A Fluorescent Probe-labeled *Escherichia coli* Aspartate Transcarbamoylase That Monitors the Allosteric Conformational State*

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A new system has been developed capable of monitoring conformational changes of the 240s loop of aspartate transcarbamoylase, which are tightly correlated with the quaternary structural transition, with high sensitivity in solution. Pyrene, a fluorescent probe, was conjugated to residue 241 in the 240s loop of aspartate transcarbamoylase to monitor changes in conformation by fluorescence spectroscopy. Pyrene maleimide was conjugated to a cysteine residue on the 240s loop of a previously constructed double catalytic chain mutant version of the enzyme, C47A/A241C. The pyrene-labeled enzyme undergoes the normal T to R structural transition, as demonstrated by small-angle x-ray scattering. Like the wild-type enzyme, the pyrene-labeled enzyme exhibits cooperativity toward aspartate, and is activated by ATP and inhibited by CTP at subsaturating concentrations of aspartate. The binding of the bi-substrate analogue N-(phosphonoacetyl)-L-aspartate (PALA), or the aspartate analogue succinate, in the presence of saturating carbamoyl phosphate, to the pyrene-labeled enzyme caused a sigmoidal change in the fluorescence emission. Saturation with ATP and CTP (in the presence of either subsaturating amounts of PALA or succinate and carbamoyl phosphate) caused a hyperbolic increase and decrease, respectively, in the fluorescence emission. The half-saturation values from the fluorescence saturation curves and kinetic saturation curves were, within error, identical. Fluorescence and small-angle x-ray scattering stopped-flow experiments, using aspartate and carbamoyl phosphate, confirm that the change in excimer fluorescence and the quaternary structure change correlate. These results in conjunction with previous studies suggest that the allosteric transition involves both global and local conformational changes and that the heterotropic effect of the nucleotides may be exerted through local conformational changes and that the heterotropic effect of the nucleotide effectors bind to the same site on each of the regulatory chains (4–8).

Allosteric regulation of enzymatic activity is manifested by the ability of the enzyme to exist in at least two different structural and functional forms (1). Enzymatic activity can therefore be modulated by changing the form in which the enzyme exists, by altering the dynamic equilibrium between multiple forms at a given time, or by causing more localized changes in the structure. Allosteric enzymes are also characterized by the regulation of their activity by effectors that bind at sites remote from the active sites.

*Escherichia coli* aspartate transcarbamoylase (EC 2.1.3.2) is a paradigm of allosteric enzymes in the study of allosteric regulation. This enzyme catalyzes the committed step of pyrimidine biosynthesis, the carbamoylation of the amino group of L-aspartate by carbamoyl phosphate to form N-carbamoyl-L-aspartate and inorganic phosphate (2). Allosteric regulation is manifested in two different ways: homotropic cooperativity for the substrate L-aspartate and heterotropic regulation by ATP, CTP (2), and UTP in the presence of CTP (3). Aspartate transcarbamoylase from *E. coli* is a dodecamer composed of six catalytic (C)1 chains organized as two trimeric subunits, and six regulatory (R) chains organized as three dimeric subunits. The active sites are shared between catalytic chains on the same trimer, and the nucleotide effectors bind to the same site on each of the regulatory chains (4–8).

The two different structural and functional states of aspartate transcarbamoylase are the low affinity, low activity conformation of the enzyme, or the T state, and the high affinity, high activity conformation of the enzyme, or the R state (9–12). Physical studies, including sedimentation velocity (9), x-ray crystallography (13, 14), and small-angle x-ray solution scattering (SAXS) (15, 16), have provided evidence of a quaternary structural change upon the binding of the natural substrates or substrate analogues. In the Thr to Arg quaternary structural transition, the enzyme elongates by at least 11 Å along the 3-fold axis, the upper and lower catalytic trimers rotate 15° relative to one another, and the regulatory dimers rotate 15° around their respective 2-fold axes (17, 18). In addition to these

