Sugar Transport by the Bacterial Phosphotransferase System

FLUORESCENCE STUDIES OF SUBUNIT INTERACTIONS OF ENZYME I*

Myun K. Han§, Jay R. Knutson‡, Saul Roseman, and Ludwig Brand¶

From the Department of Biology and the McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland 21218 and the Laboratory of Technical Development, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892

Enzyme I of the bacterial phosphoenolpyruvate:glycose phosphotransferase system (PTS) exhibits a temperature-dependent monomer/dimer equilibrium. The accompanying paper (Han, M. K., Roseman, S., and Brand, L. (1990) J. Biol. Chem. 265, 1985-1995) shows that the C-terminal -SH residue (Cys-575) can be modified specifically with fluorescent probes such as pyrene maleimide. The derivative retains full enzyme activity, and is capable of forming dimers at room temperature. In the present studies, Enzyme I labeled in this way is found to exhibit a temperature-, concentration-, and pH-dependent monomer/dimer association. The kinetics of dimer formation of Enzyme I in the following way. A derivatized Enzyme I sample is prepared with a pyrene moiety irreversibly attached to the C-terminal -SH residue and 5,5'-dithiobis-2-nitrobenzoic acid reversibly attached to the other 3 -SH residues. This modified enzyme does not form dimers at room temperature. Addition of dithiothreitol results in total release of the thionitrobenzoate anion within 2 min. After the three -SH groups are unblocked, steady-state and nanosecond time-resolved emission anisotropy measurements indicate the dimer equilibrium (9). However, tryptophan fluorescence is not a desirable probe for fluorescence anisotropy studies of large molecules. (For example, the average lifetime of tryptophan is 5-6 ns and the rotational correlation time of the enzyme is 80-160 ns.) The utilization of extrinsic covalent probes is an alternative choice for fluorescence emission anisotropy studies.

As described in the accompanying paper (10), Enzyme I contains four sulfhydryl groups which are classified into two groups: one (C-terminal Cys-575) reacts 10 times faster than the other three -SH groups with appropriate reagents such as DTNB. Chemical modification of the C-terminal cysteine residue does not change the activity of the enzyme, whereas the modification of the three other -SH groups results in an inactive and monomeric enzyme. Modification of the enzyme with a long-lived fluorescent probe at a unique (nonessential) site makes it possible to obtain specific structural insights over a time scale of seconds. Some of the information potentially available from the study of fluorescent-labeled enzymes are the following: accurate hydrodynamic properties of the functional enzyme, changes in conformation, subunit-subunit association, protein-protein interactions, and structure-function correlation. In this paper, we present characteristics of pyrene maleimide-labeled Enzyme I and studies of the influence of concentration, temperature, and pH on the relative quantities of monomer and dimer of Enzyme I labeled at Cys-575.

EXPERIMENTAL PROCEDURES

Materials
DTNB, melatonin, and DTT were obtained from Sigma, N-acetyltryptophanamide from Vega Chemical Co., Tucson, AZ, indole from

1996
Aldrich, and pyrene maleimide from Molecular Probes, Eugene, OR. Enzyme I was purified from an overproducing strain of Escherichia coli (11) as described (10). The protein concentration of Enzyme I was determined spectrophotometrically using a molar extinction coefficient of \( \varepsilon_{280} = 4.0 \) for 10 mg/ml of protein (8). The Bradford method using bovine serum albumin as a standard (12) was also employed.

**Methods**

Spectroscopic Measurements—Absorption spectra were measured with a Varian Cary 219 recording spectrophotometer. Steady-state fluorescence spectra and kinetic data were collected with a Perkin-Elmer MP4 spectrophotofluorometer, and a SLM 8000 photon-counting spectrophotometer with 10-nm Glan-Thompson polarizers operated under “magic angle” conditions to avoid rotational effects (13). The temperatures of the samples were regulated with a Neslab Instruments, Inc. T.E.Q. temperature controller and a PBC4 bath cooler. The absorbances of all fluorescence samples were less than 0.1 at the wavelength of excitation to avoid innerfilter effects (14).

Time-resolved fluorescence was measured on single-photon counting instruments similar to those described by Badesa and Brand (15). Excitation was accomplished by either the output of a PRA thyratron-gated nitrogen flashlamp or by a synchronously pulsed, mode-locked, cavity-dumped dye laser (Spectra-Physics 5000 Nd:Yag) capable of producing 20-ps (fwhm) pulses at a frequency of 4 MHz. For the arc source, excitation light was passed through a polarization scrambler plate, and the emitted fluorescence was measured through a polarizer oriented at 54.7° (the magic angle) from the horizontal symmetry axis. For the laser systems, vertical excitation and emission 54.7° from the vertical symmetry axis were used. The excitation wavelength was 295 nm, and the emission wavelengths were selected via a stepper motor-driven monochromator. Indole, N-acetyltryptophanamide, and melatonin were used as fluorescence standards to verify functioning of the instrument and to correct for the wave-length-dependent transit time of the Amperex 56 DUVP-03 SPL photomultiplier (15) and Hamamatsu R955. All fluorescence measurements were performed with the samples in 100 mM potassium phosphate buffer, \( \text{pH} 7.5 \), and 1 mM EDTA. Pyrene maleimide was dissolved in acetone and added to Enzyme I solution for the labeling.

