Unlike the Quaternary Structure Transition, the Tertiary Structure Change of the 240s Loop in Allosteric Aspartate Transcarbamylase Requires Active Site Saturation by Substrate for Completion†

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ABSTRACT: The quaternary structural change associated with the homotropic cooperative interactions in *Escherichia coli* aspartate transcarbamylase (ATCase) is accompanied by various tertiary structural modifications; the most notable one involves the 240s loop formed by residues 230−245 of the catalytic chain. In order to monitor local conformational changes in this region by fluorescence spectroscopy, Tyr-240 has been replaced by a Trp residue, in a mutant enzyme, in which both naturally occurring Trp residues in positions 209 and 284 of the catalytic chains had previously been substituted by Phe residues. This F209F284W240-ATCase still displays homotropic cooperativity for aspartate and undergoes the same T to R quaternary structure change as does the wild-type enzyme. Upon binding of the bisubstrate analogue *N*-(phosphonoacetyl)-L-aspartate, the fluorescence emission spectrum of this mutant shows a red shift directly proportional to the fraction of catalytic sites occupied by this compound, a maximum value of 4 nm being attained when all six active sites are ligated. An identical shift is observed with the catalytic subunits of this modified enzyme, when all three active sites are occupied. In contrast, the quaternary structural change of the F209F284W240-ATCase, monitored by small-angle X-ray scattering, is complete when only four out of six catalytic sites are occupied. Thus, the 240s loop adopts its final conformation only when the neighboring active site is bound.

Cooperative ligand binding is an important mechanism of biological regulation (Monod et al., 1963; Perutz, 1990). The interactions between the binding sites of allosteric enzymes involve conformational changes, based mainly on the rearrangement of the interfaces between the subunits, as formalized in the concerted (Monod et al., 1965) and sequential (Koshland et al., 1966) models. A major question remains the relationship between tertiary and quaternary structural changes which lead to the modification of distant binding site properties.

The regulatory enzyme aspartate transcarbamylase (ATCase; EC 2.1.3.2)[1] from *Escherichia coli*, whose structure and properties have recently been reviewed (Hervé, 1989; Kantrowitz & Lipscomb, 1990; Lipscomb, 1992, 1994), is one of the most studied allosteric enzymes. It catalyzes the first step of the pyrimidine biosynthetic pathway, the carbamylation of the amino group of aspartate by carbamylphosphate (Reichard & Hanshoff, 1956). ATCase is comprised of two catalytic trimers and three regulatory dimers, $(c_3)_2(r_2)_3$, which can be readily dissociated upon treatment with mercurials (Gerhart & Holoubek, 1967). The isolated catalytic trimers which carry three active sites are fully active although devoid of cooperativity for substrate binding and of regulatory properties, whereas the regulatory dimers bear the binding sites for the nucleotide effectors, but do not exhibit any catalytic activity (Gerhart & Schachman, 1965). The holoenzyme shows homotropic cooperativity between the six active sites for the binding of aspartate (Gerhart & Pardee, 1962; Bethell et al., 1968). This cooperativity can be reasonably well explained in the framework of the concerted model (Monod et al., 1965), *i.e.*, by a transition of the enzyme from a conformation having a low affinity for aspartate (T-state) to a conformation having a high affinity for this substrate (R-state) (Changeux & Rubin, 1968; Howlett et al., 1977).

The occurrence of an important quaternary structural change upon substrate binding has been evidenced by a variety of physical studies, including sedimentation velocity measurements (Kirschner & Schachman, 1973; Howlett & Schachman, 1977), X-ray crystallography (Monaco et al., 1978; Ladner et al., 1982) and X-ray solution scattering (Moody et al., 1979). These structural changes have been further documented by comparing the structure of the enzyme complexed with the bisubstrate analogue *N*-(phosphonoacetyl)-L-aspartate (PALA) with that of the unliganded enzyme (Honzatko et al., 1982; Ke et al., 1984, 1988; Krause et al., 1987). Upon the T to R structural transition, the two catalytic

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[1] Abbreviations: ATCase, aspartate transcarbamylase; PALA, *N*-(phosphonoacetyl)-L-aspartate; NATA, *N*-acetyl-L-tryptophanamide; W240, ATCase mutant in which Tyr-240 in the catalytic chain has been replaced by a Trp residue; F209F284, ATCase mutant in which both naturally occurring tryptophans, Trp-209 and Trp-284, have been replaced by Phe residues; F209F284W240, F209F284 mutant in which Tyr-240 has been replaced by a Trp residue; cs, catalytic subunit; SVD, singular value decomposition.

