Fluorescence Energy Transfer between Ligand Binding Sites on Chloroplast Coupling Factor 1†

Lewis C. Cantley, Jr.,‡ and Gordon G. Hammes*

ABSTRACT: The method of fluorescence energy transfer is used to measure the distance from the tight nucleotide binding sites to the 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole reactive sites on solubilized spinach chloroplast coupling factor 1 (CF_1). The fluorescent adenine nucleotide analogs 1,N^6-ethenoadenosine diphosphate and 1,N^6-ethenoadenylyl imidodiphosphate were used as donors and 4-nitrobenzo-2-oxa-1,3-diazole bound to a tyrosine group and to an amino group were used as acceptors of energy transfer. Using three different donor–acceptor pairs, the distance measured varied from 38 to 43 Å assuming both donor sites are equidistant from the acceptor site. A model is proposed for the location of the tight nucleotide binding sites and the active site on the α and β subunits of CF_1.

The purified coupling factor (CF_1)[1] solubilized from spinach chloroplasts has a molecular weight of 325,000 (Farron, 1970) and can be separated into five different subunits by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Lien et al., 1972). The α, β, γ, δ, and ϵ subunits have molecular weights of 59,000, 56,000, 37,000, 17,500, and 13,000, respectively. Electron microscopy studies have revealed that solubilized CF_1 is spherical in shape with a diameter of approximately 100 Å (Howell and Moudrianakis, 1967). Studies with subunit specific antibodies suggest that the α and γ subunits are intimately involved in the photophosphorylation activity of CF_1 (Nelson et al., 1973); however, a trypsin-digested enzyme containing only the α and β subunits is capable of high ATPase activity (Deters et al., 1975). The ATPase activity of CF_1 can be abolished by reacting NBD-Cl with one or two tyrosine groups on the β subunit (Deters et al., 1975). Binding experiments (Cantley and Hammes, 1975) have suggested a single active site for ATP hydrolysis on the heat-activated CF_1. The solubilized enzyme also contains two tight, noncatalytic binding sites for ADP, ϵADP, AMP-PNP, and ϵAMP-PNP which have been postulated to act as allosteric conformational switches for the ATPase activity (Cantley and Hammes, 1975).

In this work, fluorescence energy transfer was used to measure the distance from the tight nucleotide binding sites to the NBD-Cl reactive site on solubilized CF_1. The fluorescent nucleotide analogs, ϵADP and ϵAMP-PNP, were used as donors and NBD bound to a tyrosine group and bound to an amino group were used as acceptors of energy transfer. A distance of approximately 40 Å was measured from the tight nucleotide binding sites to the NBD site and a model is proposed for the location of the tight nucleotide binding sites and active site on the α and β subunits of CF_1.

Experimental Section

Materials. The [^{3}H]ϵADP and [^{3}H]ϵAMP-PNP were prepared as previously described (Cantley and Hammes, 1975). The quinine sulfate was purchased from Aldrich Chemical Company and the NBD-Cl from Pierce Chemical Company. The [^{3}H]NBD-Cl (8 Ci/mol) was a gift from Dr. D. Deters. The Ludox HS-30 colloidal silica was obtained from E. I. duPont de Nemours and Company. All other chemicals were the best available commercial grade, and all solutions were prepared with deionized distilled water.

CF_1, NBD-Tyr-CF_1, and NBD-N-CF_1 Preparations. Spinach coupling factor 1, CF_1, was prepared by known procedures (Lien and Racker, 1971). An extinction coefficient of 0.476 ml/(mg cm) at 280 nm in 0.1 *M* NaCl–50 m*M* Tris-Cl–2 m*M* $MgCl_2$ (pH 8.0), 23° was used to measure protein concentrations of CF_1 solutions free of exchangeable nucleotides. A molecular weight of 325,000 (Farron, 1970) was used to determine molar concentrations of enzyme.

The NBD-Cl modified CF_1 in which the NBD moiety is attached to one or two tyrosine groups/mole of enzyme, NBD-Tyr-CF_1, was prepared as described elsewhere (Cantley and Hammes, 1975). The NBD-N-CF_1 was prepared from NBD-Tyr-CF_1 as follows. An ammonium sulfate (50% saturated) suspension of NBD-Tyr-CF_1 (~1.3 mol/mol) was centrifugated for 10 min at 4°, 18,000*g*. The pellet was dissolved in a minimum amount of 0.025 *M* sucrose–10 m*M* Tris-Cl–2 m*M* EDTA (pH 8.0) and eluted through a Sephadex G-25 (medium) column (1.0 cm i.d. × 50 cm) equilibrated with the same buffer. The effluent was monitored by passing it through a microflow cell (Helma Cell, Inc., type 178-QS, 10-mm path length) in a Cary 14 recording spectrophotometer, and the protein fraction absorbing at 280 nm was collected. Sufficient ATP (0.2 *M*, pH 7.5) was added to the protein fraction to give a final concentration of 5 m*M* ATP, and the pH was raised to 9.0 by titrating with 0.5 *M* NaOH. The solution was incubated for 8 hr at 37°, then 5 μl of 2-mercaptoethanol was added to remove any remaining NBD-Tyr. An equal volume of saturated ammonium sulfate was added, and the solution was cooled on ice and centrifugated as above. The pellet was again passed through the Sephadex column, and the protein fraction was collected, precipitated with ammonium sulfate,

† From the Department of Chemistry, Cornell University, Ithaca, New York 14853. *Received December 28, 1974.* This work was supported by a grant from the National Institutes of Health (GM 13292).

