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Fluorescence Energy Transfer Measurements in Rabbit Muscle Phosphofructokinase†

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ABSTRACT: The distance between the citrate site and the most reactive sulfhydryl group in rabbit muscle phosphofructokinase has been determined using fluorescence resonance energy transfer. Pyridoxal 5′-phosphate and sodium borohydride reduction were used to specifically label a lysine residue at the citrate site, while 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) or 4-dimethylamino-4′-maleimidostilbene (NSM) was reacted with the most reactive sulfhydryl group of the monomeric enzyme (molecular weight 80 000). Using pyridoxamine 5′-phosphate as the fluorescent donor molecule, the measured distance was 40 Å with NBD as the acceptor molecule and 36 Å with NSM as the acceptor molecule. The magnitude of the transfer efficiency to NBD was unaltered by the presence of varying ATP concentrations (0.01–1 mM), 5 mM fructose 6-phosphate and 5 mM $MgCl_2$, and was unaffected by the aggregation state of the enzyme. Pyridoxylation of the enzyme led to depolymerization to dimer (pH 8.0), and to dimer and monomer (pH 7.0) at a protein concentration of 0.2 mg/mL, but incubation of the modified enzyme with 5 mM fructose 6-phosphate at pH 7.0 resulted in formation of the tetramer. Since the energy transfer efficiency is not dependent on the aggregation state of the enzyme, the predominant energy transfer probably occurs within a single polypeptide chain. If energy transfer occurs between subunits, the calculated distances are lower bounds to the distance within a single subunit.

Rabbit skeletal muscle phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) is an important regulatory enzyme. The activity of the enzyme is dependent upon the pH, the protein concentration, and the presence or absence of several effectors (Passoneau and Lowry, 1962; Aaronson and Frieden, 1972; Pavelich and Hammes, 1973; Lad et al., 1973). Phosphofructokinase is a multisubunit protein containing identical polypeptide chains with a monomer molecular weight of 80 000 (Pavelich and Hammes, 1973; Coffee et al., 1973), and the specific activity of the enzyme is dependent on its aggregation state. Several models have been proposed to explain the regulatory properties of the enzyme (Kemp et al., 1976; Bock and Frieden, 1976).

In this work, fluorescence energy transfer measurements have been used to determine the distance from the citrate binding site to the most reactive sulfhydryl group (Kemp and Forest, 1968) in the presence of various effectors. Pyridoxamine 5′-phosphate (PMP),[1] which has been shown to bind specifically to a lysine residue at the citrate site (Colombo and Kemp, 1976), was used as the fluorescent donor while NBD-Cl and NSM reacted with the thiol group were used as the energy acceptrs. The distance between the two sites was found to be about 40 Å for the enzyme over a range of aggregation states, in the presence of varying ATP concentrations, in the presence of fructose 6-phosphate, and in the presence of $MgCl_2$.

Experimental Section

Materials. The ATP, fructose 6-phosphate, dithiothreitol, aldolase, glucose oxidase, α-glycerophosphate dehydrogenase, pyruvate kinase, triosephosphate isomerase, serum albumin (bovine), and pyridoxal 5′-phosphate were purchased from Sigma Chemical Co. The NBD-Cl was obtained from Pierce Chemical Co., the NSM from Eastman Kodak, the [^{3}H]NEM from New England Nuclear, the quinine sulfate from Aldrich Chemical Co., and the Ludox HS-30 colloidal silica from E. I. du Pont de Nemours and Co. All other chemicals were the best available commercial grade, and all solutions were prepared with deionized-distilled water.

Phosphofructokinase. Rabbit muscle phosphofructokinase was obtained from Sigma Chemical Co. as an ammonium sulfate suspension with 4 mM ATP present (lot 126(-9690)). The enzyme concentration was calculated from the ratio of the absorbances at 280 and 260 nm and from the absorbance at 280 nm using an extinction coefficient of 1.02 mL mg^{-1} cm^{-1} (Parmeggiani et al., 1966) for the native enzyme at 280 nm and correcting for ATP absorption. Absorbances were measured with a Zeiss PMQII spectrophotometer.