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The abbreviations used are: C, catalytic chain; R, regulatory chain; SAXS, small-angle X-ray scattering; PALA, N-(phosphonoacetyl)-L-aspartate; [Asp]n, the aspartate concentration at half the maximal observed specific activity; C47A/A241C, the double mutant aspartate transcarbamoylase holoenzyme with Cys-47 replaced by Ala and Ala-241 replaced by Cys in each of the catalytic chains; C47A/A241C-pyrene, the double mutant labeled with pyrene; C47A/A241C-c-pyrene, the double mutant aspartate transcarbamoylase catalytic subunit with Cys-47 replaced by Ala and Ala-241 replaced by Cys in each of the catalytic chains; C47A/A241C-c, the double mutant catalytic subunit labeled with pyrene; 240s loop, a loop in the catalytic chain of aspartate transcarbamoylase comprised of residues 232–246; F0, fluorescence coefficient.
quaternary structural changes, several tertiary changes also occur during the T to R state transition. One example of a tertiary change that is of importance to this study is the reorientation of the 240s loop, which facilitates the domain closure in the catalytic chains, resulting in the formation of high activity high affinity active sites (19). In particular, the position of the two 240s loops at the C1-C4 interface changes from lying sideways and apart in the T state, to a position in which the loops are stacked on top of one another in the R state (see Fig. 1). Thus, the quaternary structural transition is tightly correlated with the conformational change of the 240s loop.

To create a fluorescent-labeled enzyme, which could monitor the allosteric transition, we conjugated a fluorophore site specifically via a Cys residue at position 241 of the 240s loop (Fig. 1). Previous studies have shown that when Ala-241 in the catalytic subunit, respectively, were previously constructed in this laboratory (25). The concentration of the C47A/A241C-pyrene-labeled enzyme was determined by the Bio-Rad version of the Bradford dye binding assay (30).

Efficiency of Pyrene Attachment—The completeness of the labeling of the C47A/A241C enzyme with pyrene was assessed by mass spectrometry and also by determination of untreated thiol with Ellman’s reagent (31). The C47A/A241C and C47A/A241C-pyrene holoenzymes were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry using a Micromass ToFSpec-2E mass spectrometer operating in positive mode and using a sinapic acid matrix.

Aspartate Transcarbamoylase Assay—The aspartate transcarbamoylase activity was measured at 25 °C by the colorimetric method (32). Saturation curves were performed in duplicate, and data points shown in the figures are the average values. Assays were performed in 50 mM Tris acetate buffer, pH 8.3, in the presence of saturating carbamoyl phosphate (4.5 mM). Data analysis of the steady-state kinetics was carried out as described previously (33). Fitting of the experimental data to theoretical equations was accomplished by non-linear regression. When substrate inhibition was negligible, data were fit to the Hill equation. If substrate inhibition was significant, data were analyzed using an extension of the Hill equation that included a term for substrate inhibition (34). The nucleotide saturation curves were fit to a hyperbolic binding isotherm by non-linear regression.

Fluorescence Emission Spectra—Fluorescence emission spectra were recorded at 20 ± 1 °C in 50 mM Tris acetate buffer, pH 8.3, on a Shimadzu 1000 spectrofluorimeter. The excitation wavelength was 338 nm; the emission wavelength collected was 360–560 nm; the excitation and emission bandwidths were set to 5 nm. To approximate the degree of quaternary structure change for the enzyme population from the fluorescence emission spectra, a unitless fluorescence coefficient ($F'$) was calculated from the following expression,
where \( I_\lambda \) is the fluorescence intensity at wavelength \( \lambda \) sampled over the entire emission spectrum at 0.4-nm intervals. The ratio of the sums of the intensities of the excimer to the monomer fluorescence was used to significantly reduce the signal to noise. All fluorescence experiments were performed with the enzyme in 50 mM Tris acetate buffer, pH 8.3.

Saturation curves with the bisubstrate analogue PALA and aspartate analogue succinate (plus saturating carbamoyl phosphate (4.8 mM)) were performed by incremental addition to the pyrene-labeled enzyme ([C47A/A241C-pyrene] = 1 \( \mu \)M) until there was no further change in the fluorescence emission spectrum. To normalize the data, the \( F_c \) for all data points was divided by the \( F_c \) at 0 ligand (PALA or succinate) concentration, and then subtracted by 1. The experimental data were analyzed in the same manner as the data from the activity assays.

