Proximity of Reactive Cysteine Residue and Flavin in *Escherichia coli* Pyruvate Oxidase As Estimated by Fluorescence Energy Transfer†

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ABSTRACT: Pyruvate oxidase of *Escherichia coli* possesses a reactive cysteine residue believed to be associated with the thiamin pyrophosphate (TPP) binding site. This residue is not reactive in the presence of TPP. Exposure of the enzyme to cysteine-directed fluorescent reagents results in the formation of fluorescent protein conjugates. Although these reagents do not react solely with the TPP-protectable cysteine residue, the fluorescence emission spectrum of a probe attached to this residue can be obtained by a difference technique. It was determined that the fluorescence emission of probes at the TPP-protectable site is very low due to energy transfer to the FAD coenzyme and that this fluorescence is greatly enhanced upon reduction or extraction of the flavin. Application of fluorescence energy transfer theory enabled the determination of an upper limit for the distance between the probes at the TPP-protectable site and the flavin adenine dinucleotide (FAD) (roughly 20 Å). Thus, the TPP binding site and the FAD coenzyme are likely in close proximity.

Pyruvate oxidase is a peripheral membrane enzyme from *Escherichia coli* that catalyzes the oxidative decarboxylation of pyruvate to yield acetate, CO_2, and reducing equivalents (Hager, 1957). Located on the inner surface of the cytoplasmic membrane, the enzyme donates electrons to the aerobic respiratory chain of *E. coli* (Hager, 1957; Shaw-Goldstein et al., 1978). The purified enzyme is assayed with the aid of artificial electron acceptors such as ferricyanide.

Pyruvate oxidase is a tetramer composed of identical 60 000-dalton subunits (O'Brien et al., 1976). Each of these subunits possesses a tightly bound flavin adenine dinucleotide (FAD)[1] cofactor (Williams & Hager, 1966) and binds a second coenzyme, thiamin pyrophosphate (TPP), in the form of a divalent metal ion complex (O'Brien et al., 1977).

The initial step of pyruvate oxidase catalysis is the formation of a pyruvate–TPP adduct in the TPP binding site. It has been postulated that this adduct is first decarboxylated and then directly oxidized by the FAD coenzyme (R. Blake, II, M. Mather, L. P. Hager, M. H. O'Leary, and R. B. Gennis, unpublished results). A close proximity between the TPP binding site and the tightly bound flavin would be consistent with this scheme. Recent chemical modification studies have demonstrated the presence of a single reactive cysteine residue that is somehow involved in the binding of TPP to pyruvate oxidase (Koland & Gennis, 1982). This paper describes the labeling of this TPP-protectable cysteine residue with fluorescent thiol reagents and the estimation of the distance between this cysteine residue and the FAD coenzyme by fluorescence energy transfer methods.

Materials and Methods

The reagents MIANS and 1,5-IAEDANS were obtained from Molecular Probes and Aldrich, respectively. Purity was verified by thin-layer chromatography. All manipulations involving the reagent 1,5-IAEDANS were performed under red light. Quinine sulfate and Rhodamine B were purchased from Eastman, and TPP was from Sigma.

Pyruvate oxidase was isolated as previously described (O'Brien et al., 1976) and enzymic activity determined by the standard ferricyanide assay (Russell et al., 1977). All chemical modifications were performed at 25 °C.

Flavin Extraction. The FAD chromophore of pyruvate oxidase was extracted by the acid–ammonium sulfate procedure of M. Recny and L. P. Hager (unpublished results). Complete extraction of the flavin requires a repetition of this procedure, which inevitably leads to a significant denaturation of the protein. Thus, only one extraction was performed on each sample, which resulted in the removal of approximately 80% of the original FAD coenzyme. After extraction, the apoenzyme was redissolved in 0.1 M sodium phosphate buffer at pH 7.0.

Absorption and Fluorescence Emission Spectra. For all optical absorption and fluorescence emission measurements samples were held in thermostated cells at 25 °C. A Varian Cary 219 absorption spectrophotometer and a Perkin-Elmer MPF-44A fluorescence spectrophotometer were used in these studies. Both instruments were interfaced with an LSI-11 minicomputer, which enabled digitization of spectra at 0.5-nm intervals and facilitated subsequent manipulations of absorption and emission spectra.