‡ National Science Foundation Predoctoral Fellow, 1971–1974. National Institutes of Health Predoctoral Trainee (GM 00834), 1974–1975.

[1] Abbreviations used are: CF_1, chloroplast coupling factor 1; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; AMP-PNP, adenylyl imidodiphosphate; ϵADP, 1,N^6-ethenoadenosine diphosphate; ϵAMP-PNP, 1,N^6-ethenoadenylyl imidodiphosphate; NBD-Tyr-CF_1, NBD-Cl reacted with the phenolic oxygen of a tyrosine moiety on CF_1; NBD-N-CF_1, NBD-Cl reacted with an amino group on CF_1.

and centrifugated as above. The pellet was dissolved in a minimum amount of 0.1 *M* NaCl–50 m*M* Tris-Cl–2 m*M* $MgCl_2$ (pH 8.0) and eluted through a small Sephadex G-25 (medium) column (0.7 cm i.d. × 25 cm) equilibrated with the same buffer. The protein fraction was collected in a final volume of approximately 2 ml. This procedure gave an enzyme solution (~5 μM) with an absorbance peak at 485 nm, but with no 390-nm absorbance peak, indicating that NBD has transferred to an amino group on the protein. A difference spectrum extinction coefficient of 1.62×10^4 M^{-1} cm^{-1} at 470 nm was determined for NBD-N using [^{3}H]NBD-Cl of known specific activity.

Steady-State Fluorescence Measurements. The steady-state fluorescence measurements were made with a Hitachi-Perkin-Elmer MPF-3 fluorescence spectrophotometer equipped with corrected spectrum and polarization accessories. A comparative method (Parker and Rees, 1966) was used to determine the quantum yield of ϵADP in 0.1 *M* NaCl–50 m*M* Tris-Cl–2 m*M* $MgCl_2$ at pH 8.0, 23°. Equation 1 gives the ratio of quantum yields, Q_i, as a function of

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

the area of the corrected emission spectrum, F_i, and the absorbance at the exciting wavelength, A_i, for two different fluorescing compounds. Quinine sulfate in 0.1 *N* H_2SO_4 was used as a standard and was assumed to have an absolute quantum yield of 0.70 (Scott et al., 1970) at 23°. Both samples had an absorbance less than 0.005 at the exciting wavelength, 320 nm, and the area of the corrected emission spectrum was determined by cutting out and weighing the recorded spectrum.

In the fluorescence polarization measurements, a correction was made for unequal transmission of horizontally and vertically polarized light by the emission monochromator grating (Azumi and McGlynn, 1962). In polarization measurements of fluorescent probes in the presence of enzyme, corrections were made for light scattering. For fluorescence polarization measurements involving ϵADP or ϵAMP-PNP, the excitation wavelength was 320 nm, and the emission was monitored at 400 nm.

The change in fluorescence (320-nm excitation, 400-nm emission) with time of [^{3}H]ϵADP or [^{3}H]ϵAMP-PNP accompanying binding to CF_1, NBD-Tyr-CF_1, or NBD-N-CF_1 was measured as previously described (Cantley and Hammes, 1975). After 2 hr, the free ligand and total ligand concentrations were measured (in about 10 min) using the forced dialysis technique (Cantley and Hammes, 1973). Diaflow XM-50 membranes were used and the radioactivity in the solutions was measured in 10 ml of scintillation fluid (Bray, 1960) using a Beckman LS-255 liquid scintillation counter.

Fluorescence Lifetime Measurements. Fluorescence lifetimes were measured with an ORTEC Model 9200-nsec fluorescence spectrophotometer as previously described (Matsumoto and Hammes, 1975). The excitation wavelength, 320 nm, was selected with a monochromator, and a 3-cavity interference filter centered at 435.8 nm (Ditric Optics, Inc.) was used to filter the emitted light. Triangular (1 cm × 1 cm × 1.4 cm) and square micro (0.3 cm × 0.3 cm) cuvets were thermostated at 23°. A colloidal silica (Ludox HS-30) solution was used to determine the lamp spectrum. In measurements requiring long periods of counting time (>30 min), a correction was made for noise picked up randomly across all channels. A scattering correction was made for measurements carried out in the presence of enzyme by photon counting an enzyme solution in the absence of the fluorescing probe for an equal period of time and subtracting the counts from those of the sample. Data were analyzed on a PDP-11 computer with the deconvolution and convolution programs provided by ORTEC.