The specific activity of the enzyme was determined using coupled enzyme reactions as previously described (Ling et al., 1966; Lad et al., 1973). Assays were run under the following conditions: pH 8.0, 33 mM Tris-Cl, 2 mM ATP, 5 mM $MgCl_2$, 2 mM fructose 6-phosphate, 0.1 mM NADH, 2 mM dithiothreitol, 0.20 unit/mL of aldolase, 35 units/mL of triosephosphate isomerase, 3.2 units/mL of α-glycerophosphate dehydrogenase, and 0.03–0.13 μg/mL of phosphofructokinase in a total volume of 3.0 mL. Assays were initiated by the addition of phosphofructokinase, and the reaction velocity was followed by monitoring the change in absorption at 340 nm on a Cary 118 spectrophotometer thermostated at 23 °C.

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[1] Abbreviations used are: NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; NSM, 4-dimethylamino-4′-maleimidostilbene; [^{3}H]NEM, *N*-ethyl[2-^{3}H]maleimide; PMP, pyridoxamine 5′-phosphate; EDTA, ethylenediaminetetraacetic acid; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

The specific activity of the native enzyme in 0.1 M potassium phosphate, 1 mM EDTA, pH 8.0, was 90–120 units/mg at 23 °C. This apparent specific activity is 20–40 units lower than the actual activity due to the presence of ammonium sulfate in the coupling enzymes (Lad et al., 1973). A unit of enzyme activity is defined as the production of 1 μmol of product per min.

Modification of Phosphofructokinase. Phosphofructokinase was modified with pyridoxal 5′-phosphate using a slightly modified procedure of Colombo and Kemp (1976). The enzyme was dialyzed for 24 h at 4 °C against 50 mM Tes, 50 mM KCl, 1.0 mM ATP, 1.0 mM $MgCl_2$, 1.0 mM dithiothreitol, 0.1 mM EDTA (pH 7.0), and then diluted to 3.0 mL with the same buffer to a final protein concentration of 2.5–3.0 mg/mL. Two 3.0-mL partitioned cuvettes were filled with 1.0 mL of the protein solution in one compartment and 1.0 mL of the buffer in the other. Then 100 μL of a freshly prepared solution of 3.19 mM pyridoxal 5′-phosphate in water was added to the buffer side of the reference cell and to the protein side of the sample cell. This represents a tenfold molar ratio of pyridoxal 5′-phosphate to enzyme. The cells were thermostated at 4 °C, and the Schiff base formation was monitored by following the absorbance at 422 nm on a Cary 118 spectrophotometer. The light beams were blocked between measurements in order to minimize photodecomposition, and dry nitrogen was circulated in the chamber to prevent condensation. The reaction was quenched by the addition of 100 μL of a cold, freshly prepared solution of sodium borohydride in water (15 mg/mL). The solution was dialyzed overnight against 0.1 M potassium phosphate, 1 mM EDTA (pH 8.0), and the number of moles of pyridoxal 5′-phosphate incorporated was determined using a molar extinction coefficient of 10 150 at 325 nm for N^{ϵ}-(phosphopyridoxyl)lysine (Fischer et al., 1963).

Circular dichroism spectra of both native (0.49 mg/mL) and PMP-labeled phosphofructokinase (0.16 mg/mL) in 0.1 M potassium phosphate, 1 mM EDTA (pH 8.0), were determined at 25 °C using a Cary 60 spectrophotometer. Cells with a 2.5-cm path length were used for the 300–400-nm wavelength region, while 0.3-cm path length cells were used from 220 to 300 nm.

The modification of phosphofructokinase with NBD-Cl is described elsewhere (Lad et al., 1977).

The enzyme was modified with NSM by the following procedure. Phosphofructokinase (0.8–1.0 mg/mL) was incubated at 25 °C in 25 mM diglycine, 25 mM potassium phosphate, 5 mM ATP, 5 mM fructose 6-phosphate, 1 mM EDTA (pH 7.0), for 1 h. A 1.5 molar ratio of NSM to enzyme was added from a 10 mM dimethyl sulfoxide solution, and aliquots were removed after various time intervals to follow the course of the reaction. The reaction was quenched by the addition of dithiothreitol (>0.5 mM), which reacts with unreacted NSM, and the solution was passed through a glass Pasteur disposable pipette filled with Sephadex G-25, which had been equilibrated with 0.1 M potassium phosphate, 1 mM EDTA (pH 8.0). The protein solution was dialyzed overnight at 4 °C against the same buffer, and the number of moles of enzyme linked NSM was determined using a molar extinction coefficient of 22 750 M^{-1} cm^{-1} at 350 nm and correcting for protein absorption (Papadakis and Hammes, 1977).