Fluorescence emission spectra were first corrected by subtraction of the spectrum of a solvent blank. When quantum yields were to be estimated, emission spectra were also corrected for the variation in response of the instrument as a function of emission wavelength. A method analogous to that of Parker (1968) was used, employing a Rhodamine B quantum counter and a magnesium oxide reflector. Quantum yields were then determined by comparison of the integrated intensities of the corrected emission spectra of the fluorophore in question with that of a quinine sulfate standard of equal optical density. The value 0.55 was used for the quantum yield of the standard (Melhuish, 1961).

Fluorescence Polarization Measurements. Fluorescence polarization was measured with a T-format photon-counting

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[1] Abbreviations: FAD, flavin adenine dinucleotide; TPP, thiamin pyrophosphate; MIANS, 6-(4-maleimidylanilino)naphthalene-2-sulfonic acid; 1,5-IAEDANS, 5-[[[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid; Q, observed quantum yield of fluorescence; Q_0, hypothetical quantum yield of fluorescence in the absence of excitation energy transfer; r, distance in angstroms between fluorophore and energy acceptor; R_0, critical energy transfer distance in angstroms (see eq 2); J, spectral overlap integral (see eq 3); κ, dipole–dipole orientation factor [see Förster (1948)]; n, index of refraction; f, normalized fluorescence yield; ϵ, molar extinction coefficient; λ, wavelength of light in centimeters.

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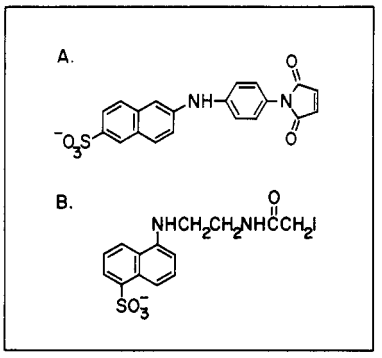

FIGURE 1: Chemical structures of fluorescent probes used in these investigations: (A) MIANS and (B) 1,5-IAEDANS.

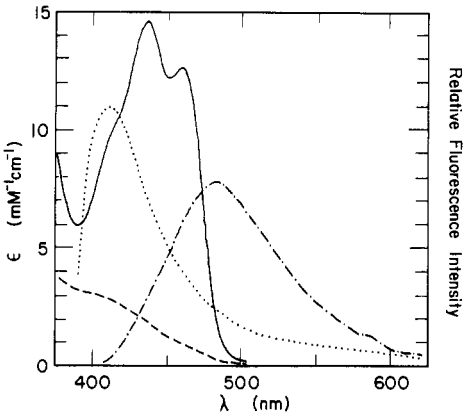

FIGURE 2: Absorption spectra of oxidized (—) and reduced (– –) pyruvate oxidase and fluorescence emission spectra of MIANS-labeled (···) and 1,5-IAEDANS-labeled (–·–) pyruvate oxidase. All samples were in 0.1 M sodium phosphate, pH 7.0, buffer. Each emission spectrum shown was obtained by subtracting the spectrum of enzyme labeled in the presence of TPP from that of enzyme labeled in its absence (see legend to Figure 3). These spectra represent the fluorescence emission of probes at the TPP-protectable site. The emission spectra were corrected for variation in the response of the instrument as a function of emission wavelength as described under Materials and Methods. In both cases, fluorescence was excited at 375 nm with 5-nm excitation and emission band-passes.

polarization photometer (Jameson et al., 1978) equipped with Glan prisms. The excitation wavelength was 350 nm with a 5-nm band-pass. Emission due to 1,5-IAEDANS was selected with appropriate cutoff filters. Solvent blank scattering, which was less than 10% of the signal, was subtracted before calculation of polarization values. Results shown are averages of three determinations. During these measurements the sample temperature was held at 25 °C.

Phase and Modulation Lifetime Measurements. Fluorescence lifetimes were determined by using the cross-correlation phase and modulation fluorometer of Spencer & Weber (1969) with improved electronics from SLM Instruments (Urbana, IL). Phase shifts and modulations were measured relative to a scattering solution (Spencer & Weber, 1969). The modulation frequency was 18 MHz. Results shown are averages of at least ten determinations.

Results and Discussion

The cysteine conjugates of 6-(4-maleimidylanilino)-naphthalene-2-sulfonic acid (MIANS) and 5-[[[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid (1,5-IAEDANS) have fluorescence emission spectra that overlap the FAD absorption spectrum of pyruvate oxidase (see Figures 1 and 2). This indicated that it might be possible to estimate the distance between the cysteine residue at the TPP-protectable site and the FAD coenzyme of pyruvate oxidase via fluorescence energy transfer measurements.

