The Tryptophan Residues of Aspartate Transcarbamylase: Site-Directed Mutagenesis and Time-Resolved Fluorescence Spectroscopy†

Luc Fetler,‡ Patrick Tauc,§ Guy Hervé,‡ Moncef M. Ladjimi,‡ and Jean-Claude Brochon*,§

Laboratoire d'Enzymologie, Centre National de la Recherche Scientifique, 91198 Gif-Sur-Yvette, France, and Laboratoire pour l'Utilisation du Rayonnement Electromagnétique, CNRS-MEN-CEA, Centre Universitaire Paris—Sud, 91405 Orsay Cedex, France

Received June 25, 1992; Revised Manuscript Received September 11, 1992

ABSTRACT: Aspartate transcarbamylase (EC 2.1.3.2) contains two tryptophan residues in position 209 and 284 of the catalytic chains (c) and no such chromophore in the regulatory chains (r). Thus, as a dodecamer $[(c_3)^2(r_2)^3]$ the native enzyme molecule contains 12 tryptophan residues. The present study of the regulatory conformational changes in this enzyme is based on the fluorescence properties of these intrinsic probes. Site-directed mutagenesis was used in order to differentiate the respective contributions of the two tryptophans to the fluorescence properties of the enzyme and to identify the mobility of their environment in the course of the different regulatory processes. Each of these tryptophan residues gives two independent fluorescence decays, suggesting that the catalytic subunit exists in two slightly different conformational states. The binding of the substrate analog *N*-phosphonacetyl-L-aspartate promotes the same fluorescence signal whether or not the catalytic subunits are associated with the regulatory subunits, suggesting that the substrate-induced conformational change of the catalytic subunit is the essential trigger for the quaternary structure transition involved in cooperativity. The binding of the substrate analog affects mostly the environment of tryptophan 284, while the binding of the activator ATP affects mostly the environment of tryptophan 209, confirming that this activator acts through a mechanism different from that involved in homotropic cooperativity.

Escherichia coli aspartate transcarbamylase (ATCase)[1] catalyzes the first reaction of the pyrimidine biosynthetic pathway, the carbamylation of the amino group of aspartate by carbamyl phosphate (Reichard & Hanshoff, 1956). This reaction proceeds through an ordered mechanism in which carbamyl phosphate binds first, followed by aspartate, with release of the products in the order carbamylaspartate then phosphate (Porter et al., 1969; Collins & Stark, 1969; Issaly et al., 1982; Hsuanyu & Wedler, 1987).

The structure and properties of ATCase have been recently reviewed (Allewell, 1989; Hervé, 1989; Kantrowitz & Lipscomb, 1990). Numerous studies showed the allosteric nature of this enzyme whose activity is feedback-inhibited by CTP and UTP, the end products of the pyrimidine pathway (Gerhart & Pardee, 1962; Wild et al., 1989; Zhang & Kantrowitz, 1991), and stimulated by the purine nucleotide ATP (Gerhard & Pardee, 1962; Thiry & Hervé, 1978). Furthermore, ATCase exhibits homotropic cooperativity between the catalytic sites for the binding of the substrate aspartate (Gerhart & Pardee, 1962; Bethell et al., 1968). This cooperative behavior is explained by a transition of the enzyme from a conformation which has a low affinity for aspartate (T state) to a conformation which has a high affinity for this substrate (R state) (Howlett & Schachman, 1977; Moody et al., 1979). The crystallographic structure of these two extreme conformations is known with a resolution of 2.5 Å (Honzatko et al., 1982; Ke et al., 1984; Krause et al., 1987). ATP and CTP affect the enzyme activity through mechanisms which are distinct from those involved in the homotropic cooperative interactions. The binding of these nucleotides to the regulatory sites promotes local conformational changes which are transmitted either to the catalytic sites ("primary–secondary effects mechanism"; Thiry & Hervé, 1978; Tauc et al., 1982; Hervé et al., 1985) or only to the interfaces between regulatory and catalytic subunits ("effector-modulated transition"; Xi et al., 1991). On the basis of the crystallographic structure of ATCase liganded with either ATP or CTP, it was recently proposed that the regulatory signals promoted by the binding of these two effectors involve also an indirect effect transmitted through the interface of the regulatory chains (Stevens & Lipscomb, 1992).

ATCase, which is composed of two trimeric catalytic subunits and three dimeric regulatory subunits, can be readily dissociated upon treatment by mercurials (Gerhart & Holoubek, 1967). The isolated catalytic subunits which carry the active sites (three per subunit) are fully active although they are devoid of regulatory properties and cooperativity for substrate binding. The regulatory subunits bear the binding sites for the effectors CTP and ATP (two per subunit) but do not exhibit any catalytic activity.

A method currently used to monitor protein conformational changes is intrinsic fluorescence spectroscopy, based on the fact that tryptophan is highly fluorescent compared to the other aromatic amino acids and that its fluorescence is very sensitive to a wide variety of environmental conditions (Creed, 1984). ATCase contains only two tryptophan residues per catalytic chain, in position 209 and 284, whereas the regulatory

† This work was supported by the Centre National de la Recherche Scientifique and by grants from the European Economic Community (BAP-0478-F) and NATO (0364/88).

* To whom correspondence should be addressed.

‡ CNRS.

§ Centre Universitaire Paris—Sud.

[1] Abbreviations: ATCase, aspartate transcarbamylase; PALA, *N*-phosphonacetyl-L-aspartate; NATA, *N*-acetyl-L-tryptophanamide; MEM, maximum entropy method; ACO, anneau de collision d'Orsay; WT, wild-type aspartate transcarbamylase; W284F-ATCase, ATCase mutant in which tryptophan 284 has been replaced by a phenylalanine residue; W209F-ATCase, ATCase mutant in which tryptophan 209 has been replaced by a phenylalanine residue; cat.su, catalytic subunit; QY, quantum yield.

0006-2960/92/0431-12504$03.00/0

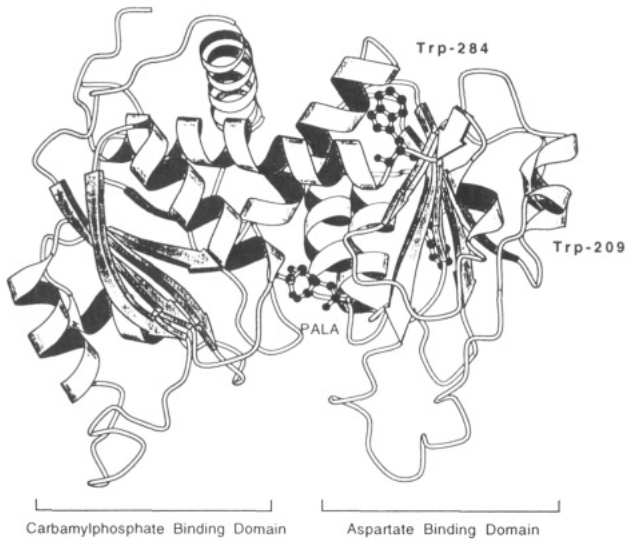

FIGURE 1: Ribbon diagram of the tridimensional structure of one catalytic chain from ATCase in the R-state. Tryptophans 209 and 284 are represented by ball-and-stick, as is the position of a PALA molecule in the active site. The graphics program MolScript (Kraulis, 1991) was used for the drawing.

chains do not contain this amino acid (Weber, 1968; Konigsberg & Henderson, 1983; Schachman et al., 1984). Thus, native ATCase contains twelve tryptophan residues. Both tryptophans 209 and 284 are located in the aspartate binding domain of the catalytic chain (Figure 1). The crystallographic structure of ATCase reveals that tryptophans 284 and 209 are located at about 17 and 25 Å, respectively, from the nearest catalytic site. Both tryptophan residues are about 50 Å away from the nearest regulatory site (Honzatko et al., 1982). In this respect, it was reported previously that neither the mean lifetime nor the polarization of the tryptophan residues of ATCase was significantly altered by the binding of the ligands (Maliwal et al., 1984). However, the results reported by Royer et al. (1987) suggested that the dynamics of the two tryptophan residues are differently affected by the binding of either the bisubstrate analog *N*-phosponacetyl-L-aspartate (PALA) or the effectors CTP and ATP. The aim of the present study was to differentiate between the signals coming from each of these two tryptophan residues and to compare the fluorescence properties of the unliganded enzyme to those of the PALA-, ATP-, or CTP-liganded enzymes. For this purpose, site-directed mutagenesis was used to replace one or the other of these residues by a phenylalanine. Using these modified enzymes, steady-state and time-resolved fluorescence measurements were performed. Lifetime distributions were then determined using a method based on entropy maximization (Livesey et al., 1986, 1987; Livesey & Brochon, 1987).

