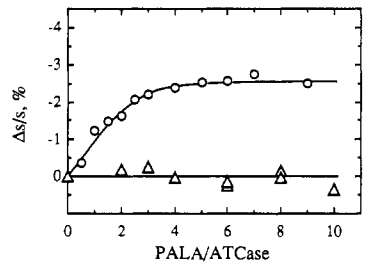

FIGURE 2: Effect of carbamoyl phosphate on the PALA-promoted change in sedimentation coefficient for r143Ala ATCase. Results are presented as the percentage change in sedimentation coefficient, $\Delta s/s$, versus the molar ratio of PALA/ATCase in the absence (○) and presence (△) of 1 mM carbamoyl phosphate. Protein concentration was approximately 3.5 mg/mL in phosphate buffer, pH 7.0.

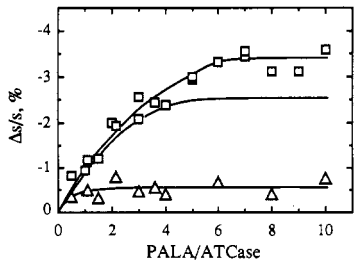

FIGURE 3: Effect of nucleotides on the quaternary conformation of r143Ala ATCase. The percent change in sedimentation coefficient versus molar ratio of PALA/ATCase is plotted for r143Ala ATCase at approximately 3.5 mg/mL in phosphate buffer, pH 7.0, in the presence of either 2 mM CTP (□) or 2 mM ATP (△). Effects of nucleotides were measured in the presence of 2 mM magnesium acetate. The solid line plateauing at $\Delta s/s = -2.55\%$ represents a reference curve for data in the absence of nucleotides shown in Figure 2.

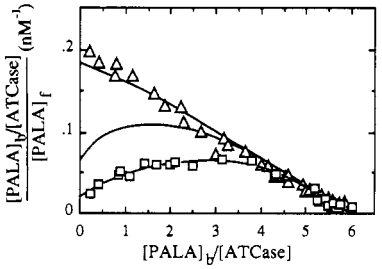

FIGURE 4: Scatchard plots showing the effects of nucleotides on PALA binding to r143Ala ATCase. Equilibrium dialysis measurements of PALA binding were performed as described in the legend to Figure 1B in the presence of 2 mM magnesium acetate and either 2 mM CTP (□) or 2 mM ATP (△). Theoretical curves were obtained as described in the legend to Figure 1B. The curve without data points is from Figure 1B and corresponds to the binding of PALA to r143 Ala ATCase in the absence of nucleotides.

no cooperativity is observed in enzyme kinetics when the concentration of aspartate is varied in the presence of saturating carbamoyl phosphate. From the value for the PALA-promoted $\Delta s/s$ in the absence of carbamoyl phosphate we calculate a value of 2.7 for the allosteric equilibrium constant, L, for r143Ala ATCase.

The Quaternary Structure of r143Ala ATCase Is Altered by the Addition of CTP and ATP. Since the PALA-promoted change in the sedimentation coefficient of r143Ala ATCase is intermediate between that observed for wild-type ATCase, which is almost completely in the T conformation, and for mutants predominantly in the R state, it was of interest to determine whether the effect of nucleotides on the quaternary structure could be measured directly by changes in the sedimentation coefficient. As seen in Figure 3, the addition of PALA to r143Ala ATCase in the presence of CTP caused a 3.4% decrease in the sedimentation coefficient, significantly larger than the 2.55% decrease observed in the absence of nucleotides. Moreover, when ATP is present, the value of $\Delta s/s$ upon PALA binding is only −0.55%. Thus, CTP and ATP have clearly demonstrable and opposite effects on the average global conformation of the mutant enzyme. In the presence of CTP the population of molecules is shifted substantially toward the T conformation, and therefore, a larger value of $\Delta s/s$ is observed upon the addition of PALA. In contrast, the bulk of the enzyme in the presence of ATP is in the R state, and consequently, there is little change in the average sedimentation coefficient when PALA is bound. For r143Ala ATCase in the presence of CTP, L is estimated as 34, and in the presence of ATP, L is 0.2 as compared to 2.7 in the absence of nucleotides.

