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Fluorescence Energy Transfer between Heterologous Active Sites of Affinity-Labeled Aspartokinase of *Escherichia coli*†

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ABSTRACT: The distance between aspartokinase and homoserine dehydrogenase active sites was determined using fluorescence energy transfer between modified substrates. The fluorescent 1,*N*(6)-ethenoadenosine 5′-triphosphate was bound at the kinase active site by Co(III) affinity labeling. Reduced thionicotinamide adenine dinucleotide phosphate quenched the fluorescence of bound nucleotide. Fluorescence depolarization measurements led to a delimitation of the value of the dipolar orientation factor to the range 0.3 to 2.8. The distance between the fluorescent probe and the quencher was 29 ± 4 Å. In the presence of threonine, this distance increased to 36 ± 5 Å. Threonine binding either increased the intersite distance by ca. 7 Å or caused a reorientation of the probe at the dehydrogenase site.

The two catalytic activities of the threonine-sensitive aspartokinase–homoserine dehydrogenase are sequentially located along each of four identical polypeptide chains (Véron et al., 1972, 1973). Each subunit is organized into two discrete domains of enzymatic activity (Véron et al., 1972; Mackall and Neet, 1974). The aspartokinase activity (ATP:L-aspartate 4-phosphotransferase, EC 2.7.2.4) is located in the amino-terminal region. The homoserine dehydrogenase activity (L-homoserine:NADP oxidoreductase, EC 1.1.1.3) resides in a domain at the carboxyl terminus (Véron et al., 1972). Véron et al. (1973) and Mackall and Neet (1974) have proposed models for the quaternary structure of the complex. Both activities are inhibited by threonine, a product of aspartate metabolism (Cohen, 1969). The aspartokinase active site can be affinity labeled with Co(III) (Ryzewski and Takahashi, 1975). This labeling procedure can also be used to attach threonine, aspartate, and ATP (Wright et al., 1976a); such enzyme derivatives lack aspartokinase activity but still retain a partially threonine-sensitive dehydrogenase activity (Ryzewski and Takahashi, 1975; Wright et al., 1976a). The native enzyme complex contains eight binding sites per tetramer for threonine the feedback inhibitor (Falcoz-Kelly et al., 1972; Véron et al., 1973). The threonine–Co(III)–aspartokinase ternary adduct binds only four additional molecules of threonine, one-half the number bound to native enzyme. These affinity-labeled enzymes are, however, appropriate derivatives for investigating the threonine-induced conformational changes in the enzyme complex. Threonine binding to the subclass of regulatory sites not modified by the labeling procedure is apparently respon-

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sible for conformational changes observed (Wright and Takahashi, 1976). Threonine binding increases the size of the nonexchangeable-hydrogen core (Takahashi and Westhead, 1971) and protects all enzyme thiol groups against modification (Truffa-Bachi et al., 1968). Native enzyme binds ATP (Ehrlich and Takahashi, 1973; Wright et al., 1976a) and NADPH (Janin et al., 1969) with nearly identical affinities in the presence and absence of threonine.

The distinctive, spectroscopic qualities of analogue substrates 1,N(6)-ethenoadenosine 5′-triphosphate (ϵATP)[1] and thionicotinamide adenine dinucleotide phosphate (TNADPH) have been used to measure the separation of the kinase and dehydrogenase active sites and the effect of the feedback inhibitor on this distance. The relatively long fluorescent lifetime of ϵATP makes this probe especially suitable for these studies. The fluorescence excitation and emission spectra of ϵATP contain regions which do not overlap the corresponding protein spectra. The fluorophore has a high quantum yield (0.6) and exhibits activity as an adenine nucleotide replacement in several systems (Secrist et al., 1972). Resonance energy transfer observed between the fluorescent ATP analogue immobilized at the kinase site by Co(III) affinity labeling and the analogue of NADPH modified in the nicotinamide ring forms the basis for the experiments described below.

Theory

Förster Energy Transfer. The dipolar interaction between an electronically excited fluorescent donor and an acceptor luminophore leading to long-range resonance energy transfer has been described by Förster (1951, 1959)

$$k_T = \frac{1}{\tau_D}\left(\frac{R_0}{R}\right)^6 \tag{1}$$

where k_T is the rate of energy transfer, τ_D the excited state lifetime in the absence of the acceptor, R the interluminophore separation, and R_0 a characteristic distance of the system. R_0 is dependent on electronic and orientational parameters of the donor–acceptor pair (Förster, 1951, 1959):