The PMP-labeled enzyme was further modified with several sulfhydryl group reagents, NBD-Cl, NSM, and [^{3}H]NEM. The labeled enzyme was first dialyzed at 4 °C against 0.1 M potassium phosphate, 1.0 mM EDTA (pH 7.0), plus any added ligands to remove dithiothreitol, and then dialyzed at 4 °C against 25 mM diglycine, 25 mM potassium phosphate, 5 mM ATP, 5 mM fructose 6-phosphate, 1 mM EDTA (pH 7.0). The PMP-modified enzyme was diluted with this same buffer to a protein concentration of 1.3–1.5 mg/mL. The solution (~6 mL) was then split into two portions, one portion serving as a control. In the case of NBD-Cl, a 2.5-fold molar excess of reagent was added from a stock solution of 10 mM NBD-Cl in dimethyl sulfoxide, and the solution was incubated at 25 °C for 2–3 h. The reaction progress was monitored by measuring the increase in absorption at 420 nm. For the NSM modification, a 1.5 molar ratio of NSM to enzyme was added using a stock solution of 5 mM NSM in dimethyl sulfoxide and allowed to react for 2.5–3.5 h at 25 °C. The stoichiometry of labeling was determined as described above. Finally with [^{3}H]NEM, a 7.5-fold excess of the reagent was used and allowed to react at 25 °C for 2.5 h. Three 10-μL samples were taken, and the radioactivity was determined in Bray's scintillation fluid (Bray, 1960) using a Beckman LS 255 liquid scintillation counter. The concentration of [^{3}H]NEM was calculated from the known specific activity of the stock [^{3}H]NEM solution. In all cases, the resulting double labeled enzyme was dialyzed for 24 h at 4 °C against 0.1 M potassium phosphate, 1 mM EDTA, pH 7.0 or 8.0, and any additional ligands. Prior to use, the enzyme solutions were filtered through 0.45 μm pore size Millipore filters to remove any accumulated particles. The final concentration of doubly labeled phosphofructokinase ranged from 0.5 to 0.7 mg/mL (6–9 μM).

Gel Chromatography. The average molecular weight of the modified enzyme species was determined using column chromatography on agarose (5-m resin, Bio-Rad Laboratories; 1.5 cm i.d. × 50 cm); 0.8-cm plugs of Sephadex G-25 were present on the top and bottom of the column bed to maintain stable agarose interfaces. The volume of protein used for the elution ranged from 45 to 70 mL. Aldolase (mol wt 150 000), glucose oxidase (mol wt 186 000), pyruvate kinase (mol wt 240 000), and native phosphofructokinase (0.19 mg/mL, mol wt 320 000) in pH 8.0 potassium phosphate were used as molecular weight standards. The elutions were carried out at 4 °C, and 2-mL fractions were collected for analysis. Samples were prepared as described earlier and then dialyzed into the appropriate buffer. Elution profiles were constructed by measuring the absorbance at 280 nm; the elution volume was taken as the eluted volume at which the protein concentration was one-half the concentration in the plateau region.

Steady-State Fluorescence Measurements. Steady-state fluorescence measurements were made with a Hitachi Perkin-Elmer MPF-3 fluorescence spectrophotometer equipped with corrected spectrum and polarization accessories. An excitation wavelength of 335 nm was used for all measurements. The quantum yield of bound PMP was determined at 23 °C using the comparative method (Parker and Rees, 1966); quinine bisulfate in 0.1 N H_2SO_4 was used as a standard and was assumed to have an absolute quantum yield of 0.70 at 23 °C (Scott et al., 1970). Equation 1 gives the ratio of quantum yields, Q_i, as a function of the area of the corrected emission spectrum, F_i, and the absorbance at the exciting wavelength, A_i, for two different fluorescent labels.

$$\frac{Q_2}{Q_1} = \frac{F_2}{F_1}\frac{A_1}{A_2} \tag{1}$$

For measuring quantum yields, the absorbance at the exciting wavelength, 335 nm, was less than 0.005. The areas under the corrected emission spectra were calculated using a computer. For polarization measurements, a correction was made for unequal transmission of horizontally and vertically polarized light by the emission monochromator grating (Azumi and McGlynn, 1962). Energy transfer efficiencies were determined at 25 °C by monitoring the fluorescence intensity of the PMP

