Fluorescence Energy Transfer between Ligand Binding Sites on Aspartate Transcarbamylase†

Steven Matsumoto and Gordon G. Hammes*

ABSTRACT: The method of fluorescence energy transfer is used to measure the distances between several sites on aspartate transcarbamylase. Both fluorescence steady-state and lifetime techniques are used. When the tryptophans on the catalytic subunit are the fluorescent donor groups, either pyridoxamine phosphate, covalently bound to an amino group at the active site, or 8-anilino-1-naphthalenesulfonate, noncovalently bound at the active site, is the acceptor group. The distance between tryptophan and the active site is calculated to be 23 Å assuming that the fluorescence of only one tryptophan per catalytic polypeptide chain is quenched by the acceptor or 27 Å assuming that both tryptophans on a catalytic chain are equally quenched. The pyridoxamine phosphate label is also used as the fluorescent donor with mercurinitrophenol bound to the sulfhydryl group of the catalytic subunit as the energy acceptor. For this pair of labels the active site is determined to be very close to the sulfhydryl group on the same catalytic chain and 26 Å from the sulfhydryl groups on the other chains of the catalytic trimer. In experiments with pyridoxamine phosphate at the active site as the donor and 8-anilino-1-naphthalenesulfonate at the active site as the acceptor, a distance of 26 Å between active sites of a catalytic trimer is found. No energy transfer is observed from pyridoxamine phosphate at the active site to a fluorescamine derivative of cytidine 5′-triphosphate at the regulatory site. This implies that these groups are separated by at least 42 Å in the native enzyme. All of the distances are calculated using the assumption of rapid rotation of donor and acceptor dipole moments relative to the donor fluorescence lifetime. Fluorescence polarization measurements suggest this assumption does not produce a significant error in the calculated distances. The distances between the various sites are related to the subunit structure of aspartate transcarbamylase.

Aspartate transcarbamylase from *Escherichia coli* is feedback regulated by nucleotides (Yates and Pardee, 1956; Gerhart and Pardee, 1962). The substrates of this enzyme bind to six catalytic sites (Hammes *et al.*, 1970; Rosenbusch and Weber, 1971; Rosenbusch and Griffin, 1973) and the nucleotide effectors bind to six regulatory sites (Hammes *et al.*, 1970; Winlund and Chamberlin, 1970; Buckman, 1970; Cook, 1972; Matsumoto and Hammes, 1973; Gray *et al.*, 1973) per enzyme molecule. The catalytic sites are on two trimeric subunits and the regulatory sites are on three dimeric subunits (Gerhart and Schachman, 1965; Changeux *et al.*, 1968; Weber, 1968b,c; Davies and Stark, 1970; Meighen *et al.*, 1970; Rosenbusch and Weber, 1971; Cohlberg *et al.*, 1972). The structural arrangement of the subunits in the native enzyme has been established by X-ray diffraction studies (Wiley *et al.*, 1971; Evans *et al.*, 1972, 1973; Warren *et al.*, 1973) and electron microscopy (Richards and Williams, 1972). There is only indirect evidence regarding the location of the catalytic and regulatory sites with respect to the overall subunit structure. The positions of the sulfhydryl groups of the catalytic subunits and the positions of the six zinc atoms in the regulatory subunits are known from the X-ray diffraction studies. The proximity of the catalytic sites to the sulfhydryl groups has been inferred from the inactivity of enzyme derivatives with bulky or negatively charged substituents at the sulfhydryl group (Vanaman and Stark, 1970; Benisek, 1971; Jacobson and Stark, 1973). The regulatory sites do not directly involve the zinc atoms since nucleotides bind to metal-free regulatory subunits (Rosenbusch and Weber, 1971) and nucleotide binding has no effect on the circular dichroism spectrum of the cadmium-substituted enzyme (Griffin *et al.*, 1973). Intramolecular distances on macromolecules can be directly measured from the energy transfer between a fluorescent donor label and an absorbing acceptor label (*cf.* Weber, 1968a; Stryer, 1968; Yguerabide, 1972). Two site-specific chromophores have been attached to aspartate transcarbamylase: pyridoxamine phosphate at the catalytic site (Greenwell *et al.*, 1973) and mercurinitrophenol at the sulfhydryl groups on the catalytic subunit (Evans *et al.*, 1972). In this study fluorescence energy transfer between these and other labels is used to measure the distances from a catalytic site to another catalytic site, to the tryptophan polypeptide residues, to adjacent and nonadjacent sulfhydryl groups and to the nucleotide binding site.

Materials and Methods

Chemicals. The pyridoxal 5′-phosphate and other biochemicals were obtained from the Sigma Chemical Company. The 8-anilino-1-naphthalenesulfonate (ANS[1]) was converted to the magnesium salt and recrystallized four times from water. The 2-chloromercuri-4-nitrophenol was obtained from Eastman Organic Chemicals. The fluorescamine, 4-phenylspiro[furan-2(3*H*),1′-phthalan]-3,3′-dione, was a product of Roche Diagnostics. All other chemicals were of reagent grade. Solvents for nucleotide synthesis were distilled and stored over molecular sieves.

Aspartate Transcarbamylase and Derivatives. Aspartate transcarbamylase was prepared from a mutant strain of *E. coli* grown at the New England Enzyme Center by the method of Gerhart and Holoubek (1967). The enzyme was

† From the Department of Chemistry, Cornell University, Ithaca, New York 14850. *Received September 6, 1974.* This work was supported by a grant from the National Institutes of Health (GM 13292).

[1] Abbreviations used are: ANS, 8-anilino-1-naphthalenesulfonate; Cp_4F, structure in Figure 1; EDTA, ethylenediaminetetraacetic acid.

dissociated into its subunits using neohydrin and DEAE-cellulose column chromatography (Schachman, 1972). Zinc acetate was added to stabilize the isolated regulatory subunit (Nelbach *et al.*, 1972). Protein concentrations were determined from the absorbance at 280 nm using extinction coefficients of 0.59 ml/(mg cm) for the native enzyme, 0.72 ml/(mg cm) for the unmodified catalytic subunit, and 0.32 ml/(mg cm) for the zinc regulatory subunit (Gerhart and Holoubek, 1967; Nelbach *et al.*, 1972). Pyridoxamine phosphate conjugates of the catalytic subunit were prepared by sodium borohydride reduction of the Schiff base formed between pyridoxal phosphate and an amino group on the protein (Greenwell *et al.*, 1973). The extent of labeling was determined from the absorbance at 325 nm using a molar extinction coefficient of 5350 M^{-1} cm^{-1} after correction for protein absorbance at that wavelength (Greenwell *et al.*, 1973). Several derivatives were made containing a range of 1.6–3.2 mol of pyridoxamine phosphate/mol of catalytic trimer. Renatured enzyme labeled with pyridoxamine phosphate was made by mixing catalytic subunit derivatives labeled with approximately 3 mol of pyridoxamine phosphate/mol of trimer with excess (20% with respect to polypeptide chains) zinc-regulatory subunit. The mixture of subunits at protein concentrations of about 1 mg/ml in 0.05 M Tris-acetate buffer (pH 8) with 2 mM mercaptoethanol and 0.1 mM zinc acetate was incubated for 30 min at 30° (Greenwell *et al.*, 1973). After precipitation with ammonium sulfate and dialysis against 0.05 M Tris-acetate buffer (pH 8) containing 2 mM mercaptoethanol and 0.2 mM EDTA, the enzyme solution was chromatographed on a 3 cm i.d. × 40 cm column of Sephadex G-200 in the same buffer at 4°. The 280-nm absorbance of the column effluent was monitored and the identity of the peaks determined by polyacrylamide gel electrophoresis in 25 mM Tris–0.2 M glycine (pH 9). The major peak corresponded to reconstituted aspartate transcarbamylase. It was preceded by unidentified high-molecular weight material and followed by the excess regulatory subunit. Little or no catalytic subunit could be detected in the column effluent. As judged by absorbance, 5.7 mol of pyridoxamine phosphate was present/mol of reconstituted enzyme. The specific activity was measured by the colorimetric method of Prescott and Jones (1969) at saturating levels of aspartate and carbamyl phosphate. The modified enzyme had approximately 3% of the activity of unmodified enzyme. Doubly labeled catalytic subunits were prepared by reacting pyridoxamine phosphate derivatives with the sulfhydryl reagent, 2-chloromercuri-4-nitrophenol (Evans *et al.*, 1972). The reaction was carried out in 0.05 M triethanolamine-acetate (pH 7). After extensive dialysis against the same buffer, the degree of labeling was determined from the absorbance of the covalently attached label using an extinction coefficient of 2.09 × 10^4 M^{-1} cm^{-1} (Evans *et al.*, 1972). A maximum of 2 mol of the mercurial was bound/mol of catalytic trimer which already contained 3 mol of pyridoxamine phosphate. All protein solutions were passed through a Millipore HA 0.45-μ filter prior to optical measurements. Pyridoxamine derivatives were stored in the dark in aluminum foil covered containers.