Table I: Spectral Overlap Integrals[a]

fluorophore	$J_{ox} \times 10^{14}$ (cm^3 M^{-1})[b]	$J_{red} \times 10^{14}$ (cm^3 M^{-1})[b]	J_{ox}/J_{red}
MIANS	3.0	0.60	5.0
1,5-IAEDANS	2.0	0.23	8.8

[a] Integrals (see eq 3) were estimated by summation. The absorption and relative fluorescence emission spectra of Figure 2 were digitized at 0.5-nm intervals (see Materials and Methods), and the approximate formula $J = \Sigma_i f(\lambda_i)\epsilon(\lambda_i)\lambda_i^4/\Sigma_i f(\lambda_i)$ was used. Here ϵ has units of M^{-1} cm^{-1} and λ is given in cm. [b] J_{ox} and J_{red} were calculated by using the absorption spectra of oxidized and reduced pyruvate oxidase, respectively.

According to the theory of Förster (1948), the observed quantum yield of a fluorescent probe near a chromophore is given by

$$Q = Q_0 \frac{(r/R_0)^6}{1 + (r/R_0)^6} \quad (1)$$

where Q and Q_0 are the quantum yields of the probe in the presence and absence of the chromophore, respectively, r is the distance (in angstroms) between probe and chromophore, and R_0 is the critical energy transfer distance (in angstroms) defined by

$$R_0 = (9.79 \times 10^3)(J\kappa^2 Q_0 n^{-4})^{1/6} \quad (2)$$

Here κ is the dipole–dipole orientation factor, n is the refractive index of the medium, and J is the spectral overlap integral:

$$J = \int f(\lambda)\epsilon(\lambda)\lambda^4 \, d\lambda \quad (3)$$

In this integral $f(\lambda)$ is the normalized fluorescence yield of the probe and $\epsilon(\lambda)$ is the molar extinction coefficient of the chromophore, both given as functions of wavelength in centimeters. The overlap integrals of the fluorescence emission spectra of both MIANS–protein and 1,5-IAEDANS–protein conjugates with the FAD absorption spectra of pyruvate oxidase are presented in Table I. The integrals are smaller when the spectrum of the reduced flavoprotein is used in the calculation. Therefore, if either fluorescent probe were close enough to the FAD chromophore to transfer a significant quantity of its excitation energy to the flavin, the quantum yield of the probe would be enhanced upon reduction of the flavin. This enhancement is given quantitatively by the equation:

$$\frac{Q_{red}}{Q_{ox}} = \left(\frac{R_0^{ox}}{R_0^{red}}\right)^6 \left[\frac{1 + (r/R_0^{ox})^6}{1 + (r/R_0^{red})^6}\right] \quad (4)$$

At very small distances ($r \ll R_0^{red}$ and R_0^{ox}) this enhancement would approach the ratio J_{ox}/J_{red} given in Table I, assuming that Q_0 and κ are independent of the flavin oxidation state. At larger distances the enhancement would be less than this ratio. These considerations prompted an attempt to experimentally determine the distance between the cysteine residue at the TPP-protectable site and the FAD coenzyme. Energy transfer to the flavin chromophore cannot be measured directly, because the FAD chromophore of pyruvate oxidase is nonfluorescent.

Reactions of MIANS and 1,5-IAEDANS with Pyruvate Oxidase. The enzyme was exposed to MIANS and 1,5-IAEDANS in attempts to label the reactive cysteine residue at the TPP-protectable site. Previous experiments had shown

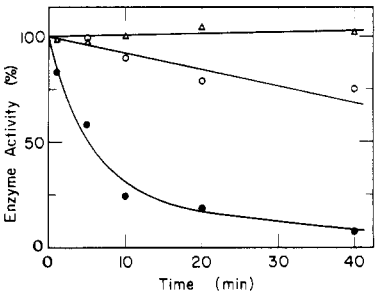

FIGURE 3: Inactivation of pyruvate oxidase by MIANS and protection by TPP. Pyruvate oxidase (3.0 mg/mL) was treated with 1.0 mM MIANS in a 0.1 M sodium phosphate and 10 mM magnesium chloride solution at pH 7.0 and activity monitored at intervals (●). Another sample was treated identically except that 1 mM TPP was included in the medium (○). In a control experiment (△), MIANS and TPP were omitted. Stoichiometry measurements (Table II) and recording of absorption and emission spectra of the protein conjugates were performed after a 40-min exposure to MIANS. Excess reagent was removed by gel filtration through a Sephadex G-25 column (Pharmacia), equilibrated and eluted with 0.1 M sodium phosphate buffer, pH 7.0. All manipulations were performed at room temperature (~25 °C).