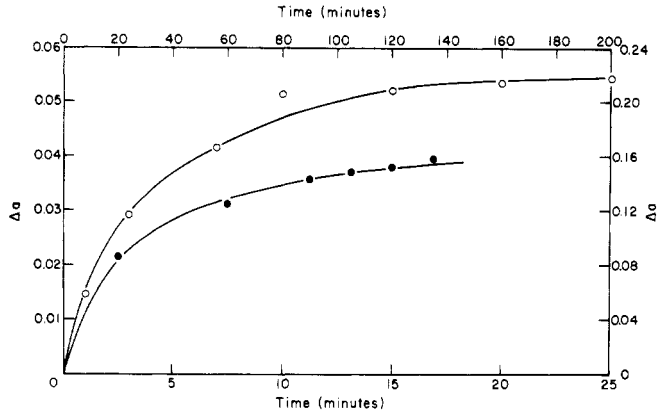

FIGURE 1: (Upper curve) A plot of the absorbance change at 422 nm, Δa, vs. time for the reaction of phosphofructokinase (2.5 mg/mL) with a 10-fold molar excess of pyridoxal 5′-phosphate in 50 mM Tes, 50 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, 0.1 mM EDTA, 1 mM dithiothreitol, pH 7.0 at 4 °C (open circles, left ordinate, lower abscissa). (Bottom curve) A plot of the absorbance change at 420 nm, Δa, vs. time for the reaction of PMP labeled enzyme (1.4 mg/mL) with a 2.5-fold molar excess of NBD in 25 mM diglycine, 25 mM potassium phosphate, 5 mM ATP, 5 mM fructose 6-phosphate, 1 mM EDTA, pH 7.0 at 25 °C (filled circles, right ordinate, upper abscissa).

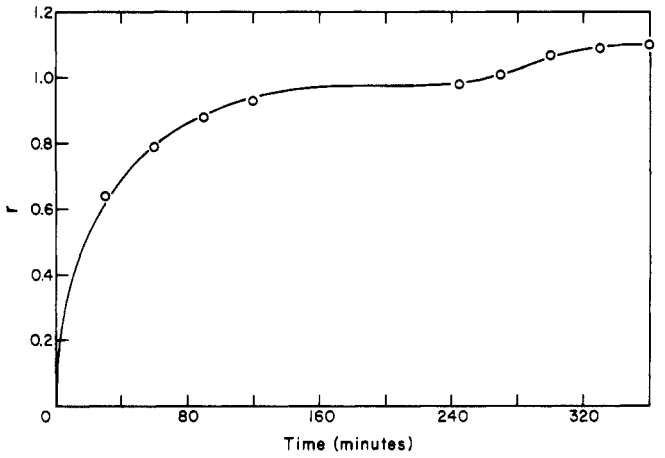

FIGURE 2: A plot of the moles of NSM reacted per 80 000 molecular weight protein, r, vs. time for the modification of native enzyme (0.8 mg/mL) with a 1.5-fold excess of NSM in 25 mM diglycine, 25 mM potassium phosphate, 5 mM ATP, 5 mM fructose 6-phosphate, 1 mM EDTA, pH 7.0 at 25 °C.

immediately before and after displacement of the NBD with 25 mM β-mercaptoethanol, using 390 nm as the emission wavelength. The excitation slit was kept open for only a few seconds to minimize photodecomposition.

Fluorescence Lifetime Measurements. Fluorescence lifetimes were measured with an Ortec Model 9200 nanosecond fluorescence spectrophotometer (Matsumoto and Hammes, 1975). The excitation and emission wavelengths, 334 and 390 nm, respectively, were selected using interference filters (Ditric Optics, Inc.). A colloidal silica (Ludox HS-30) solution was used to determine the lamp spectrum. All decay spectra were counted for the same analysis time in square (1 × 1 cm) cuvettes at 25 °C. A correction was made for protein scattering by photon counting a solution of native enzyme for the same time and subtracting the counts from those of the samples. All data were analyzed on a PDP-11 computer with the deconvolution and convolution programs supplied by Ortec.

Results

Modification of Phosphofructokinase. The time course of the modification of native enzyme with pyridoxal 5′-phosphate is shown in Figure 1. The absorbance levels off after 30–35 min, and 1.0–1.1 mol of PMP per 80 000 molecular weight of protein were found to be incorporated. The specific activity of the modified enzyme was less than 10% of a sample treated in an identical manner except for the addition of pyridoxal 5′-phosphate.