Fluorescent Nucleotide Derivative. The synthesis of the nucleotide Cp_4F (Figure 1), in which cytidine is linked through a polyphosphate chain to a fluorescent pyrrolinone, was based on the techniques of Moffatt and Khorana (1961) using the commercial fluorescamine reagent (Udenfriend *et al.*, 1972; Weigele *et al.*, 1972a,b). CTP-morpholidate was synthesized in a manner analogous to that described for ATP-morpholidate (Wehrli *et al.*, 1965). Phosphoethanolamine (1 mmol) and tributylamine (3 mmol) were dissolved in 200 ml of water and mixed with a solution of fluorescamine (1 mmol) in 200 ml of Spectroscopic grade acetone. After standing 1 hr at room temperature the mixture was evaporated to a syrup *in vacuo*. To this was added a methanol solution of 1 mmol of CTP-morpholidate as the dicyclohexylcarboxamidine salt. The mixture was evaporated twice from anhydrous methanol, four times from anhydrous pyridine, and twice from anhydrous benzene. The resulting solid residue was dissolved in 2 ml of anhydrous dimethyl sulfoxide in a glass-stoppered flask and allowed to stand 4 days at room temperature in a desiccator. The solution was dissolved in water and applied to a 1.5 cm i.d. × 40 cm column of DEAE-cellulose (bicarbonate form) at 4°. After washing with water, the column was eluted with a linear gradient made with 1 l. of water and 1 l. of 0.35 M triethylammonium bicarbonate. Overlapping peaks of the monophosphate derivative of fluorescamine, CTP, and the conjugate (Cp_4F) were identified by ultraviolet absorbance and thin-layer chromatography on cellulose sheets (Eastman Organic Chemicals) using the solvent system 0.15 M citrate (pH 4)–ethanol–1-butanol in proportions of 6:10:1. In this thin-layer system the monophosphate derivative of fluorescamine gave a yellow-green fluorescent spot near the solvent front, CTP gave an ultraviolet absorbing spot with R_F 0.1, and the compound Cp_4F gave a yellow-green fluorescent spot with R_F 0.8. The fraction containing Cp_4F was evaporated to dryness and dissolved in water. The compound Cp_4F has an absorption band at 385 nm with an extinction coefficient of 3 × 10^3 M^{-1} cm^{-1} (±15%) at this wavelength based on phosphate analysis (Chen *et al.*, 1956) and dry weight determinations. It has a fluorescence emission maxima at 480 nm with excitation maxima at 290 and 382 nm.

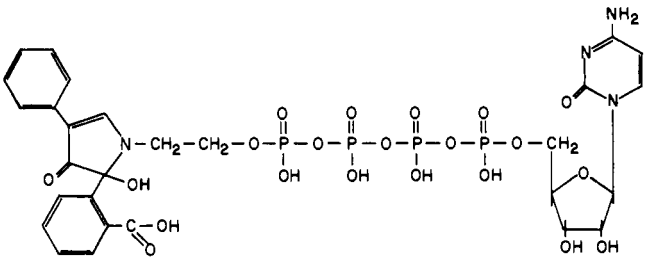

FIGURE 1: Structural formula of Cp_4F.

Equilibrium Binding Studies. The binding of Cp_4F to the native enzyme was measured by equilibrium dialysis at 4°. The specially constructed plexiglass dialysis cells have a volume of 100 μl on each side of a cellulose dialysis membrane. The dialysis membrane was freed of an ultraviolet absorbing contaminant by boiling in 0.5 M $NaHCO_3$ and 0.5 mM EDTA, followed by rinsing with distilled, deionized water. A microliter syringe was used to add 80 μl of enzyme or buffer solution on one side of the membrane and the same volume of ligand solution or buffer to the other side. The buffer was 0.05 M triethanolamine-acetate (pH 7), 2 mM mercaptoethanol, 0.2 mM EDTA, and 10 mM carbamyl phosphate. The dialysis apparatus was rotated at 4° for 20 hr by which time complete equilibration was attained. Control experiments indicated that 20–30% of ligand was bound to the membrane. The concentration of ligand in solution on both sides of the membrane was determined from the absorbance at 385 nm of diluted aliquots. The differ-

ence in ligand concentrations was used to calculate the amount of binding. The enzyme concentration was approximately 20 mg/ml and a correction was made for volume excluded by the protein.

Spectroscopic Measurements. Absorbance measurements were made with Zeiss PMQ II or Cary 14 spectrophotometers.

Steady-state fluorescence measurements were made with a Hitachi-Perkin-Elmer MPF-3 fluorescence spectrometer equipped with corrected spectrum and polarization accessories. The square (1 cm × 1 cm) or triangular (1 cm × 1 cm × 1.4 cm) cuvets were thermostated at the desired temperature. Pyridoxamine derivatives were found to be light sensitive; therefore a very narrow excitation slit width (2 nm) was used to prevent the decrease of fluorescence intensity with time.

Relative quantum yields were determined by the comparative method (Parker and Rees, 1966) using

$$Q_1/Q_2 = (F_1/F_2)(A_2/A_1) \quad (1)$$

In eq 1, Q is the quantum yield, F is the area of the corrected emission spectrum, and A is the absorbance at the exciting wavelength. The subscripts 1 and 2 refer to the sample and reference, respectively. Areas were measured by cutting out and weighing the corrected emission spectra of solutions having absorbances of less than 0.01 at the exciting wavelength. Samples were compared with quinine sulfate in 0.1 N H_2SO_4 at 23° which was assumed to have an absolute quantum yield of 0.70 (Scott *et al.*, 1970). The areas of the tryptophan emission spectra were compared with that for quinine sulfate excited at 295 nm, while pyridoxamine fluorescence was compared with quinine sulfate fluorescence excited at 325 nm. The quantum yield of quinine sulfate is constant between 250 and 366 nm (Chen, 1967).

In the polarization measurements a correction was made for unequal transmission of the horizontal and vertical components of polarized light by the emission monochromator grating (Azumi and McGlynn, 1962). The polarization at infinite viscosity was determined by extrapolation (Perrin, 1926) from measurements in 80 and 85% glycerol solutions at temperatures between 20 and 30° using tabulated viscosity data (Sheely, 1932).

Energy transfer was determined from the quenching of the fluorescence of a donor molecule by an acceptor molecule using eq 7. Inner filter effects were present when the acceptor absorbed exciting light, and in these cases a displacement method was used. The fluorescence of the donor was measured in the presence of acceptor bound to the protein, then an excess of a displacing agent was added to the cuvet and the fluorescence of the solution was remeasured. The displacing agent was either a competitive ligand (carbamyl phosphate displaces ANS, CTP displaces Cp_4F), or a reagent which cleaved the acceptor–protein covalent bond (mercaptoethanol displaces mercurinitrophenol). Since the same concentration of acceptor was present in both measurements, corrections for inner filter effects were unnecessary. Changes in the solution volume and in the acceptor extinction coefficient at the exciting wavelength were negligible. Control experiments showed that the displacing agents had no effect on donor fluorescence. The method also provides evidence that the change in donor fluorescence is due to displacement of acceptor molecules from specific sites on the protein.

Fluorescence lifetime measurements were made on an ORTEC Model 9200 nanosecond fluorescence spectrophotometer interfaced with a PDP 11/20 computer (Digital Equipment Corp.). We would like to thank Professor Juan Yguerabide for the special design and construction of the sample compartment. Exciting light was passed through a Bausch and Lomb 250-mm grating monochromator. Light emitted from the sample was passed through 3-cavity interference filters (Ditric Optics Inc.). Square (1 cm × 1 cm), triangular (1 cm × 1 cm × 1.4 cm), and square micro (0.3 cm × 0.3 cm) cuvets were thermostated at the desired temperature. A dilute solution of colloidial silica (Ludox, DuPont) in buffer was used to determine the scattering spectrum. Data were analyzed with the deconvolution and convolution programs provided by ORTEC, Inc., and with a least-squares fitting procedure devised by Mr. Gary Shepherd. Because of the short lifetimes of tryptophan and pyridoxamine in the enzyme derivatives, fluorescence decay curves were analyzed in terms of a single apparent lifetime. The efficiency of transfer was calculated from eq 6.