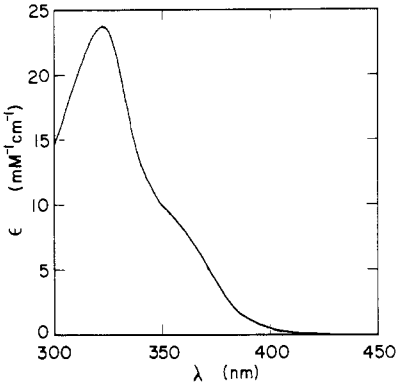

FIGURE 4: Absorption spectrum of conjugate of MIANS with *N*-acetylcysteine. Solid MIANS was dissolved in a 0.1 M solution of *N*-acetylcysteine in 0.1 M sodium phosphate, pH 7.0. The 1.20 mM MIANS–*N*-acetylcysteine solution was diluted 100-fold and the optical absorption spectrum taken against a suitable blank.

that the activity of pyruvate oxidase is rapidly lost upon exposure to thiol reagents, such as *N*-ethylmaleimide, unless this single cysteine residue is blocked as in the presence of the cofactor TPP (Koland & Gennis, 1982). Figure 3 shows that pyruvate oxidase is rapidly inactivated upon exposure to 1.0 mM MIANS at pH 7.0. A similar result was obtained with 1,5-IAEDANS at a 5.0 mM concentration. The presence of TPP at a saturating concentration results in a significant protection of the enzyme against inactivation by either reagent, although residual inactivation occurs (see Figure 3). This residual inactivation indicates that these reagents do not react solely with the cysteine residue at the TPP-protectable site.

The extent to which pyruvate oxidase is labeled by these fluorophore reagents was conveniently determined by analysis of the optical absorption spectra of the conjugates. Subtraction of the known absorption spectrum of pyruvate oxidase from that of the conjugates yielded spectra closely resembling those of the reaction products of the reagents with free *N*-acetylcysteine [see Figure 4 and Hudson & Weber (1973)]. The extents of labeling observed upon exposure of the enzyme to these reagents, in the presence and absence of TPP, are given in Table II. As anticipated, residual labeling does occur in the presence of TPP, most noticeably in the case of MIANS.

Attempts to prepare an enzyme species labeled only at the TPP binding site were unsuccessful. The relative amount of

Table II: Labeling of Pyruvate Oxidase by MIANS and 1,5-IAEDANS in the Presence and Absence of TPP[a]

fluorophore	+TPP[b]	−TPP[b]	Δ*l*[c]
MIANS	1.8	2.4	0.6
1,5-IAEDANS	0.1	0.4	0.3

[a] Extents of labeling observed upon exposure of pyruvate oxidase to the fluorophore reagents in the presence and absence of the cofactor TPP. Units are moles of fluorophore per mole of subunit (see legend of Figure 3 and the text). [b] +TPP and −TPP refer to labeling ratios measured when TPP was present and absent in the reaction medium. [c] Δ*l* is the enhancement of labeling observed when TPP is omitted from the reaction medium.

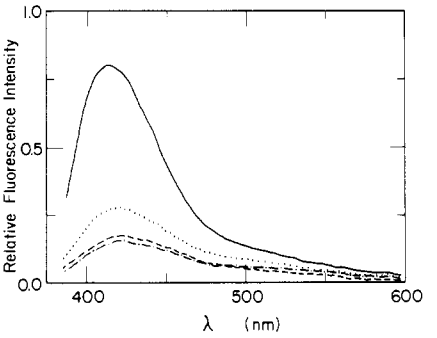

FIGURE 5: Fluorescence emission spectra of MIANS–pyruvate oxidase conjugates. Pyruvate oxidase samples were conjugated with MIANS as described in the legend of Figure 3. Spectra were recorded with samples in 0.1 M sodium phosphate, pH 7.0, in a sealed cuvette under argon before and after reduction of the flavoprotein by the addition of a minimal amount of a sodium dithionite solution. Samples analyzed were labeled either in the presence of TPP [(–·–) FAD oxidized and (···) FAD reduced] or in the absence of TPP [(––) FAD oxidized and (—) FAD reduced]. The excitation wavelength was 375 nm with 5-nm excitation and emission band-passes. These spectra are not corrected; however, the spectrum of a solvent blank was subtracted from each spectrum shown (see Materials and Methods).