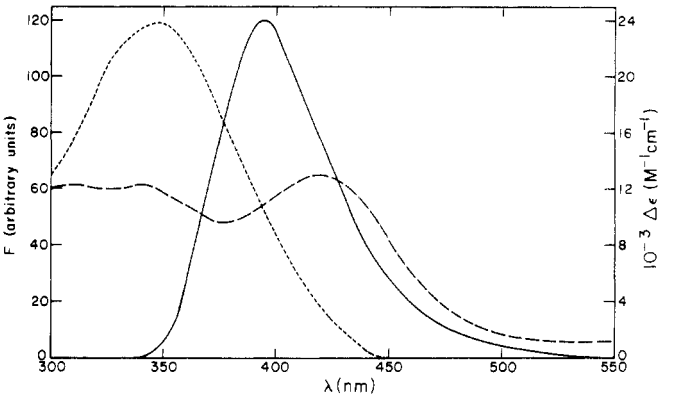

FIGURE 3: Overlap of the corrected fluorescence emission spectrum, F, of enzyme bound PMP (—), the difference extinction coefficient, $\Delta\epsilon$, of enzyme bound NBD (– –), and the difference extinction coefficient, $\Delta\epsilon$, of enzyme bound NSM (- - -).

The reaction of native phosphofructokinase with NBD-Cl is specific for the single (per 80 000 molecular weight) highly reactive sulfhydryl group (Lad et al., 1977), and this is also true for the PMP-labeled enzyme. The time course of the reaction of NBD-Cl with the PMP-labeled enzyme is included in Figure 1. The reaction is somewhat slower than for the native enzyme, but the final stoichiometry was 0.8–1.2 mol of NBD per 80 000 molecular weight protein. A decrease in the sulfhydryl group reactivity of the PMP-labeled enzyme has been reported previously (Colombo and Kemp, 1976).

The time course of the modification of native enzyme with a 1.5 molar ratio of NSM to enzyme is shown in Figure 2. Using a larger excess of this reagent results in modification of other, more slowly reacting thiol groups. The stoichiometry of bound NSM was 1.0–1.1 mol per 80 000 molecular weight protein. The specific activity was about 60% of that of the unmodified enzyme. The stoichiometry of NSM bound to the PMP-labeled enzyme was 0.5–0.8 mol per 80 000 molecular weight protein.

The reaction of PMP-labeled enzyme with [^{3}H]NEM led to the incorporation of 0.8–1.0 mol per 80 000 molecular weight protein. The reaction of [^{3}H]NEM with the unmodified enzyme was complete in about 20 min, as seen by the almost immediate loss of enzyme activity (80%) followed by a very slow activity decrease over a 2-h period (<10%).

Properties of Modified Derivatives. The corrected fluorescence emission spectrum of enzyme bound PMP is shown in Figure 3 along with difference spectra of the NBD and NSM modified enzyme. The excitation wavelength was 335 nm. The quantum yield of the enzyme bound PMP was found to be 0.36 in the singly labeled enzyme and 0.30 for a doubly labeled enzyme in which the NBD had been displaced by β-mercaptoethanol. The polarization of the enzyme bound PMP was found to be 0.28 for all of the modified enzyme species.

The circular dichroism spectrum of the PMP labeled enzyme at pH 8.0 was found to be essentially identical with that of the native enzyme over the wavelength region 220–400 nm indicating that the chemical modification has not produced large structural changes.

The molecular weights of some of the chemically modified

TABLE I: Fluorescence Energy Transfer Parameters.[a]

Enzyme (μM)	ATP (mM)	pH	Acceptor	τ_D[b] (ns)	τ_D[c] (ns)	$\tau_{D\rightarrow A}$[d] (ns)	E[e]	E[f]
6.50	0.016	7.0	NBD	4.52	3.65	2.96	0.16	0.19
6.80	0.060	7.0	NBD	4.03	3.73	3.02	0.17	0.19
7.91	0.120	7.0	NBD	4.71	4.27	3.64	0.21	0.15
7.26	1.00 (5 mM $MgCl_2$)	7.0	NBD	3.80	3.52	3.05	0.13	0.13
7.17	0.022 (5 mM fructose 6-phosphate)	7.0	NBD	5.15	4.72	3.84	0.17	0.19
7.83	1.00	7.0	NBD	4.48	4.15	3.33	0.18	0.20
8.10	0.020	8.0	NBD	3.90	3.26	2.84	0.20	0.13
8.42	0.010	7.0	NSM	4.19	—	2.97	—	0.29