Energy Transfer Calculations. The measurement of molecular distances by fluorescence energy transfer is based on the theory of Förster (1959, 1965). This theory describes nonradiative, intermolecular transfer of energy in terms of an interaction between the emission dipole moment of a fluorescent donor in an excited singlet state and the absorption dipole moment of an acceptor molecule in its electronic ground state. Because of the dipole–dipole nature of the interaction, the rate constant for transfer, $k_{D\to A}$, is inversely proportional to the sixth power of the distance, R, between the dipoles (eq 2). In eq 2, τ_D is the experimental lifetime of

$$k_{D\to A} = (1/\tau_D)(R_0/R)^6 \quad (2)$$

the donor in the absence of transfer and R_0 is the "critical transfer distance." At the critical transfer distance the rate of transfer is equal to the net rate of all other processes which depopulate the donor excited state, $k_{D\to A} = 1/\tau_D$, and transfer is 50% efficient. In the Förster theory, R_0 is given by eq 3 where K^2 is the dipole orientation factor, Q_D

$$R_0 = (9.79 \times 10^3)(JK^2Q_Dn^{-4})^{1/6}\ \text{Å} \quad (3)$$

is the quantum yield of the donor in the absence of transfer, n is the refractive index of the medium, and J is the spectral overlap integral of donor fluorescence and acceptor absorbance. The dipole orientation factor cannot be directly measured. In the calculations that follow a value of $K^2 = 2/3$ has been used. This value is correct if both donor and acceptor dipoles rotate rapidly compared to the donor fluorescence lifetime when bound to the enzyme. The validity and consequences of this assumption are discussed in greater detail below. The donor quantum yields were measured as described. The refractive index of water, $n = 1.4$, was used. The overlap integral was approximated by

$$J = \sum_\lambda F_D(\lambda)\epsilon_A(\lambda)\lambda^4\Delta\lambda \Big/ \sum_\lambda F_D(\lambda)\Delta\lambda \quad (4)$$

where the terms are summed over 10-nm intervals, $F_D(\lambda)$ is the corrected fluorescence of the donor, and $\epsilon_A(\lambda)$ is the extinction coefficient of the acceptor. The quantum yield and lifetime can be written according to eq 5 in terms of the rate

$$Q = k_f \Big/ \Big(\sum_i k_i\Big);\ 1/\tau = \sum_i k_i \quad (5)$$

constants of all deexcitation processes, k_i, which includes k_f, the rate constant of fluorescence, and $k_{D\to A}$, the rate constant for transfer. The efficiency of energy transfer, E, can also be written in terms of the rate constants and can be

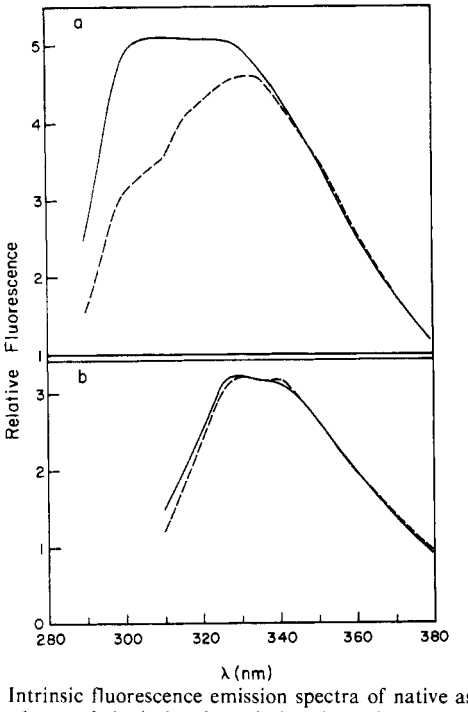

FIGURE 2: Intrinsic fluorescence emission spectra of native aspartate transcarbamylase and the isolated catalytic subunit in 0.05 M triethanolamine-acetate (pH 7), 2 mM mercaptoethanol, and 0.2 mM EDTA at 23°. (a) Excitation wavelength 280 nm; (—) native enzyme; (- - -) catalytic subunit; (b) excitation wavelength 295 nm; (—) native enzyme; (- - -) catalytic subunit.

related to the measured quantum yields and lifetimes by eq 6. In eq 6 $Q_{D\to A}$ and Q_D are the donor quantum yields in

$$E = k_{D\to A}\Big/\left(\sum_i k_i\right) = 1 - Q_{D\to A}/Q_D = 1 - \tau_{D\to A}/\tau_D \quad (6)$$

the presence and absence of energy transfer, and $\tau_{D\to A}$ and τ_D are the donor lifetimes in the presence and absence of energy transfer. The fluorescence intensities measured at given concentrations and instrument settings are proportional to the quantum yield so the efficiency can also be written as in eq 7. In eq 7 $F_{D\to A}$ and F_D are the donor fluo-

$$E = 1 - F_{D\to A}/F_D \quad (7)$$

rescence intensities at a given wavelength in the presence and absence of energy transfer. It follows from eq 2, 5, and 6 that for a single donor–acceptor pair, the efficiency of energy transfer is related to the distance between the donor and acceptor by

$$E = (R_0/R)^6/[1 + (R_0/R)^6] \quad (8)$$

Results

Intrinsic Protein Fluorescence. Native aspartate transcarbamylase and the underivatized catalytic trimer exhibit fluorescence due to aromatic amino acid residues. Each catalytic polypeptide chain of molecular weight 33,000 contains eight tyrosines and two tryptophans, while each regulatory chain of molecular weight 17,000 contains three tyrosines and no tryptophans (Weber, 1968c). Both tryptophan and tyrosine fluoresce when excited at a wavelength of 275 or 280 nm, but tryptophan fluoresces almost exclusively when excited at a wavelength of 295 nm (Tu and McCormick, 1974). Comparison of the fluorescence emission spectra of the native enzyme and the catalytic trimer shows that the native enzyme has a broader spectrum when excited at

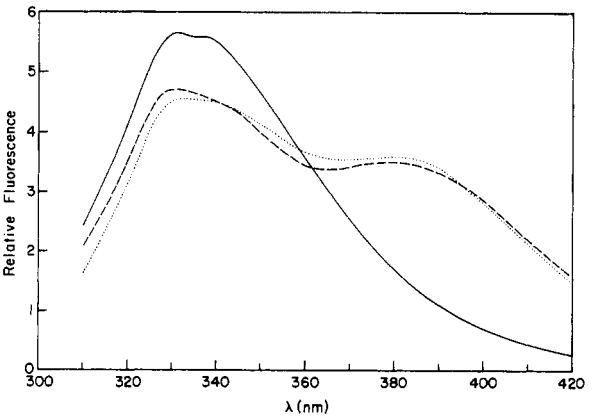

FIGURE 3: Fluorescence emission spectra of native aspartate transcarbamylase (—), aspartate transcarbamylase containing 5.7 mol of pyridoxamine phosphate/mol of enzyme (- - - -), and catalytic subunit containing 3.2 mol of pyridoxamine phosphate/mol of trimer (. . .); excitation wavelength 295 nm in 0.05 M triethanolamine-acetate (pH 7), 2 mM mercaptoethanol, and 0.2 mM EDTA at 23°.

275 nm reflecting its larger proportion of tyrosines (Figure 2a). However, the fluorescence emission spectra are identical with a maximum at 330–340 nm when excited at 295 nm (Figure 2b). Quantum yields of the tryptophan fluorescence (excited at 295 nm) at 23° in 0.05 M triethanolamine-acetate buffer (pH 7) were determined to be 0.07 for native enzyme and 0.10 for the catalytic trimer. Under the same conditions the lifetime of tryptophan in both the native enzyme and catalytic trimer was approximately 2 nsec. Saturating concentrations (10 mM) of the substrate, carbamyl phosphate, produced a slight quenching of tryptophan fluorescence which was too small to quantitate. Further addition of succinate, an aspartate analog, at a concentration of 10 mM had no effect on tryptophan fluorescence. Polarization of tryptophan fluorescence was 0.30 in the native enzyme at 23° and pH 7.

Quenching of Tryptophan Fluorescence by Pyridoxamine Phosphate. When pyridoxamine phosphate derivatives of the enzyme and the catalytic trimer are excited at 295 nm, the fluorescence emission spectra exhibit two maxima (Figure 3). The peak at 330–340 nm is due to tryptophan fluorescence and the shoulder at 380–390 nm is due to pyridoxamine fluorescence. The spectrum of pyridoxamine bound to the catalytic subunit has a maximum absorbance at 325 nm which overlaps with the tryptophan fluorescence spectrum (Figure 4). Calculation of the critical transfer distance, R_0, from eq 3 using $Q = 0.10$ for tryptophan and an extinction coefficient for bound pyridoxamine of 5350 M^{-1} cm^{-1} at 325 nm yields $R_0 = 19.7$ Å. In this set of experiments the labeling ratio was always close to three pyridoxamines per catalytic trimer and the pyridoxamine in the protein derivatives had negligible absorption at 295 nm or fluorescence at 330 nm compared to the protein itself. The efficiency of quenching was determined from the tryptophan fluorescent intensities (excitation 295 nm, emission 330 nm) of derivatized and underivatized protein after normalization with respect to concentration. The average efficiency of transfer was 0.14 (±5%) for both native enzyme and the catalytic trimer. The percentage in parentheses which follows this and other efficiency values gives the mean deviation for several different measurements. The decrease in fluorescence lifetime (excitation 295 nm, emission 340 nm) was measured for the catalytic trimer derivative only and also gave an efficiency of 0.14 (±10%). If the as-

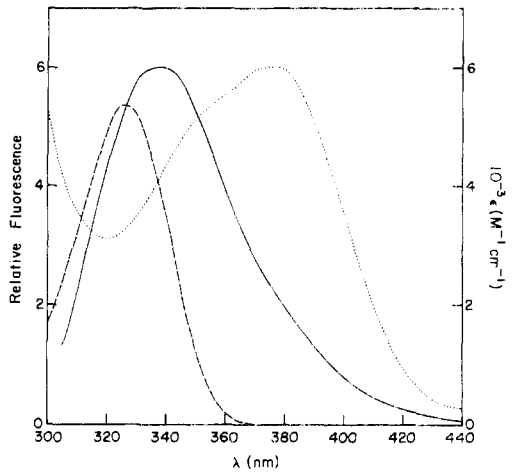

FIGURE 4: Overlap of the tryptophan corrected emission spectrum of the catalytic subunit excited at a wavelength of 295 nm (—) with the extinction coefficient (ϵ) of enzyme-bound pyridoxamine phosphate (- - -) and with the extinction coefficient (ϵ) of enzyme-bound ANS (· · ·) in 0.05 M triethanolamine-acetate (pH 7), 2 mM mercaptoethanol, and 0.2 mM EDTA at 23°.