Results

The theory of energy transfer between fluorescing and absorbing species was developed by Förster (1959, 1965). The energy transfer which occurs is related to the distance between the donor–acceptor pair according to eq 2. In this

$$R = R_0(E^{-1} - 1)^{1/6} \quad (2)$$

equation E is the efficiency of energy transfer (defined below) and R_0 is the "critical transfer distance" at which E is 50%. The value of R_0 may be calculated from known properties of the energy donor and acceptor by eq 3. In this

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

equation, n is the refractive index of the medium, Q_D is the quantum yield of the donor, K is an orientation parameter dependent on the relative orientations of the donor emission dipole and the acceptor excitation dipole, and J is the spectral overlap integral of donor fluorescence and acceptor absorbance. In the calculations to follow K^2 was assumed to have a value of ⅔ which is the calculated value if the donor and acceptor dipoles rotate rapidly compared to the fluorescence lifetime of the donor (Förster, 1959). The validity of this assumption is discussed later. The quantum yield of the donor when bound to the enzyme, Q_D, was used to calculate R_0. The value of n was taken as the refractive index of water, 1.4, and the overlap integral was approximated by eq 4, where the summation was carried out over 5-nm intervals. In this equation, $F_D(\lambda)$ is the corrected fluorescence emission of the donor and $\epsilon_A(\lambda)$ is the extinction coefficient of the acceptor. The efficiency of energy transfer is defined in terms of the rate constants for deexcitation processes, k_i, and may be written in terms of the quantum yield or lifetime of the donor in the presence and absence of energy transfer as in eq 5. In this equation, $k_{D\to A}$ is the rate constant for energy transfer, the sum is over all deexcitation processes, $Q_{D\to A}$ and $\tau_{D\to A}$ are the quantum yield and fluorescence lifetime of the donor in the presence of the acceptor, and Q_D and τ_D are the quantum yield and fluorescence lifetime of the donor in the absence of transfer.

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

$$E = k_{D\to A}/\left(\sum_i k_i\right) = 1 - Q_{D\to A}/Q_D = 1 - \tau_{D\to A}/\tau_D \quad (5)$$

Both ϵADP and ϵAMP-PNP have been shown to bind to CF_1 at two apparently identical "tight" sites. Reacting 1 mol of NBD-Cl/mol of CF_1 (NBD-Tyr-CF_1) only slightly alters the dissociation constant for ϵAMP-PNP binding to these sites, but causes some heterogeneity in the ϵADP binding (Cantley and Hammes, 1975). The steady-state fluorescence quenching of ϵAMP-PNP by both CF_1 and NBD-Tyr-CF_1 was measured by utilizing the slow rate of ϵAMP-PNP binding to the two tight sites of the enzyme. Figure 1 shows the results of a typical steady-state fluorescence quenching experiment: the change in fluorescence with time is shown after mixing ϵAMP-PNP and CF_1 in 0.1 *M* NaCl–50 m*M* Tris-Cl–2 m*M* $MgCl_2$ at pH 8.0, 23°. The quantum yield of ϵAMP-PNP bound to the enzyme,

Table I: Energy Transfer Parameters.

Donor	Q_D[a]	τ_D[a] (nsec)	Acceptor	R_0[b] (Å)	E[c]	R_2[d] (Å)	R_1[e] (Å)	Method
ϵADP	0.40	15	NBD-Tyr	32.4	0.17	42.2	36.2	Fluorescence quenching
			NBD-N	35.6	0.39	38.3	28.8	Fluorescence quenching
ϵAMP-PNP	0.47	20	NBD-Tyr	33.4	0.21	41.4	34.9	Fluorescence quenching
					0.19	42.7	36.3	Fluorescence lifetime

[a] From measurements of donor bound to CF_1 in absence of acceptor. [b] Assuming $K^2 = 2/3$. [c] Efficiency per mole of acceptor assuming equal quenching of both donors. [d] Assuming two donor sites equidistant from acceptor site (efficiency given in table). [e] Assuming only one donor site close to acceptor site (twice efficiency given in table).

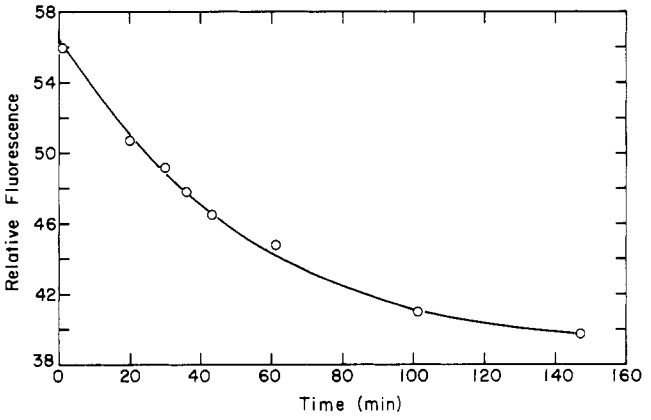


FIGURE 1: The change in fluorescence at 400 nm (exciting at 320 nm) accompanying the binding of ϵAMP-PNP (4.5 μM initial concentration) to CF_1 (23.7 μM initial concentration) vs. time in 0.1 M NaCl-50 mM Tris-Cl-2 mM $MgCl_2$ (pH 8.0), 23°.