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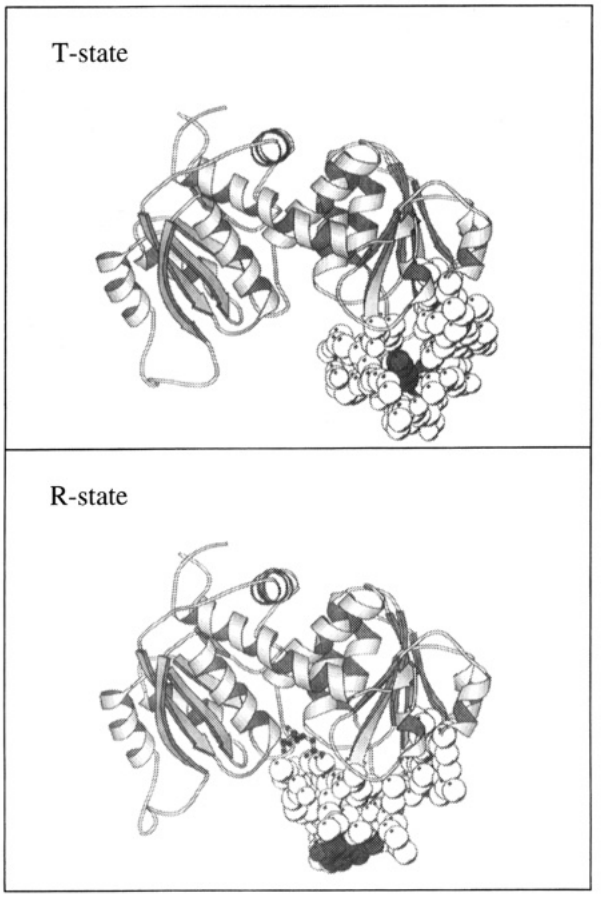

FIGURE 1: Ribbon diagram of the three-dimensional structure of one catalytic chain (c_1) from ATCase in the T-state and the R-state. PALA is represented by balls-and-sticks, and a space-filling representation is used for residues 230−250, with Tyr-240 being highlighted in grey. This figure has been produced with the graphics program Molscript (Kraulis, 1991).

trimers separate by 11 Å along the 3-fold axis and mutually reorient by 10° around that axis, while each regulatory dimer rotates by about 15° around the 2-fold axis. Moreover, within each catalytic chain the aspartate binding domain moves by 3 Å, as a rigid body, toward the carbamyl phosphate domain, accompanied by a substantial conformational change of a small region composed of residues 230−245 (the "240s loop") in the aspartate binding domain (Figure 1).

The movement of the 240s loop has been postulated to play a crucial role in the formation of a high-affinity active site and thus in the quaternary structural transition (Ladjimi et al., 1988). According to this hypothesis, the domain closure induced by aspartate binding to one active site triggers a concerted quaternary structure change that promotes the domain closure of all the remaining active sites, primarily by rupture of the interactions between the 240s loops of the two catalytic subunits (Ladjimi & Kantrowitz, 1988). Although the importance of this domain closure has been confirmed by numerous mutagenesis studies (Stevens et al., 1991; Lipscomb, 1994), no direct experimental information relates the tertiary structural change of the 240s loop to the quaternary structure transition. In order to clarify this relationship, two complementary methods were used. The local conformational changes were monitored using an intrinsic fluorescence probe, sensitive to a wide variety of environmental conditions (Creed, 1984), located in the 240s loop, and the global conformational changes were followed by solution X-ray scattering, the signal being specifically related to the quaternary structure of the enzyme (Altman et al., 1982; Hervé et al., 1985).

EXPERIMENTAL PROCEDURES

Chemicals. Carbamyl phosphate (lithium salt) and L-aspartate were purchased from Sigma Chemical Co.; tris-(hydroxymethyl)aminomethane (Tris) was from Merck, and L-[U-^{14}C]aspartate (300 mCi/mmol) was from CEA-Saclay. PALA was a generous gift from Drs. V. Narayanan and L. Kedda of the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, NIH, Silver Spring, MD. Restriction enzymes were from New England Biolabs. T4 polynucleotide kinase, T4 ligase, and the Klenow fragment of DNA polymerase I were from Pharmacia. The plasmid pUC119 and the phage M13K07 were obtained from J. Messing, Rutgers University.