The results show that binding of PALA or nucleotides affects distinctly the two tryptophan regions in spite of the distances between binding sites and fluorescence probes. Furthermore, the fluorescence signals obtained in the presence of PALA are similar, whether or not the catalytic subunits are associated with the regulatory subunits.

MATERIALS AND METHODS

Chemicals. Carbamyl phosphate (lithium salt), L-aspartate, adenosine triphosphate (sodium salt), and cytidine triphosphate (sodium salt) were purchased from Sigma Chemical Co.; tris(hydroxymethyl)aminomethane (Tris) was from Merck, and L-[U-^{14}C]aspartate (300 mCi/mmol) was from CEN-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 two mutant enzymes, W209F and W284F, containing a single tryptophan residue in position 284 and 209, respectively, 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 as previously described (Perbal & Hervé, 1972). The enzyme concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard and taking into account the 20% overestimate that is given by this method (Kerbiriou et al., 1977). The influence of ATP and CTP on the rate of the reaction was determined as previously described (Kerbiriou & Hervé, 1972; Thiry & Hervé, 1978) and expressed according to Tauc et al. (1982).

Steady-State Fluorescence. Fluorescence emission spectra were recorded at 20.0 ± 0.1 °C in 50 mM Tris–acetate buffer, pH 7.0, on a SLM 8000 spectrofluorometer in L-format configuration, interfaced to a Mac/SE. The fluorescence spectra were recorded with both polarizers set to the vertical position. This is valid because the very slow rotation of such a large molecule as ATCase, during the few nanoseconds of the fluorescence emission, does not significantly affect the fluorescence intensity at any polarizer position (formula 3 and 4) as the rotation of such a large molecule as ATCase is not significant during the nanosecond time domain over which the fluorescence emission is measured. The excitation wavelength was 300 nm. The excitation and emission bandwidths were set respectively at 4 and 2 nm. The fluorescence was collected through a 1 M $CuSO_4$ filter (1-cm optical path). Rhodamine was used to correct fluctuations of the excitation light. Each fluorescence spectrum, $F(\lambda)$, was corrected for the Raman peak and the background noise by substracting a corresponding buffer spectrum, $R(\lambda)$. In order to correct any instrumental drift, the spectra were normalized against NATA spectra, $N(\lambda)$. The center of gravity (in nanometers) of emission spectra $F(\lambda)$ was calculated as follows:

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

with the wavelength λ_i sampled over the entire emission spectrum in 0.5-nm intervals. The fluorescence emission spectra of the different enzymes in the presence of PALA, CTP, or ATP were directly corrected for dilution and inner filter effects. This correction factor was calculated in measuring fluorescence emission of a NATA solution containing the same concentration of substrate analog or effector. The quantum yields QY were obtained using NATA as a reference with $QY_{NATA} = 0.14$ (Werner & Forster, 1979), considering the normalized spectra and respective optical

densities as follows:

$$QY = QY_{NATA} \frac{OD^{300}{}_{NATA}}{OD^{300}{}_{PROT}} \frac{\sum_{\lambda_i=305}^{455} F(\lambda_i)}{\sum_{\lambda_i=305}^{455} N(\lambda_i)} \quad (1)$$

Time-Resolved Fluorescence Measurements. Fluorescence decay was measured by the single photoelectron counting method (O'Connor & Philips, 1987). The experimental setup was installed on the SA1 beam line of the synchrotron radiation machine SUPER-ACO at Laboratoire pour l'Utilisation du Rayonnement Electromagnétique (LURE), Orsay. This storage ring provides currently a light pulse of 500 ps (full width at half-maximum) at a frequency of 8.33 MHz for a double-bunch mode. The optical and electronic parts of the instrumental setup are described elsewhere (Brochon et al., 1992a) As detector, a Hamamatsu microchannel plate R1564U-06 was used. In all experiments the excitation and emission wavelengths were respectively set at 300 and 350 nm with Jobin-Yvon H25 monochromators with bandwidths of 3 and 5 nm, respectively. Time sampling was 35 ps per channel and 1024 channels were used. The fluorescence of a 1-mL sample was measured in a 5 × 10-mm quartz cuvette. Data for Ivv(t) and Ivh(t) were stored in separate memories of a plug-in multichannel analyzer card (Canberra) in a Deskpro 286E microcomputer. The correction factor β for the polarization bias was determined from the depolarized fluorescence of NATA at 20 °C under identical optical conditions:

$$\beta(\lambda em) = \frac{\sum_{k=k_0}^{n} Ivv(k)}{\sum_{k=k_0}^{n} Ivh(k)} \quad (2)$$

k_0 being the channel at which the fluorescence of NATA is fully depolarized and any excitation scattered light vanishes.

Routinely 5 × 10^6 to 10^7 counts were stored for the Ivv(t) fluorescence intensity decay. The instrumental response function was determined by measuring the light scattered by a Ludox solution close to the emission wavelength.

Data Analysis. Data analysis of the total intensity decay was performed by the maximum entropy method (Livesey & Brochon, 1987) using FAME and FAME-QT programs (MEDC Ltd., U.K.).

After excitation by a vertically polarized pulse of light, the complete expression of the measured parallel Ivv(t) and perpendicular Ivh(t) components of the fluorescence intensity at time t after the start of the excitation are (Livesey & Brochon, 1987) as follows:

$$Ivv(t) = {}^1/_3 E_\lambda(t) * \int_0^\infty \int_0^\infty \int_{-0.2}^{0.4} \gamma(\tau,\theta,A) e^{-t/\tau} (1 + 2Ae^{-t/\theta}) \, d\tau \, d\theta \, dA \quad (3)$$

and

$$Ivh(t) = {}^1/_3 E_\lambda(t) * \int_0^\infty \int_0^\infty \int_{-0.2}^{0.4} \gamma(\tau,\theta,A) e^{-t/\tau} (1 - Ae^{-t/\theta}) \, d\tau \, d\theta \, dA \quad (4)$$

where $E_\lambda(t)$ is the temporal shape of the instrumental function, * denotes a convolution product, and $\gamma(\tau,\theta,A)$ represents the number of fluorophores with fluorescence lifetime τ, correlation time θ, and initial anisotropy A. If only interested in the determination of the total intensity decay parameters, one can simplify the analysis by summing the parallel and perpendicular components:

$$T(t) = Ivv(t) + 2\beta Ivh(t) = E_\lambda(t) * \int_0^\infty \alpha(\tau) e^{-t/\tau} \, d\tau \quad (5)$$

where $\alpha(\tau)$ is the lifetime distribution given by

$$\alpha(\tau) = \int_0^\infty \int_{-0.2}^{0.4} \gamma(\tau,\theta,A) \, d\theta \, dA \quad (6)$$

The degree of structure of the distribution $\alpha(\tau)$ is characterized by an "entropy" function S defined by Skilling-Jaynes (Jaynes, 1983) as

$$S = \int_0^\infty \alpha(\tau) - m(\tau) - \alpha(\tau) \log \frac{\alpha(\tau)}{m(\tau)} \, d\tau \quad (7)$$

where $m(\tau)$ encoded prior knowledge on the distribution and then should be flat if all lifetimes are equally probable before analysis (Livesey & Brochon, 1987). The entropy of the distribution $\alpha(\tau)$ is progressively decreased [i.e., the structure of the distribution $\alpha(\tau)$ is increased]. In order to ensure that the recovered distribution agrees with the data, this procedure was subjected to the following simultaneous constraint of minimization of χ^2 criteria:

$$\sum_{k=1}^{M} \frac{(T^{calc}{}_k - T^{obs}{}_k)^2}{\sigma^2{}_k} \leq M \quad (8)$$

where $T^{calc}{}_k$ and $T^{obs}{}_k$ are the kth calculated and observed intensities. $\sigma^2{}_k$ is the variance of the kth point ($\sigma^2{}_k = \sigma^2{}_{k,vv} + 4\beta^2\sigma^2{}_{k,vh}$; Wahl, 1979). M is the total number of observations. A lifetime domain spanning 150 values equally spaced on a logarithmic scale between 0.05 and 20 ns was routinely used. A channel of a null lifetime corresponding to the scatter was added. Routinely, up to 200 iterations were used for these analyses.