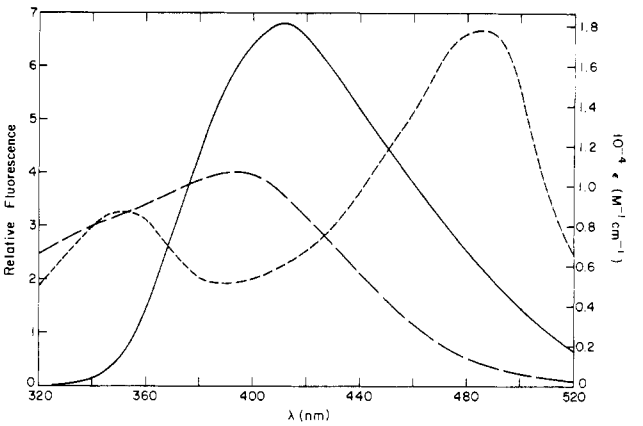


FIGURE 2: Overlap of the ϵADP corrected emission spectrum excited at 320 nm (—) with the difference extinction coefficient (ϵ) of NBD-Tyr (- -) and with the difference extinction coefficient (ϵ) of NBD-N (- - - -) in 0.1 M NaCl-50 mM Tris-Cl-2 mM $MgCl_2$ (pH 8.0), 23°.

Q_b, was calculated with eq 6 where Q_f is the quantum yield

$$Q_b = [Q_f/(L_b)][(L_T)F_t/F_0 - (L_f)]/[1 - r_b/4] \quad (6)$$

of ϵAMP-PNP free in solution, L_b, L_f, and L_T are the bound ligand concentration, free ligand concentration, and total ligand concentration, F_t and F_0 are the fluorescence emission of ϵAMP-PNP at the time of the binding measurement and at the time of mixing, respectively, and r_b is the anisotropy of the bound ligand. The factor $[1 - r_b/4]$ is a correction for polarized emission (Shinitzky, 1972). The anisotropy was calculated from the polarization, P, using the relationship $r = 2P/(3 - P)$, and the polarization was calculated as described below. The quantum yield of bound ϵAMP-PNP was measured on both CF_1 and NBD-Tyr-CF_1 under conditions where the number of moles of ϵAMP-PNP bound per mole of enzyme varied from 0.13 to 1.1. The quantum yield of ϵAMP-PNP in 0.1 M NaCl-50 mM Tris-Cl-2 mM $MgCl_2$ (pH 8.0), 23°, is 0.59. The quantum yield of ϵAMP-PNP bound to CF_1 in the above buffer is 0.47, and the quantum yield of ϵAMP-PNP bound to NBD-Tyr-CF_1 (1.36 mol/mol; NBD/CF_1) is 0.34. For ϵAMP-PNP binding to both CF_1 and NBD-Tyr-CF_1, a slight increase in the quantum yield occurred as the number of moles bound per mole of enzyme increased; however, this change was within the experimental uncertainty (<10%). Using eq 5 and assuming equal quenching of both donors, the efficiency of energy transfer is 0.28. As will be discussed later, the two NBD sites are probably very close together; therefore the efficiency of transfer per mole of acceptor is $0.28/1.36 \simeq 0.21$.

The fluorescence lifetime measurements were complicated by the fact that the lifetime of the free ϵAMP-PNP is longer than the lifetime of ϵAMP-PNP bound to either CF_1 or NBD-Tyr-CF_1. A double exponential deconvolution of the data allows a good fit to the longer lifetime but a very poor fit to the shorter lifetime. Again use was made of the slow dissociation rate of ϵAMP-PNP from the enzyme. The enzyme (either CF_1 or NBD-Tyr-CF_1) was equilibrated with saturating amounts of [^{3}H]ϵAMP-PNP (~200 μM) in 0.1 M NaCl-50 mM Tris-Cl-2 mM $MgCl_2$ at pH 8.0, 23° overnight. The enzyme was then precipitated with ammonium sulfate, centrifugated, and passed through the small Sephadex column as described in the Experimental Section. The fluorescence lifetime was immediately measured (photon counting time <3 hr). After the lifetime measurement, the ratio of free ϵAMP-PNP concentration to bound ϵAMP-PNP concentration was measured by the forced dialysis technique (Cantley and Hammes, 1973). During this period of time, less than 10% of the ligand dissociated from the enzyme, and the number of moles of ϵAMP-PNP bound per mole of enzyme was typically 0.3. The lifetime was determined using the best single exponential reconvoluted fit to the data, which implicitly assumes both donors are equally quenched. The lifetime of ϵAMP-PNP in 0.1 M NaCl-50 mM Tris-Cl-2 mM $MgCl_2$ at pH 8.0, 23° is 25.8 nsec. The lifetime of ϵAMP-PNP bound to CF_1 in the above buffer is 20 nsec, and the lifetime of ϵAMP-PNP bound to NBD-Tyr-CF_1 (1.33 mol/mol; NBD/CF_1) is 15 nsec. According to eq 5, the efficiency of transfer is 0.25, and the efficiency of transfer per mole of acceptor (NBD-Tyr) is 0.19.