[a] 0.1 M potassium phosphate, 1 mM EDTA, plus indicated ligands at the pH listed. [b] Lifetime of the PMP in singly labeled enzyme. [c] Lifetime of the PMP in enzyme labeled with PMP and NBD after displacement of the NBD with β-mercaptoethanol. [d] Lifetime of the PMP in enzyme labeled with PMP and NBD. [e] Energy transfer efficiency calculated from changes in fluorescence intensity. [f] Energy transfer efficiency calculated from changes in the PMP fluorescence lifetime.

enzyme species were determined by gel chromatography in 0.1 M potassium phosphate, 1 mM EDTA, 0.2 mg/mL enzyme at 4 °C. The PMP-labeled enzyme had an apparent molecular weight of 135 000 at pH 7.0 and 200 000 at pH 8. When 5 mM fructose 6-phosphate was added at pH 7.0, the molecular weight was found to be 345 000. The enzyme labeled with both PMP and NBD had a molecular weight of 200 000 at pH 8.0. Since modification of the enzyme with PMP depolymerizes the enzyme, the depolymerization may be responsible for the loss of activity rather than the chemical modification. This possibility was tested by incubating the enzyme for 24 h at 4 °C in 0.1 M potassium phosphate, 1.0 mM EDTA, 5.0 mM fructose 6-phosphate, pH 7.0. Under these conditions the PMP-labeled enzyme is tetrameric, but it still possessed only 15% of the activity of a native enzyme sample treated in a similar fashion. Therefore, the chemical modification itself is inactivating the enzyme, unless the enzyme depolymerizes very rapidly under steady-state assay conditions.

Energy Transfer Measurements. The transfer efficiency for a fluorescing energy donor and absorbing energy acceptor is defined by eq 2 (Förster, 1959)

$$E = 1 - Q_{D\rightarrow A}/Q_D = 1 - \tau_{D\rightarrow A}/\tau_D \qquad (2)$$

where Q_D and τ_D are the quantum yield and fluorescence lifetime in the absence of acceptor, and $Q_{D\rightarrow A}$ and $\tau_{D\rightarrow A}$ are the same quantities in the presence of the acceptor. In the majority of the experiments, enzyme bound PMP was the energy donor and enzyme bound NBD was the energy acceptor. In a few cases, enzyme bound NSM was the energy acceptor.

In the cases where NBD was the acceptor, the lifetime and quantum yield were measured before and after displacing the NBD with β-mercaptoethanol (25 mM). Since fluorescence energy transfer efficiencies are then determined with a single solution in the same cuvette, possible systematic errors are eliminated. The displacement of NBD is essentially instantaneous as judged by the loss of the absorption at 420 nm. The energy transfer efficiency was determined both from changes in fluorescence intensity at 390 nm and from changes in fluorescence lifetimes. The fluorescence intensity is assumed to be proportional to the quantum yield since the shape of the fluorescence spectrum is unaltered by the acceptor molecule. The nanosecond fluorescence decay curves were fit to the two exponential equation

$$F(t) = C_1 e^{-t/\tau_1} + C_2 e^{-t/\tau_2} \qquad (3)$$

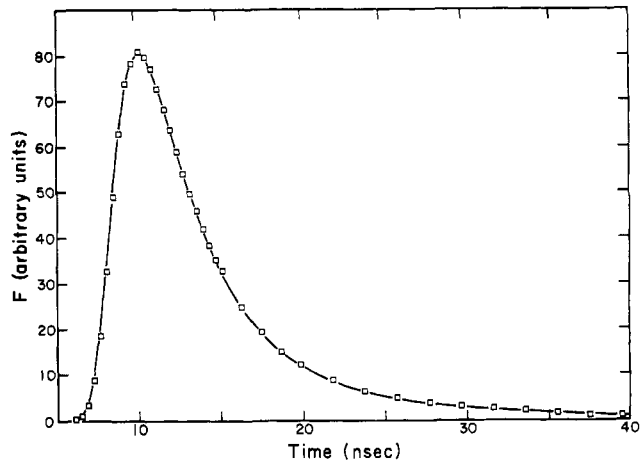

FIGURE 4: A plot of the experimental (squares) and theoretical (line) nanosecond fluorescence decay of enzyme bound PMP. The enzyme concentration was 7.6 μM in 0.1 M potassium phosphate, 5.0 mM $MgCl_2$, 1.0 mM ATP, 1.0 mM EDTA (pH 7.0). The best values for the parameters of eq 3, which were used to calculate the theoretical curve, are $C_1 = 5.797$, $\tau_1 = 2.73$ ns, $C_2 = 1.511$, and $\tau_2 = 6.94$ ns.