The center $\langle\tau_j\rangle$ of a single class j of lifetimes over the $\alpha_i(\tau_i)$ distribution is defined as

$$\langle\tau_j\rangle = \frac{\sum_{i_1}^{i_2} \alpha_i(\tau_i) \, \tau_i}{\sum_{i=n}^{i_2} \alpha_i(\tau_i)} \quad (9)$$

the summation being performed on the significant values of the $\alpha_i(\tau_i)$ for the j class. C_j is the normalized contribution of the lifetime class j:

$$C_j = \frac{\sum_{i_1}^{i_2} \alpha_i(\tau_i)}{\sum_{i=1}^{150} \alpha_i(\tau_i)} \quad (10)$$

Fluorescence Anisotropy Decays. The fluorescence anisotropy decays were analyzed in a one-dimensional way using the maximum entropy method as detailed previously (Brochon et al., 1992b).

RESULTS

Kinetic Properties of the W284F and W209F Mutant ATCases and Their Isolated Catalytic Subunits. (a) Aspartate Saturation Curves of the Modified Enzymes. The

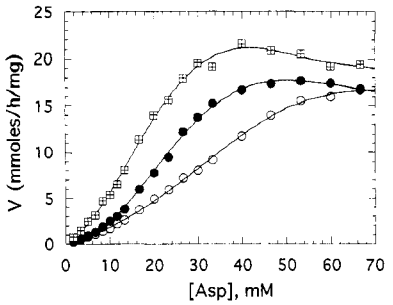

FIGURE 2: Aspartate saturation curves of ATCase and its modified forms. The ATCase activity was measured as indicated in Materials and Methods. (●) Wild-type ATCase; (○) W209F-ATCase; (⊞) W284F-ATCase.

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

ATCase	V_m [mmol h^{-1} (mg of protein)$^{-1}$]	$S_{[0.5]}$ (mM)	n_H	PALA (rel act.)
WT	25.3 ± 0.5[b]	19.7 ± 2.1	2.7 ± 0.3	3.9
W284F	20.7 ± 2.7	15.4 ± 1.8	2.3 ± 0.1	1.7
W209F	23.4 ± 1.4	34.7 ± 5.1	2.6 ± 0.5	2.8
cat.su WT	37.5 ± 1.0[b]	19.6 ± 3.9	1	
cat.su W284F	53.8 ± 4.7	21.2 ± 2.9	1	
cat.su W209F	41.2 ± 3.7	33.9 ± 2.5	1	

[a] These parameters were calculated as described in Materials and Methods through a computer fit by a nonlinear least-squares procedure to either the Hill equation or the Michaelis–Menten equation. The standard deviations were determined on the basis of 3–4 different aspartate saturation curves in the presence of 5 mM carbamyl phosphate. 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 made 1. [b] Taken from previous work (Xi et al., 1990).

aspartate saturation curves of the modified ATCases are shown in Figure 2 and the deduced kinetic parameters are presented in Table I. It appears that the replacement of either W209 or W284 by a phenylalanine does not affect significantly the maximal velocity. The affinity for aspartate, as reflected by $S_{[0.5]}$, is lowered in W209F-ATCase when compared to that of the wild-type enzyme. As shown in Table I, the Hill coefficients of both mutant enzymes are similar to that of the WT enzyme, indicating that the replacement of one or the other tryptophan residue by a phenylalanine does not alter the homotropic cooperative interactions between the catalytic sites.

(b) Aspartate Saturation Curves of the Isolated Catalytic Subunits. Since the mutation is located in the catalytic chain, it was essential to determine its influence on the kinetic parameters of the isolated catalytic subunits. The constants derived from the aspartate saturation curves are shown in Table I. Replacement of tryptophan 209 by a phenylalanine residue provokes an increase of the K_m for aspartate, as observed in the native mutant enzyme. The catalytic subunit from W284F-ATCase exhibits a significantly higher maximal velocity than the WT catalytic subunit.

(c) Stimulation of the Enzyme Activity by the Bisubstrate Analog PALA. In the following investigation, PALA is used to assess the effect of substrate binding, and possibly of the T to R transition, on the environment of the tryptophans of the catalytic chain. Since it is well-known that, at low concentration, the bisubstrate analog PALA increases the rate of the reaction by promoting the T to R transition (Collins & Stark, 1971), the extent of the stimulation of the mutant enzymes' activity by this compound was determined in the presence of an aspartate concentration adjusted to one-tenth

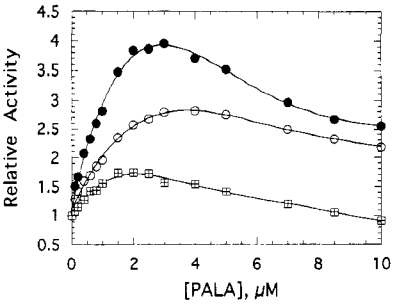

FIGURE 3: Stimulation of the ATCase reaction by PALA. The reaction was performed as indicated under Materials and Methods in the presence of 5 mM carbamyl phosphate and 2 mM L-aspartate for WT enzyme, 3.5 mM L-aspartate for W209F mutant, and 1.5 mM L-aspartate for W284F mutant, in the presence of increasing concentrations of PALA. The relative activity in the presence of PALA is expressed as compared to the reaction in the absence of this compound. (●) Wild-type ATCase; (○) W209F-ATCase; (⊞) W284F-ATCase.

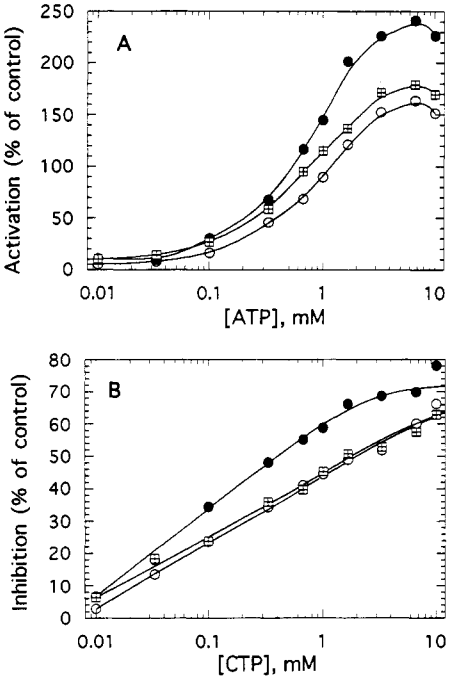

FIGURE 4: Influence of nucleotide triphosphate on the activity of ATCase and its modified forms. The influence of ATP (A) and CTP (B) on the rate of reaction were determined, and the percent stimulation were calculated as described in Materials and Methods. (●) Wild-type ATCase; (○) W209F-ATCase; (⊞) W284F-ATCase.

the $S_{[0.5]}$ value. Figure 3 shows that the two mutant enzymes are stimulated by low concentrations of PALA, although to a lesser extent than the WT enzyme, indicating that these modified enzymes are able to undergo the T or R transition.