Construction of Mutant ATCases. The mutant enzymes W240-ATCase, containing a tryptophan residue instead of a tyrosine in position 240, F209F284-ATCase, in which both naturally occurring tryptophans in positions 284 and 209 were replaced by phenylalanines, and F209F284W240-ATCase, containing a single tryptophan per catalytic chain, were prepared by site-directed mutagenesis as described previously (Ladjimi & Kantrowitz, 1988).

Enzyme Preparation and Assay. The wild-type and modified forms of the enzyme were purified from the overproducing strains according to Nowlan and Kantrowitz (1985). The corresponding isolated catalytic subunits were prepared by the method of Gerhart and Holoubek (1967).

The ATCase activity was measured in 50 mM Tris-HCl buffer at pH 8.0, in the presence of saturating (5 mM) carbamyl phosphate using a radioactive assay (Perbal & Hervé, 1972). Enzyme concentration was determined by the method of Lowry (Lowry et al., 1951) using wild-type ATCase as a standard.

Steady-State Fluorescence. Fluorescence emission spectra were recorded at 20.0 ± 0.1 °C in 50 mM Tris-HCl buffer, pH 7.0, on an SLM 8000 spectrofluorometer in the L-format configuration. The excitation wavelength was 300 nm; the excitation and emission bandwidths were set respectively at 4 nm and 2 nm. The fluorescence was collected through a 1 M CuSO4 filter (1 cm optical path). Rhodamine was used to correct fluctuations of the excitation light. In order to correct any instrumental drift, the spectra were normalized against NATA spectra, $N(\lambda)$. The fluorescence emission spectra were directly corrected for dilution and inner filter effects, and the positions of the center of gravity (in nanometers) of emission spectra, $F(\lambda)$, were calculated as previously described (Fetler et al., 1992):

$$CG = \frac{\sum_{\lambda_i=n}^{m} \lambda_i^{-2} F(\lambda_i)}{\sum_{\lambda_i=n}^{m} \lambda_i^{-3} F(\lambda_i)} \quad (1)$$

with the wavelength λ_i sampled over the entire emission spectrum in 0.5 nm intervals.

The bisubstrate analogue PALA was used to titrate the active sites. Since the concentration of active sites used in

Table 1: Kinetic Parameters of the Wild-Type and Modified Forms of ATCase[a]

ATCase	V_m [mmol h^{-1}/(mg of protein)$^{-1}$]	$S^{Asp}_{[0.5]}$ (mM)	n_H	PALA (rel act.)
WT	24.5 ± 0.5	16.1 ± 0.5	2.9 ± 0.2	3.9
W240	14.5 ± 0.6	41.7 ± 2.0	2.6 ± 0.3	5.8
F209F284	28.3 ± 1.1	25.7 ± 1.2	2.6 ± 0.2	3.7
F209F284W240	15.7 ± 0.5	52.1 ± 2.4	2.3 ± 0.2	3.4
cs WT	37.5 ± 1.0[b]	19.6 ± 3.9	1	
cs W240	34.4 ± 0.6	30.1 ± 1.2	1	
cs F209F284W240	36.5 ± 0.5	33.7 ± 1.6	1	

[a] These parameters were calculated through a computer fit by a nonlinear least-squares procedure to either the Hill equation or the Michaelis–Menten equation. n_H is the Hill coefficient. PALA stimulation is expressed as relative activity. The values correspond to the highest stimulation observed when the reaction velocity in the absence of PALA is 1. The reaction was performed in the presence of 5 mM carbamyl phosphate and one-tenth of the $S^{Asp}_{[0.5]}$ value. [b] Taken from previous work (Xi et al., 1990).

these experiments was about 2000-fold higher than the dissociation constant of PALA from the wild-type enzyme (K_D = 30 nM) (Collins & Stark, 1971; Jacobson & Stark, 1973), assumed to be close to that of the mutant enzyme, virtually no PALA was left free in solution. Thus, the fractional saturation function $\bar{Y}$ can be directly related to the concentration of PALA added.