nonspecific labeling could not be reduced by decreasing the duration of the incubation of reagent and enzyme. Although in one instance the enzyme was preincubated with excess *N*-ethylmaleimide in the presence of TPP in order to block all reactive residues other than the TPP binding site cysteine, even this pretreatment did not result in a significant decrease in the amount of nonspecific incorporation of the fluorescent probe. Apparently the fluorescent thiol reagents employed react less specifically than *N*-ethylmaleimide. It is not known whether the nonspecific labeling observed is due to the reaction of these probes with cysteine, lysine, or terminal amino groups.

Fluorescence Emission Spectra of MIANS–Pyruvate Oxidase and 1,5-IAEDANS–Pyruvate Oxidase Conjugates. The fluorescence emission spectra of MIANS-labeled pyruvate oxidase are shown in Figure 5. Spectra are shown for enzyme labeled in the presence and absence of TPP under the conditions described in the caption of Figure 3. When the FAD chromophore is oxidized, these emission spectra are nearly identical. Upon reduction of the FAD cofactor by dithionite (see Figure 5), the emission of both species is enhanced; however, the fluorescence intensity of the species labeled in the absence of TPP is enhanced to a much greater degree.

Previous work has demonstrated that TPP protects a single cysteine residue (Koland & Gennis, 1982). Thus, the fluorescence emission spectrum of the probe bound to this cysteine residue can be obtained by subtraction of the spectrum of enzyme labeled in the presence of TPP from that of enzyme labeled in its absence. This was done with the spectra of MIANS-labeled pyruvate oxidase for both the reduced and oxidized states of the flavin (see Figure 6). Apparently the

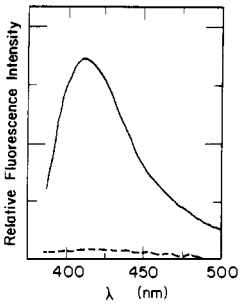

FIGURE 6: Fluorescence emission of MIANS bound to the TPP-protectable cysteine residue of pyruvate oxidase. The emission spectrum of MIANS bound at the TPP-protectable site was obtained by subtracting the spectrum of enzyme labeled in the presence of TPP from that of enzyme labeled in its absence (see Figure 5). This was done with spectra corresponding to the oxidized (– –) and reduced (—) states of the flavin. After subtraction, the spectra were corrected for the variation of instrument response with changing emission wavelength (see Materials and Methods).

fluorescence yield of the probe at the TPP-protectable site is very small when the FAD coenzyme is oxidized and is greatly enhanced when the flavin is reduced.

Absolute quantum yields were determined by comparing the integrated intensities of spectra such as those of Figure 6 to that of a quinine sulfate standard of equal optical density (see Materials and Methods). The quantum yield of MIANS bound at the TPP-protectable site was estimated to be less than 0.0012 with the flavin oxidized and equal to 0.027 ± 0.002 when the flavin is reduced. The quantum yield of MIANS bound at the TPP-protectable site is thus enhanced at least 23-fold upon flavin reduction. In a completely analogous manner it was determined that the fluorescence yield of 1,5-IAEDANS bound at this site is less than 0.007 and is enhanced at least 10-fold upon reduction of the FAD coenzyme. These estimates rely upon the assumption that the difference spectra in Figure 6 and the analogous spectra obtained in the case of 1,5-IAEDANS accurately reflect the fluorescence properties of the probes bound at the TPP-protectable site. Only upper limits are given for the quantum yields of these probes in the presence of oxidized flavin because the values obtained were not much greater than the experimental variation.

The fluorescence emission spectrum attributed to MIANS bound at the TPP-protectable site (Figure 6) is blue shifted relative to that of the MIANS fluorescence observed in the presence of oxidized FAD (Figure 5). This latter fluorescence is attributed mainly to nonspecifically bound probes. The observed blue shift is consistent with the known sensitivity of the fluorescence emission spectra of MIANS–cysteine conjugates to solvent polarity (Gupte & Lane, 1979) and the assumption that the spectra shown in Figure 6 are those of the MIANS probe in the hydrophobic TPP binding site. A blue shift was also observed with 1,5-IAEDANS-labeled enzyme upon flavin reduction or flavin extraction (see below). The cysteine conjugate of this reagent also exhibits an enhanced quantum yield and blue-shifted emission spectrum in nonpolar media (Hudson & Weber, 1973).