(d) Influence of the Allosteric Effectors ATP and CTP on the Activity of the WT and Mutant Enzymes. The influence of ATP and CTP on the rate of the reaction catalyzed by the two modified forms of ATCase was determined. Figure 4 shows that the mutant enzymes are stimulated by ATP and inhibited by CTP, to a slightly lower extent than the WT enzyme.

Steady-State Fluorescence. The steady-state fluorescence spectra of the WT and modified forms of ATCase are very similar in terms of their center of gravity, located at 356 ± 1.5 nm (results not shown). The same is observed in the case of the corresponding isolated catalytic subunits, in the presence or absence of their different ligands. The values of the quantum yields are given in Table II. The quantum yields of

Table II: Spectroscopic Features of WT ATCase and Single Tryptophan-Containing Mutants in the Absence or Presence of Different Ligands[a]

	WT		W209F		W284F	
	QY	QY/τ_m	QY	QY/τ_m	QY	QY/τ_m
unliganded	0.028	0.02	0.008	0.09	0.021	0.01
PALA	0.026	0.02	0.005	0.08	0.022	0.01
ATP	0.026	0.02	0.007	0.08	0.020	0.01
CTP	0.016	0.01	0.005	0.06	0.016	0.007

[a] The experiments were performed as described under Materials and Methods. QY is the quantum yield, taking the quantum yield of NATA (0.14) as a reference (Werner & Forster, 1979) with a lifetime of 3.0 ns (Szabo & Rayner, 1980). τ_m is the fluorescence mean lifetime.

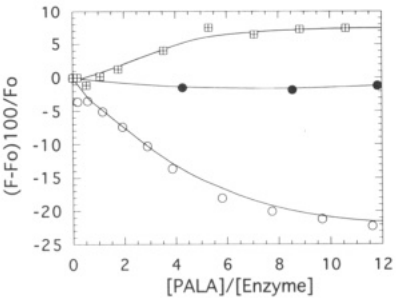

FIGURE 5: Relative fluorescence intensity of ATCase and its modified forms as a function of PALA concentration. The relative fluorescence is based upon integration of emission spectra in the presence (F) and absence (F_0) of PALA. The experimental conditions are described under Materials and Methods. (●) Wild-type ATCase; (○) W209F-ATCase; (⊞) W284F-ATCase.

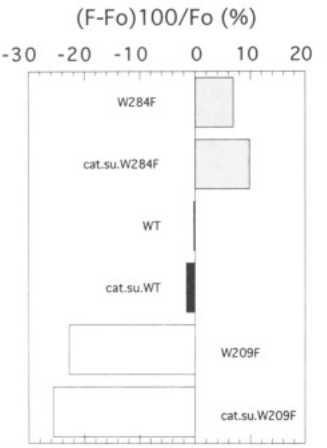

FIGURE 6: Relative fluorescence intensity of ATCase and its modified forms in the presence of saturating PALA. The relative fluorescence is based upon integration of emission spectra in the presence (F) and absence (F_0) of PALA (3.3×10^{-4} M). The experimental conditions are described under Materials and Methods.

the mutant ATCases are lower than those of the WT enzyme. The relative variation of the fluorescence intensity observed upon progressive saturation of the different enzymes by PALA is shown in Figure 5. According to previous reports (Maliwal et al., 1984; Royer et al., 1987) the binding of this bisubstrate analog has virtually no influence on the fluorescence intensity of the WT enzyme. However, the results obtained with both mutants show that tryptophans 209 and 284 react in opposite ways to PALA binding. Their fluorescence intensity is increased by about 6% and decreased by 20%, respectively. The same determination was made on the catalytic subunits isolated from the WT and the modified enzymes. Figure 6 shows that the binding of PALA induces the same variations of the tryptophan fluorescence, whether or not these catalytic subunits are associated with the regulatory subunits.

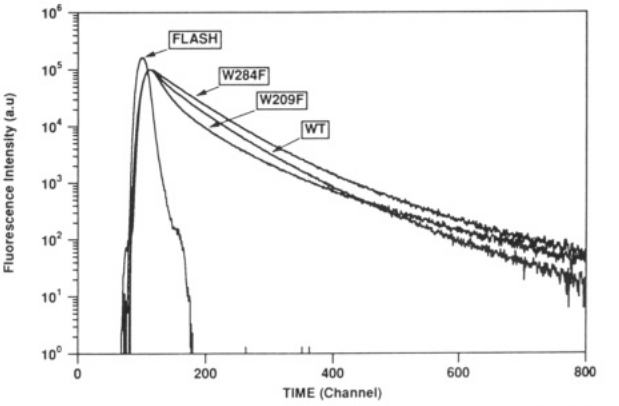

FIGURE 7: Normalized total fluorescence intensity decays of ATCase and its modified forms. λ_{exc} = 300 nm; λ_{em} = 350 nm; Flash = instrumental response function. One channel equals 35 ps. The experimental conditions are described under Materials and Methods.

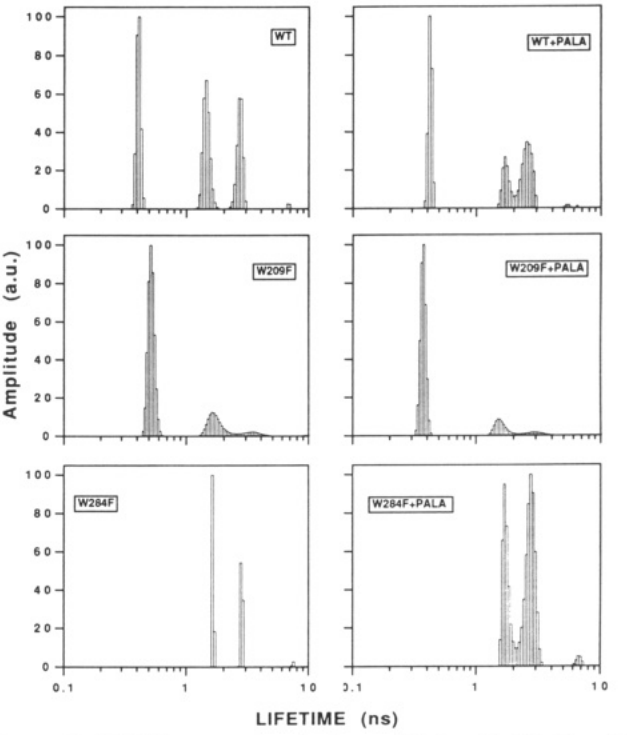

FIGURE 8: MEM-recovered excited-state lifetime distribution of ATCase and its modified forms in the absence and presence of PALA. PALA concentration was 3.3×10^{-4} M. The experimental conditions are described under Materials and Methods.

The binding of ATP has no significant influence on the quantum yields of both tryptophans, whereas CTP binding lowers it by 40% (Table II). Except for CTP, the addition of ligands has no detectable effect on the ratio of quantum yield over mean lifetime, indicating that no additional static quenching occurs.

Time-Resolved Fluorescence. In order to further investigate the respective contribution of each of the two tryptophans to the fluorescence properties of the enzyme, time-resolved fluorescence measurements were performed on the three proteins.

(*a*) *Unliganded Enzymes.* The normalized fluorescence intensity decay of the wild-type and the two mutant enzymes are presented in Figure 7, and the corresponding τ distribution is shown in Figure 8. Fluorescence lifetime distributions obtained with the WT enzyme display three main components centered at 0.4, 1.4, and 2.6 ns. The values of the relative areas of these components are close to 33 ± 5% (Table III).

Table III: Effects of Ligand Binding on the Total Intensity Decay Parameters of the WT ATCase and the Corresponding Catalytic Subunits

	lifetimes (ns) and amplitudes (%)[a]				
enzyme	τ_1 (c_1)	τ_2 (c_2)	τ_3 (c_3)	mean lifetime (ns)	χ^2
WT	0.39 (38)	1.41 (35)	2.60 (27)	1.34	1.03
WT + PALA	0.41 (42)	1.64 (20)	2.44 (38)	1.42	1.08
WT + ATP	0.38 (38)	1.33 (29)	2.40 (32)	1.35	1.02
WT + CTP	0.45 (42)	1.35 (23)	2.28 (37)	1.33	1.06
cat.su WT	0.57 (41)	1.48 (21)	2.37 (38)	1.44	0.98
cat.su WT + PALA	0.39 (44)	1.63 (23)	2.65 (33)	1.42	1.07

[a] τ_j values are the barycenters of the lifetime class j, and c_j values are the normalized areas over each class.