Solution X-ray Scattering. Samples of the wild-type and F209F284W240 enzymes were prepared from a stock solution in 50 mM Tris-HCl buffer, pH 8.3, 0.1 mM EDTA, and 0.1 mM dithiothreitol as described previously (Hervé et al., 1985). X-ray scattering curves were recorded on the small-angle scattering instrument D24 using synchrotron radiation at LURE-DCI, Orsay. The instrument (Depautex et al., 1987), the data acquisition system (Bordas et al., 1980), and the experimental procedures (Fetler et al., 1995) were described previously. The protein concentration of X-ray samples was 7 mg/mL (corresponding to 0.14 mM in active sites), close to the concentration used for fluorescence measurements. Eight successive frames of 100 s were recorded for each sample. After visual inspection of each frame, the average and the standard deviation were computed. The value of the radius of gyration was derived from a Guinier analysis for the two curves recorded in the absence of substrates and in the presence of a 2-fold molar excess of PALA (Guinier & Fournet, 1955).

The singular value decomposition method used for data analysis has already been presented in detail (Fowler et al., 1983; Provencher & Glöckner, 1983; Fetler et al., 1995). Briefly, scattering curves can be considered as vectors with as many coordinates as data points. A titration experiment thus yields a two-dimensional data set, the two dimensions being the scattering angle and the total substrate concentration. The singular value decomposition analysis determines the minimum number of components that are required to account for the data within experimental error in the sense of least squares (Provencher & Glöckner, 1983), using the χ^2 expression:

$$\chi^2_n = \sum_{k=1}^{N}\sum_{i=1}^{M} \frac{[I^k_{exp}(s_i) - I^k_{calc,n}(s_i)]^2}{\sigma^k(s_i)^2} = \sum_{k=1}^{N}\sum_{i=1}^{M} [r^k_n(s_i)]^2 \quad (2)$$

where $I^k_{calc,n}(s)$ is the best reconstruction of curve $I^k_{exp}(s)$ using n components and $r^k_n(s_i)$ are the reduced residuals between observed and calculated values using n components. The dimension of the data set is thus taken as the minimum value of n for which χ^2_n is close to unity. For each curve, the $r^k_n(s_i)$ are examined and checked to follow a reasonably random distribution between +1 and −1.

This number of components is equal to the number of different quaternary structures in solution. In the particular case of only two components, *i.e.*, of only two structures, the variation of the projection of each curve onto the second component is directly related to the fraction of one species in solution. So, after proper scaling, using the initial and the final state, the analysis yields the $\bar{R}$ state function, which is the fraction of molecules in the R quaternary structure, as a function of the total PALA concentration. Furthermore, since virtually all of the PALA added in substoichiometric amount is bound to the enzyme, the saturation function $\bar{Y}$ is known for each concentration of PALA.

RESULTS

Rationale for the Preparation of the Fluorescence Probe. Escherichia coli ATCase contains two naturally occurring Trp residues at positions 209 and 284 of each catalytic chain (Weber, 1968; Königsberg & Henderson, 1983; Schachman et al., 1984). Prior to introducing a fluorescence probe in the 240s loop, the protein was made tryptophan-free by replacing these residues with phenylalanines. Then a Trp was substituted for Tyr-240 in the double mutant, F209F284-ATCase. In the resulting mutant, F209F284W240-ATCase, the fluorescence signal changes observed upon substrate binding can thus be unambiguously attributed to the chromophore located in the 240s loop.

Kinetic Properties of the Mutant ATCases and Their Isolated Catalytic Subunits. The kinetic parameters of the mutant enzymes are presented in Table 1. It appears that in F209F284-ATCase, in which both naturally occurring Trps were replaced by Phe residues, the maximal velocity and the Hill coefficient are not significantly affected, indicating that the homotropic cooperative interactions between the catalytic sites are not altered. However, the affinity for aspartate, as reflected by the $S_{[0.5]}$, is slightly lowered as compared to that of the wild-type enzyme. Since the kinetic properties of the double mutant are essentially unchanged as compared to those of the wild-type enzyme, this protein has been used to insert a Trp residue in the 240s loop. Replacement of Tyr-240 by Trp in the F209F284-ATCase mutant did not significantly affect the homotropic cooperativity, as illustrated by the minor reduction of the Hill number, but reduced both the maximal velocity and the affinity for aspartate.