The effects of CTP and ATP in perturbing the T ⇌ R equilibrium for r143Ala ATCase were also determined directly in an independent sedimentation velocity experiment without the use of PALA. When the sedimentation coefficient of ATP-saturated enzyme was measured relative to that of the enzyme in the presence of CTP, the value of $\Delta s/s$ was −3.0%. This result is virtually identical with the difference in the maximum values of $\Delta s/s$ in Figure 3 for the effects of PALA on the enzyme in the presence of ATP as contrasted to CTP.

The Affinity of r143Ala ATCase for PALA Is Markedly Affected by CTP and ATP. Although the sedimentation velocity experiments demonstrate that r143Ala ATCase undergoes changes in quaternary structure upon the addition of nucleotides, the results do not indicate whether CTP and ATP shift only the T ⇌ R equilibrium or, in addition, affect the binding of PALA. Hence, equilibrium dialysis experiments were performed to study the affinity of the mutant enzyme in the absence and presence of the nucleotides, and the results are summarized in Figure 4. As seen in the Scatchard plots, CTP increases the cooperativity of PALA binding over that in the absence of nucleotides. This change is demonstrated by the shift in the maximum of the concave curve to higher saturation (Dahlquist, 1978) and the increase in n_H from 1.4 to 2.0. In contrast, ATP virtually eliminates cooperativity in the binding of PALA, as seen by the nearly straight Scatchard plot and a value of 1.0 for n_H.

Table I: Effect of Nucleotides on the Quaternary Structure of r143Lys→Ala ATCase

ligand	sedimentation velocity[a] [T]/[R]	PALA binding[b] [T]/[R]
none	2.7 (2.0–3.6)[c]	2.6 (1.7–3.6)[d]
ATP	0.20 (0.10–0.30)[c]	0.14 (0–1.2)[d]
CTP	34 (8.0–>70)[c]	37 (28–48)[d]

[a] Values were derived from the maximum PALA-promoted $\Delta s/s$ as described under Materials and Methods. [b] Values were derived by fitting the two-state model to the binding data by nonlinear least-squares methods as described in the text. [c] Error limits were estimated from range of values observed for the maximal $\Delta s/s$. [d] Error limits represent the 65% confidence limits for the fitting routine.

To compare the results for L from the sedimentation velocity experiments with those from the binding studies, the two-state model of Monod et al. (1965) was used to fit the entire set of binding data in the absence and presence of nucleotides. A good fit was obtained with $K_R = 29.5$ nM, $c = 0.085$, $L = 2.6$ in the absence of effectors, $L = 37$ in the presence of CTP, and $L = 0.14$ in the presence of ATP.[3] These values of L deduced from the binding data are in excellent agreement with those determined from the difference sedimentation velocity experiments (Table I).

Discussion

r143Ala ATCase Is a Mixture of T and R Quaternary Structures. Of the various models proposed to account for

[3] For wild-type ATCase the values for L evaluated by analyzing the structural and functional properties of the enzyme in terms of the two-state model are 250 in the absence of effectors, 1250 in the presence of CTP, and 70 in the presence of ATP. These values of L are reduced to 7, 35, and 2, respectively, when carbamoyl phosphate is present. It should be noted that these values for wild-type ATCase were based on data from sedimentation velocity and enzyme kinetics experiments (Howlett et al., 1977). Also, no magnesium ions were added when the effects of CTP and ATP were determined. In a more recent study (Newell et al., 1989) based solely on equilibrium dialysis measurements of PALA binding to wild-type ATCase, a value of 45 nM was obtained for K_R and the changes in L due to nucleotides were much larger. Whether this is due to use of the magnesium salts or limitations in the fitting procedure for the two-state model is not clear.