Using 0.47 for the donor quantum yield and the overlap integral calculated from the spectra in Figure 2, and assuming $K^2 = 2/3$, the value of R_0 for the donor-acceptor pair is 33.4 Å. The distances calculated with this value of R_0 and eq 2 assuming both donor sites equidistant from the acceptor, R_2, and assuming one donor site much closer to the acceptor, R_1, are summarized in Table I.

If the polarization of the fluorescence of the ligand free in solution is small compared with the polarization of the fluorescence of the ligand bound to the enzyme, then the polarization of bound ligand fluorescence may be calculated from eq 7. In this equation, P is the measured polarization,

$$1/P = 1/P_b + \frac{(I_V + I_H)_f}{(I_V + I_H)_b P_b}((L_f)/(L_b)) \quad (7)$$

P_b is the polarization of the bound ligand, $(I_V + I_H)_f$ and $(I_V + I_H)_b$ are the sums of the horizontal and vertical components of the fluorescence of the ligand free in solution and bound to the protein, respectively, and (L_f) and (L_b) are the concentrations of the ligand free in solution and bound to the protein, respectively. In Figure 3, a plot of $1/P$ vs. $(L_f)/(L_b)$ is presented for ϵAMP-PNP binding to CF_1. The line was determined by a least-squares fit to the data, and a value of P_b of 0.25 was determined from the intercept. The fluorescence of NBD-Tyr is too small to measure the polarization accurately because of light scattering from the protein.

The steady-state fluorescence quenching of ϵADP on binding to CF_1, NBD-Tyr-CF_1, and NBD-N-CF_1 was measured in an analogous manner to the quenching of ϵAMP-PNP. The quantum yield of bound ϵADP was measured on all three proteins with the number of moles of ϵADP per mole of protein varying from 0.23 to 1.3. The quantum yield of ϵADP in 0.1 M NaCl–50 mM Tris-Cl–2 mM $MgCl_2$ (pH 8.0), 23°, is 0.59. The quantum yield of ϵADP bound to CF_1 under the same conditions is 0.40, the quantum yield of ϵADP bound to NBD-Tyr-CF_1 (1.03 mol/mol; NBD/CF_1) is 0.33, and the quantum yield of ϵADP bound to NBD-N-CF_1 (0.56 mol/mol; NBD/CF_1) is 0.31. A slight increase (~15%) in the quantum yield of ϵADP bound to NBD-Tyr-CF_1 occurred as the number of moles of ϵADP bound per mole of protein increased. This change in quantum yield may reflect the heterogeneity of ϵADP binding to NBD-Tyr-CF_1 (Cantley and Hammes, 1975). A smaller change (<10%) in the quantum yield of bound ϵADP as a function of moles of ligand bound per mole of protein was found for the other two proteins, CF_1 and NBD-N-CF_1. According to eq 5 and again assuming both donors are quenched equally, the efficiency of energy transfer from ϵADP to NBD-Tyr is 0.17, and the efficiency per mole of acceptor is 0.165. The efficiency of energy transfer from ϵADP to NBD-N is 0.22, and the efficiency per mole of acceptor is 0.39.

Because of a slow decomposition of ϵADP by the enzyme (Cantley and Hammes, 1975) it was not possible to measure the fluorescence lifetime of ϵADP bound to the enzyme in the absence of free ligand as was done with ϵAMP-PNP. The best fit double exponential reconvolution to the data gave lifetimes of 15 and 26 nsec for ϵADP equilibrated with CF_1 in 0.1 M NaCl–50 mM Tris-Cl–2 mM $MgCl_2$ (pH 8.0) at 23°. The lifetime of ϵADP in the above buffer is 25.8 nsec, in agreement with the double exponential fit; however, the error in the shorter lifetime (20%) made an accurate determination of energy transfer impossible. If the donor quantum yield is taken as 0.40, the overlap integral is calculated from the spectra in Figure 2, and K^2 is assumed to have a value of $\frac{2}{3}$, the values of R_0 for the donor–acceptor pairs ϵADP-NBD-Tyr and ϵADP-NBD-N are 32.4 and 35.6 Å, respectively. The distances calculated with these parameters and eq 2 assuming both donor sites equidistant from the acceptor, R_2, and assuming one donor site much closer to the acceptor, R_1, are included in Table I. The po-

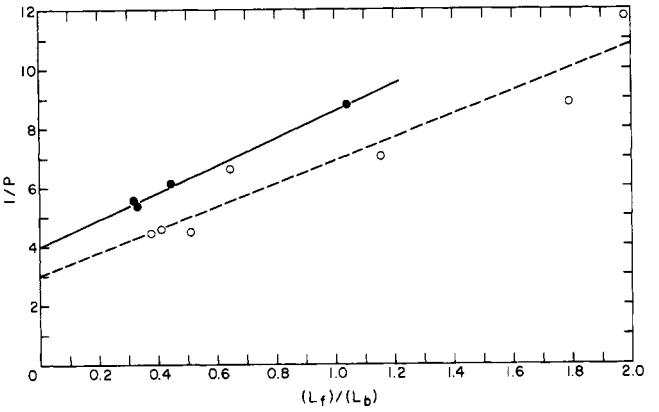


FIGURE 3: A plot of $1/P$ vs. $(L_f)/(L_b)$ accompanying the binding of ϵAMP-PNP (●) and ϵADP (○) to CF_1 in 0.1 M NaCl–50 mM Tris-Cl–2 mM $MgCl_2$ (pH 8.0), 23°. Here P is the measured polarization and (L_f) and (L_b) are the concentration of ligand free in solution and the concentration of ligand bound to the enzyme, respectively. The protein concentration varied from 5.5 to 7.5 mg/ml and the total ligand concentration varied from 3 to 65 μM.

