rabarti et al., 1974), may prove a success if based on carbene rather than on nitrene chemistry.

Acknowledgments

We thank S. J. Abbott, Dr. S. R. Jones, and D. N. Standring for their advice and help.

References

Bayley, H. (1974), Part II Thesis, Oxford University.
Bayley, H., and Knowles, J. R. (1978), *Biochemistry 17* (preceding paper in this issue).
Bradley, G. F., Evans, W. B. L., and Stevens, I. D. R. (1977), *J. Chem. Soc., Perkin Trans. 2,* 1214.
Chakrabarti, P., Paisley, K., and Khorana, H. G. (1974), Abstracts, 168th National Meeting of the American Chemical Society, Atlantic City, N.J., No. BIOL-150.
Chowdhury, V., Vaughan, R., and Westheimer, F. H. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 1406.
Closs, R. L., and Moss, R. A. (1964), *J. Am. Chem. Soc. 86,* 4042.
Galardy, R. E., Craig, L. C., and Printz, M. P. (1973), *Nature (London), New Biol. 242,* 127.
Glass, R. L. (1971), *Lipids 6,* 919.
Gutsche, C. D., Bachman, G. L., and Coffey, R. S. (1962), *Tetrahedron 18,* 617.
Isaev, S. D., Yurchenko, A. G., Stepanov, F. N., Kolyada, G. G., Novikov, S. S., and Karpenko, N. F. (1973), *Zh. Org. Chim. 9,* 724.
Isaev, S. D., Yurchenko, A. G., Murzinova, Z. N., Stepanov, F. N., Kolyada, G. G., and Novikov, S. S. (1974), *Zh. Org. Khim. 10,* 1338.
Jones, M., Jr., and Moss, R. A. (1973), Carbenes, Volume I, New York, N.Y., Wiley-Interscience.
Marchesi, V. T., Furthmayr, H., and Tomita, M. (1976), *Annu. Rev. Biochem. 45,* 667.
Mattheson, R. R., Van Wart, H. E., Burgess, A. W., Weinstein, L. I., and Scheraga, H. A. (1977), *Biochemistry 16,* 396.
Moss, R. A., and Jones, M., Jr. (1975), Carbenes, Volume II, New York, N.Y., Wiley-Interscience.
Schmitz, E. (1962), *Chem. Ber. 95,* 688.
Smith, R. A. G., and Knowles, J. R. (1975), *J. Chem. Soc., Perkin Trans. 2,* 686.
Udding, A. C., Strating, J., Wynberg, H., and Schlatmann, J. L. M. A. (1966), *J. Chem. Soc., Chem. Commun.,* 657.

Structural Mapping of Aspartate Transcarbamoylase by Fluorescence Energy-Transfer Measurements: Determination of the Distance between Catalytic Sites of Different Subunits†

Liang-Hsien E. Hahn and Gordon G. Hammes*

ABSTRACT: Fluorescence energy transfer measurements have been used to determine the distances between active sites on different catalytic subunits of aspartate transcarbamoylase and between active sites and sulfhydryl groups (which are adjacent to active sites) on different catalytic subunits. Catalytic subunits were covalently labeled with pyridoxamine phosphate, which binds at the active sites, and reconstituted with regulatory subunits and with either unmodified catalytic subunits or catalytic subunits with sulfhydryl groups modified with 2-mercuri-4-nitrophenol. The unmodified subunit was subsequently modified with pyridoxal phosphate, which binds at the catalytic site, and fluorescence energy transfer was measured between pyridoxamine phosphate and pyridoxal phosphate by steady state fluorescence (quantum yield) and fluorescence lifetime measurements. The energy transfer between pyridoxamine phosphate and 2-mercuri-4-nitrophenol also was determined by steady-state fluorescence measurements. In both cases significant energy transfer was measured, and control experiments indicated conformational changes were not causing the observed changes in fluorescent properties. No significant changes in fluorescent properties or in energy transfer were detected in the presence of the allosteric effectors cytidine 5′-triphosphate or adenosine 5′-triphosphate or in the presence of the substrate carbamoyl phosphate alone or with the substrate analogue succinate. If the active sites of each catalytic subunit are assumed to define an equilateral triangle, with the two planes of the triangles being parallel, the distance between pyridoxamine phosphate and the closest pyridoxal phosphate is approximately 33 Å. The distance between pyridoxamine phosphate and the closest mercurinitrophenol is 28 Å. Thus the distance between catalytic sites on different subunits is concluded to be about 30 Å.

Aspartate transcarbamoylase catalyzes the formation of carbamoyl-L-aspartate and is the first enzyme in the biosynthetic pathway leading to the formation of pyrimidine nucleotides (Jacobson & Stark, 1973a; Gerhart, 1970). The enzyme is subject to feedback regulation by nucleotides (Gerhart & Pardee, 1962). Reaction with mercurials results in dissociation of the enzyme into two distinct kinds of subunits: the catalytic subunit, which is more active than the native enzyme, and the regulatory subunit, which binds nucleotides (Gerhart & Holoubek, 1967). The catalytic subunit has a molecular weight of 100 000 and is a trimer. The regulatory subunit has a molecular weight of 34 000 and is a dimer (Rosenbusch & Weber, 1971a). The intact enzyme has a molecular weight of 300 000 (Rosenbusch & Weber, 1971b) and is composed of two catalytic trimers and three regulatory dimers. The struc-

† From the Department of Chemistry, Cornell University, Ithaca, New York 14853. *Received May 10, 1977; revised manuscript received December 5, 1977.* This work was supported by a grant from the National Institutes of Health (GM 13292).