Nucleotide saturation curves ([C47A/A241C-pyrene] = 1 \( \mu \)M) were performed at a subsaturating PALA concentration ([PALA]:[C47A/A241C-pyrene] = 1), a subsaturating succinate concentration ([succinate] = 1 mM and [carbamoyl phosphate] = 4.8 mM), and also in the absence of ligands. ATP or CTP were added incrementally until the enzyme was completely saturated with nucleotide, as indicated by no further change in the fluorescence spectrum. To normalize the data, the \( F_c \) for all data points was divided by the \( F_c \) at 0 nucleotide concentration.

**RESULTS**

**A Cys Residue on the 240s Loop for Covalent Attachment of a Fluorescent Probe**—The distance between identical residues in the 240s loop of the C1 and C4 chains of aspartate transcarbamoylase changes significantly in the transition from the T to R states (13, 24). Therefore, the 240s loop was chosen as the location for placement of a fluorescent probe that is sensitive to conformation. We chose to conjugate pyrene site specifically to our previously constructed 240s loop mutant, C47A/A241C (20). The substitution of the one naturally occurring cysteine in the catalytic chain, Cys-47, by alanine ensured site-specific labeling.

After labeling the C47A/A241C holoenzyme with pyrene, the degree of labeling as determined by mass spectrometry appeared nearly complete. The molecular mass of the catalytic chain for the C47A/A241C holoenzyme, as determined by mass spectrometry, was 33,958 Da. The mass spectrum for the C47A/A241C-pyrene enzyme had a molecular mass of 34,292 Da. This is a difference in molecular mass of 342 Da, close to the molecular mass of pyrene maleimide (297.3). The degree of labeling with pyrene, as determined by the amount of unreacted thiol using Ellman's reagent, was 70 \pm 10\%.

The fluorescence emission spectrum of the C47A/A241C-pyrene-labeled holoenzyme is shown in Fig. 2A.

**Steady-state Kinetics of the Wild-type and Fluorophore-labeled Enzymes**—The kinetic parameters calculated from the aspartate saturation curves shown in Fig. 3A are displayed in Table I. The parameters for wild-type holoenzyme were a maximal activity of 17.5 mmol h\(^{-1}\) mg\(^{-1}\), an [Asp]\(_{0.5}\) of 13.0 mM, and Hill coefficient of 2.4. The C47A/A241C-pyrene-labeled holoenzyme exhibited a sigmoidal aspartate saturation curve similar to that of the wild-type curve, with a maximal activity of 15.8 mmol h\(^{-1}\) mg\(^{-1}\), an [Asp]\(_{0.5}\) of 12.5 mM, and somewhat reduced cooperativity with a Hill coefficient of 1.4.

**Influence of the Allosteric Effectors on the Wild-type and Labeled Enzymes**—Nucleotide saturation curves with either CTP or ATP were determined for the wild-type and C47A/A241C-pyrene-labeled holoenzymes at one-half the [Asp]\(_{0.5}\). ATP or CTP was added incrementally until the enzyme was completely saturated with nucleotide, as indicated by no further change in the fluorescence spectrum. To normalize the data, the \( F_c \) for all data points was divided by the \( F_c \) at 0 nucleotide concentration.

**Small Angle X-ray Scattering—**The SAXS experiments were performed at 0 nucleotide concentration. The experimental data were analyzed in the same manner as the data from the activity assays.