the properties of allosteric proteins, the two-state model of Monod, Wyman, and Changeux (Monod et al., 1965) has been most useful for interpreting the cooperative ligand binding and enzyme kinetics for ATCase as well as its regulation by the heterotropic effectors CTP and ATP (Schachman, 1988). Several postulates of the model, however, have been refractory to direct inquiry. Foremost among these is the postulate of an equilibrium between the T and R quaternary conformations in the absence of ligands. The principal obstacle to determining whether there is a dynamic equilibrium is the predominance of one conformational state over the other under accessible experimental conditions. With wild-type ATCase, the small population of enzyme molecules in the R state is virtually undetectable by physical measurements. In addition, the two-state model requires that heterotropic effectors bind preferentially to one or the other structural forms, thereby regulating enzyme activity by shifting the putative allosteric equilibrium between the two quaternary states. If one global structure predominates, then small shifts in the population of enzyme molecules are extremely difficult to measure experimentally. These limitations have been overcome with the mutant enzyme, r143Ala ATCase, which exhibits solution properties of a system containing both T and R conformations.

Evidence for the view that r143Ala ATCase contains molecules in both the T and R conformations stems directly from both the sedimentation velocity and PALA-binding experiments. The 2.55% decrease in the sedimentation coefficient of the enzyme upon PALA binding, which indicates an alteration in quaternary structure, is significantly less than the 3.5% decrease observed for the wild-type enzyme which is predominantly in the T state. The value of −2.55% for $\Delta s/s$ for the mutant enzyme is most readily interpreted in terms of a mixture of T and R conformations. We estimate a [T]/[R] ratio of 2.7 on the assumption that only two conformations are present for which $\Delta s/s = 0\%$ for the R form and $\Delta s/s = -3.5\%$ for the T structure. The inferred value of L is independent of all other postulates of the model. Analysis of the PALA-binding isotherm of r143Ala ATCase, according to the two-state model (Monod et al., 1965), yields an independently determined value for L of 2.6. The agreement in the values of L deduced by the two methods, one based on structure and the other on functional properties, is excellent.

Although it could be argued that r143Ala ATCase has a unique structure intermediate between that of unliganded T state and liganded R state wild-type ATCase, such a proposal would require a fortuitous set of circumstances to produce equivalent results from both the PALA-promoted changes in the sedimentation coefficient and the cooperativity in the binding of PALA. It would also be dependent on the assumption that there is a stable "half-swollen" structure with physical properties intermediate between those of the T and R conformations. Although recently a crystal form of ATCase has been reported for the wild-type enzyme in the presence of phosphate and aspartate for which one unit cell dimension is an average of those for the crystals of CTP-liganded and PALA-liganded enzyme, no molecular refinement of the data has been presented (Gouaux & Lipscomb, 1989).[4] Moreover, various observations can be cited as evidence that a static, stable, half-swollen structure is unlikely to exist in solution. First, the free energy difference between the T and R conformations of wild-type ATCase is of the order of 3.3 kcal/mol. For the r143Ala mutant, it is probably even less, thereby facilitating the interconversion of the two forms. Second, only two high-resolution structures have been deduced from X-ray crystallographic studies, the CTP-liganded enzyme corresponding to the T state (Kim et al., 1987) and the PALA-liganded enzyme representative of the R state (Ke et al., 1988). No compelling evidence, from either crystallographic or solution studies, has yet been presented for a structure of an unliganded enzyme in an intermediate conformation. Third, there is abundant evidence for a concerted transition of the wild-type T structure to the R conformation upon ligand binding at the active sites (Yang & Schachman, 1980; Foote & Schachman, 1985; Schachman, 1988; Werner & Schachman, 1989). Fourth, several amino acid substitutions in widely diverse regions of the molecule promote the conversion from almost exclusively the T conformation to predominantly the R form, thereby indicating the ease of the transition from one state to the other (Eisenstein et al., 1989; Newell & Schachman, unpublished results; Wente and Schachman, unpublished results). Fifth, solutions of wild-type ATCase partially liganded with PALA (about 2 PALA/ATCase) have been shown to contain both T- and R-state molecules in approximately equimolar amounts (Werner & Schachman, 1989). These studies showed also that molecules of intermediate quaternary structure were not present in amounts exceeding 10% of the total population.