where $F(t)$ is the fluorescence intensity, C_1 and C_2 are amplitude parameters, τ_1 and τ_2 are fluorescence lifetimes, and t is the time. A typical fluorescence decay curve and the calculated curve are shown in Figure 4. A single exponential equation does not provide a satisfactory fit of the data. Under all conditions investigated, the ratio C_2/C_1 is about 4. For the purpose of calculating energy transfer efficiencies, an average lifetime, τ_{av}, was defined as

$$\tau_{av} = \frac{C_1\tau_1 + C_2\tau_2}{C_1 + C_2} \qquad (4)$$

The use of either of the individual lifetimes gives about the same efficiency, but the use of τ_{av} has the advantage of combining all of the data in a single parameter. A summary of the observed energy transfer from PMP to NBD in the presence of a variety of ligands is given in Table I. Also included are the average fluorescence lifetimes for the PMP-labeled enzyme, the PMP–NBD-labeled enzyme, and the PMP–NBD enzyme after displacement of NBD with β-mercaptoethanol. Although some variations in the lifetimes and efficiencies are observed, they do not show any obvious trend. Thus the average efficiency measured by changes in fluorescence intensity is 0.17

± 0.02 and that measured from the average fluorescence lifetime is 0.17 ± 0.03.

The energy transfer efficiency for the donor-acceptor pair PMP-NSM is included in Table I; the value reported is the average of three experiments. Since the amount of NSM incorporated varied from 0.5 to 0.8 mol per 80 000 molecular weight protein, the value of the measured energy transfer efficiency was normalized to one acceptor per polypeptide chain. In this case, of course, the NSM cannot be displaced from the enzyme.

As a control experiment, the average fluorescence lifetime of PMP-labeled enzyme modified with NEM was determined at pH 7.0. No significant change relative to the PMP-labeled enzyme was observed. This result indicates the observed changes in fluorescence lifetimes are due to energy transfer and not to conformational changes since NEM modifies the same sulfhydryl group as NBD and NSM but does not have an absorption spectrum overlapping the fluorescence emission of enzyme bound PMP.

The energy transfer efficiency is related to the distance between an isolated acceptor and donor, R, by (Förster, 1959)

$$E = \frac{R^{-6}}{R^{-6} + R_0^{-6}} \tag{5}$$

where R_0 is the distance at which the efficiency is 0.5. The parameter R_0 can be calculated from the equation (Stryer and Haugland, 1967)

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

where J is the overlap integral, n is the refractive index of the medium, and K^2 is a dipole-dipole orientation factor. The overlap integral was calculated as previously described (Cantley and Hammes, 1975) from the results shown in Figure 3, and n was assumed to be 1.4. The quantity K^2 was assumed to be $^2/_3$ which is the value calculated assuming that the donor and acceptor molecules rotate rapidly relative to the fluorescence lifetime. The calculated values of R_0 are 33.1 Å for PMP-NBD and 32.0 Å for PMP-NSM. If the energy transfer efficiency between enzyme bound PMP and NBD is assumed to be 0.17 (Table I), the distance between the two labels is 40 Å; the distance between enzyme bound PMP and NSM is calculated to be 36 Å from the observed energy transfer efficiency.