0006-2960/78/0417-2423$01.00/0

tural arrangement of the subunits in the native enzyme has been established by x-ray diffraction (Warren et al., 1973) and electron microscopy (Richards & Williams, 1972). The catalytic subunit trimers are eclipsed and are connected by regulatory subunit dimers which join to catalytic chains displaced by 120° in different trimers (Cohlberg et al., 1972). The overall dimensions of the molecule are about 90 Å along the threefold axis and 110 Å along the twofold axis. The assembled molecule has a central cavity about 25 Å along the threefold direction and about 50 Å along the twofold direction (Warren et al., 1973). The distances between catalytic sites on the catalytic trimer and between the catalytic site and the sulfhydryl groups (1 per catalytic polypeptide chain) have been determined using fluorescence resonance energy transfer (Matsumoto & Hammes, 1975). The distance between succinate bound at the catalytic site and the metal ion which is located at the interface between regulatory and catalytic subunits also has been determined using nuclear magnetic resonance (Fan et al., 1975). This study continues the structural mapping of aspartate transcarbamoylase by using fluorescence energy-transfer measurements to determine the distances between the catalytic sites of different catalytic subunits within a native enzyme molecule.

Experimental Section

Chemicals. The PLP,[1] PHMB (sodium salt), and biochemicals were obtained from Sigma Chemical Co. The MNP was obtained from Eastman Organic Chemicals. All other chemicals were reagent grade, and deionized distilled water was used in all solutions.

Aspartate Transcarbamoylase and Derivatives. The enzyme was prepared from a mutant strain of *E. coli* grown at the New England Enzyme Center by the method of Gerhart & Holoubek (1967). It was dissociated into its subunits using neohydrin and DEAE-cellulose column chromatography (Schachman, 1972). Zinc acetate (2 mM) 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 & Holoubek, 1967; Nelbach et al., 1972). In some cases the concentrations of the enzyme or its derivatives were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Activity measurements of the native enzyme, catalytic subunit and derivatives were done colorimetrically (Prescott & Jones, 1969). The activity of a given derivative always was determined simultaneously with that of native enzyme (or catalytic subunit) in order to provide a precise comparison.

A derivative of the enzyme containing one catalytic trimer modified with pyridoxamine phosphate (one per catalytic site) and one catalytic trimer unmodified, $C_{PMP}C_nR_3$, was prepared by the method of Gibbons et al. (1974) with minor changes. PMP conjugates of the catalytic subunit were prepared by sodium borohydride reduction of the Schiff base formed between PLP and an amino group at the catalytic site (Greenwell et al., 1973). Acylation of catalytic subunit with H_4pht anhydride was carried out in 50 mM potassium phosphate (pH 8.2) with 2 mM EDTA and 2 mM mercaptoethanol. The final concentration ratio of H_4pht anhydride to lysine residues was 0.6 (Gibbons et al., 1974). Equal amounts of the PMP modified catalytic subunit and the H_4pht anhydride modified catalytic subunit were mixed, and a slight excess (about 15%) of regulatory subunits was added. The mixture of subunits, at a protein concentration of about 1 mg/mL in 50 mM Tris-acetate (pH 8) with 2 mM mercaptoethanol and 0.1 mM zinc acetate was incubated for 30 min at room temperature (Greenwell et al., 1973). After concentration over Amicon PM-30 membrane to a protein concentration of about 10 mg/mL, the solution was dialyzed against 50 mM Tris-HCl (pH 7.5) containing 0.2 M KCl and 2 mM EDTA. After dialysis it was loaded onto a DEAE-Sephadex A-50 column (2 cm i.d. × 50 cm) equilibrated with the same buffer. The column was eluted with a linear gradient of KCl varying from 0.2 to 0.8 M in the same buffer. The time duration between acylation of the catalytic subunit and the column chromatography was always 24 h or less in order to prevent extensive deacylation. The 280 nm absorbance of the column effluent was monitored, and the peak containing reconstituted enzyme with one catalytic trimer modified with pyridoxamine phosphate and the other catalytic trimer modified with THP, $C_{PMP}C_{THP}R_3$, was collected. The derivative was deacylated at pH 6.0 (Gibbons & Schachman, 1976). The activity of the derivative was 51% of the activity of native enzyme. A ratio of 3.2 mol of PMP/mol of derivative was determined spectrophotometrically using an extinction coefficient of 5.8×10^3 M^{-1} cm^{-1} at 325 nm and pH 7.0 (Blackburn & Schachman, 1976).