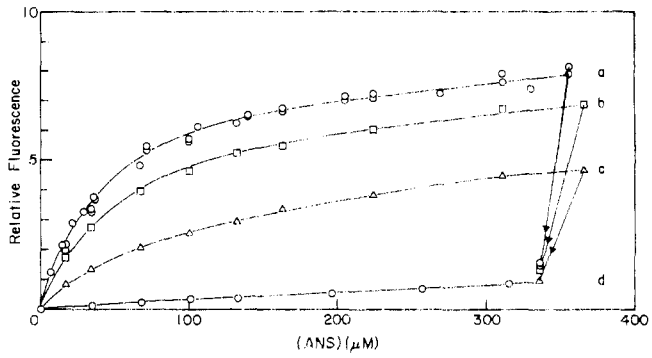

FIGURE 5: Fluorescence titration curve of catalytic subunit with ANS at an emission wavelength of 470 nm and an excitation wavelength of 375 nm in 0.05 M triethanolamine-acetate (pH 7) at 23°. (a) Catalytic subunit; (b) catalytic subunit containing 1.6 mol of pyridoxamine phosphate/mol of trimer; (c) catalytic subunit containing 2.8 mol of pyridoxamine phosphate/mol of trimer; (d) catalytic subunit in the presence of 10 mM carbamyl phosphate. The titration curves are normalized by dividing the relative fluorescence by the protein concentration. The concentration of catalytic subunit ranged from 0.6 to 1.2 mg/ml. The arrows indicate the effect of carbamyl phosphate addition to a final concentration of 10 mM.

sumption is made that the two tryptophans per catalytic chain are equally quenched, their average distance from the one pyridoxamine phosphate on the catalytic chain is calculated to be 26.6 Å (eq 6–8). Alternatively, if one tryptophan is much closer to the pyridoxamine so as to account entirely for the decrease in fluorescence intensity, the efficiency of its quenching is twice the apparent efficiency and the distance between this closest donor–acceptor pair would be 23.0 Å.

Quenching of Tryptophan Fluorescence by ANS. Titration of the underivatized catalytic trimer with ANS can be followed by exciting the solution at 375 nm and observing the increase in fluorescence at 470 nm due to ANS binding (Figure 5). The quantum yield of ANS is over 100-fold larger in nonpolar media than in water (Stryer, 1965; Turner and Brand, 1968). When the titration is carried out in the presence of the substrate, carbamyl phosphate, or when this substrate is added at any point, the fluorescence of ANS is markedly reduced suggesting the ANS is binding to the catalytic site and is displaced by carbamyl phosphate (Figure 5). The absorbance spectrum of ANS bound to the catalytic trimer (Figure 4) is red shifted approximately 25 nm relative to the spectrum of free ANS. An extinction coefficient of 5970 M^{-1} cm^{-1} at 375 nm was calculated for bound ANS based on a value of 4950 M^{-1} cm^{-1} at 350 nm for ANS in water solutions (Weber and Young, 1964). The polarization of enzyme-bound ANS is 0.33 compared to an extrapolated polarization of 0.396 for ANS at infinite viscosity (Stryer, 1965). The ANS absorbance overlaps the tryptophan fluorescence (Figure 4) and using a quantum yield of 0.10 for tryptophan fluorescence in the catalytic subunit the critical transfer distance is calculated to be R_0 = 21.6 Å from eq 3. The intensity of tryptophan fluorescence (excitation 295 nm, emission 340 nm) was measured in solutions of catalytic trimer plus saturating (350 μM) ANS at 23° in 0.05 M triethanolamine-acetate (pH 7). After addition of 10 mM carbamyl phosphate the fluorescence intensity was found to increase, due to the displacement of ANS from the catalytic site. From these measurements, the efficiency of transfer was determined to be 0.22 (±5%). The change in tryptophan lifetime (excitation 295 nm, emission 340 nm) was measured for solutions of catalytic trimer and ANS before and after adding carbamyl phosphate. The efficiency of transfer was 0.23 (±13%). For the assumption that both tryptophans are equally quenched the average distance is 26.6 Å from the intensity measurements and 26.4 from the lifetime measurements. If only one close tryptophan is quenched, the distance calculated is 22.5 Å from the intensity measurements and 22.2 Å from the lifetime measurements.

Fluorescence of Pyridoxamine Derivatives. Pyridoxamine phosphate covalently bound to native enzyme or the catalytic trimer has a fluorescence excitation maximum at 325 nm and a fluorescence emission maximum at 390 nm, identical with those of pyridoxamine phosphate alone in solution. Little, if any, change in the fluorescent intensity of pyridoxamine in the protein conjugates occurs after the addition of carbamyl phosphate, succinate, CTP, or ATP, singly or in combination, at concentrations which saturate the catalytic and regulatory sites of unmodified enzymes. The quantum yield of enzyme bound pyridoxamine phosphate was determined to be 0.23 at 23° and 0.40 at 4° in 0.05 M triethanolamine-acetate (pH 7). For comparison, the quantum yield of pyridoxamine phosphate alone in 0.05 M triethanolamine acetate (pH 7) is 0.15 at 23° and 0.26 at 4°. The lifetimes of bound pyridoxamine phosphate fluorescence are 3.8 nsec at 23° and 4.4 nsec at 4° for the enzyme derivative, and 4.0 nsec at 23° in the catalytic trimer derivative. For pyridoxamine phosphate alone in buffer the lifetime is 1.9 nsec at 23°. The polarization of pyridoxamine phosphate is approximately 0.28 in the enzyme derivative at both 4 and 23°. In the presence of 2 mM carbamyl phosphate and 10 mM succinate, this polarization dropped to about 0.25. Pyridoxamine phosphate has a limiting polarization of 0.41 at infinite viscosity (Churchich, 1965).

Quenching of Pyridoxamine Fluorescence by Mercurinitrophenol. It was possible to simultaneously label two amino acid residues located near the active site. After reacting each of the three amino groups of the catalytic trimer with pyridoxal phosphate, various amounts of 2-chloromercuri-4-nitrophenol could be reacted with the single sulfhydryl group of the catalytic polypeptide chain. The absorption spectrum of covalently bound mercurinitrophenol overlaps with the fluorescence spectrum of the pyridoxamine phosphate derivative excited at 325 nm (Figure 6). A critical transfer distance of 32.8 Å was calculated from eq 3,

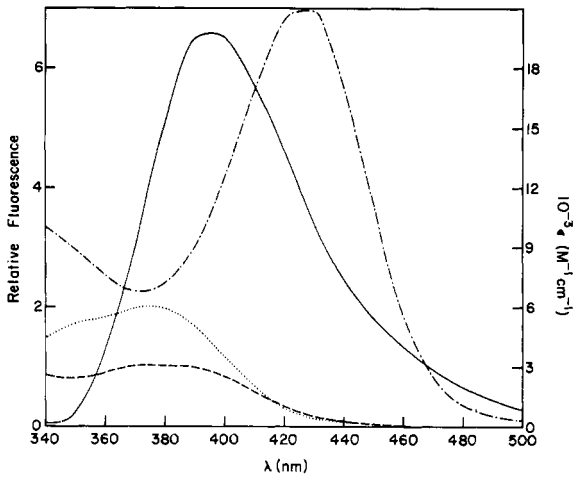

FIGURE 6: Overlap of the pyridoxamine phosphate corrected emission spectrum of aspartate transcarbamylase containing 5.7 mol of pyridoxamine phosphate/mol of enzyme excited at a wavelength of 325 nm (—) with the extinction coefficient of Cp_4F (- - -), of enzyme-bound ANS (· · ·), and of enzyme-bound mercurinitrophenol (- · -) in 0.05 M triethanolamine-acetate (pH 7) at 23°. In addition 2 mM mercaptoethanol and 0.2 mM EDTA were present for all spectra except that of the mercurinitrophenol.