Table IV: Effects of Ligand Binding on the Total Intensity Decay Parameters of the W209F ATCase and the Corresponding Catalytic Subunits

	lifetimes (ns) and amplitudes (%)[a]				
enzyme	τ_1 (c_1)	τ_2 (c_2)	τ_3 (c_3)	mean lifetime (ns)	χ^2
W209F	0.51 (75)	1.67 (19)	3.45 (5)	0.93	1.08
W209F + PALA	0.36 (80)	1.47 (13)	2.70 (5)	0.70	1.12
W209F + ATP	0.51 (75)	1.70 (18)	3.17 (6)	0.90	1.14
W209F + CTP	0.44 (74)	1.41 (19)	2.97 (7)	0.80	1.05
cat.su W209F	0.50 (70)	1.45 (24)	2.94 (6)	0.87	1.07
cat.su W209F + PALA	0.35 (80)	1.23 (13)	2.64 (7)	0.62	1.04

[a] τ_j and c_j values are defined in Table III.

Table V: Effects of Ligand Binding on the Total Intensity Decay Parameters of the W284F ATCase and the Corresponding Catalytic Subunits

	lifetimes (ns) and amplitudes (%)[a]				
enzyme	τ_1 (c_1)	τ_2 (c_2)	τ_3 (c_3)	mean lifetime (ns)	χ^2
W284F		1.60 (57)	2.72 (43)	2.08	1.32
W284F + PALA		1.68 (39)	2.63 (61)	2.26	1.50
W284F + ATP		1.46 (47)	2.44 (53)	1.98	1.31
W284F + CTP	0.48 (7)	1.70 (69)	2.77 (24)	1.83	1.06
cat.su W284F		1.50 (42)	2.46 (58)	2.05	1.03
cat.su W284F + PALA		1.50 (37)	2.40 (63)	2.06	1.04

[a] τ_j and c_j values are defined in Table III.

In all measurements an additional longer lifetime was found, ranging from 5.5 to 7.5 ns and having a relative contribution of 1–4%. Occasionally, a shorter lifetime around 0.1 ns, which corresponds to the limit of the present instrumental resolution, was recorded. These later components were regarded as artifactual and are no longer presented in the further results. The lifetime distribution obtained for the single tryptophan in W209F mutant is different (Table IV). It is mainly characterized by two well-separated major peaks centered at 0.5 and 1.67 ns. The relative area of the first one is strongly dominant (75%). A third peak, centered at 3.45 ns, contributes only 5% to the total fluorescence decay. The single tryptophan residue in the W284F mutant exhibits biexponential fluorescence decay, with a lifetime centered at 1.6 ns (55%), well-separated from a second lifetime at 2.7 ns (45%) (Table V).

The same kind of analysis was performed on the isolated catalytic subunits. The results show qualitatively the same pattern of lifetime distributions, with some small quantitative variations (Tables III–V). The catalytic subunits prepared from the WT enzyme exhibit a similar lifetime distribution to the native enzyme, comprising three principal peaks around 0.6 ns (40%), 1.5 ns (20%), and 2.4 ns (40%). In catalytic subunits from the W209F mutant, a short lifetime of 0.5 ns (70%) is also present, whereas both longer lifetimes equal 1.45 ns (25%) and 2.9 ns (5%). The W284F mutant catalytic subunits differ from the corresponding entire enzyme only by a change in the relative contributions of these two components, with the first one accounting for 40% and the second one for 60%.

(*b*) *Influence of PALA Binding.* At saturation, the bisubstrate analog PALA increases the mean lifetime of WT enzyme fluorescence by 5% and that of W284F by 10%. On the contrary, under the same conditions W209F-ATCase responds by a 20% decrease of the mean lifetime. Figure 8 shows the influence of PALA binding on the fluorescence lifetime distributions of the three enzyme species. This binding has a strong effect on the lifetime distribution in the WT enzyme. Although the shorter peak τ_1 does not change, one observes a slight increase of the middle lifetime, τ_2, from 1.4 to 1.65 ns, its contribution being lowered from 35% to 20%. The longest lifetime component, τ_3, decreases from 2.6 to 2.4 ns, whereas its relative contribution changes from 24% to 38% (Table III). In the case of the mutant W209F, upon PALA binding the position of the τ_2 lifetime peak is lowered by 0.2 ns (Table IV), and the longer one, τ_3, is diminished by 0.75 ns. In the case of the W284F mutant the position of the lifetime peaks is not significantly affected. The relative contribution of τ_2 is decreased from 57% to 39%, and that of τ_3 is increased from 43% to 61% (Table V).

The results obtained in the case of the isolated catalytic subunits are presented in Tables III–V. Considering the mean lifetimes of the catalytic subunits, one notices that only the W209F mutant is affected by the presence of PALA. In contrast with the results obtained in the case of the entire enzyme, PALA binding on the WT catalytic subunits shows a slight effect on the three lifetime components without any change on their relative contribution. The shortest lifetime component decreases from 0.57 to 0.40 ns, and an increase of about 0.1–0.2 ns for the two other components is observed (Table III). In the case of the W209F catalytic subunits, PALA binding provokes the same fluorescence variations as in the native mutant enzyme. No influence on the fluorescence

parameters of the W284F catalytic subunits is observed upon PALA binding.

(*c*) *Influence of the Allosteric Effectors.* Upon binding of the effector ATP to the WT enzyme, neither the mean lifetime nor the distribution of lifetimes is significantly affected. The fluorescence of W209F is not sensitive to the binding of ATP, whereas the W284F shows a decrease of the longest lifetime from 2.72 to 2.44 ns, with an inversion of the relative areas of the two lifetime peaks.

The binding of CTP does not significantly change the fluorescence mean lifetimes of the WT and the two mutant enzymes. Nevertheless, the longer lifetime of WT enzyme, centered at 2.6 ns, moves to 2.3 ns, and its relative contribution changes from 27% to 37%. In contrast, in W284F τ_2 and τ_3 are insensitive to the presence of CTP. Surprisingly, a short lifetime peak τ_1 of 0.5 ns, accounting for only 7%, appears upon CTP ligation. In W209F, tryptophan 284 exhibits τ_2 and τ_3 peak lifetime positions that are lowered by 0.26 and 0.5 ns, respectively.

Anisotropy Decays. The fluorescence anisotropy decays were measured under the same conditions as above, in the presence and absence of the ligands. In the case of the three enzymes and their catalytic subunits, a single rotational correlation time without any evidence for internal flexibilities in the nanosecond domain was found. In addition, the values r_0 of initial anisotropy at time zero were close to 0.3, as expected from immobilized tryptophans (Valeur & Weber, 1977).

DISCUSSION

In order to analyze the relative contribution of each of the two tryptophan residues of ATCase to the fluorescence properties of this enzyme, single-tryptophan-containing enzymes were prepared by site-directed mutagenesis. The tryptophan in position 209 or 284 was replaced by another aromatic residue, phenylalanine. These mutations do not alter significantly the catalytic and regulatory properties of the enzyme, allowing the direct comparison of the fluorescence parameters of these different enzymes. The only alteration observed is the increase of $S_{[0.5]}$ for aspartate of W209F by a factor of 2. A similar observation was made by Smith et al. (1986) upon replacement of tryptophan 209 by a series of other amino acids. These authors did not observe an influence of the replacement of tryptophan 209 by glutamic acid, tyrosine, serine, lysine, or glutamine on the sensitivity of the enzyme to the effectors ATP and CTP. In the present work, replacement of this amino acid provokes a 30% decrease of the normal stimulation by ATP.