A derivative of aspartate transcarbamoylase modified either with PMP or MNP at the active sites also was prepared. The catalytic trimer labeled with PMP was obtained as described above. The reaction of MNP with catalytic subunit was carried out by adding a twofold excess of MNP ($\sim 2 \times 10^{-4}$ M in 1 mM KOH) to catalytic subunit at a concentration of about 2 mg/mL in 40 mM potassium phosphate (pH 7; Evans et al., 1972). After incubation at room temperature for 1 h, the reaction mixture was dialyzed against the same buffer at 4 °C to eliminate unreacted MNP. (Each catalytic polypeptide chain has only one sulfhydryl group (Jacobson & Stark, 1973b).) The resultant modified catalytic subunit has no enzymatic activity, but the addition of mercaptoethanol restores full activity. Reconstitution was done by mixing equal amounts of the MNP and PMP modified catalytic trimers followed by a slight excess of zinc regulatory subunit. Great care was taken to ensure that the protein solution was free from the presence of reducing reagents such as mercaptoethanol. The mixture was dialyzed against 50 mM imidazole-acetate and 0.1 mM zinc acetate (pH 7) at 4 °C overnight (Fan et al., 1975). During the dialysis nitrogen was bubbled into the buffer solution to minimize the oxidation of protein sulfhydryl groups. After concentration over Amicon PM-30 membrane, the protein solution was loaded onto a Sephadex G-200 column (3 cm i.d. × 85 cm) and eluted with 40 mM potassium phosphate (pH 7.0). The identity of the peaks was determined by electrophoresis in 7% polyacrylamide gels in 25 mM Tris-0.2 M glycine (pH 9) (Matsumoto & Hammes, 1975). The peak corresponding to the reconstituted enzyme was collected. The degree of modification of the reconstituted enzyme by MNP, and hence its activity, was quite variable because of the fact that MNP dissociated during the reconstitution and purification. The activity of the reconstituted enzyme ranged from 10 to 30% of that of the unmodified enzyme. After incubation with 2 mM mercaptoethanol, the enzymatic activity was re-

[1] Abbreviations used: H_4pht, 3,4,5,6-tetrahydrophthalic; MNP, 2-mercuri-4-nitrophenol; PHMB, *p*-hydroxymercuribenzoate; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; THP, 3,4,5,6-tetrahydrophthaloyl; $C_iC_jR_3$, reconstituted enzyme with C representing a catalytic (trimer) subunit, R a regulatory (dimer) subunit, and i and j the modification of the catalytic subunit (i, j = PMP, MNP, PHMB, THP, or n (unmodified)).

stored to 50% of the activity of native enzyme indicating that all the MNP on the enzyme was cleaved by the reducing reagent. Because of the instability of this modified enzyme, it was not possible to separate the three species present, i.e., enzyme labeled only with PMP, only with MNP, and with both PMP and MNP.

A statistical mixture of reconstituted enzyme derivatives, $C_{PMP}C_{PMP}R_3$, $C_{PHMB}C_{PHMB}R_3$, and $C_{PMP}C_{PHMB}R_3$ (1:1:2), was prepared by similar procedures. Derivatives containing all six catalytic chains modified with PMP or with MNP also were prepared.

Preparation of all derivatives containing PMP was done in the dark to prevent photochemical decomposition.

Binding Measurements. The binding of PLP to the active sites of the enzyme derivative $C_{PMP}C_nR_3$ was studied by titration of the enzyme with PLP and conversion of the enzyme bound PLP to covalently bound PMP by reduction with $NaBH_4$. Different amounts of PLP were added to the enzyme (1.50 mg/mL) in 50 mM imidazole hydrochloride, 2 mM mercaptoethanol (pH 7), 23 °C, and a 300-fold molar excess of sodium borohydride was added after a 30-min incubation period. The enzyme solution was dialyzed against the same buffer, and the enzymatic activity was measured. The measured activity was compared with that of $C_{PMP}C_nR_3$ subjected to identical treatment except for the addition of PLP. Since modification of the catalytic site with PMP completely abolishes the enzymatic activity (Greenwell et al., 1973), the fraction of activity remaining is equal to the fraction of unliganded catalytic sites at a given concentration of PLP. Polyacrylamide gel electrophoresis (7% cross-linking) of the enzyme after treatment with PLP and $NaBH_4$ was carried out in 25 mM Tris-0.2 M glycine (pH 9) to check for subunit dissociation (Matsumoto & Hammes, 1975).

Spectroscopic Measurements. Absorbance measurements were made either with a Zeiss PMQII or Cary 118 spectrophotometer. All protein solutions were dialyzed against the desired buffer and passed through an HA 0.45-μm Millipore filter before each spectroscopic experiment to remove particulate material. Steady-state fluorescence measurements were carried out with a Hitachi Perkin-Elmer MPF-3 fluorescence spectrometer equipped with corrected spectrum and polarization accessories. Square micro (0.3 × 0.3 cm) or triangular (1 × 1 × 1.4 cm) cuvettes were thermostated at 23 °C. Because of the light sensitivity of pyridoxamine derivatives, a narrow slit width (<2 nm) was used and exposure to the light source was minimized. The absorbance of the solutions used was kept under 0.05 at the exciting wavelength, 325 nm, and the fluorescence emission was observed at 395 nm.

Relative quantum yields were determined by a comparative method (Parker & Rees, 1966) using

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

In eq 1, Q_i is the quantum yield, F_i is the area of the corrected emission spectrum, and A_i is the absorbance at the exciting wavelength. The subscripts 1 and 2 refer to the sample and reference, respectively. The areas of the corrected emission spectra were determined with a computer. The reference was quinine sulfate in 0.1 N H_2SO_4 at 23 °C which was assumed to have an absolute quantum yield of 0.70 (Scott et al., 1970).

In polarization measurements a correction was made for unequal transmission of the horizontal and vertical components of polarized light by the emission monochromator grating (Azumi & McGlynn, 1962).