It is particularly relevant that the replacement of Lys at position 143 in the r chains by Ala as well as other substitutions (Eisenstein et al., 1989) at the c1–r4 interface destabilize the T state relative to the R conformation. These observations are in accord with the suggestions based on the crystallographic studies (Kim et al., 1987; Krause et al., 1987; Ke et al., 1988) that interactions at the c1–r4 interface contribute substantially to the stability of the T state relative to the R conformation.

Carbamoyl Phosphate Promotes the T → R Transition of r143Ala ATCase. Both the enzyme kinetics measurements and the sedimentation velocity experiments demonstrate that carbamoyl phosphate, the first substrate to bind in an ordered binding pathway (Porter et al., 1969; Wedler & Gasser, 1974; Hsuanyu & Wedler, 1987), promotes the conversion of r143Ala ATCase to the R conformation. As shown by the Eadie plot in Figure 1A, the mutant enzyme is essentially devoid of cooperativity, consistent with the view that the enzyme–carbamoyl phosphate complex is in the high-affinity R form (K_m = 2.5 mM aspartate). Moreover, PALA has no effect on the sedimentation coefficient of r143Ala ATCase in the presence of carbamoyl phosphate. These results are in accord with earlier observations indicating that carbamoyl phosphate promotes the T → R transition of wild-type ATCase as well as a chemically modified enzyme (Howlett & Schachman, 1977; Hensley & Schachman, 1979). With wild-type ATCase the [T]/[R] ratio is estimated to decrease from about 2×10^2 to 7 upon the binding of carbamoyl phosphate (Howlett et al., 1977). Hence, even in the presence of the substrate, the enzyme is largely in the T state. Consequently, the effect of carbamoyl phosphate alone on the average sedimentation coefficient of wild-type enzyme is very small. When, however, amino acid substitutions are made in ATCase that decrease the free energy for the T → R transition, as in r143Ala ATCase, the effect of carbamoyl phosphate is detected more readily, as seen in Figure 2.

The results of both the structural and the binding studies on r143Ala ATCase illustrate the pitfalls in using enzyme

[4] While this paper was in press the crystal structure of a mutant form of ATCase was described by Gouaux et al. (1989) in terms of an intermediate quaternary structure. It should be noted that the unliganded mutant differed only very slightly from the T state of wild-type ATCase, and the authors raise the question that the structure of the mutant enzyme in the crystal might be different from that in solution.

kinetics alone to draw conclusions about the allosteric properties of ATCase. In the absence of carbamoyl phosphate, a substantial fraction of the mutant enzyme is in the T state and the binding of PALA is cooperative and leads to the conversion of those molecules to the R conformation. In contrast, r143Ala ATCase, in the presence of carbamoyl phosphate, is almost completely in the R conformation and, therefore, no cooperativity in enzyme kinetics is detectable.

CTP and ATP Promote Opposite Changes in the Quaternary Structure of r143Ala ATCase. Wild-type ATCase as well as chemically modified derivatives and mutant forms that exhibit cooperativity in enzyme kinetics or PALA binding are inhibited by CTP and activated by ATP (Gerhart & Pardee, 1962; Gerhart & Schachman, 1968; Howlett et al., 1977; Hensley & Schachman, 1979; Yang & Schachman, 1980; Vickers et al., 1984; Robey et al., 1986; Newell et al., 1989). These observations have been interpreted in terms of preferential binding of the effectors (CTP to the T state and ATP to the R state), thereby perturbing the putative T $\rightleftharpoons$ R equilibrium. Other workers, however, attribute ATCase regulation by nucleotides to a direct effect on the active site (Thiry & Hervé, 1978; Tauc et al., 1982; Hervé et al., 1985; Hsuanyu & Wedler, 1988). These workers proposed that the "primary effect" of nucleotides is to change the dissociation constant for aspartate without an alteration in the quaternary structure of the enzyme. Changes in the global conformation of the enzyme, in this model, are a consequence of a "secondary effect" resulting from a redistribution of ligands at the active sites. The two-state model based on linked equilibria predicts that effectors would alter the T $\rightleftharpoons$ R equilibrium in the absence of aspartate analogues, whereas the model based on primary and secondary effects predicts that nucleotides would not alter the global conformation under these conditions. Thus, we can test these alternative models directly by determining whether heterotropic effectors alone can alter the quaternary structure of the enzyme.