Are ATCase Tryptophans Fluorescing Independently? The W284F mutant shows a biexponential decay and the W209F mutant a triexponential decay, while the fluorescence decay of WT ATCase is described by only three exponentials. This apparent discrepancy results from the fact that the two tryptophans have overlapping lifetimes. The question arises whether WT fluorescence decay can be described as the sum of the mutants' protein signals, despite slight variations of the lifetime values. In fluorescence decay, the preexponential coefficient is proportional to $\epsilon_i[M_i]/\tau_0$ (ϵ_i is the molecular absorbance and $[M_i]$ is the molecular concentration of each fluorophore i). Assuming that the natural lifetime τ_0 and ϵ_0 for the tryptophan is the same in all proteins and taking into account the preexponential amplitudes c_j, given in Tables III–V, one can reasonably state that the sum of the two mutant enzyme coefficients c_j fits the values of the WT enzyme. Similar results were reported for the *lac* repressor (Royer et al., 1991) and the glutamine-binding protein (Axelsen et al., 1991).

The results of the PALA titration experiments lead to the same conclusion. In the mutants the two tryptophans react in opposite ways to the binding of the bisubstrate analog. Taking into account the quantum yields of the two tryptophans, the result obtained from the wild-type enzyme upon PALA saturation is roughly the sum of the signals recovered from the two mutants under the same conditions. In addition, fluorescence polarization decays do not provide evidence for any energy transfer between excited tryptophans which fluoresce independently in various environments.

Heterogeneous Decays of Single Tryptophan ATCase Mutants. For both single-tryptophan mutant enzymes, the fluorescence decays are not fit by single exponentials. Such heterogeneity can be explained by a local variability of the three-dimensional structure. There is now a large set of experimental data showing that single-tryptophan-containing proteins generally exhibit multiexponential fluorescence decays (Beechem & Brand, 1985; Vincent et al., 1988; Mérola et al., 1989; Szabo et al., 1989; Royer et al., 1990; Gentin et al., 1990; Kuipers et al., 1991; Brochon et al., 1992b). Only a few examples of quasi-monoexponential fluorescence decays (preexponential factor > 90%) in single-tryptophan-containing proteins are known (Chen et al., 1987; Hedstrom et al., 1988; Brochon et al., 1992a,b). The complex decays are modulated by the interactions of the indole ring of the tryptophan residue with the amino acid side chains present in its vicinity. Although theoretical work has put the emphasis on the multiplicity of conformational substates in proteins (Edler & Karplus, 1988), there is no general agreement about the molecular phenomena underlying this heterogeneity. Different hypotheses were put forward, such as the existence of a quasi-continuum of thermally equilibrated excited-state populations (James & Ware, 1985, 1986; James et al., 1987; Alcala et al., 1987a,b), the presence of discrete species of conformations (Szabo & Rayner, 1980; Chang et al., 1983; Engh et al., 1986; Colucci et al., 1990), and the existence of conformational substates with various energy barrier levels (Frauenfelder et al., 1988). The fluorescence anisotropy decay parameters of ATCase allow us to state that the tryptophan residues are immobile in the subnanosecond time domain with respect to the protein matrix. Thus, the heterogeneity of the fluorescence lifetime distribution is assigned to a local flexibility of this matrix around the indole ring. This heterogeneity might be related to the slight asymmetry between the two catalytic trimers which was observed by crystallography (Kim et al., 1987) or to the existence of two conformers of the catalytic monomer.

Environment of the Tryptophan Residues. Although the WT ATCase molecule contains 12 tryptophan residues (two per catalytic chain), its fluorescence decay is satisfactorly described by three lifetime classes, in favor of a homogeneity of the catalytic chains in the dodecamer. The steady-state and time-resolved fluorescence properties and the lifetime distributions of native ATCase and its isolated catalytic subunits are very similar. Thus, the association of the catalytic and regulatory subunits does not significantly affect the local environments of the tryptophan residues. The steady-state fluorescence emission spectra of the two single-tryptophan-containing mutants present a center of gravity around 356 nm, indicating that the two tryptophans are located in a polar environment (Van Durren, 1961). This observation is consistent with the crystallographic data (Honzatko et al., 1982), which show that the two tryptophans of ATCase reside in solvent-accessible environments at the exterior shell of the catalytic subunit and are not involved in interfaces between chains or domains. In the mutant W284F, the τ_3 value is

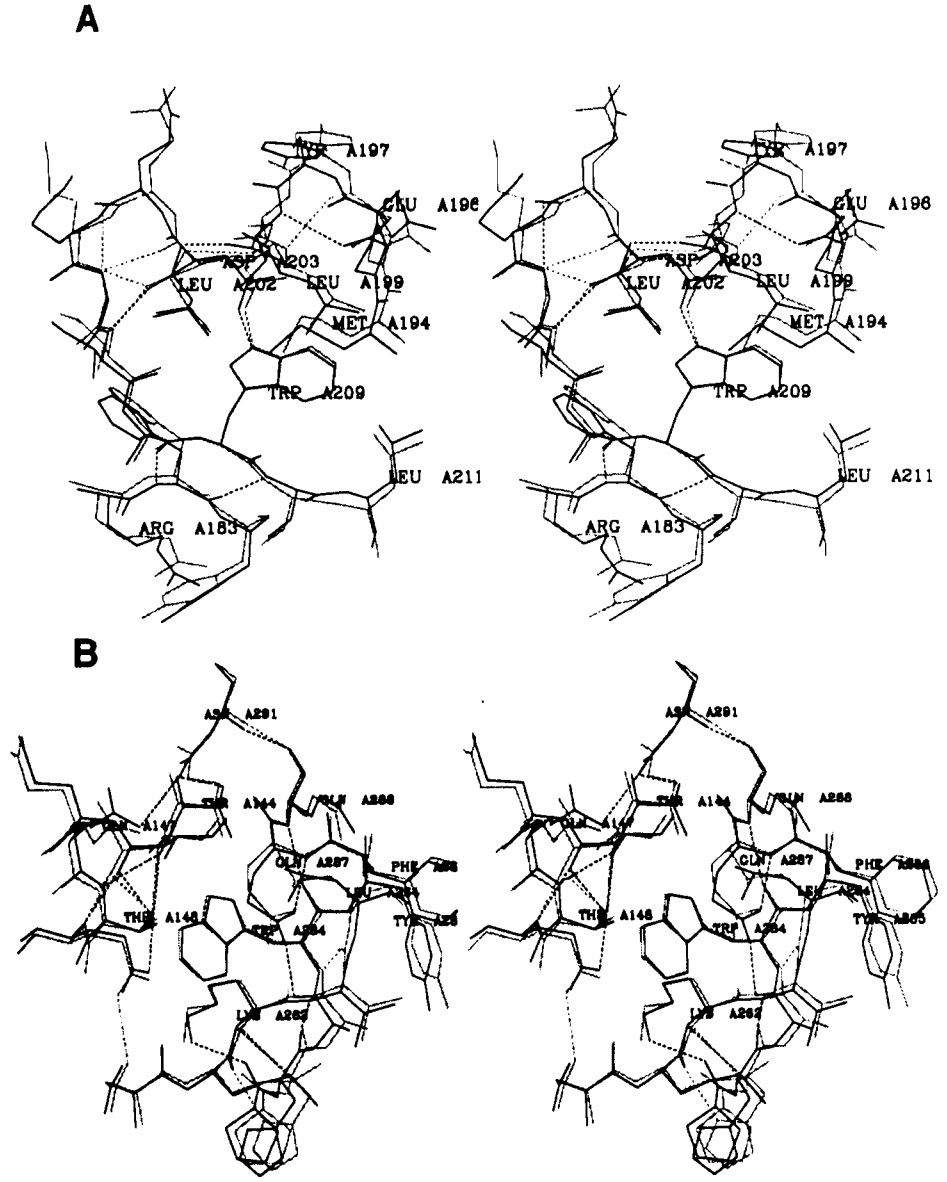

FIGURE 9: Close-up stereoview of the environment of the two tryptophan residues. The comparison between the T state (black line) and the R state (gray line) was obtained by superimposing the indole rings. H-bonds are represented as discontinuous lines. (A) Tryptophan 209; (B) tryptophan 284.

close to the NATA lifetime in water (Szabo & Rayner, 1980), suggesting that the corresponding conformational substate does not involve interaction of the indole ring with the surrounding protein matrix. The other lifetime of W284F, τ_2, corresponds to a quenched tryptophan and reports for another environment. Indeed, the X-ray crystallographic structure shows that tryptophan 209 is hydrogen-bonded to Asp 203, thus stabilizing a bend connecting strand S8 to helix H7 (Figure 9A). In the W209F mutant, though, quantum yield and lifetimes τ_1 or τ_2, much lower than 3.0 ns, indicate the existence of a dynamic quenching from the protein matrix. From the crystallographic data (Figure 9B) it appears that tryptophan 284 may interact with Thr 244, Lys 262, and Gln 287, all located within 5 Å of the indole ring (Krause et al., 1987; Ke et al., 1988). In the three enzymes, the ratio QY/τ_m (Table II), much smaller than in the case of NATA in water, indicates the existence of a static quenching or a very fast dynamic quenching in the picosecond time domain. That is particularly evident for W209F.