Fluorescence Lifetime Measurements. Lifetimes were measured with an ORTEC Model 9200 nanosecond fluorescence spectrophotometer interfaced with a PDP 11/20 computer (Digital Equipment Corp.) as previously described (Matsumoto & Hammes, 1975). A colloidal silica (Ludox, HS-30) solution was used to determine the lamp spectrum. A scattering correction was made for measurements carried out in the presence of enzyme by subtracting the photon counts observed from an enzyme solution in the absence of the fluorescing probe from the counts of the sample; equal counting periods were used in both cases. The data were analyzed with the deconvolution and convolution programs provided by ORTEC Inc. in terms of one or two fluorescence lifetimes.

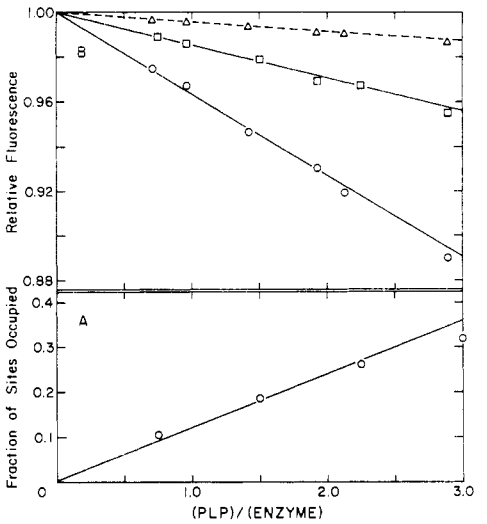

FIGURE 1: Titration of the modified enzymes (1.50 mg/mL) $C_{PMP}C_nR_3$ and $C_{PMP}C_{PMP}R_3$ with PLP in 50 mM imidazole hydrochloride, 2 mM mercaptoethanol (pH 7) at 23 °C. (A) Fraction of catalytic sites occupied vs. the molar ratio of PLP to $C_{PMP}C_nR_3$. (B) Relative fluorescence of $C_{PMP}C_nR_3$ (○) and $C_{PMP}C_{PMP}R_3$ (□) vs. the molar ratio of added PLP to enzyme. The dashed line is the corrected curve for the fluorescence quenching of $C_{PMP}C_{PMP}R_3$ (see the text for details).

Results

Binding Measurements. The addition of PLP to the native enzyme in 50 mM imidazole hydrochloride (pH 7), 2 mM mercaptoethanol at 23 °C showed a maximum change in absorbance at 432 nm with an isosbestic point at 404 nm when the spectrum was compared with that of PLP in buffer. This spectral change reflects binding to both the catalytic and regulatory subunits. The binding of PLP to the active sites of $C_{PMP}C_nR_3$ was studied as described in the Experimental Section, and the results obtained are shown in Figure 1A, where the fraction of catalytic sites occupied is plotted vs. the molar ratio of PLP to $C_{PMP}C_nR_3$. Measurements at a molar ratio greater than 3 were not carried out to avoid extensive labeling of the regulatory subunits which interferes with the energy-transfer measurements and might alter the enzymatic activity. Polyacrylamide gel electrophoresis of the enzyme after treatment with PLP and $NaBH_4$ indicated the native structure of the enzyme was intact.

Spectroscopic Properties of Enzyme Derivatives. A difference spectrum between the enzyme with bound PLP and enzyme without PLP was determined with 21.4 μM enzyme and 28.5 μM PLP; the spectrum of the bound PLP is included in Figure 2. A molar extinction coefficient of 4200 cm^{-1} M^{-1} at 410 nm was calculated assuming two binding sites with a dissociation constant of 4 μM, 6 sites with a dissociation constant of 75 μM, and 12 sites with a dissociation constant of 100 μM, per molecule of enzyme (Suter & Rosenbusch, 1975). A molar extinction coefficient of 3850 M^{-1} cm^{-1} was reported

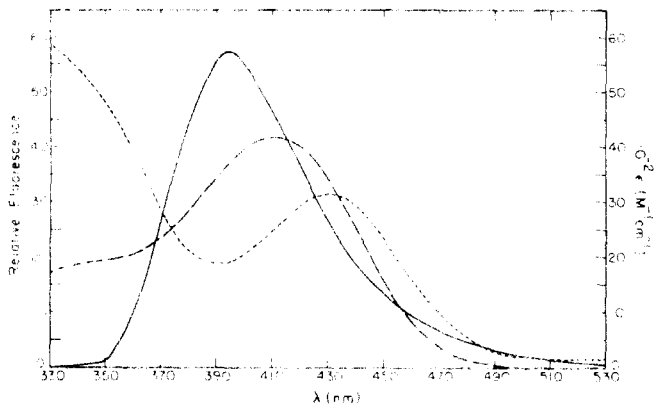

FIGURE 2: Overlap of the pyridoxamine phosphate corrected emission spectrum of enzyme bound PMP (—) and the extinction coefficients of enzyme bound MNP (- - -) and of enzyme bound PLP (- · -) in 50 mM imidazole hydrochloride (pH 7) at 23 °C.