$$R_0^6 = \frac{9(\ln 10)\kappa^2\phi_D J(\lambda)}{128\pi^5 n^4 N} \tag{2}$$

Here ϕ_D is the quantum yield of the donor in the absence of the acceptor, $J(\lambda)$ is the overlap integral of the normalized donor fluorescence emission spectrum with the absorption spectrum of the energy acceptor, n is the refractive index of the intervening medium, N is Avogadro's number, and κ^2 is the orientation factor for the dipole–dipole interaction. The overlap integral is given by

$$J(\lambda) = \frac{\int_0^\infty a_m(\lambda)F(\lambda)\lambda^4 d\lambda}{\int_0^\infty F(\lambda)d\lambda} \tag{3a}$$

where $a_m(\lambda)$ is the molar absorptivity of the acceptor and $F(\lambda)$ is the relative fluorescent intensity of the donor at wavelength λ. The orientation factor κ^2 reflects the mutual orientation of donor and acceptor with respect to the interluminophore separation vector and is given by

$$\kappa^2 = [\hat{D}\cdot\hat{A} - 3(\hat{D}\cdot\hat{R})(\hat{A}\cdot\hat{R})]^2 \tag{4}$$

where $\hat{D}$, $\hat{A}$, and $\hat{R}$ are unit vectors along the directions of the transition dipole moments of donor and acceptor and the separation vector, respectively. The limiting values of κ^2 are 0 and 4.

Energy transfer results in a decrease in the steady-state fluorescence intensity of the donor in the presence of the acceptor. The ratio of the observed steady-state fluorescence of the donor in the absence (F_D) and presence ($F_{D,A}$) of the acceptor is related to the donor–acceptor separation (R) and the characteristic distance:

$$\frac{F_D}{F_{D,A}} = 1 + \left(\frac{R_0}{R}\right)^6 \tag{5}$$

Fluorescence Polarization. Previous studies using energy transfer for determining the separation distances of probes on macromolecules have been hampered by an inability to specify suitable estimates of κ^2. Dale and Eisinger (1974, 1975) and Blumberg et al. (1974) have described using fluorescence depolarization measurements to reduce the uncertainty in κ^2 for a variety of reasonable model geometries.

The emission anisotropy r of a fluorescent system excited by linearly polarized light (cf. Dale and Eisinger, 1974, 1975) is defined as:

$$r = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp} \tag{6}$$

The axes of the orthogonal components of the fluorescence emission are parallel ($\parallel$) or perpendicular ($\perp$) to the polarization of the exciting light.

In addition to the quenching of donor fluorescence, three measurements of fluorescence polarization are required: the dynamic depolarization $\langle d'\rangle_d$ of donor and acceptor and the dynamic transfer depolarization $\langle d_T\rangle_d$. The donor dynamic depolarization in this case arises from the noncoincidence of donor absorption and emission transition moments due to the rapid motion of the fluorophore with respect to the macromolecule to which the donor is conjugated. The measured steady state emission anisotropy r (eq 6) is related to r_{0_m} the limiting emission anisotropy in the absence of macromolecular reorientation by

$$r = r_{0_m}\frac{\tau_m}{\tau + \tau_m} \tag{7}$$

where τ_m is the rotational correlation time for the macromolecule and τ is the decay time of total emission.

Finally, r_{0_m} is related to the dynamic depolarization by

$$r_{0_m} = r_f\langle d'\rangle_d \tag{8}$$

where r_f, fundamental anisotropy, is a measure of the noncoincidence of absorption and emission moments within the molecular framework of the donor. The fundamental anisotropy may range from 0.4 (parallel moments) to −0.2. If the dynamic depolarization is interpreted by a model in which the emission dipole moment is free to reorient over the surface of a cone (due to rotation of the fluorophore about a single bond), then $\langle d'\rangle_d$ is related to the half-angle of this cone, Ψ:

$$\langle d'\rangle_d = \left(\frac{3}{2}\cos^2\psi - \frac{1}{2}\right)^2 \tag{9}$$

In a similar fashion one can measure the emission anisotropy of the sensitized fluorescence of the acceptor where the donor has been excited by polarized light. The observed emission anisotropy, a reflection of the angular separation between donor absorption–acceptor emission transition dipole moments, gives the transfer depolarization directly:

[1] Abbreviations used: AK, aspartokinase; ϵ-ADP, 1,N(6)-ethenoadenosine 5′-diphosphate; ϵ-ATP, 1,N(6)-ethenoadenosine 5′-triphosphate; TNADP (H) (reduced), thionicotinamide adenine dinucleotide phosphate; PEI, polyethylenimine.

$$r = 0.4\langle d_T \rangle_d \quad (10)$$

Determination of the parameters ψ and $\langle d_T \rangle_d$ and selection of a suitable model to describe the energy-transfer system enables one to delimit κ^2 extrema. Fluorescence quenching and depolarization measurements were obtained on aspartokinase affinity labeled with a fluorescent substrate.