In order to determine whether these altered properties were due to a direct effect of the mutation on the active site, the

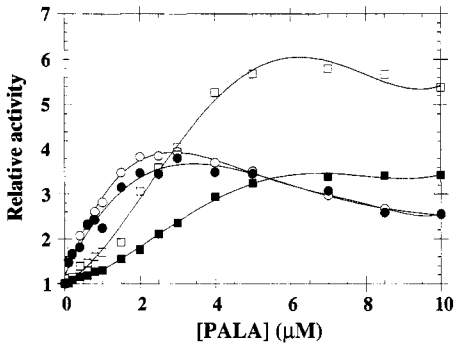

FIGURE 2: Stimulation of the ATCase reaction by PALA. The reaction was performed as indicated under Experimental Procedures in the presence of saturating carbamyl phosphate (5 mM) and one-tenth of $S_{[0.5]}$ for aspartate (1.5 mM for the wild-type enzyme, 4.0 mM for the W240 mutant, 2.5 mM for the F209F284 mutant, and 5.0 mM for the F209F284W240 mutant). The relative activity in the presence of PALA is expressed as the ratio A_P/A where A_P is the activity in the presence of PALA and A is the activity in the absence of this compound. (○) Wild-type ATCase; (●) F209F284-ATCase; (□) W240-ATCase; (■) F209F284W240-ATCase.

isolated catalytic subunits were also analyzed. The kinetic parameters derived from the corresponding aspartate saturation curves and reported in Table 1 show that the catalytic subunits exhibit a decreased affinity for aspartate, as compared to the wild-type catalytic subunits, indicating that the replacement of Tyr-240 by Trp in F209F284-ATCase slightly affects the conformation of the active site, in accordance with the differences in affinity for aspartate reported above.

It is well-known that, at low concentration, PALA increases the rate of the reaction (Collins & Stark, 1971) by promoting the conformational transition from the low-affinity T-state to the high-affinity R-state in the wild-type enzyme (Gerhart & Schachman, 1968; Blackburn & Schachman, 1977; Howlett et al., 1977; Howlett & Schachman, 1977). The extent of stimulation of the mutant enzyme activity by this bisubstrate analogue was determined. As shown in Figure 2, all the mutants are stimulated by PALA, although the maximal stimulation required higher concentrations of PALA for the mutants in which Tyr-240 has been replaced by Trp.

Monitoring of Local Changes in the 240s Loop by Steady-State Fluorescence. Steady-state fluorescence emission spectra were recorded for the F209F284W240 mutant ATCase in the absence and the presence of saturating amounts of PALA. A red-shift by about 4 nm was observed in the presence of a 2 molar excess of PALA. This red-shift signal, indicative of an enhanced exposure of the Trp residue to the solvent, has been used in titration experiments with increasing concentrations of PALA, allowing one to monitor local changes in the 240s loop. The saturation function $\bar{Y}$ can be directly related to the concentration of PALA added (see Experimental Procedures). As shown in Figure 3, the titration of F209F284W240-ATCase with PALA indicates that the change in exposure of Trp to the solvent is proportional to the degree of saturation of the active sites, the maximum value being attained for six PALA per ATCase molecule. To confirm this relationship between PALA binding and the fluorescence signal, similar spectra were recorded with the corresponding mutant catalytic subunits, which do not exhibit homotropic cooperativity. In this case, the maximal change was found to occur when all three active

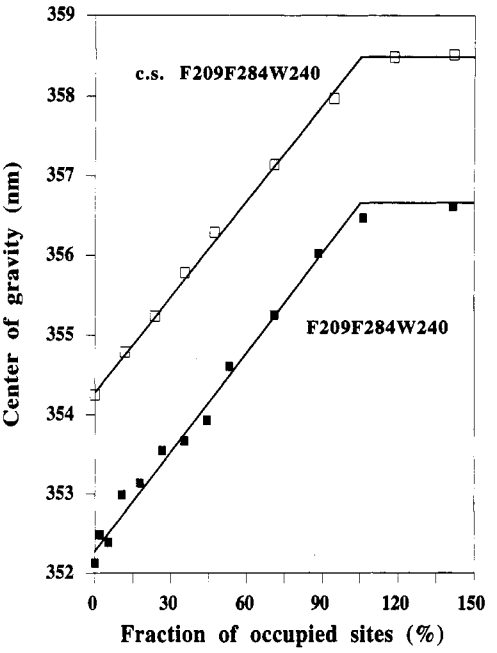

FIGURE 3: Position of the center of gravity of the fluorescence emission spectra of the F209F284W240-ATCase holoenzyme and the corresponding catalytic subunit as a function of the fractional saturation of the active sites by PALA. [(■) F209F284W240-ATCase; (□) F209F284W240-ATCase catalytic subunit].

sites were occupied (Figure 3). Thus, the same variation in the red-shift of the fluorescence emission spectra upon ligand binding was observed in both the cooperative holoenzyme and the noncooperative catalytic subunits.