for the PLP catalytic subunit Schiff base at pH 8.0 in triethanolamine (Greenwell et al., 1973). PMP covalently bound to the catalytic subunit of aspartate transcarbamylase has an absorption maximum at 325 nm in 50 mM imidazole hydrochloride (pH 7), and the absorption maximum shifts to near 320 nm in 50 mM sodium borate (pH 8.0). Similar effects of pH on the spectra were observed by Kempe & Stark (1975). When excited at 325 nm, the fluorescence emission maximum was at 395 nm for both pH values. The emission spectrum at pH 7 is included in Figure 2. The quantum yield of the bound PMP on the derivative $C_{PMP}C_{PMP}R_3$ was determined to be 0.23 at pH 7 and 0.20 at pH 8. Essentially no change (<5%) in the fluorescence at 395 nm was observed upon addition of carbamoyl phosphate (up to 0.1 mM), succinate (up to 10 mM), and CTP (up to 1 mM) singly or in combination. At high concentrations (5 mM), ATP significantly increased the fluorescent intensity, especially at pH 8. This same effect was observed with catalytic subunit modified with PMP at the active sites. The absorption maximum also was shifted from near 320 nm to 325 nm after the addition of ATP. Both the fluorescence and absorption changes probably are due to the direct interaction of ATP with the bound PMP rather than to a conformational change (Porter et al., 1969). A similar, but smaller, effect occurs at the highest carbamoyl phosphate concentration used.

The polarization of enzyme bound PMP is 0.28 ± 0.01 for all derivatives at both pH 7 and 8. The limiting polarization of PMP at high viscosities is 0.41 (Churchich, 1965). No significant change in polarization of the bound PMP occurs in the presence of substrates or allosteric effectors (Kempe & Stark, 1975).

The MNP bound to the enzyme has an ionization constant of about 8, and only the ionized form absorbs appreciably at 430 nm (Evans et al., 1972; McMurray et al., 1969). The absorption spectrum of the bound MNP was determined using the reconstituted enzyme having all 6 sulfhydryl groups on the catalytic subunits modified with MNP. A plot of the extinction coefficient vs. wavelength at pH 7 (50 mM imidazole hydrochloride) is shown in Figure 2. Similar data were obtained at pH 8 (20 mM sodium borate), the spectrum obtained is similar to that at pH 7, but the extinction coefficients associated with the bound MNP centered at 430 nm are larger. The extinction coefficient at 430 nm is 8900 M^{-1} cm^{-1} at pH 8 and 3100 M^{-1} cm^{-1} at pH 7.

Fluorescence Lifetime Measurements. Although the nanosecond fluorescence decay curves for PMP bound to ATCase can be described reasonably well by a single lifetime, a more precise fitting is obtained by analyzing the data in terms of two lifetimes according to the equation

$$F(t) = C_1 e^{-t/\tau_1} + C_2 e^{-t/\tau_2} \quad (2)$$

where $F(t)$ is the fluorescence; C_1 and C_2 are the relative amplitudes; and τ_1 and τ_2 are fluorescence lifetimes. An average lifetime, τ_{av}, can be defined as

$$\tau_{av} = (C_1\tau_1 + C_2\tau_2)/(C_1 + C_2) \quad (3)$$

The sum $C_1 + C_2$ was arbitrarily set equal to 1 in the data analysis. The average lifetime of PMP in the absence of PLP (3.2 ns; $C_1 = 0.74$, $\tau_1 = 2.6$ ns, $C_2 = 0.26$, $\tau_2 = 4.8$ ns) is somewhat shorter than the lifetime obtained assuming a single exponential decay (3.8 ns; Mattingly & Hammes, 1975).

Energy-Transfer Measurements. The efficiency of energy transfer, E, from a fluorescent donor to an absorbing acceptor is

$$E = 1 - \frac{Q_{D:A}}{Q_D} = 1 - \frac{F_{D:A}}{F_D} = 1 - \frac{\tau_{D:A}}{\tau_D} \quad (4)$$

where Q, F, and τ denote the quantum yield, fluorescent intensity, and fluorescence lifetime of the donor, and the subscripts D:A and D refer to measurements made in the presence and absence of acceptor, respectively. If the shape of the fluorescence emission spectrum remains constant, the fluorescence (F) at a particular wavelength can be measured rather than the quantum yield (Q). The most precise measurements of energy transfer can be made by an experiment in which the fluorescence (or quantum yield or nanosecond decay curve) is measured, the acceptor is displaced by an optically inert ligand, and the fluorescence characteristics are remeasured, or by an experiment in which the quenching agent is added and binds specifically, and the fluorescence properties are remeasured. If this is done within a single cuvette, systematic errors due to inner filter effects, instrument geometry, etc. are eliminated. In the present instance, carbamoyl phosphate (1 mM) can be used to displace PLP, and mercaptoethanol (5 mM) can be used to displace MNP and PHMB.

Fluorescence quenching titrations were done with the derivative of native enzyme containing one catalytic trimer modified with PMP and the other unmodified. PLP was added to the enzyme solution (1.50 mg/mL) in 50 mM imidazole hydrochloride (pH 7) and 2 mM mercaptoethanol at 23 °C. The fluorescence of bound PMP on the derivative at 395 nm (325 nm excitation) was quenched upon the addition of PLP as shown in Figure 1B. When carbamoyl phosphate (~1 mM) was added at the end of titration, the fluorescence intensity went back to approximately 103% of the original value. When the derivative of the enzyme with all six catalytic sites modified with PMP was titrated with PLP, a much smaller quenching occurs (Figure 1B). Addition of carbamoyl phosphate restored the fluorescence intensity to 102% of its original value. The small quenching occurring in the titration of $C_{PMP}C_{PMP}R_3$ presumably is due to the nonspecific binding of PLP to the regulatory subunit. There are 12 binding sites on the regulatory polypeptides (2 per chain) per enzyme molecule with an approximate dissociation constant of 100 μM (Suter & Rosenbusch, 1975). The amount of PLP bound to the regulatory subunits of $C_{PMP}C_nR_3$ is somewhat less than that bound to $C_{PMP}C_{PMP}R_3$ for a given total amount of PLP since some of the PLP is bound to the catalytic site, and half as many donor (PMP) molecules are present on $C_{PMP}C_nR_3$. If the measured quenching curve for $C_{PMP}C_{PMP}R_3$ is corrected for these fac-

TABLE I: Summary of Energy-Transfer Measurements.