The T to R Transition. The binding of the bisubstrate analog PALA, which promotes the transition from T to R (Collins & Stark, 1969; Howlett & Schachman, 1977; Moody et al., 1979; Hervé et al., 1985; Krause et al., 1987; Ke et al., 1988), affects the fluorescence properties of both tryptophans in spite of the fact that these residues are far from the binding sites. Tryptophan 209 is slightly affected by PALA binding, the total fluorescence intensity and the mean lifetime increasing by about 6%. The fluorescence intensity and mean lifetime of tryptophan 284 are more affected, decreasing by 20% upon PALA binding. This observation suggests that during the T to R transition, either a polar group or water molecules become able to reach this residue. The crystallographic data concerning the vicinity of the two tryptophan residues show that the differences between the T and R states concern mainly the environment of tryptophan 284 (Figure 9B). That includes a rotation of about 90° around the C_α–C_β axis of the side chain of Gln 287 and the disappearance of hydrogen bonds in the R state (Ke et al., 1988).

The heterogeneity of the time-resolved fluorescence signals of the two mutant enzymes could indicate that the catalytic chains might exist in slightly different conformations. These conformations cannot correspond to the T and R states since the binding of PALA does not simplify the fluorescence pattern. Upon PALA binding the tryptophan fluorescence signals are

the same in the entire enzyme and the isolated catalytic subunits. Although the crystallographic structure of the isolated catalytic trimer is not yet available, these results indicate that PALA binding has the same influence on the tertiary structure of the catalytic subunits whether or not they are associated with the regulatory subunits. The same conclusion was reached when PALA binding was studied by UV difference spectroscopy (Kerbiriou et al., 1977). Thus, in the entire enzyme this change of the tertiary structure of the catalytic chain would be sufficient to trigger the quaternary structure change, as already reported by Ladjimi and Kantrowitz (1988). This interpretation is consistent with the fact that, in ATCase, cooperative interactions between the catalytic sites cannot be explained by a simple two-state equilibrium and that an "induced fit" promoted by the substrate is involved. This conclusion is based on the observation that some pseudosubstrates like L-cysteine sulfinate (Foote et al., 1985) and L-alanosine (Baillon et al., 1985), as well as carbamylaspartate in the reverse reaction (Foote & Lipscomb, 1981), are used as substrates by the T form but are unable to promote the T to R transition.

Influence of the Allosteric Effectors. The fluorescence variation observed following the binding of ATP to the enzyme is different from that consecutive to the binding of PALA and concerns tryptophan 209. This results are in accordance with the conclusion that ATP acts through a mechanism different from that involved in the homotropic cooperative interactions between the catalytic sites (Thiry & Hervé, 1978; Tauc et al., 1982; Hervé et al., 1985). The implication of the different interfaces between the regulatory and catalytic chains in the mechanisms of ATCase allosteric regulation is currently investigated. On the basis of the results obtained, it was recently proposed that the transmission of the CTP regulatory signal involves the R1C4 interface and that the transmission of the ATP signal rather involves the R1C1 interface (Xi et al., 1991). An implication of such a mechanism is that the CTP signal would reach the catalytic site through the aspartate binding domain of the catalytic chain, whereas the ATP signal would reach this site through the carbamyl phosphate binding domain. Consequently, and since the two tryptophan residues are located in the aspartate binding domain, it would be expected that the environment of these residues would be more sensitive to CTP binding than to that of ATP. This is what is observed in the present work. This rationale would not apply to the recently proposed "indirect effect" (Stevens & Lipscomb, 1992).

In conclusion, the present study of a large oligomeric molecule containing a single tryptophan per catalytic chain demonstrates again the possible structural heterogeneity of proteins in solution. The influence of the bisubstrate analog on these heterogeneous fluorescence properties of ATCase and its isolated catalytic subunits provides information about the mechanism of cooperativity in this enzyme. Further investigation is needed to relate these fluorescence properties to the mechanism of catalysis and regulation in this enzyme.

ACKNOWLEDGMENT

We thank M. Vincent for interfacing the SLM 8000 to a Mac/SE. We appreciate the technical staff of LURE for running the synchrotron machine during the beam-time session and the technical assistance of S. Tosti. We thank Dr. P. Kraulis for the program Molscript.

REFERENCES

Alcala, J. R., Gratton, E., & Prendergast, F. G. (1987a) *Biophys. J. 51*, 597–604.

Alcala, J. R., Gratton, E., & Prendergast, F. G. (1987b) *Biophys. J. 51*, 925–936.

Allewell, N. M. (1989) *Annu. Rev. Biophys. Biophys. Chem. 18*, 71–92.

Axelsen, P. H., Bajzer, Z., Prendergast, F. G., Cottam, P. F., & Ho, C. (1991) *Biophys. J. 60*, 650–659.

Baillon, J., Tauc, P., & Hervé, G. (1985) *Biochemistry 24*, 7182–7187.

Beechem, J. M., & Brand, L. (1985) *Annu. Rev. Biochem. 54*, 43–71.

Bethell, M. R., Smith, K. E., White, J. S., & Jones, M. E. (1968) *Proc. Natl. Acad. Sci. U.S.A. 60*, 1442–1446.

Brochon, J. C., Tauc, P., Mérola, F., & Schoot, B. (1992a) *Biophys. J. 61*, A179.

Brochon, J. C., Mérola, F., & Livesey, A. K. (1992b) in *Synchrotron Radiation and Dynamic Phenomena*, Conference Proceedings No. 258 (Beswick, A., Ed.) pp 435–452, American Institute of Physics, New York.

Chang, M. C., Petrich, J. W., Mc Donald, D. B., & Fleming, G. R. (1983) *J. Am. Chem. Soc. 105*, 3819–3824.

Chen, L. X. Q., Longworth, J. W., & Fleming, G. R. (1987) *Biophys. J. 51*, 865–873.

Collins, K. D., & Stark, G. R. (1969) *J. Biol. Chem. 244*, 1869–1877.

Collins, K. D., & Stark, G. R. (1971) *J. Biol. Chem. 246*, 6599–6605.

Colucci, W. J., Tilstra, L., Sattler, M. C., Fronczek, F. R., & Barkley, M. D. (1990) *J. Am. Chem. Soc. 112*, 9182–9190.

Creed, D. (1984) *Photochem. Photobiol. 39*, 537–562.

Elber, R., & Karplus, M. (1987) *Science 235*, 318–321.

Engh, R. A. L., Chen, X. Q., & Fleming, G. R. (1986) *Chem. Phys. Lett. 126*, 365–372.

Foote, J., & Lipscomb, W. N. (1981) *J. Biol. Chem. 256*, 11428–11433.

Foote, J., Lauritzen, A. M., & Lipscomb, W. N. (1985) *J. Biol. Chem. 260*, 9624–9629.

Frauenfelder, H., Parak, F., & Young, R. D. (1988) *Annu. Rev. Biophys. Biophys. Chem. 17*, 451–479.