Materials and Methods

Enzymes. Aspartokinase-homoserine dehydrogenase was isolated from *E. coli* K12 Tir-8 by previously described procedures (Ehrlich and Takahashi, 1973). Purified enzyme was stored as an ammonium sulfate (50% saturation) slurry at 4 °C. The ϵATP-Co(III)-aspartokinase complex was synthesized in a manner similar to the preparation of the corresponding ATP complex (Wright et al., 1976a). Aspartokinase (11 μM) was treated with 12 mM ϵATP-Co(II) and 3 mM H_2O_2 overnight. The extent of modification was determined by measuring the incorporation of [^{14}C]ϵATP. For fluorescence studies, samples of enzyme were dialyzed into the appropriate buffers for 1-2 h (Englander and Crowe, 1965). The buffers used were: 10 mM tris(hydroxymethyl)aminomethane-0.15 M KCl-5 mM threonine-50 μM dithiothreitol (pH 7.6) for fluorescence measurements in the presence of threonine and 10 mM tris(hydroxymethyl)aminomethane-0.5 M KCl-50 μM dithiothreitol (pH 7.6) for studies in the absence of threonine. The higher concentration of potassium in the second buffer is necessary to stabilize the enzyme in the absence of the inhibitor. At pH 7.6 the enzyme exists principally in tetrameric form (Mackall and Neet, 1973).

Analogue Substrates. The ϵATP used for kinetic measurement was obtained from Sigma. The [^{14}C]ϵATP used in formation of Co(III) adducts was synthesized by treating solutions of ATP containing [U-^{14}C]ATP (New England Nuclear) with chloroacetaldehyde at pH 4.2 (Kochetkov et al., 1971; Secrist et al., 1972). The reaction was allowed to proceed until no ATP could be detected by thin-layer chromatography. The product was analyzed spectroscopically using published molar absorptivities (Secrist et al., 1972; P-L Biochemicals Circular OR-10, 1969). The product isolated from several preparations consisted of 96-90% pure ϵATP. Thus the reported stoichiometries for affinity labeling reflect both bound ϵATP and ATP. In kinetic and affinity-labeling studies, the concentration of nucleotide was determined using a molar absorptivity for ϵATP of 2.9 mM^{-1} cm^{-1} at 294 nm (Secrist et al., 1972). Although there was no direct evidence for photodecomposition of ϵATP or its complexes, all solutions were kept in the dark until use.

TNADPH was synthesized enzymatically from TNADP. A procedure similar to that of Anderson and Nordlie (1968) was employed. To a solution containing 50 mM glucose 6-phosphate (Sigma), 2 mg/mL TNADP (Sigma), and 50 mM tris(hydroxymethyl)aminomethane was added baker's yeast glucose-6-phosphate dehydrogenase, EC 1.1.1.49 (type XV, sulfate-free; Sigma), in 20 mM sodium citrate buffer (pH 7.7) to a final concentration of 21 μg/mL. The concentration of TNADPH was determined using a molar absorptivity of 11.7 mM^{-1} cm^{-1} at 399 nm (Stein et al., 1963). Because of the photolability of the reduced cofactor (Anderson et al., 1963), TNADPH was prepared immediately before use and was stored in the dark. The spectrum of TNADPH prepared as described above was identical with published spectra (Stein et al., 1963; P-L Biochemicals Circular OR-18, 1971).

Kinetic Studies. The utilization of ϵATP as a substrate for aspartokinase was followed by a coupled assay (Wampler and Westhead, 1968) since ϵADP is a substrate for pyruvate kinase (Secrist et al., 1972). The amount of pyruvate kinase in the coupling mixture was twice the normal amount. Duplicate assays were performed. This assay cannot discriminate between ϵATP and ADP utilization. The production of ϵADP was confirmed by isolating this product by thin-layer chromatography. The kinase activity was assayed by the hydroxamate procedure (Wampler and Westhead, 1968) except that $FeCl_3$ was not added to the reaction mixture. Aliquots of assay mixtures and controls were spotted on PEI-cellulose plates (F-22, Brinkman). Plates were developed for 90 min (20 cm) by ascending chromatography in the solvent 0.8 M LiCl plus 0.8 M acetic acid. The ϵADP which has a larger R_f than ϵATP was visualized by ultraviolet illumination.

Homoserine dehydrogenase was assayed in the nonphysiological direction using TNADP as substrate. The assay mixture contained 0.1 M tris(hydroxymethyl)aminomethane-0.8 M KCl-25 mM DL-homoserine (pH 9.0) (cf. Olgilvie et al., 1969). The production of TNADPH was monitored at 399 nm. Assays were performed in triplicate. The velocities for both activities are reported as specific activities in units of μmol of product min^{-1} (mg protein)$^{-1}$. Both enzymes were assayed at 30 °C.