**Monitoring the Structural Change by Steady-state Fluorescence—**To determine whether the fluorescence of the C47A/A241C-pyrene-labeled holoenzyme was sensitive to alterations in quaternary structure, steady-state fluorescence emission spectra were recorded in the absence and presence of PALA. There was a significant change in the fluorescence emission peaks with increasing PALA concentrations (Fig. 2A). The intensity of the peak at 380 nm decreased, concomitant with the increasing intensity of a broad peak at \( \approx 480 \) nm. Shown in Fig. 2B is a plot of the \( F_c \) of the C47A/A241C-pyrene-labeled holoenzyme as a function of PALA concentration. The sigmoidal data fit to the Hill equation that gave a Hill coefficient
of 1.8. The $F_c$ increased until a ratio of approximately six PALA molecules per molecule was attained. At higher concentrations of PALA there was only a slight increase in the $F_c$. When PALA was added to the C47A/A241C-C-pyrene-labeled catalytic subunit no change in fluorescence was observed (data not shown).

The change in fluorescence emission of the C47A/A241C-pyrene-labeled holoenzyme was measured as a function of succinate concentration in the presence of saturating levels of carbamoyl phosphate (4.8 mM). The values reported are the average deviation of three determinations.

| Enzyme             | $V_{max}$ (µmol·min⁻¹·mg⁻¹) | [Asp]₀.₅ (mM) | $n_H$ |
|--------------------|------------------------------|--------------|------|
| Wild-type          | 17.5 ± 1.5                   | 13.0 ± 0.9   | 2.4 ± 0.2 |
| C47A/A241C        | 25.4 ± 2.3                   | 10.8 ± 0.8   | 1.3 ± 0.1 |
| C47A/A241C-pyrene | 15.8 ± 2.5                   | 12.5 ± 0.5   | 1.4 ± 0.2 |

The data for the C47A/A241C is reported under reducing conditions that prevent the cysteines from forming a disulfide bond between corresponding residues in the upper and lower catalytic subunits (20).

The fluorescence and activity data were scaled so that the maximum change at infinite nucleotide was the same. Data were fit to a simple Langmuir binding isotherm.

**Fig. 3.** A, aspartate saturation curves for the wild-type holoenzyme (■) and the C47A/A241C-pyrene enzyme (○). Colorimetric assays were performed at 25 °C in 50 mM Tris acetate buffer, pH 8.3, at saturating concentrations of carbamoyl phosphate (4.8 mM). B, succinate saturation curve for the C47A/A241C-pyrene enzyme (○), measured as the change in fluorescence coefficient. The fluorescence spectra were recorded at 20 °C in 50 mM Tris acetate buffer, pH 8.3, at a saturating concentration of carbamoyl phosphate (4.8 mM). The fluorescence coefficient is normalized to 0 for [succinate] = 0. The curves shown correspond to the fit of the data to the Hill equation with an additional term for substrate inhibition (34).

**Table I**

Kinetic parameters of the wild-type and modified forms of aspartate transcarbamoylase

These data were determined from the aspartate saturation curves (Fig. 4). Colorimetric assays were performed at 25 °C in 50 mM Tris acetate buffer, pH 8.3, and saturating levels of carbamoyl phosphate (4.8 mM). The values reported are the average deviation of three determinations.

| Enzyme             | $V_{max}$ (µmol·min⁻¹·mg⁻¹) | [Asp]₀.₅ (mM) | $n_H$ |
|--------------------|------------------------------|--------------|------|
| Wild-type          | 17.5 ± 1.5                   | 13.0 ± 0.9   | 2.4 ± 0.2 |
| C47A/A241C        | 25.4 ± 2.3                   | 10.8 ± 0.8   | 1.3 ± 0.1 |
| C47A/A241C-pyrene | 15.8 ± 2.5                   | 12.5 ± 0.5   | 1.4 ± 0.2 |

**Fig. 4.** Influence of ATP (circles) and CTP (squares) on the activity (filled symbols) and relative fluorescence coefficient (open symbols) of the C47A/A241C-pyrene enzyme. Colorimetric assays were performed at 25 °C in 50 mM Tris acetate buffer, pH 8.3, at saturating concentrations of carbamoyl phosphate (4.8 mM). The aspartate concentration was held constant at half the [Asp]₀.₅. The fluorescence spectra for the saturation of C47A/A241C-pyrene with nucleotides was performed at 20 °C in 50 mM Tris acetate buffer, pH 8.3, and the [PALA]/[C47A/A241C-pyrene] = 1. The fluorescence and activity curves shown correspond to the fit of the data to the Hill equation with an additional term for substrate inhibition (34).