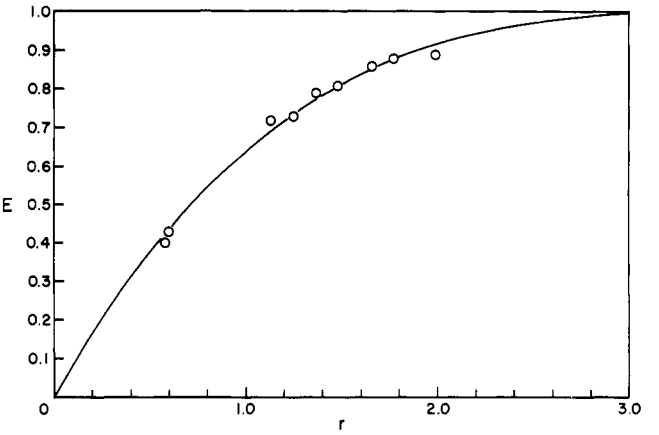

FIGURE 7: A plot of the efficiency of transfer, E, of the fluorescence at 390 nm excited at 325 nm of the catalytic subunit containing 2.7 or 2.8 mol of pyridoxamine phosphate/mol of trimer as a function of r, the moles of mercurinitrophenol bound per mole of trimer, in 0.05 M triethanolamine-acetate (pH 7) at 23°. The concentration of catalytic subunit ranged from 0.3 to 0.6 mg/ml.

using an extinction coefficient of 2.09×10^4 M^{-1} cm^{-1} at 430 nm for bound mercurinitrophenol (Evans *et al.*, 1972) and a quantum yield of 0.23 for the pyridoxamine derivative at 23°. In derivatives containing three molecules of pyridoxamine per catalytic trimer and various amounts of mercurinitrophenol, the pyridoxamine fluorescence (excitation 325 nm, emission 390 nm) was quenched. Addition of 10 mM mercaptoethanol to these solutions caused pyridoxamine fluorescence to increase as a result of the displacement of bound mercurinitrophenol. The efficiency of transfer of pyridoxamine fluorescence increases with the amount of mercurinitrophenol bound (Figure 7). These data can be explained in terms of a model of three equidistant catalytic sites on the catalytic trimer with adjacent amino and sulfhydryl groups at each site. The two amino acid residues are assumed to be sufficiently close so that complete quenching of a bound pyridoxamine molecule by an adjacent bound mercurinitrophenol molecule occurs. Quenching also occurs between labels on nonadjacent sites. This is shown in Figure 7 where the efficiency of transfer is greater than one-third when only a single mercurinitrophenol is bound. If random binding of the mercurinitrophenol is assumed, the fractions of the species with zero, one, two, or three bound mercurinitrophenols can be calculated using the binomial distribution (eq 9), where f_i is the fraction of a species with i bound

$$f_i = \frac{3!}{i!(3-i)!}\left(\frac{r}{3}\right)^i\left(1-\frac{r}{3}\right)^{3-i} \qquad (9)$$

mercurinitrophenols at a given value of r, the average number of moles of mercurial bound per mole of trimer, with a maximum of three binding sites. For each of these species the efficiency of quenching can be written as

$$E_i = \frac{1}{3}\sum_{m=1}^{3}\left\{\sum_{n=1}^{i}(R_0/R_{mn})^6\Big/\left[1+\sum_{n=1}^{i}(R_0/R_{mn})^6\right]\right\} \qquad (10)$$

In eq 10 E_i is the efficiency of transfer for the ith species, m is an index running over all donors, n is an index running over all acceptors present, R_0 is the critical transfer distance for the donor-acceptor pair, and R_{mn} is the distance between a given m,n donor-acceptor pair (*cf.* Gennis and Cantor, 1972; Moe *et al.*, 1974). For the symmetrical model under consideration all terms involving adjacent donor-acceptor pairs are equal to unity and only the distance between donor and acceptor on separate catalytic sites enters explicitly. The efficiencies of each species, E_i, and the measured efficiency, E, are given by

$$E_1 = \left[1+\frac{2(R_0/R)^6}{1+(R_0/R)^6}\right]\Big/3$$

$$E_2 = \left[2+\frac{2(R_0/R)^6}{1+2(R_0/R)^6}\right]\Big/3 \qquad (11)$$

$$E_3 = 1$$

$$E = f_1E_1 + f_2E_2 + f_3E_3$$

Using these equations a value for R was calculated for each point in Figure 7. The average value of R is 25.9 Å and the theoretical curve shown in Figure 7 was calculated for this average distance with eq 9 and 11. The fluorescence lifetimes of pyridoxamine in the doubly labeled derivatives before and after mercaptoethanol addition were determined. The fluorescence was found to decay faster in the presence of mercurinitrophenol, but this information cannot be used to calculate distances. Several lifetimes are possible for the different pyridoxamines in the four doubly labeled species, and it was not possible to experimentally resolve the fluorescence decay curves into a sum of several exponential decays. However, the postulated model and the data from the equilibrium fluorescence quenching experiments were used to calculate a sum of exponential decays which was consistent with the observed fluorescence decay. This indicates the interpretation of the steady-state fluorescence data is consistent with the fluorescence lifetime measurements, but does not provide a unique interpretation of the lifetime measurements.

Quenching of Pyridoxamine Fluorescence by ANS. The fluorescence titration curves of ANS (excitation 375 nm, emission 470 nm) with catalytic trimer containing 0, 1.6, or 2.8 molecules of pyridoxamine phosphate show that the presence of pyridoxamine phosphate decreases ANS binding, but does not abolish it completely (Figure 5). The addition of carbamyl phosphate caused ANS binding to drop to a similar background level for all catalytic trimer derivatives. The fluorescent intensity after subtraction of the

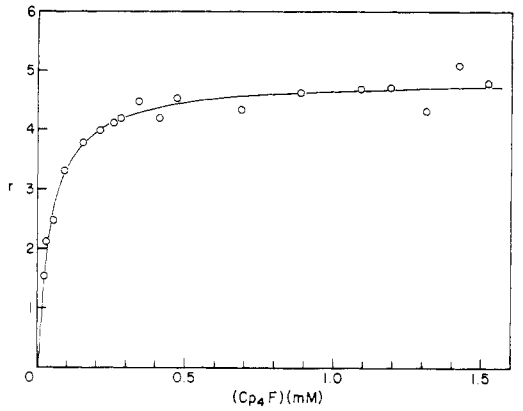

FIGURE 8: A plot of the number of moles of Cp_4F bound per mole of aspartate transcarbamylase, r, *vs.* the concentration of free Cp_4F, (Cp_4F), in 0.05 M triethanolamine-acetate (pH 7), 2 mM mercaptoethanol, and 0.2 mM EDTA as determined by equilibrium dialysis. Ligand concentrations were measured using an extinction coefficient for Cp_4F of 3×10^3 M^{-1} cm^{-1} at 385 nm. The protein concentration was approximately 20 mg/ml.

background value was taken to be proportional to the concentration of bound ANS. Assuming that at a concentration of 350 μM ANS, three ANS molecules are bound to the unmodified subunit (Figure 5), the amount of ANS bound to the derivatives can be calculated. At an ANS concentration of 350 μM an average of 2.5 molecules of ANS are bound per catalytic trimer with 1.6 pyridoxamines and an average of 1.7 molecules of ANS are bound per catalytic trimer with 2.8 pyridoxamines. The fluorescence (excitation 325 nm, emission 390 nm) of pyridoxamine phosphate in the derivatives is quenched by ANS binding. The efficiency of transfer was determined from the pyridoxamine fluorescence in the presence of 350 μM ANS at 23° in 0.05 M triethanolamine-acetate (pH 7), before and after addition of carbamyl phosphate. For the catalytic trimer derivative containing 2.8 molecules of pyridoxamine phosphate, the efficiency of transfer was 0.72. The critical transfer distance was calculated to be R_0 = 25.0 Å using the overlap of bound pyridoxamine phosphate fluorescence and bound ANS absorbance (Figure 6) with a quantum yield of 0.23 for the pyridoxamine fluorescence and an extinction coefficient of 5970 M^{-1} cm^{-1} at 375 nm for the ANS absorbance. The distance between pyridoxamine and ANS on nonadjacent catalytic sites can be calculated using the model described for mercurinitrophenol quenching of pyridoxamine. Again, the assumptions are made that pyridoxamine is completely quenched by an adjacent ANS molecule and that the binding of ANS is random. The distance calculated is 26.1 Å between a pyridoxamine phosphate molecule at one catalytic site and an ANS molecule at another. For the catalytic trimer containing 1.6 molecules of pyridoxamine phosphate the efficiency of transfer is 0.78. Because the pyridoxal phosphate sites are not fully occupied in this derivative, there are ten different species involving the two ligands, pyridoxamine phosphate and ANS. This case is too complex to analyze quantitatively.