Spectra. Absorbance measurements and kinetic analyses were made with a Gilford 240 spectrometer. Spectra were obtained on a Cary 118. Fluorescence measurements were made on a Schoeffel fluorometer equipped with a 1000-W xenon lamp and double monochromators for excitation. The instrument was operated in the ratio mode with the correction function generator. Excitation and emission spectra were corrected. The overlap integral was calculated using eq 3b over the range 340-490 nm with 5-nm intervals:

$$J = \frac{\sum_{\lambda_1}^{\lambda_2} a_m(\lambda) F(\lambda) \lambda^4}{\sum_{\lambda_1}^{\lambda_2} F(\lambda)} \quad (3b)$$

Fluorescence spectra used in quenching measurements were obtained with 325-nm excitation. In quenching experiments the observed fluorescence was corrected for inner filter effects (Brand and Witholt, 1967) and dilution. The change in relative intensity was calculated using a solution containing an identical concentration of native enzyme and TNADPH. The enzyme concentrations were 2-4 μM. Fluorescence polarization spectra were obtained with an air-gap, ultraviolet-grade prism polarizer and a polarizing filter analyzer. Emission anisotropies were corrected for instrumental polarization (Azumi and McGlynn, 1962). All measurements were made at room temperature, ca. 22 °C. Quantum yields were estimated from lifetime measurements made on an Ortec 9200 nanosecond fluorometer.

Results

Both analogues selected for the energy-transfer experiments were demonstrated to function as substrates. Ethenoadenosine 5′-triphosphate (ϵATP) has a K_m of ca. 0.1 mM and a maximal velocity of 0.14 μmol min^{-1} (mg of protein)$^{-1}$ compared with values of (Wright, 1976) 0.2 mM and 13 μmol min^{-1} (mg of protein)$^{-1}$ for ATP (cf. Wampler and Westhead, 1968). The utilization of ϵATP as a substrate was confirmed by the separation of ϵADP from ϵATP by thin-layer chromatography. The reaction is completely inhibited by 7 mM threonine. Thionicotinamide adenine dinucleotide phosphate (TNADP) was assayed as a substrate for homoserine dehydrogenase in the nonphysiological direction. The maximal velocity found was

TABLE I: Ternary ϵATP-Co(III)-Aspartokinase Complexes.

Derivative	Stoichiometry[a]	Specific Activity[b] AK	Specific Activity[b] Dehydrogenase	Inhibition[c] (%)
1	0.85	1.2	40	31
2	1.04	0.1	37	35
3	0.91	0.8	42	32
4	0.88	1.1	35	30

[a] Adenine equivalents incorporated per monomer. [b] In units of μmol min^{-1} (mg of protein)$^{-1}$. The values for native enzyme are ca. 13 for aspartokinase (AK) and ca. 100 for homoserine dehydrogenase. [c] Maximal threonine inhibition of residual homoserine dehydrogenase (85-88% for native enzyme).

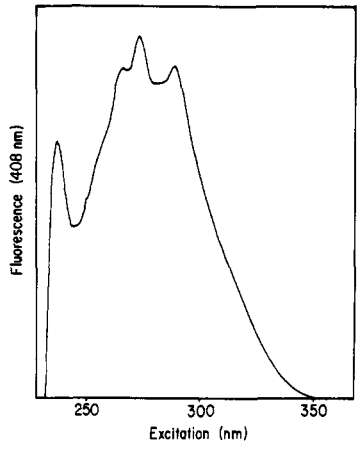

FIGURE 1: The corrected fluorescence excitation spectrum of the ϵATP-Co(III)-aspartokinase complex monitored by emission at 408 nm. This spectrum was obtained in the presence of 0.15 M KCl plus 5 mM threonine. The substitution of 0.5 M KCl produced a spectrum which is identical except for a slight decrease in the peak at 291 nm.

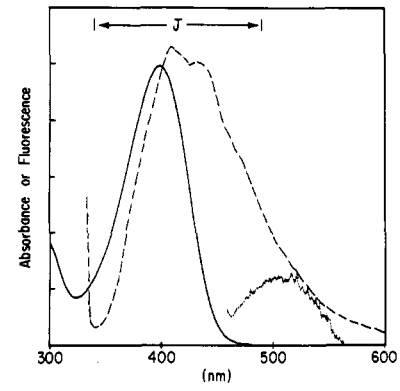

FIGURE 2: The corrected fluorescence emission spectrum of the ϵATP-Co(III)-aspartokinase complex (- - - -). The maxima were at 408 and 432 nm. The spectrum did not change upon substitution of 0.5 M KCl for 0.15 M KCl plus 5 mM threonine in the buffer. The excitation wavelength was 325 nm. Also presented are the absorption (—) and corrected fluorescence emission (···) spectra of TNADPH. The molar absorptivity of TNADPH at 399 nm was 11.7 mM^{-1} cm^{-1}. The region of overlap between the fluorescence emission spectrum of the ϵATP-Co(III)-aspartokinase complex (J) and the absorption spectrum of TNADPH are indicated above the spectra. The value of the overlap integral J was 14.8×10^{36} Å^6/mol. The fluorescence emission spectrum of TNADPH was obtained by excitation at 400 nm in the presence of aspartokinase.