Derivative	pH	R_0 (Å)	E	R_1 (Å)[c]
$C_{PMP}C_{PLP}R_3$	7.0	25.8	0.27[a]	33 (33)
	7.0	25.8	0.36[b]	30 (31)
$C_{PMP}C_{MNP}R_3$	7.0	24.7	0.41[a]	27 (28)
	8.0	27.9	0.56[a]	28 (29)

[a] Determined by changes in quantum yield. [b] Determined by changes in the average fluorescence lifetime. [c] Calculated assuming R_3 = 26 Å or 22 Å (in parentheses).

tors, the dashed line shown in Figure 1 is obtained.[2] The difference between these two curves is the quenching due to binding at the catalytic sites. A plot of the energy transfer efficiency vs. the fraction of catalytic sites occupied by PLP on the derivative $C_{PMP}C_nR_3$ constructed from the difference in the quenching curves is presented in Figure 3. Extrapolation to saturation of catalytic sites with PLP gives an energy-transfer effciency of 0.27. Fluorescence quenching titrations also were carried out in the presence of 0.32 mM CTP or 0.1 mM ATP; within experimental error the transfer efficiency was not altered by these ligands.

Fluorescence lifetime measurements were made before and after addition of PLP. If the average lifetime is assumed to be a measure of energy transfer, an efficiency of 0.36 is calculated when extrapolated to saturation of the catalytic sites, in reasonable agreement with the result obtained from steady state measurements. In this case, corrections for nonspecific binding of PLP cannot be made.

Energy-transfer measurements with the enzyme derivative modified with MNP and PMP is complicated by the instability of the derivative with respect to dissociation of MNP. This prevented isolation of the species $C_{PMP}C_{MNP}R_3$. If the reconstitution of the enzyme is assumed to occur statistically, i.e., $C_{PMP}C_{PMP}R_3$:$C_{MNP}C_{MNP}R_3$:$C_{MNP}C_{PMP}R_3$ = 1:1:2, and, if the MNP is assumed to dissociate equally well from all species, an interpretation of energy transfer measurements can be made. The addition of mercaptoethanol yields a derivative that is 50% active indicating the correct stoichiometry of PMP and MNP. The extent of energy transfer was measured by determining the fluorescence at 395 nm or the quantum yield of the modified enzyme before and after the addition of mercaptoethanol (5 mM). The observed energy transfer was linearly extrapolated to 100% of the catalytic subunit sulfhydryl groups modified with MNP and multiplied by two since only 50% of the PMP is on an enzyme species in which energy transfer is possible. The energy-transfer efficiencies obtained this way were found to be 0.41 at pH 7 (50 mM imidazole hydrochloride) and 0.56 at pH 8 (20 mM sodium borate). As a control, a similar experiment was carried out with a derivative in which PHMB replaced the MNP. The absorption of PHMB does not overlap the fluorescence emission of PMP. The quantum yield and fluorescence intensity were unchanged when PHMB was displaced by mercaptoethanol. Fluorescence lifetime measurements were not carried out with these derivatives.

[2] The quenching correction is made by subtracting the amount of PLP bound to catalytic sites from the total concentration; the quenching of $C_{PMP}C_{PMP}R_3$ for this new concentration can be obtained from the experimentally determined curve in Figure 1. This quenching is then divided by two to correct for the fact that twice as many PMP molecules are present per enzyme molecule in the control experiment. This assumes energy transfer between PMP on one catalytic trimer and a regulatory subunit associated with the other catalytic trimer is negligible.

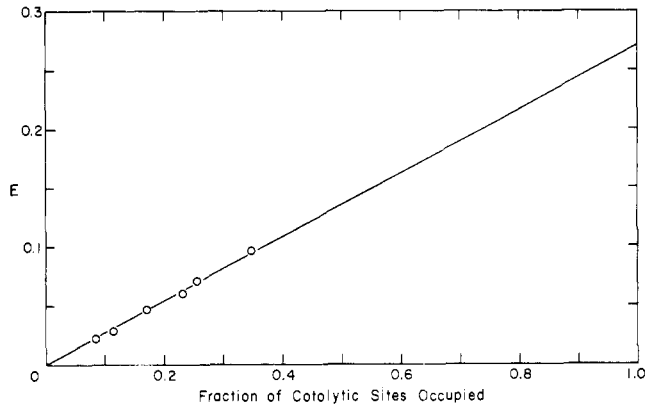

FIGURE 3: A plot of the efficiency of energy transfer, E, of the fluorescence at 395 nm (325 nm excitation) of the derivative $C_{PMP}C_nR_3$ as a function of the percent saturation of the three unmodified active sites with PLP, as derived from Figure 1B, in 50 mM imidazole hydrochlorate, 2 mM mercaptoethanol (pH 7) at 23 °C.