Quenching of Pyridoxamine Fluorescence by Cp_4F. The nucleotide Cp_4F binds to aspartate transcarbamylase in the presence of 10 mM carbamyl phosphate, 0.05 M triethanolamine-acetate (pH 7), 2 mM mercaptoethanol, and 0.2 mM EDTA at 4° (Figure 8). The data were fit to eq 12, which is

$$r = nK(\mathrm{L})/[1 + K(\mathrm{L})] \qquad (12)$$

valid for binding to identical independent sites, by a nonlinear least-squares analysis. In eq 12, r is the average number of moles of ligand bound per mole of enzyme, (L) is the ligand concentration, n is the total number of ligand-binding sites, and K is the binding constant for the ligand–enzyme interaction. The solid line in Figure 8 is the theoretical curve calculated with eq 12 and the best fit parameters, $n = 5$ and $K = 2 \times 10^4$ M^{-1}. The saturation of the binding curve at 5 rather than 6 mol of ligand bound/mol of enzyme is probably due to the uncertainty in the value of the extinction coefficient used to calculate the concentration of the nucleotide. It should be mentioned that the use of the published extinction coefficients for other fluorescamine derivatives (5450–6000 M^{-1} cm^{-1}; Weigele *et al.*, 1972a,b) results in a saturation of the binding curve at approximately 3 mol of ligand bound/mol of enzyme. The pyridoxamine derivative of the enzyme was not obtained in sufficient quantity for a complete binding study, but its ability to bind the nucleotide as determined by equilibrium dialysis is similar to that of the underivatized enzyme. When assayed in the presence of 5 mM aspartate and 3.6 mM carbamyl phosphate at 23° and pH 7, a 0.38 mM concentration of Cp_4F inhibits the native enzyme by about 60%, which is similar to the maximal inhibition by CTP under the same conditions. The compound Cp_4F does not inhibit the activity of the catalytic subunit at the same concentration. The fluorescence of Cp_4F (excitation 385 nm, emission 475 nm) can be used to measure its polarization, P, when bound to the native enzyme at 4° and pH 7 in the presence of 10 mM carbamyl phosphate. A plot of the ratio of free to bound ligand concentrations vs. $1/P$ was extrapolated to zero to give a polarization of 0.33 for the bound ligand. Measurements of the polarization of Cp_4F in glycerol solutions were used to construct a plot (Perrin, 1926) of $(1/P) - \frac{1}{3}$ *vs.* T/η where η is the viscosity. Extrapolation to infinite viscosity gave P_0 = 0.34. Thus, the compound Cp_4F is not free to rotate when bound to the enzyme and its polarization is a measure of its binding. On addition of CTP, the polarization of Cp_4F in the presence of enzyme decreases almost to the value found in buffer solution. The spectral overlap of bound pyridoxamine fluorescence and the absorbance of Cp_4F is shown in Figure 6. The overlap integral along with a quantum yield of 0.40 for bound pyridoxamine phosphate at 4° and an extinction coefficient of 3×10^3 M^{-1} cm^{-1} at 385 nm for Cp_4F were used to calculate a critical transfer distance of 25.5 Å at 4°. Using an enzyme derivative with 5.7 molecules of pyridoxamine phosphate bound per enzyme molecule in the presence of up to 0.32 mM Cp_4F, a search was made for energy transfer. Neither measurements of pyridoxamine fluorescence intensity nor measurements of fluorescence lifetime provided any evidence for energy transfer at 4 or 23°. If the experimental sensitivity is assumed to be about 5%, the best mean deviation obtained in other efficiency measurements, then no effect would be observed for efficiencies less than 0.05. Using this value a lower bound of 42 Å for the distance of separation between nearest neighbor catalytic and regulatory sites can be calculated.

The fluorescence properties of tryptophan and enzyme-bound pyridoxamine phosphate and the values of R_0, E, and R for all donor–acceptor pairs are summarized in Table I.

Discussion

Förster's theory of fluorescence energy transfer has been experimentally tested and shown to be valid for several systems of known geometry (Latt *et al.*, 1965; Stryer and

Table I: Fluorescence Properties of Donors and Energy Transfer Parameters for Donor–Acceptor Pairs.

Donor	Excitation λ(nm)	Emission λ(nm)	Q	τ(nsec)	Acceptor	R_0(Å)[a]	E	R(Å)[a]
Tryptophan	295	340	0.10(23°)	2(23°)	1. Pyridoxamine phosphate	19.7	0.14	26.6[b] 23.0[c]
					2. ANS	21.6	0.22	26.6[b] 22.5[c]
Pyridoxamine phosphate	325	390	0.23(23°) 0.40(4°)	3.8(23°) 4.4(4°)	1. Mercurinitrophenol	32.8(23°)	0.40–0.89	25.9[d]
					2. ANS	25.0(23°)	0.72	26.1[d]
					3. Cp_4F	25.5(4°)	<0.05	>42

[a] Assuming $K^2 = 2/3$. [b] Assuming two equidistant tryptophans are donors. [c] Assuming only one tryptophan is the donor. [d] Assuming the structural model described in the Results section.

Haugland, 1967). The applicability of the theory to the experiments reported here depends upon three criteria. The first is that the experimental measurements must give the true energy transfer efficiency. Secondly, the correct molecular constants, *i.e.*, quantum yield, extinction coefficient, overlap integral, and dipole orientation factor, must be used to calculate R_0, the critical transfer distance. The third criterion is that the correct geometrical arrangement of sites must be described by the model. Each of these will be discussed in turn for the experiments with aspartate transcarbamylase.

Several factors other than energy transfer can effect the observed fluorescence quenching. Fluorescence intensity is decreased by inner filter effects. The displacement method used to measure donor fluorescence when acceptor is bound and then released eliminates this problem. Due to instrumental limitations, fluorescence lifetimes could not be analyzed in terms of a sum of several exponential decays. For this reason, the pyridoxamine lifetime measurements were not used to calculate transfer efficiencies. When analyzed as a single exponential decay, the change in tryptophan lifetime gave the same efficiency of transfer as that obtained from the fluorescence intensity measurements. This suggests that the two tryptophans per catalytic chain are approximately equally quenched and thus are at approximately equal distances from the active site. An erroneous interpretation of the fluorescence quenching can result if there is nonspecific binding of the donor or acceptor molecules. The site specificity of the pyridoxamine phosphate labeling has been demonstrated through inhibition studies, spectral measurements, and amino acid analysis (Greenwell *et al.*, 1973). The stoichiometric binding and specific location of mercurinitrophenol have been demonstrated in titration and X-ray diffraction studies (Evans *et al.*, 1972). Although ANS binds nonspecifically to the catalytic subunit as well as at the active site, only those ANS molecules displaced by carbamyl phosphate contribute to the measured quenching. The carbamyl phosphate is assumed to bind specifically at the active site where it displaces ANS directly by steric obstruction of that site and not indirectly, for example, by inducing a conformational change. Other studies have shown that anions such as sulfate, nitrate, and arsenate also inhibit enzyme activity (Kleppe, 1966). The evidence for the specific binding of the nucleotide Cp_4F is the saturation of the binding curve at a small value (approximately five) of the moles of ligand bound per mole of enzyme, the similarity of its ability to inhibit enzyme activity with that of CTP, and the displacement of bound ligand by CTP as measured by the decrease in polarization. The binding curve of the nucleotide, Cp_4F, can be described in terms of a single class of identical independent sites, a situation which differs from the negative cooperativity in CTP binding to the enzyme (Winlund and Chamberlin, 1970; Buckman, 1970; Cook, 1972; Matsumoto and Hammes, 1973; Gray *et al.*, 1973). The weakness of the binding of Cp_4F and the uncertainty in its extinction coefficient do not permit detailed conclusions with regard to stoichiometry and binding mechanism to be drawn from the binding data obtained. A conformational change in the enzyme caused by the binding of acceptor could produce a change in the environment of the donor to change its fluorescent intensity. This possibility cannot be definitely excluded, but it has been shown that tryptophan and bound pyridoxamine fluorescence are little affected by substrates, substrate analogs, or nucleotides.

The electronic dipole interaction parameters of a given donor–acceptor pair are combined in R_0, the critical transfer distance (eq 3). The value of R_0 must be determined independently of the efficiency measurements and is subject to additional sources of error. The quantum yields of the two donors were determined by comparison with that of quinine sulfate. An absolute quantum yield of quinine sulfate of 0.70 (Scott *et al.*, 1970) was used for the calculations rather than the older and more widely quoted value of 0.55 (Melhuisch, 1961). The quantum yield of tryptophan in proteins has been reported to vary from 0.06 to 0.27 based on a quantum yield of 0.20 for free tryptophan (Kronman and Holmes, 1971). No known relationship between tryptophan quantum yields and the nature of their environment in proteins exists. The tryptophan quantum yields of 0.07 for the native enzyme and 0.10 for the catalytic subunit and the tryptophan lifetime of 2 nsec are similar to the values found for tryptophan in lysozyme and trypsin (Kronman and Holmes, 1971). Pyridoxamine phosphate has been reported to have a quantum yield of 0.14 ± 0.01 at pH 7 and 25° with respect to a quantum yield of 0.55 for quinine sulfate (Chen, 1965) and a lifetime of 4.3 ± 0.5 nsec at pH 7 and 23° (Chen *et al.*, 1967). The quantum yield of 0.15 obtained here is in good agreement with the reported value. The lifetime of 1.9 nsec obtained here is shorter than that previously reported. Both lifetime values were determined by a nanosecond pulse method, although different instrumentation and fitting techniques were employed. Short lifetimes are difficult to measure with this method because the exciting light pulse is itself several nanoseconds in duration. The use of a monochromator and interference fil-

ter in the present work eliminates the possible interfering fluorescence of the dyes in the color filters generally used. Regardless of the absolute accuracy of the measurements, both the quantum yield and lifetime of pyridoxamine phosphate bound to the enzyme are larger than the values found for pyridoxamine phosphate in solution. This increase cannot be explained by the nonpolarity of the binding site, because the quantum yield of pyridoxamine phosphate decreases in dimethylformamide (Cortijo and Shaltiel, 1972). The fluorescence intensity of pyridoxamine is pH dependent (Bridges *et al.*, 1966). In solution at a given pH, pyridoxamine exists as an equilibrium mixture of ionic forms with different quantum yields. One of the forms, the dipolar ion, is nonfluorescent (Bridges *et al.*, 1966). For pyridoxamine phosphate covalently bound to aspartate transcarbamylase, the equilibria among the different ionic forms may be shifted to give a higher net quantum yield.