larization of ϵADP fluorescence bound to CF_1 was determined from a least-squares fit of the data in Figure 3 to eq 7. The value of P_b obtained from the intercept is 0.33. The scatter in the data reflects a decrease in the sensitivity of this plot as the quantum yield of the bound ligand decreases with respect to the quantum yield of the free ligand. The polarization of the fluorescence of NBD-N-CF_1 (excitation at 468 nm, emission at 524 nm) is 0.415, and the fluorescence lifetime is about 2 nsec in 0.1 M NaCl–50 mM Tris-Cl–2 mM $MgCl_2$ (pH 8.0) at 23°. The polarization of ϵADP fluorescence in the same buffer is 0.002.

Discussion

Although the theory of fluorescence energy transfer (Förster, 1959, 1965) has been tested on systems of known geometry (Latt et al., 1965; Stryer and Haugland, 1967), the practical application of the theory to measure distances between specific ligand binding sites on proteins involves several assumptions. First, the donor and acceptor probes must be bound specifically to the ligand binding sites; second, the donor fluorescence quenching must be due to energy transfer to the acceptor and not to some other change in the environment of the donor induced by the acceptor binding. Third, some assumption must be made about the dipole orientation factor, K^2, used to calculate R_0, the critical transfer distance. Finally, a model must be assumed to calculate the distances between sites in the case of multiple donor or acceptor sites. These assumptions will now be considered in detail for the system under investigation here.

In an accompanying paper (Cantley and Hammes, 1975) results were presented indicating a single active site for ATP hydrolysis exists on solubilized CF_1, and the results of both binding and steady-state kinetics experiments indicate that a single mole of NBD-Cl reacted/mol of CF_1 blocks this catalytic site. Deters et al. (1975) have demonstrated that the NBD-Tyr site is on the β subunit of CF_1. Although the subunit stoichiometry of CF_1 has not been established, the amino acid composition and molecular weights of the subunits (Nelson et al., 1973; Farron, 1970) are consistent with two α subunits (59,000 molecular weight), two β subunits (56,000 molecular weight), and one γ subunit (37,000 molecular weight). The facts that NBD-Cl reacts with a second tyrosine group at a much slower rate and that only

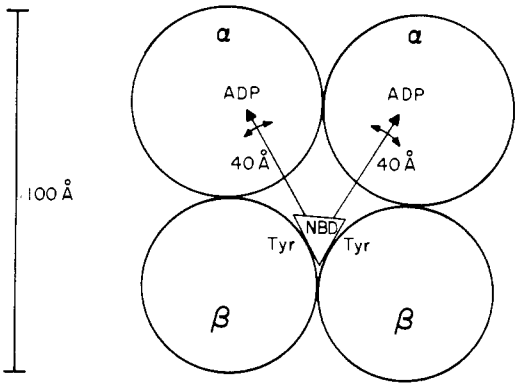


FIGURE 4: Schematic cross-sectional diagram of the α and β subunits of CF_1. The proposed active site is represented by a triangle.

one active site exists suggest that the active site is between the two β subunits, and the two tyrosine groups are close together. An intramolecular S → N transfer has been noted for NBD-Cl reacting with cysteine (Birkett et al., 1970). The kinetics of the Tyr → N transfer of NBD observed in this work is consistent with an intramolecular process indicating that the NBD-N also is close to the active site.

The results of binding experiments (Cantley and Hammes, 1975) indicate that two apparently identical "tight" sites exist on CF_1 which bind ADP, AMP-PNP, and the fluorescent analogs, ϵADP and ϵAMP-PNP. These sites are not directly active in ATP hydrolysis on the solubilized enzyme but appear to act as allosteric conformational switches for the ATPase activity of solubilized CF_1. Reacting 1 or 2 mol of NBD-Cl/mol of CF_1 only slightly increases the dissociation constant of AMP-PNP at these sites (from 1.35 to 2.34 μM; 0.1 M NaCl, 50 mM Tris-Cl, and 2 mM $MgCl_2$, pH 8.0, 23°) but induces an apparent heterogeneity in ADP or ϵADP binding to these sites. However, the binding of ϵADP to NBD-N-CF_1 is identical with the binding of ϵADP to CF_1 (unpublished results). Thus, the assumption that the immediate environment of the donor is unaffected by the presence of the acceptor seems valid in the case of the donor–acceptor pairs ϵADP-NBD-N and ϵAMP-PNP-NBD-Tyr but may be questioned for the pair ϵADP-NBD-Tyr.