**Table II**

ATP activation and CTP inhibition of the wild-type and modified forms of aspartate transcarbamoylase

These data were determined from ATP and CTP saturation curves (Fig. 5). Colorimetric assays were performed at 25 °C in 50 mM Tris acetate buffer, pH 8.3. ATP and CTP saturation curves were determined at saturating levels of carbamoyl phosphate (4.8 mM) and aspartate concentrations at one-half the [Asp]₀.₅ of the respective holoenzyme at pH 8.3. The values reported are the average deviation of three determinations.

| Enzyme             | ATP $\Delta V$ (%) | $K_{ATP}^b$ | CTP $\Delta V$ (%) | $K_{CTP}^b$ |
|--------------------|--------------------|------------|--------------------|------------|
| Wild-type          | 450                | 1.1        | 25                 | 0.12       |
| C47A/A241C        | 220                | 1.6        | 59                 | 0.033      |
| C47A/A241C-pyrene | 150                | 1.3        | 81                 | 0.029      |

*Percent activation is defined as 100 ($A_{ATP}/A$), where $A_{ATP}$ is the activity in the presence of ATP and $A$ is the activity in the absence of ATP. $K_b^*$ is the nucleotide concentration required to activate or inhibit the enzyme by 50% of the maximal effect.

*Percent residual activity is defined as 100 ($A_{CTP}/A$), where $A_{CTP}$ is the activity in the presence of CTP and $A$ is the activity in the absence of CTP.

*Values derived from fluorescence coefficient, $F_c$, measured by nucleotide saturation curves (Fig. 5) of C47A/A241C-pyrene-labeled holoenzyme liganded with an equimolar amount of PALA at 25 °C in 50 mM Tris acetate buffer, pH 8.3.
C47A/A241C-pyrene holoenzyme in the absence and presence of saturating concentrations of PALA (see Fig. 5). The data for the wild-type holoenzyme, shown in Fig. 5A, displays the characteristic change in the scattering pattern upon addition of PALA, as noted by the change in the peak position and increase in relative intensity (15) accompanying the T to R state transition. The scattering patterns of the C47A/A241C-pyrene holoenzyme are shown in Fig. 5B. The unliganded C47A/A241C-pyrene-labeled holoenzyme displayed the same characteristic change in the peak position and relative intensity of the scattering pattern as the wild-type holoenzyme from addition of PALA.

**Monitoring the Structural Change by Stopped-flow Fluorescence and SAXS**—The time evolution of the change in fluorescence induced by the binding of the natural substrates carbamoyl phosphate and aspartate to the C47A/A241C-pyrene-labeled holoenzyme was monitored by stopped-flow fluorescence. As seen in Fig. 6, when a saturating concentration of aspartate was mixed with the C47A/A241C-pyrene-labeled holoenzyme saturated with carbamoyl phosphate the fluorescence changes and then levels off. To correlate the change in fluorescence with the quaternary structural change, stopped-flow SAXS was employed (36). The relative change in the SAXS intensity, when a saturating concentration of aspartate was mixed with C47A/A241C-pyrene-labeled holoenzyme saturated with carbamoyl phosphate the fluorescence changes and that was very similar to that observed by fluorescence. The buffer and pH used for the fluorescence and stopped-flow SAXS were identical; however, the SAXS experiment required an enzyme concentration 500-fold (35 mg/ml) higher than used for the fluorescence experiments.

**DISCUSSION**

Aspartate transcarbamoylase has two allosteric states, T and R, each distinct in structure and function. The quaternary structural transition from the low affinity, low activity T state to the high affinity, high activity R state upon binding substrate analogues has been well demonstrated (9, 14, 24, 38, 39). However, details of the structural transition between the T and R states have been difficult to obtain. To be able to observe the actual T to R transition in aspartate transcarbamoylase, as well as to monitor structural changes induced by the heterotropic effectors, we have developed a fluorescent-labeled version of aspartate transcarbamoylase that not only has kinetic characteristics similar to the wild-type enzyme, but also fluoresces uniquely in the R quaternary structure.