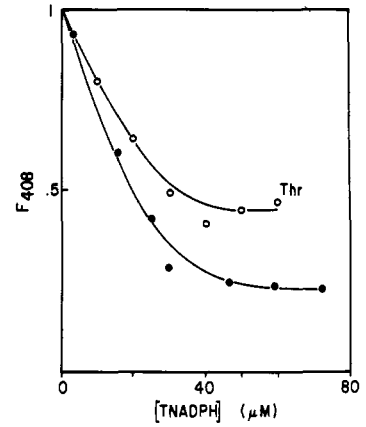

FIGURE 3: The quenching of ϵATP fluorescence at 408 nm (F_{408}) by TNADPH binding at the dehydrogenase site in the ϵATP-Co(III)-aspartokinase complex. The presence of 5 mM threonine plus 0.15 M KCl (●) reduced the efficiency of energy transfer in comparison with that in the presence of 0.5 M KCl (○). The excitation wavelength was 325 nm. The fluorescence is given in arbitrary units.

3.6 μmol min^{-1} (mg of protein)$^{-1}$ (compared with a value of 8 μmol min^{-1} (mg of protein)$^{-1}$ for NADP as the cofactor) with a K_m of 0.2 mM. The presence of 7 mM threonine reduces the maximal velocity to 0.18 μmol min^{-1} (mg of protein)$^{-1}$.

The analogue substrate ϵATP can be attached to the aspartokinase active site via a Co(III) bridge by a previously described affinity-labeling procedure (Wright et al., 1976a). Three derivatives prepared for fluorescence studies are characterized in Table I. The labeling procedure eliminates the aspartokinase activity but leaves a partially threonine-sensitive dehydrogenase activity. Affixing the fluorescent substrate to the enzyme complex eliminates complications introduced by the presence of free fluorescent ligand.

The corrected fluorescence excitation spectrum of the ϵATP-Co(III)-aspartokinase complex is presented in Figure 1. This spectrum combines features of the spectra of ethenoadenosine (Secrist et al., 1972) and aspartokinase (Janin et al., 1969). The maximum at 291 nm may indicate energy transfer from protein tryptophyl residues to ϵATP. The corrected emission spectrum of the ϵATP-Co(III)-aspartokinase complex excited at 325 nm is presented in Figure 2. The spectrum showed some fine structure with maxima at 408 and 432 nm. The emission spectrum of ethenoadenosine shows this same fine structure at pH 7.0 in phosphate buffer (Secrist et al., 1972). Also presented in Figure 2 is the portion of the absorption spectrum of TNADPH which overlaps the emission spectrum of the enzyme conjugated fluorophore. The overlap integral was equal to 14.8×10^{36} Å^6/mol. The fluorescence emission spectrum of TNADPH is also presented in Figure 2. The fluorescence excitation spectrum of TNADPH with a maximum at 400 nm is similar to its absorption spectrum. While free TNADPH is only weakly fluorescent, the fluorescence of enzyme-bound dinucleotide was enhanced (data not presented). The presence of 5 mM threonine quenches the fluorescence of this complex in the region of TNADPH emission. Threonine has a similar effect upon the fluorescence of enzyme-bound NADPH (Janin et al., 1969). In contrast, the fluorescence emission spectrum of the ternary ϵATP-Co(III)-aspartokinase complex is unaffected by the addition of threonine. Thus the quantum yield of ϵATP in the affinity-labeled enzyme is unchanged by the addition of threonine.

When freshly prepared TNADPH is added to solutions of the ternary complex, the fluorescence emission at 408 nm of the conjugated ϵATP is quenched (Figure 3). The fluorescence

TABLE II: Emission Anisotropy of Donor and Acceptor Fluorescence.