The most uncertain quantity in calculating the critical transfer distance, R_0, is the dipole orientation factor, K^2. Although theoretically K^2 can vary between 0 and 4, the extreme values require very rigid specific orientations. If both the donor and acceptor rotate rapidly in comparison to the lifetime of the donor excited state, then K^2 equals $\frac{2}{3}$ (Förster, 1959). If only the donor is free to rotate, then K^2 can vary from $\frac{1}{3}$ to $\frac{4}{3}$ (Wu and Stryer, 1972). This range of K^2 changes $R_0 \pm 11\%$ from the value obtained using $K^2 = \frac{2}{3}$. For a solid solution of fixed but random orientation of donor and acceptor dipoles, the average value of K^2 is 0.475 (Maksimov and Rozman, 1962). Using 100 Å as the spherical diameter (Howell and Moudrianakis, 1967), a rotational relaxation time of about 300 nsec can be calculated for CF_1. Since the probes studied have fluorescence lifetimes less than 20 nsec, any decrease in fluorescence polarization from the polarization in the absence of rotation, P_0, is due to local rotation. For the chromophore 1,N^6-ethenoadenine, P_0 is approximately 0.36 (320-nm excitation; Secrist et al., 1972). The fluorescence polarizations of ϵADP and ϵAMP-PNP bound to unmodified CF_1 are 0.33 and 0.25, respectively. The depolarization indicates some rotation of the chromophores occurs, especially in the case of ϵAMP-PNP. The lower polarization observed for ϵAMP-PNP is consistent with its longer fluorescence lifetime (20 nsec) and higher quantum yield (0.47) when compared to ϵADP bound to unmodified CF_1 (15-nsec lifetime; 0.40 quantum yield). These two chromophores have identical fluorescence spectra and lifetimes when free in solution and bind to the same sites on CF_1; however, they probably induce different conformational changes on binding to the enzyme, as has been suggested for the natural ligands ADP and ATP (Cantley and Hammes, 1975), causing different fluorescence properties of the bound chromophores. The fluorescence quenching of these probes on binding to CF_1 indicates a nonpolar environment at the nucleotide binding sites (Secrist et al., 1972). The fluorescence polarization of NBD bound to an amino group on CF_1 is 0.415. This is quite close to the maximum limit for polarization, 0.5, indicating very little rotation occurs during the fluorescence lifetime of NBD-N (~2 nsec), although some rotation may occur during the longer lifetime of the donor, ϵADP (15-nsec lifetime). The low fluorescence intensity of NBD-Tyr did not allow determination of its polarization. Since free rotation of both donor and acceptor cannot be assumed for this system, three sets of donor–acceptor pairs were used to measure the same distance. The good agreement between the distances measured with the three donor–acceptor pairs assuming $K^2 = \frac{2}{3}$ (Table I) suggests that K^2 is close to this average value in all cases.

In Figure 4, a minimal model is proposed for the nucleotide binding sites on CF_1. Two α subunits and two β subunits are proposed. The enzyme lacking the three smaller subunits has been shown to have a high ATPase activity (Deters et al., 1975). A single active site is indicated between the β subunits, and the tyrosine groups which react with NBD-Cl are placed adjacent to the active site. The "tight" nucleotide binding sites are located about 40 Å from the active site, consistent with the fluorescence energy transfer results, and have been tentatively located on the α subunits. The large distance between the "tight" nucleotide sites and the active site indicates that any control which these sites may manifest on the active site must involve a conformational change of the protein. Experiments are currently underway to determine the subunits containing the "tight" nucleotide binding sites and the overall subunit structure using affinity labels and cross-linking reagents.

References

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

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Birkett, D. J., Price, N. C., Radda, G. K., and Salmon, A. G. (1970), *FEBS Lett. 6*, 346.

Cantley, L. C., Jr., and Hammes, G. G. (1973), *Biochemistry 12*, 4900.

Cantley, L. C., Jr., and Hammes, G. G. (1975), *Biochemistry*, preceding paper in this issue.

Deters, D. W., Nelson, N., Nelson, H., and Racker, E. (1975), *J. Biol. Chem. 250*, 1041.

Farron, F. (1970), *Biochemistry 9*, 3823.

Förster, T. (1959), *Discuss. Faraday Soc. 27*, 7.

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

Howell, S. H., and Moudrianakis, E. N. (1967), *Proc. Natl. Acad. Sci. U.S.A. 58*, 1261.