Gentin, M., Vincent, M., Brochon, J. C., Livesey, A. K., Cittanova, N., & Gallay, J. (1990) *Biochemistry 29*, 10405–10412.

Gerhart, J. C., & Pardee, A. B. (1962) *J. Biol. Chem. 237*, 891–896.

Gerhart, J. C., & Holoubek, H. (1967) *J. Biol. Chem. 242*, 2886–2892.

Gouaux, J. E., & Lipscomb, W. N. (1990) *Biochemistry 29*, 389–402.

Hedstrom, J., Sedarou, S., & Prendergast, F. G. (1988) *Biochemistry 27*, 6203–6208.

Hervé, G. (1989) in *Allosteric Enzymes*, pp 61–79, CRC Press, Boca Raton, FL.

Hervé, G., Moody, M. F., Tauc, P., Vachette, P., & Jones, P. T. (1985) *J. Mol. Biol. 185*, 189–199.

Howlett, G. J., & Schachman, H. K. (1977) *Biochemistry 16*, 5077–5083.

Honzatko, R. B., Crawford, J. L., Monaco, H. L., Ladner, J. E., Edwards, B. F. P., Evans, D. R., Warren, S. G., Wiley, D. C., Ladner, R. C., & Lipscomb, W. N. (1982) *J. Mol. Biol. 160*, 219–263.

Hsuanyu, Y., & Wedler, F. (1987) *Arch. Biochem. Biophys. 259*, 316–330.

Issaly, I., Poiret, M., Tauc, P., Thiry, L., & Hervé, G. (1982) *Biochemistry 21*, 1612–1623.

James, D. R., & Ware, W. R. (1985) *Chem. Phys. Lett. 120*, 450–454.

James, D. R., & Ware, W. R. (1986) *Chem. Phys. Lett. 126*, 7–11.

James, D. R., Turnbull, J. R., Wagner, B. D., Ware, W. R., & Petersen, N. O. (1987) *Biochemistry 26*, 6272–6277.

Jaynes, E. T. (1983) in *Collected Works. Papers on Probability Statistics and Statistical Physics* (Rosenkrantz, R. D., Ed.) D. Reidel, Dordrecht, The Netherlands.

Kantrowitz, E. R., & Lipscomb, W. N. (1990) *Trends Biochem. Sci. 15*, 53–59.
Ke, H., Hontzatko, R. B., & Lipscomb, W. N. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4037–4040.
Ke, H., Lipscomb, W. N., Cho, Y., & Honzatko, R. G. (1988) *J. Mol. Biol. 204*, 705–747.
Kerbiriou, D., & Hervé, G. (1972) *J. Mol. Biol. 64*, 379–392.
Kerbiriou, D., Hervé, G., & Griffin, J. H. (1977) *J. Biol. Chem. 252*, 2881–2890.
Kim, K. H., Pan, Z., Hontzatko, R. B., Ke, H. M., & Lipscomb, W. N. (1987) *J. Mol. Biol. 196*, 853–875.
Konigsberg, W. H., & Henderson, L. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2467–2471.
Kraulis, P. J. (1991) *J. Appl. Crystallogr. 24*, 946–950.
Krause, K. L., Volz, K. W., & Lipscomb, W. N. (1987) *J. Mol. Biol. 193*, 527–553.
Kuipers, O. P., Vincent, M., Brochon, J. C., Verheij, H. M., De Haas, G. H., & Gallay, J. (1991) *Biochemistry 30*, 8771–8785.
Ladjimi, M., & Kantrowitz, E. R. (1988) *Biochemistry 27*, 276–283.
Ladjimi, M., Middleton, S. A., Kelleher, K. S., & Kantrowitz, E. R. (1988) *Biochemistry 27*, 268–276.
Lakowicz, J. R., Maliwal, B., Cherek, H., & Balter, A. (1983) *Biochemistry 22*, 1741–1752.
Livesey, A. K., & Brochon, J. C. (1987) *Biophys. J. 52*, 693–706.
Livesey, A. K., Licinio, P., & Delaye, M. (1986) *J. Chem. Phys. 84*, 5102–5107.
Livesey, A. K., Delaye, M., Licinio, P., & Brochon, J. C. (1987) *Faraday Discuss. Chem. Soc. 83*, paper 14.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 53*, 9097–9101.
Maliwal, B. P., Allewel, N. M., & Lakowicz, J. R. (1984) *Biophys. Chem. 81*, 209–216.
Mérola, F., Rigler, R., Holmgren, A., & Brochon, J. C. (1989) *Biochemistry 28*, 3383–3398.
Moody, M. F., Vachette, P., & Foote, A. M. (1979) *J. Mol. Biol. 133*, 517–532.
Nowlan, S. F., & Kantrowitz, E. R. (1985) *J. Biol. Chem. 260*, 14712–14716.
O'Connor, D. V., & Philips, D. (1984) in *Time-correlated single photon counting*, Academic Press, London.
Perbal, B., & Hervé, G. (1972) *J. Mol. Biol. 70*, 511–529.
Porter, R. W., Modebe, M. O., & Stark, G. R. (1969) *J. Biol. Chem. 244*, 1846–1859.
Reichard, P., & Hanshoff, G. (1956) *Acta Chem. Scand. 10*, 548–566.
Royer, C. A., Tauc, P., Hervé, G., & Brochon, J. C. (1987) *Biochemistry 26*, 6472–6478.
Royer, C. A., Gardner, J. A., Beechem, J. M., Brochon, J. C., & Matthews, K. S. (1990) *Biophys. J. 58*, 363–378.
Schachman, H. K., Pauza, C. D., Navre, M., Karels, M. J., Wu, L., & Yang, Y. R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 115–119.
Smith, K. A., Nowlan, S. F., Middleton, S. A., O'Donovan, C., & Kantrowitz, E. R. (1986) *J. Mol. Biol. 189*, 227–238.
Stevens, R. C., & Lipscomb, W. N. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 5281–5285.
Szabo, A. G. (1989) in *The Enzyme Catalysis Process* (Cooper, A., Houben, J. L., & Chien, L. C., Eds.) pp 123–139, Plenum, New York.
Szabo, A. G., & Rayner, D. M. (1980) *J. Am. Chem. Soc. 102*, 554–563.
Tauc, P., Leconte, C., Kerbiriou, D. N., Thiry, L., & Hervé, G. (1982) *J. Mol. Biol. 155*, 155–168.
Thiry, L., & Hervé, G. (1978) *J. Mol. Biol. 125*, 515–534.
Valeur, B., & Weber, G. (1977) *Photochem. Photobiol. 25*, 441–444.
Van Durren, B. L. (1961) *J. Org. Chem. 26*, 2954.
Vincent, M., Brochon, J. C., Mérola, F., Jordi, W., & Gallay, J. (1988) *Biochemistry 27*, 8752–8761.
Wahl, Ph. (1979) *Biophys. Chem. 10*, 91–104.
Weber, K. (1968) *Nature 218*, 1116–1119.
Werner, T. C., & Forster, L. S. (1979) *Photochem. Photobiol. 29*, 905–914.
Wild, J. R., Loughrey-Chen, S. J., & Corder, T. S. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 46–50.
Xi, G. X., Van Vliet, F., Ladjimi, M. M., De Wannemaeker, B., De Staerke, C., Piérard, A., Glansdorff, N., Hervé, G., & Cunin, R. (1990) *J. Mol. Biol. 216*, 375–384.
Xi, G. X., Van Vliet, F., Ladjimi, M. M., De Wannemaeker, B., De Staerke, C., Glansdorff, N., Piérard, A., Cunin, R., & Hervé, G. (1991) *J. Mol. Biol. 220*, 789–799.
Zhang, Y., & Kantrowitz, E. R. (1992) *Biochemistry 31*, 792–798.

Registry No. ATCase, 9012-49-1; Trp, 73-22-3; ATP, 56-65-5; PALA, 51321-79-0; CTP, 65-47-4; Phe, 63-91-2.