Monitoring the Quaternary Structure Change by X-ray Solution Scattering. X-ray solution scattering spectra were recorded in the absence and in the presence of saturating concentrations of PALA, for the wild-type and the F209-F284W240 mutant ATCase. Both enzymes have nearly identical radii of gyration (in the absence of substrates: 46.4 ± 0.3 Å for the F209F284W240-ATCase *vs* 46.5 ± 0.3 Å for the wild-type; in the presence of a 2-fold molar excess of PALA: 49.0 ± 0.3 Å for the F209F284W240-ATCase *vs* 49.6 ± 0.3 Å for the wild-type) and very similar scattering patterns at higher angles (Figure 4), indicating that the mutant enzyme is able to undergo the same T to R quaternary structure transition as that of the wild-type enzyme. Considering the experimental conditions used, the small differences between the scattering data observed for the mutant enzyme as compared to the wild-type (Figure 4) should not be considered as significant.

In order to titrate the quaternary structure transition, a total of 20 scattering patterns were recorded on F209F284W240-ATCase solutions containing increasing amounts of PALA. This data set was globally analyzed by the singular value decomposition method (see Experimental Procedures). The examination of the statistical parameters provided by this analysis, the global χ_n^2 and the reduced residuals, leads to the conclusion that all the experimental scattering patterns can satisfactorily be approximated by a linear combination of only two curves. In other words, the enzyme solutions only contain the two extreme quaternary structures, T and R. Thus, the analysis directly yields the variation of the $\bar{R}$ state function with the saturation function $\bar{Y}$ shown in Figure 5 (see Experimental Procedures). The quaternary conformational change is complete even though only four out of

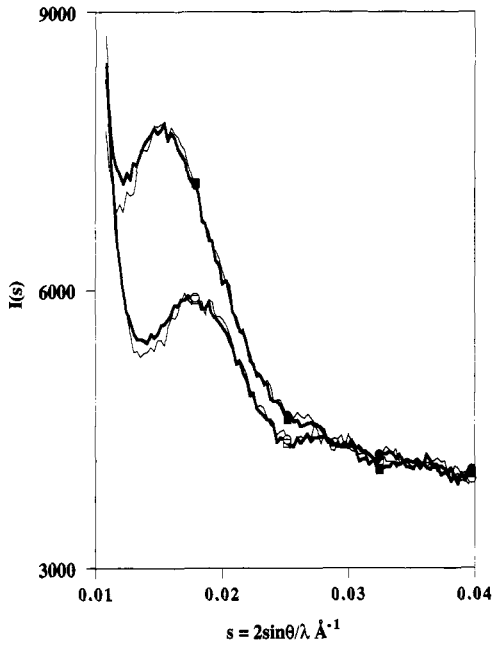

FIGURE 4: Solution X-ray scattering spectra of the wild-type and the F209F284W240 enzymes. Spectra are shown for the unligated wild-type enzyme (○, thin lines), unligated F209F284W240-ATCase (□, thick lines), wild-type enzyme in the presence of a 2 molar excess of PALA (●, thin lines), and the F209F284W240-ATCase in the presence of a 2 molar excess of PALA (■, thick lines).

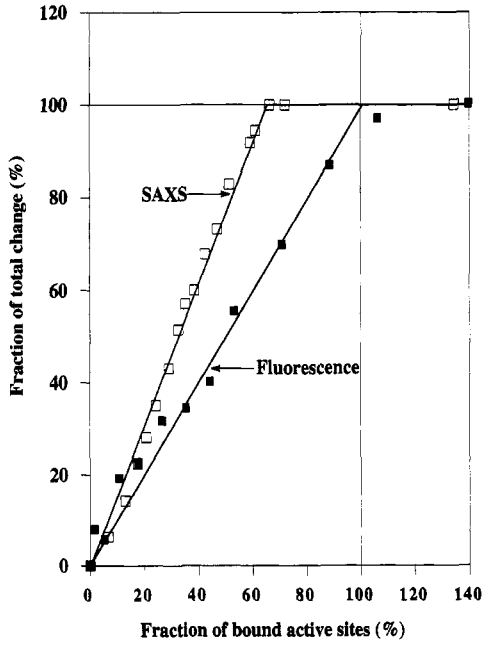

FIGURE 5: Titration curves of F209F284W240-ATCase. (□) X-ray titration ($\bar{R}$ *vs* $[PALA]_{tot}$); (■) fluorescence spectroscopy titration (same experimental data as in Figure 3). X-ray scattering data were analyzed using the singular value decomposition method (see Experimental Procedures). The propagation of the experimentally determined standard deviations leads to errors associated with the $\bar{R}$ values on the order of 2%.

six active sites are occupied by PALA. Subsequent additions of this ligand have no further effect.