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

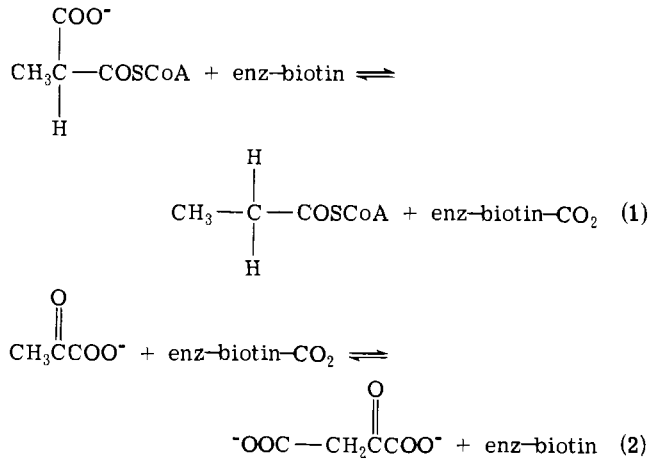
Lien, S., Berzborn, R. J., and Racker, E. (1972), *J. Biol. Chem. 247,* 3520.
Lien, S., and Racker, E. (1971), *Methods Enzymol. 23,* 547.
Maksimov, M. Z., and Rozman, I. M. (1962), *Opt. Spectrosc. (USSR) 12,* 337.
Matsumoto, S., and Hammes, G. G. (1975), *Biochemistry 14,* 214.
Nelson, N., Deters, D. W., Nelson, H., and Racker, E. (1973), *J. Biol. Chem. 248,* 2049.
Parker, C. A., and Rees, W. T. (1966), *Analyst 85,* 587.
Scott, T. G., Spencer, R. D., Leonard, N. J., and Weber, G. (1970), *J. Am. Chem. Soc. 92,* 687.
Secrist, J. A., III, Barrio, J. R., Leonard, N. J., and Weber, G. (1972), *Biochemistry 11,* 3499.
Shinitzky, M. (1972), *J. Chem. Phys. 56,* 5979.
Stryer, L., and Haugland, R. D. (1967), *Proc. Natl. Acad. Sci. U.S.A. 58,* 719.
Wu, C.-W., and Stryer, L. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1104.

Stereochemistry of Propionyl-Coenzyme A and Pyruvate Carboxylations Catalyzed by Transcarboxylase†

Yak Fa Cheung, Chien H. Fung, and Christopher Walsh*

ABSTRACT: The stereochemistry of the two half-reactions catalyzed by the biotin-containing enzyme, transcarboxylase from *Propionobacteria shermanii,* has been determined. The *pro-R* hydrogen at C-2 of propionyl-coenzyme A is replaced by CO_2 in formation of the *S* isomer of methylmalonyl-CoA, defining the process as retention of configuration. This C-2 hydrogen is abstracted at a rate identical with product formation. For the other half-reaction, pyruvate to oxalacetate, the chiral methyl group methodology of Rose (I. A. Rose (1970), *J. Biol. Chem. 245,* 6052) was employed. First, it was determined with [3-$^2H_3$]pyruvate that a kinetic deuterium isotope effect of 2.1 occurs at V_{max} in this carboxyl transfer, indicating that the necessary requirement for discrimination against heavy isotopes of hydrogen existed. Then, 3(*S*)-[3-^{2}H,^{3}H]pyruvate, generated from 3(*S*)-[3-^{2}H,^{3}H]phosphoglycerate, was carboxylated and the oxalacetate trapped as [3-^{3}H]malate using malate dehydrogenase. Exhaustive incubation of the tritiated malate ($^3H/^{14}C$ = 1.95) with fumarase to labilize the *pro-R* hydrogen at C-3 resulted in release of 65% of the tritium into water. Reisolation of the malate after fumarase action yielded a $^3H/^{14}C$ ratio of 0.67, indicating 34% retention as expected. The theoretical enantiotopic distribution for the observed k_{1H}/k_{2H} of 2.1 is 68:32. Selective enrichment of tritium in the *pro-R* position at C-3 of malate indicates enzymatic carboxylation of pyruvate with retention of configuration in this half-reaction also.

Transcarboxylase (methylmalonyl-CoA:pyruvate carboxyltransferase, EC 2.1.3.1) is a metalloenzyme, containing covalently bound biotin, which has been purified from *Propionobacteria shermanii* (Wood et al., 1969). This multimeric enzyme catalyzes the carboxyl transfers of eq 1 and 2. This sequence has been supported by kinetic studies (Northrop, 1969), by partial exchange reactions (Northrop and Wood, 1969), and by the isolation and subsequent reutilization of the carboxybiotinyl-enzyme intermediate (Wood et al., 1963). As with other biotin carboxylases [e.g., bacterial acetyl-CoA carboxylase (Polakis et al., 1974)], it is reasoned that acyl-CoA substrates bind to regions of the active site distinct from regions where keto acid substrates bind (quite probably on distinct subunits) with the biotinyl carrier protein serving as a swinging arm mechanism (Northrop, 1969).



On the other hand, transcarboxylase is an unusual biotin enzyme in at least one sense: ATP and bicarbonate are not substrates, there is no carboxyl activation step, and no energy input is required. In a mechanistic sense it is as though transcarboxylase were composed, in addition to the biotinyl carrier subunit, of the substrate specific subunits of two other well-known ATP-dependent biotin carboxylases, pyruvate carboxylase and propionyl-CoA carboxylase.

† From the Departments of Chemistry and Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 (Y.F.C. and C.W.), and the Institute for Cancer Research, Fox Chase, Pennsylvania 19111 (C.F.). *Received January 22, 1975.* This investigation was supported by Research Grant GM 20011 from the National Institutes of Health, and Sloan Research Traineeship (Y.F.C.) administered by the M.I.T. Graduate School, National Science Foundation Grant GB-27739x, and U.S. Public Health Service Postdoctoral Fellowship GM-53808 (C.H.F.).