Fluorescence Polarization and Lifetimes of 1,5-IAEDANS-Labeled Pyruvate Oxidase. The polarization of fluorescence from the 1,5-IAEDANS–pyruvate oxidase conjugates was studied in detail. Four samples were analyzed (see Materials and Methods). These samples had been labeled in either the presence or absence of TPP and had either an oxidized FAD chromophore or 80% of the flavin extracted. The effect of this partial extraction of the flavin (see below) is entirely analogous to reduction of the flavin. Polarization data are summarized in Table III. The fluorescence of the flavin-extracted sample labeled in the absence of TPP is most highly polarized. This high polarization indicates an immobilization of the probe molecule at the TPP-protectable site on the time scale of fluorescence emission and energy transfer processes. Thus, it is not possible to make any assumption about the orientation of probes at the TPP-protectable site (e.g., random) and narrow the possible limits of κ.

Table III: Polarization of Fluorescence Emission from 1,5-IAEDANS-Labeled Pyruvate Oxidase[a]

species	+FAD[b]	–FAD[b]
+TPP[c]	0.184	0.235
–TPP[c]	0.240	0.331

[a] Fluorescence polarization was measured as described under Materials and Methods, with samples in 0.1 M sodium phosphate, pH 7.0, buffer. [b] +FAD and –FAD indicate whether the 1,5-IAEDANS-labeled pyruvate oxidase sample had a full complement of the FAD chromophore or had been subjected to a partial extraction of the FAD (see Materials and Methods). In the latter case, approximately 20% of the original flavin was retained by the enzyme. [c] +TPP and –TPP indicate whether the labeling by 1,5-IAEDANS was performed in the presence or absence of the cofactor TPP (see legend of Figure 3).

Table IV: Phase and Modulation Lifetimes for 1,5-IAEDANS-Labeled Pyruvate Oxidase

species	τ_p (ns)[a]	τ_m (ns)[a]
+TPP[b]	9.2	11.3
–TPP[b]	6.9	11.5

[a] τ_p and τ_m are the lifetimes estimated by phase and modulation measurements, respectively (see Materials and Methods). Discrepancies between τ_p and τ_m are an indication of heterogeneity in the fluorescence decay. [b] +TPP and –TPP indicate whether labeling by 1,5-IAEDANS was performed in the presence or absence of the cofactor TPP (see legend of Figure 3). Lifetimes were determined with samples in 0.1 M sodium phosphate, pH 7.0, buffer.

The lifetimes of fluorescence emission from the 1,5-IAEDANS–pyruvate oxidase conjugates were measured by the phase-modulation technique (see Materials and Methods). Enzyme samples labeled in the presence and absence of TPP were analyzed. The results are given in Table IV. Some lifetime heterogeneity is observed, indicating heterogeneity in chromophore environment. This effect was greater with samples labeled in the absence of TPP. Heterogeneity prohibited the determination of the lifetime of the probe at the TPP-protectable site.

Estimation of Distance between TPP-Protectable Site and FAD Coenzyme. It was noted above that upon reduction of the FAD chromophore, fluorescence attributed to probe molecules at the TPP-protectable site is enhanced at least 23-fold in the case of MIANS and 10-fold with 1,5-IAEDANS. With the spectral overlap integrals of Table I, a simple application of eq 4 indicates that the maximum enhancement of fluorescence expected is 5.0 in the case of MIANS and 8.8 for 1,5-IAEDANS. However, in this computation it is assumed that all parameters contributing to R_0 (see eq 2) except J are unchanged upon flavin reduction. It is likely that either κ, the probe–FAD dipole–dipole orientation factor, or Q_0, the quantum yield of the probe in the absence of energy transfer, might change significantly upon reduction of the flavin.