The results with r143Ala ATCase show clearly that nucleotides cause an alteration in the global structure of the enzyme. Binding of PALA to r143Ala ATCase in the presence of ATP leads to only a 0.55% decrease in the sedimentation coefficient of the enzyme as compared to −2.55% for $\Delta s/s$ in the absence of nucleotides. In contrast, $\Delta s/s$ in the presence of CTP is −3.4%. Thus, in the absence of nucleotides, [T]/[R] is 2.7, and this ratio is decreased to 0.2 when ATP is present and increased to 34 in the presence of CTP. An independent experiment confirmed these results by demonstrating that ATP-liganded enzyme sedimented 3.0% more slowly than the CTP-liganded mutant enzyme. Thus, ATP stabilizes the swollen R state, and CTP stabilizes the more compact T state. Clearly the two-state model accounts for these observations, whereas the other model does not.

A stringent and quantitative test of this interpretation was conducted by measuring the equilibrium binding of PALA in the absence and presence of nucleotides to determine independently whether these ligands affect the [T]/[R] ratio. The Scatchard plots in Figure 4 demonstrate that the alterations in global conformation promoted by the nucleotides are reflected by changes in the cooperativity of PALA binding. In the absence of nucleotides, n_H = 1.4, and n_H is increased to 2.0 by the presence of CTP and decreased to 1.0 when ATP is present. Analysis of the binding data in terms of the two-state model (Monod et al., 1965) by nonlinear least-squares methods using multiple independent variables yielded values for the allosteric equilibrium constant, L, in the absence and presence of nucleotides. As seen in Table I, the agreement between the values for L, determined from equilibrium dialysis and from sedimentation velocity experiments, is excellent. This striking agreement between the [T]/[R] ratios, determined independently from structural and thermodynamic measurements, provides strong evidence that nucleotides, in binding to sites on the r chains, perturb the T $\rightleftharpoons$ R equilibrium and that the alteration in the average global structure underlies the effect of nucleotides on cooperativity. It should be noted that CTP and ATP cause opposite alterations in the allosteric equilibrium constant of r143Ala ATCase amounting to about 10-fold. Changes of this same magnitude have been reported for wild-type ATCase from an analysis of many experiments interpreted according to the two-state model (Howlett et al., 1977; Schachman, 1988). With the wild-type enzyme it was not possible to detect directly the effects of CTP and ATP because the T state is predominant in the absence of ligands.

Active-Site Ligands Vary in Promoting the Allosteric Transition of ATCase. Since all of the experiments reported here were performed in a buffer containing 40 mM phosphate and it is known that phosphate is bound at the active sites and probably the nucleotide binding sites of the enzyme, the results apply to the ATCase–phosphate complex. Therefore, the nucleotide-promoted changes in global conformation, which we have attributed to an alteration in the global structure of unliganded ATCase, could be interpreted alternatively as the result of nucleotides causing a change in the relative affinity of the T and R states for phosphate. Various observations can be cited in favor of the former explanation. Substrates for the reverse reaction catalyzed by ATCase, phosphate and carbamoyl aspartate, do not have a detectable effect on the quaternary structure of wild-type ATCase (Foote & Lipscomb, 1981). Hence, these ligands do not exhibit significant preferential binding to the R conformation relative to the T state. In addition, the cooperativity of ATCase in 50 mM imidazole–acetate buffer, as measured by the Hill coefficient of the enzymatically catalyzed formation of carbamoyl aspartate, is the same as that in phosphate buffer. For the measurements reported here, phosphate buffer was used because of the aggregation of ATCase in buffers lacking phosphate giving rise to anomalous sedimentation behavior (Howlett & Schachman, 1977). Although equilibrium dialysis measurements can be performed in other buffers, the presence of phosphate leads to an increase in the precision of the data, presumably because of the lowering of the affinity for PALA and the stabilization of ATCase.