DISCUSSION

On the basis of the high-resolution three-dimensional structures and site-directed mutagenesis studies, a possible mechanism for a concerted transition has been proposed (Ladjimi & Kantrowitz, 1988). In this model, the binding of substrates to one active site induces a domain closure in the remaining active sites, thereby triggering the quaternary structure change through the disruption of the 240s loop interactions between the opposed catalytic trimers. According to this hypothesis, tertiary changes in the 240s loop are linked to the quaternary structure changes (Ladjimi & Kantrowitz, 1988). Although this model is based on the dramatic rearrangement of the 240s loop upon PALA binding observed in structural studies (Krause et al., 1987; Ke et al., 1988), the relationship between this local conformational change and the quaternary structure change has not been specifically investigated in solution.

Structural and Functional Properties of the F209-F284W240-ATCase. The study of the relationship between tertiary and quaternary structure changes required a modified enzyme retaining homotropic cooperativity after introduction of an intrinsic fluorescence probe at the site of interest. Since previous work showed that neither homotropic cooperativity (Middleton & Kantrowitz, 1986) nor the quaternary structures of the T and R states (Cherfils et al., 1989; Gouaux et al., 1989) are affected by the replacement of Tyr-240 by Phe, Tyr-240 was selected for replacement by a Trp residue. Additionally, in order to relate specifically the intrinsic fluorescence signal to the environment of the 240s loop, the two naturally occurring Trps at positions 209 and 284 had first to be replaced by Phe.

The analysis of the F209F284-ATCase mutant shows that the catalytic properties are not significantly altered by the substitution of the two Trp residues. Replacing Tyr-240 by a Trp in the double mutant F209F284-ATCase leads to a decrease of about 2.5-fold in the affinity for aspartate and 2-fold in the specific activity, without significant alteration of the homotropic cooperativity. Thus, the altered kinetic properties in the F209F284W240-ATCase can essentially be ascribed to the replacement of Tyr-240 by a more bulky Trp side chain, as further confirmed by the kinetic properties of the W240-ATCase mutant (Table 1). Moreover, the fact that cooperativity, as well as PALA stimulation, was conserved in the F209F284W240-ATCase indicates that this enzyme is able to undergo the T to R quaternary transition. This is further demonstrated by the identical values of the radius of gyration and the very similar scattering patterns recorded for the wild-type enzyme and the mutant enzyme (Figure 4).

Correlation between Tertiary and Quaternary Structure Changes. The fluorescence emission spectrum of the mutant holoenzyme, F209F284W240, is blue-shifted by about 2 nm as compared to those of the corresponding catalytic subunits, reflecting the slight decrease in solvent exposure of the Trp probe due to the presence of the additional regulatory chains and the opposing catalytic subunit in the former. Indeed, the sensitivity of the indole fluorescence to the environment is well documented (Burstein et al., 1973): an increased exposure to solvent causes a red-shift of the emission spectrum [see Lakowicz (1983) and references cited therein]. Such a red-shift is precisely what is observed upon saturation of the active sites by PALA. The total amplitude of this red-shift, namely, 4 nm, is the same in the catalytic subunits and in the holoenzyme, suggesting that the local conformational changes around Trp-240 are very similar in both enzyme forms and that the quaternary structure change has no detectable effect on the indole fluorescence. The red-

shift in the fluorescence emission spectra reaches its maximal value when all the active sites are occupied by PALA, whether in the holoenzyme or in the catalytic subunits. In contrast, the change in the quaternary structure, as measured here by solution X-ray scattering, is already complete when four PALA molecules are bound per ATCase molecule, whereas in the catalytic subunits this change is proportional to substrate binding (Howlett & Schachman, 1977). Compensating effects from local and global conformational changes, which might be postulated, would thus yield different variations of the red-shift with the fractional saturation of active sites in the catalytic subunits and the holoenzyme, while identical variations are observed as shown in Figure 3. We conclude that the indole fluorescence of Trp-240 is specifically sensitive to substrate binding at the nearest active site.