The values for the extinction coefficients of pyridoxamine phosphate (Greenwell *et al.*, 1973) and of mercurinitrophenol (Evans *et al.*, 1972) bound to the enzyme were taken from published sources. The extinction coefficient of ANS bound to the enzyme was determined from its absorption spectrum when bound to the catalytic subunit based on the published extinction coefficient for ANS in solution (Weber and Young, 1964). The increased extinction coefficient and red-shifted spectrum of bound ANS are larger in magnitude than similar changes of ANS absorbance upon binding to myoglobin (Stryer, 1965). The extinction coefficient of the nucleotide, Cp_4F, is smaller than that reported for other fluorescamine derivatives (5450–6000 M^{-1} cm^{-1}; Weigele *et al.*, 1972a,b). Possible explanations are the modification of the fluorescamine moiety during the coupling reaction or the stacking interaction of cytosine and fluorescamine rings to produce hypochromicity. The extinction coefficient of this nucleotide has been assumed not to change upon binding to the enzyme. Calculation of the spectral overlap integral also utilizes the corrected fluorescence spectra and absorption spectra; in practice the overlap integral was found to be relatively insensitive to small variations in the spectra.

The dipole orientation factor, K^2, is the most uncertain quantity in the calculation of the critical transfer distance. Values for K^2 theoretically may range from 0 to 4 depending on the relative orientations of the donor emission and acceptor absorption dipoles. For a random orientation with no rotation of the dipoles, K^2 is 0.475 (Maksimov and Rozman, 1962). When the donor dipole is free to rotate and the acceptor dipole is fixed, the range of K^2 values is $\frac{1}{3}$ to $\frac{4}{3}$ (Wu and Stryer, 1972). When both donor and acceptor have complete rotational freedom $K^2 = \frac{2}{3}$ (Förster, 1959). When either or both of the dipoles are randomly oriented the possible range of K^2 is greatly limited. The calculated distances are proportional to the one-sixth power of K^2. When one of the dipoles is free to rotate, the distance calculated using $K^2 = \frac{1}{3}$ will be 80% of the distance calculated using $K^2 = \frac{4}{3}$. The distance calculated with $K^2 = \frac{2}{3}$ will be at the middle of these extremes. The polarization measurements can be used to obtain some idea about the rotational mobility of the fluorescent labels. A molecule as large as aspartate transcarbamylase rotates slowly compared to the rates of fluorescence of the chromophores attached to it. The rotational relaxation time for the enzyme at 20° in water is about 300 nsec based on a partial specific volume of 0.738 cm^3/g (Rosenbusch and Weber, 1971) or about 400 nsec based on a radius of 50 Å (Warren *et al.*, 1973). The lifetimes of the fluorescent molecules used in this study are 20 to 200 times shorter than the rotational relaxation time of the enzyme. In this situation, local rotation of the fluorescent chromophores is the major cause of a decrease in polarization with respect to that at infinite viscosity. The polarizations of enzyme-bound pyridoxamine phosphate and ANS are less than their respective polarizations at infinite viscosity. Therefore, these fluorescent labels have some degree of rotational mobility. The polarization of the enzyme-bound fluorescamine nucleotide is the same as its polarization at infinite viscosity and this label is not free to rotate when bound. At least one member of every energy transfer pair has some rotational mobility so that the uncertainty in the distances calculated with $K^2 = \frac{2}{3}$ is probably not greater than 10% due to the uncertainty in orientation. The validity of the K^2 assumption may be tested by measuring the same distance with more than one donor–acceptor pair. If the molecules are bound in fixed positions, different orientation factors should be required for different molecules (Wu and Stryer, 1972). Although the transfer efficiencies were not the same for the tryptophan–pyridoxamine pair and the tryptophan–ANS pair, identical distances were calculated using $K^2 = \frac{2}{3}$. Similarly the assumption of $K^2 = \frac{2}{3}$ resulted in the calculation of equal distances of separation for the pyridoxamine–mercurinitrophenol and pyridoxamine–ANS pairs.

When multiple donors and acceptors are present on the same protein molecule, the geometrical arrangement of the binding sites must be known or postulated in order to relate the measured efficiencies to distances. In the experiments with aspartate transcarbamylase the geometrical model was based to a large extent on known structural information. The threefold symmetry of the enzyme has been established by electron microscopy and X-ray diffraction (Richards and Williams, 1972; Warren *et al.*, 1973). The equidistant placement of the mercurinitrophenol labels on the catalytic trimer and thus of the three sulfhydryl groups has also been shown in the X-ray studies (Evans *et al.*, 1972). Indirect evidence that the sulfhydryl group is near the active site exists: when bulky or negatively charged compounds are covalently reacted with the sulfhydryl group, the enzyme is inactivated (Vanaman and Stark, 1970; Benisek, 1971; Jacobson and Stark, 1973).

Although changes in the tryptophan ultraviolet absorption and circular dichroism accompanying substrate and inhibitor binding have been interpreted in terms of a tryptophan residue (or residues) very close to the active site (Collins and Stark, 1969; Griffin *et al.*, 1972), the distances calculated from the fluorescence data suggest tryptophan is not close to the active site. (A significantly shorter distance would require a rigid donor and acceptor with identical orientation of transition moments for both tryptophan–pyridoxamine and tryptophan–ANS so as to produce a very small value of K^2.) The substrate and inhibitor induced spectral changes are most likely due to a conformational change of the catalytic polypeptide chain.

The conclusions of this study are summarized in Figure 9. The schematic cross-section of the aspartate transcarbamylase molecule shown is loosely based on the electron-density map determined by X-ray diffraction (Warren *et al.*, 1973). The locations of the sulfhydryl groups have been determined by the X-ray diffraction studies. The energy transfer measurements suggest that a sulfhydryl group is near the active site. A distance of 26 Å was found to separate the active sites from the nonadjacent sulfhydryl groups

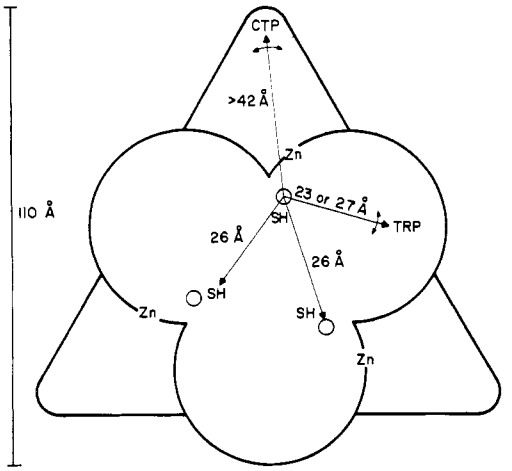

FIGURE 9: Schematic cross-sectional diagram of aspartate transcarbamylase showing the central catalytic trimer and peripheral regulatory subunits. The proposed active site positions are represented by small circles; the active site may overlap the position of the adjacent sulfhydryl group. The scale is only approximate.

compared to the distance of 22 Å between sulfhydryl groups determined by X-ray diffraction. However, the chromophores used in this study are themselves almost 10 Å in length so fine distinctions cannot be made. As discussed above, tryptophan appears not to be directly at the active site. The active sites are far from the regulatory sites (>42 Å). This result suggests the regulatory subunit is elongated, which is consistent with the X-ray and electron microscopy results. Thus no direct electrostatic or steric interactions can account for the regulation of enzyme activity by nucleotides.

References

Azumi, T., and McGlynn, S. P. (1962), *J. Chem. Phys. 37,* 2413.

Benisek, W. F. (1971), *J. Biol. Chem. 246,* 3151.

Bridges, J. W., Davies, D. W., and Williams, R. T. (1966), *Biochem. J. 98,* 451.

Buckman, T. (1970), *Biochemistry 9,* 3255.

Changeux, J.-P., Gerhart, J. C., and Schachman, H. K. (1968), *Biochemistry 7,* 531,

Chen, P. S., Jr., Toribara, T. Y., and Warner, H. (1956), *Anal. Chem. 28,* 1756.

Chen, R. F. (1965), *Science 150,* 1593.

Chen, R. F. (1967), *Anal. Biochem. 19,* 374.

Chen, R. F., Vurek, G. G., and Alexander, N. (1967), *Science 156,* 949.

Churchich, J. E. (1965), *Biochim. Biophys. Acta 102,* 280.

Cohlberg, J. A., Pigiet, V. P., and Schachman, H. K. (1972), *Biochemistry 11,* 3396.

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

Cook, R. A. (1972), *Biochemistry 11,* 3792.

Cortijo, M., and Shaltiel, S. (1972), *Eur. J. Biochem. 29,* 134.

Davies, G. E., and Stark, G. R. (1970), *Proc. Nat. Acad. Sci. U. S. 66,* 651.

Evans, D. R., McMurray, C. H., and Lipscomb, W. H. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 3638.