It is particularly noteworthy that the average quaternary structure of r143Ala ATCase in 40 mM phosphate is altered dramatically by changes in the pH of the solutions (Markby et al., 1990). At pH 6.3 the enzyme is predominantly in the R conformation (L is about 0.05), whereas at pH 8.0 the mutant enzyme is almost exclusively in the T state (L is greater than 70) compared to a population of molecules in both conformations at pH 7.0 (L = 2.7). These conclusions are based both on structural studies of the effect of PALA on the sedimentation coefficient and on the affinity of r143Ala ATCase for PALA. Moreover, other mutant forms of ATCase (Newell and Schachman, unpublished results) in the absence of phosphate are predominantly in the R conformation, exhibit Michaelis–Menten kinetics, are devoid of cooperativity in PALA binding, and show no heterotropic effects in the presence of CTP or ATP. Thus, even though ATCase doubtless binds phosphate, which probably promotes local conformational changes at the site of binding (Yang & Schachman, 1987), there is no compelling evidence that phosphate promotes a change in the [T]/[R] ratio comparable to that caused by

PALA, carbamoyl phosphate, or the combination of carbamoyl phosphate and succinate. Nonetheless, further experiments are clearly required to clarify the effect of phosphate on the structure and properties of ATCase. In this regard it is of interest that magnesium pyrophosphate has a significant effect in promoting the T → R transition (Foote & Schachman, 1985).

Previous investigations of the effect of nucleotides on the global conformation of ATCase were performed in the presence of ligands such as PALA or carbamoyl phosphate plus succinate to equalize the distribution of molecules in the T and R states. At subsaturating PALA concentrations where the allosteric transition is only partially complete, Howlett et al. (1977) detected opposite shifts in the quaternary structure upon the addition of CTP or ATP. Similarly, experiments on a nitrated derivative of ATCase in the presence of carbamoyl phosphate showed that CTP and ATP in binding to r chains had opposite effects on the absorption spectrum of the nitrotyrosyl residues on c chains and altered the distribution of the T and R states as measured by the PALA-promoted values of $\Delta s/s$ (Hensley & Schachman, 1979). These results demonstrate that nucleotides alter the global conformation of ATCase in the presence of active-site ligands that promote the allosteric transition.

In contrast, Hervé and co-workers did not observe large nucleotide-promoted changes in the low-angle X-ray scattering curves for ATCase partially ligated with PALA, although such changes were seen for the enzyme in the presence of carbamoyl phosphate and succinate. They maintained that their "direct" model could account for the differences between the effects of weakly bound ligands (carbamoyl phosphate plus succinate) and tightly bound ligands (PALA). At present, no explanation is available for the conflicting results obtained by Howlett et al. (1977) on the one hand and by Hervé et al. (1985) and Hervé (1988) on the other. It should be noted, however, that the experimental conditions, pH, and enzyme concentration used by the two groups are drastically different. Moreover, experimental data for the changes in the X-ray scattering at different concentrations of PALA have not been presented. Thus, it is possible that the conditions used by Hervé et al. (1985) were such that the effect of ATP could not be detected because the enzyme was already predominantly in the R conformation. They did, however, observe a slight change toward the T conformation upon the addition of CTP, thereby confirming the earlier observation of Howlett et al. (1977). The direct effects shown here with the mutant, r143Ala ATCase, upon the addition of CTP and ATP cannot be attributed to changes in the affinity of the enzyme for aspartate as proposed by Tauc et al. (1982), Hervé et al. (1985), and Hsuanyu and Wedler (1988) since aspartate was not present in any of the solutions.

The principle of linked equilibria inherent in the two-state model of Monod et al. (1965) requires that preferential binding of heterotropic ligands must displace the equilibrium between the T and R conformations of an allosteric protein. By stabilizing either the T or R state, heterotropic effectors modify the cooperativity in binding active-site ligands. It is our view, based on the evidence presented here with a mutant form of ATCase, that the regulation of ATCase activity by CTP and ATP is attributable to changes in the T ⇌ R equilibrium caused by their preferential binding to one or the other conformation.