In an attempt to estimate Q_0, the FAD coenzyme was extracted from 1,5-IAEDANS-labeled enzyme (see Materials and Methods). Because the extraction procedure is rather harsh, the FAD chromophore could not be totally removed without irreversibly denaturing some of the protein. Thus, 80%

of the flavin was removed in these experiments, and only a lower limit for Q_0 was obtained. By subtraction of the emission spectrum of enzyme labeled with 1,5-IAEDANS in the presence of TPP from that of enzyme labeled in its absence, it was determined that the quantum yield of 1,5-IAEDANS bound at the TPP-protectable site is less than 0.007 in the presence of oxidized flavin. From the spectra of the FAD-extracted species a lower limit of 0.140 ± 0.014 was obtained for Q_0, the quantum yield of the 1,5-IAEDANS probe in the absence of energy transfer to the flavin. Thus, upon extraction of the flavin, the quantum yield of 1,5-IAEDANS bound at the TPP-protectable site is increased at least 20-fold. Flavin extraction was not attempted with MIANS-labeled enzyme; however, it is clear from the flavin reduction experiments (see above) that the quantum yield of MIANS bound at the TPP-protectable site would also be at least 20 times greater in the absence of energy transfer to the flavin.

Attempts to estimate r, the distance between the probe at the TPP-protectable site and the FAD chromophore by application of eq 4 were frustrated by uncertainties concerning the quantities κ^2 and Q_0. However, in the case of oxidized enzyme, energy transfer quenches the intrinsic fluorescence of both probes by at least a factor of 20 ($Q/Q_0 \leq 0.05$), and a simplified form of eq 1 can be used to a very good approximation:

$$r = R_0(Q/Q_0)^{1/6} \quad (5)$$

It can be shown that this approximation introduces less than 5% error when $Q/Q_0 \leq 0.25$. Using the definition of R_0 (eq 2), we find that when $Q \ll Q_0$

$$r = (9.79 \times 10^3)(QJ\kappa^2 n^{-4})^{1/6} \quad (6)$$

Equation 6 shows that r can be estimated without explicit knowledge of Q_0. Furthermore, an absolute upper limit for r can be obtained by assuming that $\kappa^2 = 4$, the largest possible value for the orientation factor. A value of 1.33 was chosen for the refractive index of the medium.

If one uses the upper limits for the quantum yields of the two fluorescent probes when bound at the TPP-protectable site in the presence of oxidized flavin, 0.0012 and 0.007 for MIANS and 1,5-IAEDANS, respectively, and the appropriate spectral overlap integrals from Table I, two independent upper limits for r are given by eq 6. These upper limits are 19 and 23 Å for MIANS and 1,5-IAEDANS, respectively.

It should be noted that the value of r given by eq 6 is rather insensitive to possible errors in the determination of either Q or J. Increasing the value of either parameter by a factor of 2 would increase the value of r obtained by only 12%. Likewise, although it is conceivable that a fluorophore bound at one of the four TPP-protectable sites of the tetrameric protein might transfer excitation energy to more than one flavin chromophore, ignorance of this fact would lead to only a small underestimation of the probe–flavin distance. The distance obtained would then be an estimate of that between the probe and the closest of the flavin chromophores.

Conclusions

The TPP-protectable cysteine residue believed to be involved in the binding of TPP to pyruvate oxidase can be covalently labeled with either of two fluorescent probes. Other amino acid residues are unavoidably labeled in this process, but the fluorescence emission spectrum of a probe bound at the TPP-protectable site can be obtained by subtraction of the spectrum of enzyme labeled in the presence of TPP from that of enzyme labeled in its absence. The observed blue shift and high polarization of the emission attributed to the probe at the TPP-protectable site is consistent with the assumption that the probe is located in the hydrophobic TPP binding pocket.

Quantum yield determinations showed that, when bound at the TPP-protectable site, each of the probes has a highly attenuated fluorescence emission. Apparently transfer of excitation energy to the FAD chromophore is a dominant process for such probes. Through an application of the theory of excitation energy transfer (Förster, 1948), upper limits of 19 and 23 Å were obtained for the distances between the FAD chromophore and the MIANS- and 1,5-IAEDANS-labeled cysteine, respectively. Because of the relative insensitivity of these upper limits to potential underestimation of any of the parameters of eq 6, these upper limits can be considered quite rigorous. The largest possible value for κ^2, which corresponds to a rather unlikely collinear orientation of donor and acceptor transition dipole moments, was used in the calculations. Thus, it is likely that the actual probe–chromophore distances are considerably less than the upper limits given above.

These results indicate that the TPP binding site and the FAD coenzyme of pyruvate oxidase are in close proximity. This is consistent with a catalytic mechanism that includes a direct oxidation of the decarboxylated pyruvate–TPP adduct by the flavin coenzyme.

Acknowledgments

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