Evans, D. R., Warren, S. G., Edwards, B. F. P., McMurray, C. H., Bethge, P. H., Wiley, D. C., and Lipscomb, W. N. (1973), *Science 179,* 683.

Förster, T. (1959), *Discuss. Faraday Soc. No. 27,* 7.

Förster, T. (1965), *Modern Quantum Chem. Lect. Istanbul Int. Summer Sch., 1964, 3,* 93.

Gennis, R. B., and Cantor, C. R. (1972), *Biochemistry 11,* 2509.

Gerhart, J. C., and Holoubek, H. (1967), *J. Biol. Chem. 242,* 2886.

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

Gerhart, J. C., and Schachman, H. K. (1965), *Biochemistry 4,* 1054.

Gray, C. W., Chamberlin, M. J., and Gray, D. M. (1973), *J. Biol. Chem. 248,* 6071.

Greenwell, P., Jewett, S. L., and Stark, G. R. (1973), *J. Biol. Chem. 248,* 5994.

Griffin, J. H., Rosenbusch, J. P., Blout, E. R., and Weber, K. K. (1973), *J. Biol. Chem. 248,* 5057.

Griffin, J. H., Rosenbusch, J. P., Weber, K. K., and Blout, E. R. (1972), *J. Biol. Chem. 247,* 6482.

Hammes, G. G., Porter, R. W., and Wu, C.-W. (1970), *Biochemistry 9,* 2992.

Jacobson, G. R., and Stark, G. R. (1973), *J. Biol. Chem. 248,* 8003.

Kleppe, K. (1966), *Biochim. Biophys. Acta 122,* 450.

Kronman, M. J., and Holmes, L. G. (1971), *Photochem. Photobiol. 14,* 113.

Latt, S. A., Cheung, H. T., and Blout, E. R. (1965), *J. Amer. Chem. Soc. 87,* 995.

Maksimov, M. Z., and Rozman, I. M. (1962), *Opt. Spectrosc. (USSR) 12,* 337.

Matsumoto, S., and Hammes, G. G. (1973), *Biochemistry 12,* 1388.

Meighen, E. A., Pigiet, V., and Schachman, H. K. (1970), *Proc. Nat. Acad. Sci. U. S. 65,* 234.

Melhuisch, W. H. (1961), *J. Phys. Chem. 65,* 229.

Moe, O. A., Lerner, D. A., and Hammes, G. G. (1974), *Biochemistry 13,* 2552.

Moffatt, J. G., and Khorana, H. G. (1961), *J. Amer. Chem. Soc. 83,* 649.

Nelbach, M. E., Pigiet, V. P., Gerhart, J. C., and Schachman, H. K. (1972), *Biochemistry 11,* 315.

Parker, C. A., and Rees, W. T. (1966), *Analyst 85,* 587.

Perrin, F. (1926), *J. Phys. Radium 7,* 390.

Prescott, L. M., and Jones, M. E. (1969), *Anal. Biochem. 32,* 408.

Richards, K. E., and Williams, R. C. (1972), *Biochemistry 11,* 3393.

Rosenbusch, J. P., and Griffin, J. H. (1973), *J. Biol. Chem. 248,* 5063.

Rosenbusch, J. P., and Weber, K. (1971), *J. Biol. Chem. 246,* 1644.

Schachman, H. K. (1972), *in* Protein-Protein Interactions, Jaenicke, R., and Helmreich, E., Ed., West Berlin, Springer-Verlag.

Scott, T. G., Spencer, R. D., Leonard, N. J., and Weber, G. (1970), *J. Amer. Chem. Soc. 92,* 687.

Sheely, M. L. (1932), *Ind. Eng. Chem. 24,* 1060.

Stryer, L. (1965), *J. Mol. Biol. 13,* 482.

Stryer, L. (1968), *Science 162,* 526.

Stryer, L., and Haugland, R. D. (1967), *Proc. Nat. Acad. Sci. U. S. 58,* 719.

Tu, S.-C., and McCormick, D. B. (1974), *Biochemistry 13,* 893.

Turner, D. C., and Brand, L. (1968), *Biochemistry 7,* 3381.

Udenfriend, S., Stein, S., Böhlen, P., Dairman, W.,

Leimgruber, W., and Weigele, M. (1972), *Science 178,* 871.
Vanaman, T. C., and Stark, G. R. (1970), *J. Biol. Chem. 245,* 3565.
Warren, S. G., Edwards, B. F. P., Evans, D. R., Wiley, D. C., and Lipscomb, W. N. (1973), *Proc. Nat. Acad. Sci. U. S.*
Weber, G. (1968a), *in* Molecular Associations in Biology, Pullman, B., Ed., New York, N. Y., Academic Press, p 499.
Weber, K. (1968b), *Nature (London) 218,* 1116.
Weber, K. (1968c), *J. Biol. Chem. 243,* 543.
Weber, G., and Young, L. B. (1964), *J. Biol. Chem. 239,* 1415.
Wehrli, W. E., Verheyden, D. L. M., and Moffatt, J. G. (1965), *J. Amer. Chem. Soc. 87,* 2265.
Weigele, M., Blount, J. F., Tengi, J. P., Czajkowski, R. C., and Leimgruber, W. (1972a), *J. Amer. Chem. Soc. 94,* 4052.
Weigele, M., DeBernardo, S. L., Tengi, J. P., and Leimgruber, W. (1972b), *J. Amer. Chem. Soc. 94,* 5927.
Wiley, D. C., Evans, D. R., Warren, S. G., McMurray, C. H., Edwards, B. F. P., Franks, W. A., and Lipscomb, W. N. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36,* 285.
Winlund, C. C., and Chamberlin, J. J. (1970), *Biochem. Biophys. Res. Commun. 40,* 43.
Wu, C.-W., and Stryer, L. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 1104.
Yates, R. A., and Pardee, A. B. (1956), *J. Biol. Chem. 221,* 757.
Yguerabide, J. (1972), *Methods Enzymol. 26,* 498.

Calorimetric Analysis of Aspartate Transcarbamylase from *Escherichia coli:* Binding of Cytosine 5′-Triphosphate and Adenosine 5′-Triphosphate†

Norma M. Allewell,* Joan Friedland,‡ and Karl Niekamp§

ABSTRACT: The binding of CTP and ATP to aspartate transcarbamylase at pH 7.8 and 8.5 at 25° has been investigated by equilibrium dialysis and flow microcalorimetry. The binding isotherms for CTP at both pH 7.8 and 8.5 and ATP at pH 8.5 can be fit by a model which assumes three tight, three moderately tight, and six weak binding sites. The binding isotherms for ATP at pH 7.8 are best fit by a model which assumes six tight and six weaker sites. Both $\Delta H_{\text{binding}}$ and $\Delta S_{\text{binding}}$ are negative for both nucleotides at both pH values, so that the binding is enthalpy driven. For both nucleotides, ΔH is the same for the first two classes of binding sites, implying that the difference in the dissociation constants of these two classes of sites is the result of entropic effects. Direct pH measurements and calorimetric measurements in two buffers with very different heats of ionization (Tris and Hepes) indicate that the binding of both nucleotides is accompanied by the binding of protons. In the pH range 6.7–8.4, the number of moles of protons bound per mole of nucleotide increases as the pH decreases.

The allosteric regulation of enzymatic activity is generally believed to require changes in tertiary and quaternary structure. However, very little quantitative information is available at the present time on the nature or magnitude of these conformational changes. Crystallographic investigations now in progress will undoubtedly provide a substantial body of detailed structural information. However, our ability to elucidate from a crystal structure the thermodynamic principles which underlie it is still very rudimentary. Moreover the question of the extent to which the conformation of a protein differs in solution and in the crystal will be very difficult to resolve for proteins which are known to be conformationally flexible.

Because of these considerations, we have undertaken a comprehensive calorimetric analysis of aspartate transcarbamylase from *Escherichia coli,* an enzyme which has been widely used as a model system for investigating the mechanism of allosteric regulation. We hope to (a) establish thermodynamic criteria against which models of the catalytic and regulatory mechanisms may be tested, (b) evaluate the magnitude of the various conformational changes which the enzyme undergoes, and (c) clarify the nature of the forces involved in binding small molecules to the enzyme and in the interactions of its subunits.

The properties of aspartate transcarbamylase have been summarized in recent review articles (Gerhart, 1970; Jacobson and Stark, 1973). The enzyme catalyzes the first metabolic reaction unique to the synthesis of pyrimidine nucleotides, the transfer of the carbamyl group of carbamyl phosphate to the α-amino group of L-aspartic acid. At nonsaturating substrate concentrations, CTP inhibits enzymatic activity, while ATP is an activator (Gerhart and Pardee,

† From the Department of Chemistry, Polytechnic Institute of Brooklyn, Brooklyn, New York 11201 and the Department of Chemistry, Yale University, New Haven, Connecticut 06520. *Received March 11, 1974.* Supported by a grant (to N.M.A.) from the National Institute of Health (AM-15814).

* Present address: Department of Biology, Wesleyan University, Middletown, Connecticut 06457.
‡ Present address: Department of Biochemistry, Downstate Medical Center, Brooklyn, New York 11203.
§ Present address: Hanover College, Hanover, Indiana 47243.