The increased exposure of Trp to the solvent could be the result of a local structural rearrangement in the 240s loop, a movement of the indole group alone, or a combination of both. Indeed, one consequence of the T to R structure transition in the wild-type enzyme is that the α-carbon of Tyr-240 relocates 8.2 Å closer to the carbamyl phosphate domain and the average χ_1 dihedral angle changes from −169° (T) to −78° (R) (Gouaux et al., 1989), thus increasing the solvent exposure of Tyr-240 as shown in Figure 1. In the R structure in the presence of saturating PALA (Ke et al., 1988), the side chain of Tyr-240 is not constrained and seems to be free to rotate. This should still be the case even for the bulkier indole group. If the only structural consequence of substrate binding were the rotation of the indole group, this would imply that this same group would have occupied a different, solvent-protected position in the absence of substrate. Although difficult to rule out, this seems rather unlikely, and we favor the existence of a local conformational change of all or part of the 240s loop triggered by substrate binding, which provokes the increased exposure of the chromophore to the solvent. In other words, the conformation of the 240s loop is different depending upon whether the nearest active site is empty or contains a substrate molecule.

Previous studies on the relationship between local and global conformational changes, monitored respectively by difference spectroscopy using a nitrotyrosyl chromophore and analytical ultracentrifugation, showed that both structure changes are weakly linked, the gross conformational change being concerted and occurring when only a fraction of the binding sites are occupied and the local changes being sequential and strongly associated with ligand binding (Kirschner & Schachman, 1973). This work was complemented by a subsequent study with hybrid molecules formed by three native regulatory dimers, a native catalytic subunit, and one chemically modified inactive catalytic trimer, pyridoxylation preventing binding of any substrate, with a nitrotyrosyl chromophore (Yang & Schachman, 1980). These hybrids exhibited a tight link between the change in the tertiary structure of the unliganded catalytic chains and the alteration in the quaternary structure change upon ligand binding. These apparently contradictory results have been explained by partially compensating effects measured with the reporter group used: the ionization state of the nitrotyrosyl group is sensitive to the local reorganization associated with the T to R transition as well as to modifications following substrate binding. However, a shortcoming of this work was the lack of knowledge of the precise location of the chromophore in the enzyme structure, preventing a direct conclusion to be drawn on the relationship between changes in a specific region and the quaternary structure. Tyr-240 was later thought to have been the target of the nitrotyrosylation (Cohen et al., 1985). The difference between the sensitivity of two reporter groups, likely positioned at the same location in the enzyme, stresses the importance of using several probes: the nitrotyrosyl group is sensitive to the local reorganization associated with the T to R transition as well as to modifications following substrate binding, while the fluorescence of Trp is not affected by the former.

It is finally of interest to note that the X-ray scattering titration of the quaternary structure transition yields an identical titration curve to that obtained with the wild-type enzyme (Fetler et al., 1995). Together with the kinetic characterization of the triply mutated enzyme, this result shows that the F209F284W240-ATCase behaves in a very similar way as the wild-type enzyme upon substrate binding, supporting the validity of the extrapolation of our conclusions to the latter.

Implications for the Linkage between Local and Global Structural Changes. Based on crystallographic data and the results of numerous mutagenesis studies, a model for the allosteric transition has been put forward in which substrate binding at one active site causes a domain closure inducing local conformational changes, notably in the 240s loop, thus triggering the T to R quaternary structure transition (Ladjimi & Kantrowitz, 1988). Although the concerted MWC model accounts, by and large, for the homotropic cooperativity in ATCase, the previous model represents an extreme case of the MWC model. Indeed, it assumes that binding of one substrate molecule triggers the T to R transition (Foote & Schachman, 1985), leaving only the totally empty enzyme molecules in the T structure. However, it has recently been shown that one enzyme molecule has to bind two to three bisubstrate PALA molecules to be in the R quaternary structure state (Fetler et al., 1995).

The results presented here show that the structure of the 240s loop still undergoes modifications upon substrate binding, even after the T to R quaternary structure transition is complete. Thus, the final R-state configuration of the 240s loop, as described by X-ray crystallography, is only reached once the corresponding active site is occupied.

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