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Regulation of the Oxidative Phosphorylation Rate in the Intact Cell†

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ABSTRACT: The mechanisms that underlie the balance between the consumption and oxidative generation of ATP in the intact cell are not well-defined. Cytosolic inorganic phosphate (P_i) and ADP levels, the cytosolic ATP/ADP ratio, and the cytosolic phosphorylation potential (PP) have all been proposed as major regulatory variables, the latter as a component of a "near-equilibrium" thermodynamic regulatory scheme. Therefore, the potential regulatory roles of these variables in the intact cell were evaluated with ^{31}P NMR and Langendorff perfused rat hearts; in this preparation, the tissue oxygen consumption rate (MVO_2) can be varied over a wide range. When the exogenous carbon source was varied, none of the proposed regulatory parameters, i.e., the ATP/ADP ratio, PP, or cytosolic ADP level, were found to be uniquely related to MVO_2. Rather, ADP levels at a given MVO_2 decreased progressively for the exogenous carbon sources in the following order: glucose, glucose + insulin, palmitate + glucose, lactate, pyruvate + glucose, and octanoate + glucose. In the octanoate and pyruvate groups, MVO_2^{-1} was linearly dependent upon $[ADP]^{-1}$ with apparent K_m values being in the range previously observed in isolated mitochondria. A similar trend was observed in the MVO_2–$[P_i]$ relationship. The present findings suggest that exogenous carbon sources which effectuate deregulation of intramitochondrial NADH generation lower cytosolic ADP and P_i to levels which are limiting to the rate of oxidative phosphorylation. For other carbon sources, the processes controlling the rate of NADH generation also participate in determining the rate of oxidative ATP synthesis. However, this control must be exerted kinetically rather than through a near-equilibrium thermodynamic mechanism as indicated by the present data and prior kinetic studies of the ATP synthetic process in both isolated mitochondria and intact myocardium [La Noue, K. F., et al. (1986) *Biochemistry 25*, 7667–7675; Kingsley-Hickman, P., et al. (1987) *Biochemistry 26*, 7501–7510].

It is well-known that, in the intact cell, the rate of ATP production is tightly coupled to the rate of ATP consumption. In cell types that rely primarily on oxidative metabolism, this tight coupling implies that the rate of oxygen consumption by mitochondria is highly regulated by cytoplasmic processes. However, the biochemical signals that mediate this regulation and that relate the rates of ATP synthesis and utilization are unclear. Studies of isolated mitochondrial preparations have led to hypotheses that phosphate donor/acceptor status (i.e., ADP and/or P_i),[1] the ATP/ADP ratio, or phosphorylation potential (defined as $[ATP]/[P_i][ADP]$) may be regulatory (e.g., Chance and Williams (1955), Erecinska and Wilson (1982), and Tager et al. (1983)]. However, studies with isolated mitochondria are limited in that many of the parameters that may influence respiratory regulation, such as the carbon source required for TCA cycle operation and NADH generation, may be set by the experimenter to conditions that do not necessarily apply to the intact cell. Therefore, in such studies it is difficult to fully evaluate all potential regulatory influences associated with mitochondrial NADH[2] synthesis that may be of great significance in intact tissues.

In this paper, we present detailed studies of the mechanisms of respiratory regulation conducted with ^{31}P NMR spectroscopy and intact perfused hearts. The myocardium was the

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[1] Abbreviations: MVO_2, myocardial oxygen consumption rate; LV, left ventricle; EDP, left ventricular end diastolic pressure; RPP, rate pressure product; PP, phosphorylation potential; PDH, pyruvate dehydrogenase; TCA, tricarboxylic acid; P_i, inorganic phosphate; G, glucose; I, insulin; PG, pyruvate + glucose; Palm, palmitate + glucose; Oct, octanoate + glucose; Lac, lactate; NMR, nuclear magnetic resonance; gdw, gram dry weight.

[2] Unless specifically stated otherwise, NAD and NADH refer to mitochondrial contents; ATP, ADP, and P_i refer to cytosolic contents.

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