Discussion

The modification of rabbit muscle phosphofructokinase with pyridoxal 5′-phosphate has been previously reported (Uyeda, 1969; Colombo and Kemp, 1976). Under the proper conditions a single lysine residue per 80 000 molecular weight protein is modified, and this modification blocks the binding of the physiological inhibitor citrate (Colombo and Kemp, 1976). The results reported here indicate that modification with PMP causes the enzyme to depolymerize at both pH 7.0 and 8.0 at an enzyme concentration of 0.2 mg/mL. The PMP-labeled enzyme is essentially dimeric at pH 8.0 and almost monomeric at pH 7.0. The native enzyme is tetrameric under identical conditions (Lad et al., 1973). The presence of fructose 6-phosphate converts the enzyme to a tetrameric form, indicating a preferential binding of fructose 6-phosphate to the tetramer. As previously found, the modification of the enzyme with NBD-Cl has no effect on the aggregation state of the enzyme (Lad et al., 1977). This aggregation behavior is similar to that observed with the native enzyme except that depolymerization occurs at much lower concentrations with the native enzyme (Lad et al., 1973). The PMP-labeled enzyme has been reported not to be depolymerized, but the enzyme concentration used was 7-8 mg/mL, where depolymerization would not be expected to occur (Colombo and Kemp, 1976). The loss in activity of the PMP modified enzyme probably is not due to depolymerization since the tetrameric modified enzyme is still not very active. This is in accord with the finding that citrate inhibits a cross-linked tetramer (Lad and Hammes, 1974) and with results obtained with the sheep heart enzyme (Setlow and Mansour, 1972). Thus it is not clear whether polymerization-depolymerization actually plays a role in physiological regulation. Although the PMP modified enzyme is almost inactive, gross structural changes have not occurred as judged from the circular dichroism and the reactivity of specific sulfhydryl groups with NBD-Cl, NSM, and NEM. Also the modified enzyme binds fructose 6-phosphate as judged by the fructose 6-phosphate induced polymerization.

The use of fluorescence resonance energy transfer to determine distances between specific sites on macromolecules is well documented (Stryer and Haugland, 1967; Wu and Stryer, 1972; Bunting and Cathou, 1973; Matsumoto and Hammes, 1975; Cantley and Hammes, 1975). In the present case, the acceptor and donor are at specific sites so that energy transfer involving nonspecific modifications is unlikely. Since modification of the sulfhydryl group with NEM does not change the fluorescence properties of enzyme bound PMP, it is unlikely that the changes induced by NBD-Cl and NSM reacting with the sulfhydryl group are due to conformational changes. The major uncertainty in calculating the distance between the acceptor and donor is in assigning a value of $^2/_3$ to K^2. However, since the enzyme bound PMP has a polarization (0.28) only 70% of the limiting value (0.41; Churchich, 1965) and since the same distance is calculated with either NSM or NBD as an acceptor, it is unlikely that K^2 is significantly different from $^2/_3$. A further possible difficulty in interpreting the calculated distance is the presence of multiple donors and acceptors, but since the energy transfer efficiency is the same over a range of average molecular weights (Table I), the energy transfer apparently is predominantly between sites on a single polypeptide chain. However, although the PMP-labeled enzyme is mostly monomer at a concentration of 0.2 mg/mL (pH 7.0), this may not be true at the enzyme concentration used for energy transfer measurements (0.5 mg/mL). The amount of protein required to determine the molecular weight at a concentration of 0.5 mg/mL is prohibitive.

The fact that the fluorescence decay curves require two time constants to describe the data implies that PMP exists in two slightly different environments. However, it is not possible to interpret this finding in molecular terms. The average fluorescence lifetime shows a slight trend as the ligands vary (Table I): it is longest in 5 mM fructose 6-phosphate (pH 7.0) and becomes generally shorter in the presence of ATP, the shortest lifetime being observed in the presence of 1 mM MgATP. These variations may be indicative of protein conformational changes, but they are similar in magnitude to the experimental uncertainties. The displacement of NBD by β-mercaptoethanol does not fully restore the fluorescence properties of enzyme bound PMP. This irreversible aspect of NBD modification has also been noted in activity measurements: removal of NBD from NBD modified enzyme gives an enzyme that is only 60% active (Lad et al., 1977). At the present time, we have no explanation for this behavior.

Within the experimental uncertainties, the energy transfer efficiencies are identical at pH 7.0 and 8.0, at varying ATP concentrations, in the presence of fructose 6-phosphate, and in the presence of $MgCl_2$. Thus any conformational changes induced by ligand binding and protein aggregation equilibria

alter the distance between enzyme bound PMP and NBD very little (less than a few angstroms). The distance between the PMP-citrate binding site and the sulfhydryl group, which may be near the catalytic site (Jones et al., 1973), is about 40 Å. This indicates that the inhibitory action of citrate is allosteric in nature.

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