	λ_{em}	r^a
ϵATP-Co(III)-AK	408	0.088 ± 0.004
ϵATP-Co(III)-AK	432	0.089 ± 0.003
ϵATP-Co(III)-AK + Thr	408	0.092 ± 0.003
ϵATP-Co(III)-AK + Thr	432	0.095 ± 0.004
ϵATP-Co(III)-AK + Thr + TNADPH	432	0.079 ± 0.004
ϵATP-Co(III)-AK + Thr + TNADPH	517	0.079 ± 0.004
ϵATP-Co(III)-AK + TNADPH	517	0.077 ± 0.004

[a] The value of the emission anisotropy, r, measured for excitation at 325 nm and with emission at the indicated wavelength (λ_{em}). To solutions of the ϵATP-Co(III)-aspartokinase complex were added 5 mM threonine and 30 μM TNADPH. The enzyme complex fluorescence was at 408 and 432 nm. The TNADPH fluorescence was at 432 and 517 nm.

TABLE III: Summary of Data Used in Estimating κ^2.[a]

	Parameter		
Luminophore	No Thr	5 mM Thr	
	ϵATP		
$\langle r \rangle$	0.088 ± 0.004	0.092 ± 0.005	eq 6
τ	25 ns	25 ns	*b*
τ_M	550 ns	550 ns	*c*
r_{0_m}	0.092	0.096	eq 7
r_f	0.37	0.37	*d*
$\langle d' \rangle_d$	0.25	0.26	eq 8
ψ	35°	35°	eq 9
	TNADPH		
r_T	0.077 ± 0.004	0.079 ± 0.004	eq 6
$\langle d_T \rangle_d$	0.192	0.198	eq 10

[a] κ^2_{min} (θ): 0.3 (72°), 0.6 (29°). κ^2_{max} (θ): 0.9 (70°), 2.8 (22°). The values of the two permissible extrema (Dale and Eisenger, 1974) are indistinguishable for the data from systems with and without threonine. [b] An upper limit based on the fluorescence lifetime of ϵATP in the ternary complex. [c] Estimated from Heck (1974) assuming $\tau_m = \tau_{h/3}$, where τ_h is the harmonic mean relaxation time. [d] Taken from data for ethenoadenosine in propylene glycol at −50 °C, 325 nm (Secrist et al., 1972).

intensity at 432 nm was not used in energy-transfer measurements since the sensitized fluorescence of the acceptor TNADPH contributes to the observed intensity at this wavelength. This interference is not as severe in the presence of threonine, however. In the absence of threonine, the quenching or the efficiency of energy transfer is 77 ± 4%. However, in the presence of 5 mM threonine the efficiency of energy transfer is reduced to 50 ± 3%. The values of the TNADPH quenching of immobilized ϵATP fluorescence are the averages obtained with the three separate ϵATP-Co(III)-aspartokinase preparations. The magnitudes of the quenching observed in the absence and presence of threonine suggest the interluminophore separations are $0.82R_0$ and $1.00R_0$ (eq 5), respectively. R_0 is the critical distance in eq 2. The evaluation of R_0 was accomplished through fluorescence polarization measurements.

Determination of κ^2 and R_0. The emission anisotropies for the fluorescent emission at the two ϵATP maxima under different conditions are in Table II. These values are for excitation at 325 nm and represent the limiting values of the emission anisotropy at the long wavelength edge of the excitation spectrum. There was a slightly positive, monotonic dependence of emission anisotropy on exciting wavelength. A similar dependence is observed with ethenoadenosine (Secrist et al., 1972). The emission anisotropy of ϵATP of 0.09 in the presence and absence of threonine indicates that the probe still retains some reorientational freedom after attachment to the macromolecule even in the presence of the feedback inhibitor. Also, the emission spectrum of the protein conjugate is identical in shape and intensity in the presence of 0.15 M and 0.5 M KCl. Our assessment of probe mobility was supported by the observations of Secrist on ethenoadenosine. The Stokes shift (26 kcal) of this derivative is not altered by changes in solvent polarity but by changes in solvent viscosity. A decrease in the Stokes shift is viewed as arising from a restriction in nuclear reorientation with a corresponding decrease in vibrational relaxation of the excited state. Rigid positioning of the imidazopurine moiety of ϵATP at the kinase active site could evoke a similar decrease in the Stokes shift. We observe only a small hypsochromic shift of 7 nm for the ϵATP-Co(III)-aspartokinase complex relative to the fluorescence maximum of ethenoadenosine. A minimal requirement for ϵATP mobility to be manifest in the protein complex would be rotational freedom about the N(3)-C(1′) bond.

In contrast to the mobility ascribed to ϵATP, we allot no reorientational freedom to the acceptor. As the acceptor is also a substrate, albeit a poor one, the thionicotinamide ring is probably rigidly held in the dinucleotide binding cleft at the dehydrogenase active site. These assumptions regarding luminophore mobilities permit the selection of a donor-acceptor orientation model. The model employed is described as follows: one luminophore (TNADPH) is fixed, $\langle d' \rangle_d = 1$, at an angle θ to the separation vector while the other is free to reorient over the surface of a cone of half angle ψ (model 3, Dale and Eisinger, 1974).