Analysis of Energy-Transfer Results. Energy transfer is characterized by a distance R_0, which is the distance for 50% energy transfer for an isolated donor–acceptor pair (Förster, 1965; Stryer & Haugland, 1967)

$$R_0 = 9.79 \times 10^3 (Q_D \kappa^2 J n^{-4})^{1/6} \text{ Å} \quad (5)$$

In this equation, J is the spectral overlap integral for donor fluorescence and acceptor absorbance, κ^2 is a factor taking into account the orientation between transition dipoles of donor and acceptor, and n is the refractive index of the medium. The spectral overlap of the PMP fluorescence and the MNP and PLP absorbances are shown in Figure 2. The overlap integral was calculated as previously described (Matsumoto & Hammes, 1975). The dipole orientation factor is assumed to be 2/3, which is the value obtained if both the donor and acceptor rotate isotropically and rapidly relative to the fluorescence lifetime. The values of R_0 calculated are summarized in Table I, along with the measured energy-transfer efficiency.

The calculation of the distance between donors and acceptors requires some model of the enzyme structure. In the present case, the overall configuration of the enzyme is known from x-ray crystallography (Warren et al., 1973; Edwards et al., 1974), and both x-ray crystallography and fluorescence energy-transfer measurements (Matsumoto & Hammes, 1975) have been used to establish the spatial relationships between the catalytic sites and sulfhydryl groups within a catalytic trimer. The three active sites can be approximated as an equilateral triangle, and a sulfhydryl group is within a few angstroms of each active site. In the native enzyme, the two triangles of active sites are known to be parallel and for the present also are assumed to be congruent. A schematic drawing of the assumed structure is shown in Figure 4. This structure is defined by the following distances: R_1, the distance between catalytic sites on different subunits that are congruent; R_2, the distance between catalytic sites on different subunits that are not congruent; and R_3, the distance between catalytic sites within a subunit. The distance R_3 has been found to be about 22 Å from x-ray crystallography and about 26 Å from fluorescence energy-transfer measurements, and from the geometry of the model, $R_2 = \sqrt{R_1^2 + R_3^2}$. Therefore, only a single distance is unknown. The energy-transfer efficiency for this model is

$$E = \frac{(R_0/R_1)^6 + 2(R_0/R_2)^6}{1 + \left(\frac{R_0}{R_1}\right)^6 + 2\left(\frac{R_0}{R_2}\right)^6} \quad (6)$$

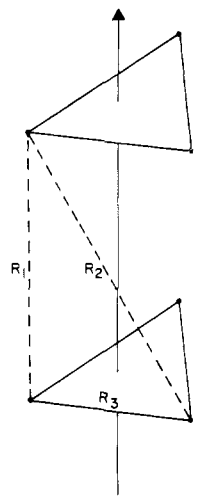

FIGURE 4: Schematic drawing of the location of catalytic sites in aspartate transcarbamoylase. Each triangle represents the three sites within a catalytic subunit, while the relative spacing of the two triangles represents spacing of the catalytic sites of different catalytic subunits within a native enzyme molecule.

The values of R_1 calculated from this equation for the two derivatives are given in Table I assuming R_3 is 26 Å. The values in parentheses assume R_3 is 22 Å.

Discussion

The extent of PLP binding to the active site of the enzyme derivative $C_{PMP}C_nR_3$ is determined by the extent of inhibition by the PLP after the Schiff base is reduced by borohydride. The validity of using the borohydride reduction to determine a binding isotherm has been demonstrated previously by Suter & Rosenbusch (1975). In this work, the additional assumption has been made that the enzyme activity can be used to assess the amount of covalent modification. The only potential problem with this assumption is the alteration of the enzyme activity by covalent modification of the regulatory subunit. However, the estimated amount of binding to regulatory subunits, using the binding constant of Suter & Rosenbusch (1975), is less than 0.7 molecule of PLP per enzyme molecule, and the energy-transfer measurements indicate the binding sites are quite far from the catalytic site. Therefore, the activity assay should provide a good estimate of the extent of PLP binding to the active sites of $C_{PMP}C_nR_3$.

The problems associated with using the Förster theory of fluorescence energy transfer to map ligand binding sites have been discussed previously (Matsumoto & Hammes, 1975; Dale & Eisinger, 1974; Hillel & Wu, 1976). Because of the inverse sixth power dependence of transfer rate on the intramolecular distance, the theory can be used to obtain a fairly good determination of distance even though some uncertainties in the parameters of the theory may exist. In this work κ^2 is assumed to have a value of 2/3, which is the correct value if both the donor and acceptor rotate isotropically and rapidly relative to the fluorescence lifetime. Minimum and maximum values can be estimated for κ^2 using the measured fluorescence polarization of PMP and the model of Dale & Eisinger (1974). The motion of PLP and PMP were approximated to motion over a volume of a cone of half angle 38°. This gives minimum and maximum values of κ^2 of 0.2 and 2.6. The range of R_1 values calculated with these values of κ^2 is 26–42 Å (with R_3 = 26 Å). For the case of energy transfer between PMP and MNP at pH 8, consideration of a similar model gives a range in R_1 values of 22–36 Å. In the present case, the actual value of κ^2 is probably closer to 2/3 than indicated by the maximum and minimum values: each donor transfers its energy to three acceptors on the other catalytic trimer which should randomize the mutual orientation of donor and acceptor.