The fluorescent label was attached to the enzyme site-specifically in the 240s loop of the catalytic chain of the enzyme because the 240s loops at the C1-C4 interface are much closer in the R state than in the T state (13, 24). For instance, the distance between side chains for a pair of identical residues in the region 236–241 at the C1-C4 interface is greater than 20 Å in the T state, whereas this distance becomes less than 10 Å in the R state. Pyrene was selected as the fluorophore because it absorbs in the near UV region, and is sensitive to the proximity of another pyrene, as a large change in the fluorescence emission spectrum occurs upon excimer formation (21). The fluorescence emission spectrum of pyrene is characterized by a monomeric emission peak at 380 nm, which decreases with a concomitant increase in a broad peak at 480 nm upon excimer formation. This excimer emission occurs only when two pyrenes are within -10 Å of one another (21). Previous fluorescence studies of aspartate transcarbamoylase were performed in which the Tyr-240 was substituted by Trp in the 240s loop; and this mutant enzyme was sensitive to the conformational change of the 240s loop (40, 41), but the fluorescence changes were not directly correlated with the quaternary structural transition as determined by SAXS.

We chose to substitute a Cys residue into the 240s loop for coherent attachment of pyrene. However, because many of the residues in the 240s loop are critical for the proper allosteric regulation of this enzyme, such as Asp-236 (42) and Glu-239 (43), and because we sought a system that closely resembled the kinetics of the wild-type enzyme, our choices for residues to mutate to Cys were limited. Therefore, we chose as the best candidate a double mutant enzyme previously created in our laboratory, C47A/A241C, which was known to have kinetic parameters very similar to that of the wild-type enzyme (20).
Site-specific labeling with pyrene at Cys-241 was assured as the single cysteine in the wild-type catalytic chain, Cys-47, had been replaced with Ala. The mass spectrometry and the determination of unreacted thiol with Ellman’s reagent suggest that most of the C47A/A241C-pyrene enzyme was labeled.

The C47A/A241C-pyrene enzyme was tested in several experiments to determine whether it behaved as does the wild-type enzyme. The aspartate saturation curve of C47A/A241C-pyrene was sigmoidal, and the maximal velocity and \( K_{\text{mAsp}} \) were close to the wild-type values (Table I). The nucleotide saturation curves of C47A/A241C-pyrene demonstrated activation by ATP and inhibition by CTP in a manner similar to that of the wild-type enzyme, albeit with lower activation by ATP and lower inhibition by CTP (Table II). The unliganded C47A/A241C-pyrene enzyme (Fig. 5B) exhibited a SAXS pattern similar to that of the wild-type enzyme in the absence of ligands (Fig. 5A). In the presence of PALA, the C47A/A241C-pyrene enzyme demonstrated the same shift in peak position and relative intensity as did the wild-type enzyme in the presence of PALA indicating that the enzyme undergoes the allosteric transition.

Because the pyrenes on the 240s loops should be much closer in the R state than the T state, as shown in our model of the C1 and C4 catalytic chains conjugated with pyrene at Cys-241 in Fig. 1, excimer formation should only occur in the R state. Excimer formation caused by the T to R state transition upon saturation with PALA or succinate (in the presence of ATP) was not observed. The excimer formation in the presence of PALA or succinate is probably caused by the quaternary structural change and closure of the domains and the movement of the 240s loops after the binding of the bisubstrate analogue PALA, and not simply the binding of PALA to the active site, because saturating concentrations of substrates were used in these experiments all of the enzyme active sites should be filled, as opposed to the data in Fig. 2 in which the observed fluorescence was monitored as a function of substrate saturating. These data support the conclusion that excimer formation directly reflects the alterations in quaternary structure of the enzyme.