Using the values for the emission anisotropy of ϵATP in the presence or absence of threonine (Table II), equations 7 to 9, and the values of additional factors reported in Table III, the value of ψ is found to be 35° in both cases.

The transfer depolarization was obtained from the emission anisotropy of the sensitized acceptor fluorescence (Table II). The transfer depolarization was calculated on the assumption that TNADPH absorption and emission transition dipole moments are parallel (eq 10). This is likely, as resonance energy transfer occurs on the long wavelength edge of the TNADPH excitation spectrum (excitation λ_{max} = 400 nm, for observation at 432 or 517 nm). We also assume that no depolarization occurs due to reorientation of an excited and previously enzyme-bound acceptor in solution; that is, that the residence time of TNADPH at the homoserine dehydrogenase active site is longer than the excited lifetime. There are no significant contributions of the directly excited fluorescence of TNADPH to the observed sensitized fluorescence since free and enzyme-bound TNADPH do not fluoresce appreciably with 325-nm excitation. The anisotropies observed at the short- and long-wavelength edges of the TNADPH emission spectrum in the presence of the affinity-labeled enzyme and threonine are identical (Table II), suggesting that a limiting value is observed.

The acceptor emission anisotropies at 517 nm for excitation with polarized light at 325 nm were also similar in the presence, 0.079, or absence of threonine, 0.077. These values correspond to transfer depolarizations of 0.198 (5 mM threonine) and 0.192 (no threonine). Using the previously determined values of ψ and the transfer depolarizations above, the maximum and minimum values of κ^2 were determined with the corresponding values of θ, the angle which the transition moment of the ac-

ceptor TNADPH makes with the separation vector (cf. model 3, Dale and Eisinger, 1974). These results, along with the data employed to estimate them, are presented in Table III. The value of κ^2 is restricted to the range 0.3–2.8. This delimitation significantly restricts the value of R, the separation distance, since this distance is proportional to the sixth root of κ^2.

The value of R_0, the characteristic distance, was calculated using eq 2. The overlap integral $J(\lambda)$ obtained was 14.8×10^{36} Å^6/mol. The determination of κ^2 (0.3–2.8) was described above. The quantum yield of the ϵATP-Co(III)-aspartokinase complex was estimated from lifetime measurements of ϵATP fluorescence in this complex. The average lifetimes of ϵATP in this complex in the presence of 0.5 M KCl or 0.15 M KCl plus 5 mM threonine were 25.0 and 24.6 ns, respectively. Using the values of 23 ns for the average lifetime of free ϵATP and 0.59 for its quantum yield (Secrist et al., 1972) and assuming that the natural lifetimes of free and bound ϵATP were the same, a value 0.64 was obtained for the quantum yield under both conditions. The value of the refractive index in the wavelength region of the energy transfer is assumed to be 1.4. The energy transfer could occur through the solvent or through the protein matrix. The refractive indices near 400 nm of water, cyclohexane, and formamide are 1.34, 1.44, and 1.47, respectively (Sober, 1968). The possible range of variation in the refractive index would alter R_0 less than 1%. The characteristic distance is 35.8 ± 5.2 Å. The range in R_0 reflects the uncertainty for κ^2.

The separation between the ϵATP at the kinase active site is 29 ± 4 Å. In the presence of threonine this distance increases to 36 ± 5 Å. If in fact both probes were rigidly fixed on the protein, a maximum value of κ^2 may be estimated from $\langle d_T \rangle_d$ (model 1, Dale and Eisinger, 1974). This value of 2.8 increases R_0 by 15% and permits only the estimation of the maximum value of R.

Discussion

The results of fluorescence quenching and fluorescence depolarization measurements interpreted using a reasonable model describing the mutual orientation of donor and acceptor fluorophores have permitted estimation of the distance between the two types of active sites. The depolarization measurements eliminated the need for a priori estimations to be made of a value for the dipole-dipole orientation factor κ^2. The delimitation of possible values of κ^2 obtained enables us to estimate the distance between a probe at the kinase active site and a dehydrogenase substrate to be 29 ± 4 Å. Heck (1974) has shown that electric birefringence and sedimention data were consistent with aspartokinase being a prolate ellipsoid of revolution with semimajor and semiminor axes of approximately 120 and 50 Å, respectively. Such hydrodynamic estimates can, of course, only give an approximate idea of the macromolecular dimensions.