With the derivative $C_{PMP}C_{MNP}R_3$, the assumption has been made that the reconstitution of the native enzyme yields a statistical distribution of possible structures. While this assumption cannot be rigorously checked, it is unlikely to be seriously in error. This difficulty is not present with the derivative $C_{PMP}C_{PLP}R_3$; a correction for nonspecific binding of PLP must be made but this is small and the necessary correction can be determined experimentally.

The possibility exists that conformational changes are causing the observed changes in fluorescence rather than energy transfer. However, this is unlikely since the fluorescence of PMP is not significantly altered in the presence of ligands binding at the catalytic (carbamoyl phosphate and succinate) or the regulatory (CTP and ATP) site. The absence of fluorescence changes when PHMB is substituted for MNP and is displaced by mercaptoethanol also indicates conformational changes are not influencing the results.

Both the quantum yield and fluorescence lifetime measurements give a similar estimate of the efficiency of energy transfer. The lifetime measurements are complicated by the presence of at least two lifetimes. The use of an average lifetime is the most convenient way to handle this situation. Other types of average lifetimes could be used, but the one we have chosen to use is proportional to the area under the fluorescence decay curve and, therefore, is proportional to the quantum yield. In actuality, a theory for energy transfer which includes a multiple exponential fluorescence decay curve is not available; the application of the simple Förster theory to the present situation represents an assumption, although probably not a serious one.

The structural model assumed, congruent triangles (Figure 4), is consistent with electron microscopy studies (Richards & Williams, 1972). A model has been proposed in which rotation of the catalytic subunits with respect to each other is responsible for regulation of the enzyme activity (Gerhart, 1970). Unfortunately no change in fluorescence or energy transfer is seen when the allosteric ligands ATP and CTP are bound to the enzyme. However, the change in energy-transfer efficiency in going from completely congruent to completely staggered triangles is only about 10% if the planes of the two triangles are 33 Å apart. Thus the structural change predicted by the rotational model may be too small to be seen by the fluorescence energy-transfer experiments. The derivatives studied also may be locked into a particular conformation by the catalytic site modification.

The calculated distance between PLP and the closest PMP on different catalytic subunits is about 33 Å and that between the closest MNP and PMP on different catalytic subunits is comparable although somewhat shorter, 28 Å. The sulfhydryl group modified by MNP has been shown to be within a few angstroms of the catalytic sites (Matsumoto & Hammes, 1975) so that MNP and PLP should bind at essentially the same site. The difference in the calculated distances may be simply a reflection of the experimental uncertainties or the nitrophenol ring of MNP may project into the central cavity bringing it closer to the PMP than the PLP ring. In fact, the similar distances calculated with two different energy acceptors is further evidence that κ^2 does not have values near the possible maxima and minima.

The distance between mercury atoms of MNP bound to sulfhydryl groups on different catalytic subunits determined from x-ray crystallography is significantly longer (42 Å) than

the distances reported here (Evans et al., 1972). The distance between the active sites on different catalytic subunits determined in this work is not much longer than the length of the central cavity measured along the threefold direction with x-ray crystallography (Warren et al., 1973). This and the fact that the distance between active sites on the same catalytic subunit is about 26 Å would mean that the active sites are buried close to the cavity.

References

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

Blackburn, M. N., & Schachman, H. K. (1976) *Biochemistry 15,* 1316.

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

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

Dale, R. E., & Eisinger, J. (1974) *Biopolymers 13,* 1573.

Edwards, B. F. P., Evans, D. R., Warren, S. G., Monaco, H. L., Landfear, S. M., Eisele, G., Crawford, J. L., Wiley, D. C., & Lipscomb, W. N. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 4437.

Evans, D. R., McMurray, C. H., & Lipscomb, W. N. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69,* 3638.

Fan, S., Harrison, L. W., & Hammes, G. G. (1975) *Biochemistry 14,* 2219.

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

Gerhart, J. C. (1970) *Curr. Top. Cell Regul. 2,* 275.

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

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

Gibbons, I., & Schachman, H. K. (1976) *Biochemistry 15,* 52.

Gibbons, I., Yang, Y. R., & Schachman, H. K. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 4452.

Greenwell, P., Jewett, S. L., & Stark, G. R. (1973) *J. Biol. Chem. 248,* 5594.

Hillel, Z., & Wu, C.-W. (1976) *Biochemistry 15,* 2105.

Jacobson, G. R., & Stark, G. R. (1973a) *Enzymes, 3rd Ed. 9,* 225-308.

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

Kempe, T. D., & Stark, G. R. (1975) *J. Biol. Chem. 250,* 6861.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193,* 265.

Matsumoto, S., & Hammes, G. G. (1975) *Biochemistry 14,* 214.

McMurray, C. H., & Trentham, D. R. (1969) *Biochem. J. 115,* 913.

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

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

Porter, R. W., Modebe, M. D., & Stark, G. R. (1969) *J. Biol. Chem. 244,* 1846.

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

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

Rosenbusch, J. P., & Weber, K. (1971a) *Proc. Natl. Acad. Sci. U.S.A. 68,* 1019.

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

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

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

Stryer, L., & Haugland, R. P. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58,* 719.

Suter, P., & Rosenbusch, J. P. (1975) *Eur. J. Biochem. 54,* 293.

Warren, S. G., Edwards, B. F. P., Evans, D. R., Wiley, D. C., & Lipscomb, W. N. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70,* 1117.