Because of the ordered binding of the substrates in aspartate transcarbamoylase, the allosteric transition is monitored as a function of aspartate concentration at a saturating concentration of carbamoyl phosphate. The binding of aspartate requires the closure of the two domains of the catalytic chain and the repositioning of the 80s and 240s loops resulting in the high activity, high affinity active site. However, this domain closure cannot take place in a single active site because the movement of the domains in an upper catalytic chain is blocked by the corresponding domains in a lower catalytic chain (44). Thus, the binding of aspartate in the presence of carbamoyl phosphate induces the allosteric transition (44). The results reported here as well as Trp fluorescence experiments (40) indicate that once the quaternary structural change has occurred, the 240s loop positions in the catalytic chains that have not bound aspartate must be a different conformation from the ones that have bound aspartate. Thus, the allosteric transition in aspartate transcarbamoylase is composed of the quaternary structural change accompanied by local conformational changes near the active sites. The local conformational changes are not induced by the quaternary structural change but rather occur on a site by site basis induced by the binding of substrates to each of the high affinity active sites created by the quaternary structural change. The change in the fluorescence observed upon addition of PALA to the C47A/A241C-pyrene-labeled holoenzyme suggests that the global structural change puts the enzyme into the R quaternary structure and primes the active sites for substrate binding. However, it is the binding of PALA, or aspartate in the presence of carbamoyl phosphate, that causes the final repositioning of the 240s loop into its catalytically active conformation. These local conformational changes may actually occur in a transient fashion to allow product release after the reaction has taken place.

To examine whether the effect exerted by the nucleotides would cause a change in the fluorescence emission, fluorescence spectra were recorded for the C47A/A241C-pyrene holoenzyme at a saturating concentration of ATP or CTP. ATP induced only a very small increase in the \( F_{5}, \) whereas CTP induced no measurable decrease in the \( F_{5}. \) This agrees with structural studies that show that ATP and CTP do not appreciably change the quaternary structure of the enzyme (46). The influence of ATP and CTP was also examined after addition of a subsaturating amount of PALA or succinate, as was done in SAXS experiments that measured the effect of the nucleotides on the wild-type enzyme (47, 48). In the first study, the authors observed an effect on the \([T]/[R]\) equilibrium for both ATP and CTP when succinate and carbamoyl phosphate were used. However, they detected no effect by ATP on the \([T]/[R]\) equilibrium when PALA was used. This equilibrium shift in the case of ATP was interpreted as a secondary effect caused by a change in affinity for the substrate analogue, succinate. As demonstrated by our fluorescence data, shown in Fig. 4, the \( F_{5} \) increased in a hyperbolic manner upon saturation with ATP in the presence of a subsaturating concentration PALA. In addition, when the fluorescence curve from the ATP saturation was normalized to the ATP saturation curve (kinetic assay), the curves could be overlaid almost exactly. There was a greater effect upon the fluorescence emission by ATP with succinate...
than with PALA, with an increase in the $F_r$ of 85%. In addition, when the fluorescence curve from the CTP saturation was normalized to the CTP saturation curve (kinetic assay), the curves overlaid exactly (Fig. 4). The effect of CTP upon the fluorescence emission was the same with succinate and PALA.

Because of the strong evidence, from the SAXS experiments (47, 48) that the nucleotide ATP does not perturb the $[T]/[R]$ equilibrium, our results suggest that the effect of ATP on the activity of aspartate transcarbamoylase is to cause a local change in the conformation of the 240s loop in the R state. We suggest that the conformational change is the closure of the 240s loops in the R state to decrease the number of catalytic chains with the fully formed active site. Thus, the fluorescence changes observed by the addition of ATP and CTP are directly related to the position of the 240s loop as it either helps to create the active site (ATP) or prevents the active site from forming (CTP).

The availability of the pyrene-labeled enzyme developed here provides a new tool for the study of aspartate transcarbamoylase and the structural changes that the molecule undergoes. For example, the quaternary conformation of the enzyme that is trapped in silica matrix sol-gels can be directly determined (49) and fluorescence stopped-flow will not only allow the direct monitoring of the allosteric conformational change but also allow a means to directly determine how the regulatory nucleotides alter the rate of the allosteric transition.

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