Our estimate based on fluorescence measurements corresponds to the separation of the ethenoadenosine and thionicotinamide moieties. Aspartokinase binds only 1 equiv of NADPH per monomer (Falcoz-Kelly et al., 1972; Heck and Truffa-Bachi, 1970), the binding site corresponding to the dehydrogenase active site. In contrast, however, we have located another class of ATP binding sites in the dehydrogenase region in addition to the kinase active sites (Wright and Takahashi, 1977). This somewhat nonspecific, lower affinity site may be the dinucleotide binding cleft. Attachment of ϵATP by Co(III) affinity labeling restricts the ϵATP probe to the kinase active site. The site of affinity labeling in fact corresponds to the aspartokinase active site because labeling of the enzyme is paralleled by kinase inactivation, and labeling also results in the specific incorporation of aspartate, a kinase cosubstrate (Wright et al., 1976a). Mild proteolysis of the labeled enzyme releases an ATP-Co(III)-peptide complex from the kinase domain (Wright et al., 1976b). We are thus confident that the probes are appropriately located at kinase and dehydrogenase active sites. This distance of 29 ± 4 Å of course represents a minimal estimate of the distance between the sites if both inter- and intrasubunit quenching occurs. We cannot easily separate out the effects of the quenching of donor fluorescence by more than one acceptor since the observed quenching may possibly represent the interaction of acceptors with different separations and different orientations.

Assuming that the 29-Å distance is the kinase-dehydrogenase separation for the enzyme in an active conformation, the separation distance in the presence of threonine (enzyme inhibited) is increased by 7 Å. This change is of the order of the uncertainty in the two distance measurements due to the residual indeterminacy in κ^2. However, the differences in quenching observed may reflect either real changes in dimension of the enzyme or changes in the donor–acceptor orientation. The actual κ^2 values for the complexes in the presence and absence of threonine may be quite similar (see also Hillel and Wu, 1976). The transition of the enzyme from the active to the inhibited form may be accompanied by a change in intersite distance. Others have observed this transition as the protection of thiol groups (Truffa-Bacchi et al., 1966, 1968), inertness to proteolytic enzymes (Véron et al., 1972), and as an increase in the size of the nonexchangeable hydrogen core (Takahashi and Westhead, 1971). The sedimentation coefficient of aspartokinase is the same in the presence and absence of threonine under conditions where the tetramer predominates (Janin et al., 1969; Wampler, 1972; Mackall and Neet, 1973). On the other hand, threonine binding may be altering κ^2. Since the emission anisotropy of the stably bound ϵATP is not significantly altered by the addition of threonine, the change in κ^2 may result from a conformational change in the dehydrogenase region. This conformational change may explain the reduced catalytic efficiency of the dehydrogenase in the presence of threonine.

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Sulphydryl Group Modification of Aspartate Aminotransferase with 3-Bromo-1,1,1-trifluoropropanone during Catalysis†

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ABSTRACT: After protection of cysteine-45 and -82 with iodoacetamide or *N*-ethylmaleimide, and in the presence of saturating concentrations of substrates, the supernatant isozyme of pig heart aspartate transaminase has been covalently modified at cysteine-390 with 3-bromo-1,1,1-trifluoropropanone. The modified enzyme retains 60–70% of the initial specific activity and is similar to native enzyme in pH and temperature stability. After tagging cysteine-390 with the fluorinated compound, the enzyme retains substrate and inhibitor binding abilities, as shown by direct spectrophotometric titration of the active-site chromophores. The ^{19}F NMR spectrum of the modified enzyme has been obtained by a Fourier transform NMR method. Although the transaminase is a dimeric enzyme, ^{19}F bound at each subunit's cysteine-390 gives rise to only a single ^{19}F resonance upfield from that of trifluoroacetic acid. The fact that the chemical shifts of the ^{19}F probe differ in native and guanidine hydrochloride (Gdn-HCl) denatured enzyme is interpreted as the effect of the native protein groups on the probe. The discordance between the changes induced by varying concentrations of Gdn-HCl on the ^{19}F resonance parameters, on the one hand, and the changes in enzyme activity and prosthetic group absorbance, on the other, suggests that, in aspartate transaminase, cysteine-390 lies in an environment dissimilar from that of the active-site components.

Chemical modification of enzymes is used as a tool to determine amino acid residues essential for activity, and for the introduction of various probes into macromolecules to obtain structural and/or functional information. Supernatant aspartate aminotransferase (EC 2.6.1.1) has five cysteine residues per subunit, but only three react with alkylating agents unless the enzyme is completely denatured. The inaccessible cysteine residues have been identified as cysteine-191 and -252 (Birchmeier et al., 1973; Wilson et al., 1974; Zufarova et al., 1973). Of the three remaining cysteines residues, cysteine-45 and -82 react 10^5 times faster than Cys-390 (Birchmeier et al., 1973; Stankewicz et al., 1971).

The relationship of the chemically accessible sulphydryl groups to enzymatic activity has been studied in detail. Cysteine residues 45 and 82 can be blocked with a variety of al-

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