Data were analyzed by the procedure described by Badesa and Brand (15) and also by the global analysis procedures described by Knutson et al. (10) and Beeskew et al. (17). A word of caution should be given regarding the analysis of the type of data presented below. Data obtained as a function of reaction time (kinetic decay measurements) often comprise multiple individual nanosecond decay curves, each composed of only a few thousand photon counts (S/N \( \approx 100 \)). The total counts under the *entire* surface (rather than each curve) will dominate, as long as Gaussian errors can be assumed. On the other hand, Poisson noise (the fundamental noise process in a photo counting experiment) only approximates Gaussian form for counts \( \approx 50 \). We have observed that “thinning” the data out into many small curves can thus lead to large “lifetime defect” errors (on the order of 0.5–2 ns). Fortunately, as suggested by Selinger and Harris (18), reanalysis with altered least-squares weighting restores the correct values (19, 20).

**RESULTS**

Reaction of Pyrene Maleimide with Enzyme I—Pyrene maleimide is an “SH-selective reagent.” It is virtually nonfluorescent in aqueous solutions, but it becomes fluorescent when conjugated to proteins. The ultraviolet absorption spectrum of pyrene overlaps with the emission spectrum of tryptophan. Reaction of pyrene maleimide with Enzyme I can be measured either by the appearance of pyrene fluorescence or by the quenching of tryptophan fluorescence (Fig. 1).

The kinetics of the reaction between pyrene maleimide and the –SH groups of Enzyme I is biphasic. The slow rate is influenced by either \( \text{Mg}^{++} \) or \( \text{Mg}^{++} \) plus \( \text{P-enolpyruvate} \), suggesting conformational changes induced by PTS ligands (10). These changes are consistent with corresponding results obtained by studying the \( \text{D1N65} \) reactions with Enzyme I (e.g. the predominant effect of PTS ligands was on the slow rate).

Nonanosecond time-resolved fluorescence decay studies were conducted to obtain site-specific information on tryptophan fluorescence during the specific binding of pyrene maleimide to the fast –SH group (Cys-575). The tryptophan emission of Enzyme I is characterized by two prominent decay times (9) of 3.7 and 7.3 ns, and a small fraction of a short-lived species of 0.6 ns (Table 1). The aim of the experiment reported here was to attempt to determine the origin of the two long decay components with respect to the 2 tryptophan residues. One possible explanation for the lifetimes, 3.7 and 7.3 ns, is that each is associated with an individual tryptophan residue. Therefore, the quenching pattern of these lifetimes by pyrene conjugated to the protein should depend on the distance between these tryptophans and Cys-575 (since the efficiency of energy transfer is inversely proportional to the sixth power of the distance between a pair of fluorophores).

Two types of experiments were performed. In one, Enzyme I was reacted with 0.5–1.2 mol of pyrene maleimide/monomer. The reactions were allowed to go to completion and tryptophan fluorescence decay was measured. In a second set of experiments, approximately 1 mol of pyrene maleimide was reacted per mol of Enzyme I monomer, and a series of decay curves were obtained as a function of reaction time.

The results obtained from the first set of experiments are
summarized in Table I. The data were analyzed simultaneously by a global procedure with the decay constants ($\tau_i$ to $\tau_d$) “linked” (16, 17), and the lifetimes recovered from this analysis are 0.7, 1.9, 3.8, and 7.1 ns.

The new value is 1.9 ns, since the fluorescence decay of native Enzyme I (Table I) can be resolved into a triple exponential with two major components of 3.7 ns (41%), preexponential) and 7.3 ns (43%), and a minor component of 0.6 ns (16%). We believe that the 1.9-ns decay component is most likely a quenched lifetime due to a resonance energy transfer from tryptophan to pyrene. The four lifetimes obtained with pyrene-conjugated Enzyme I are very similar to the lifetime values obtained from the TNB-bound enzyme (9).

Tracing the origins of the 1.9-ns component may provide valuable information for distinguishing the origins of the two native major decay components (i.e., whether they arise from the same or both tryptophan residues). The “map” of the extent of reaction (Table I) shows that the amplitude of the 7.1-ns decay ($\alpha_4$) decreases as it is “exchanged” with the amplitude of the 1.9-ns decay ($\alpha_3$). On the other hand, the amplitude of the 3.8-ns component is apparently unchanged. Thus, these results suggest that the 1.9-ns component is a quenched decay arising “solely” from the 7.3-ns species of native Enzyme I, and therefore the 3.7- and 7.3-ns decay components originate independently from the two different tryptophan residues.

The second type of experiment (time-resolved reaction kinetics of the interaction of pyrene with Enzyme I), was performed to test this conclusion.

For kinetic studies, the rapid collection of decay curves during the reaction is essential and can be achieved with a single photon-counting pulse fluorometer with a synchronously pumped, mode-locked, cavity-dumped dye laser as the excitation source. Approximately one pyrene maleimide was reacted with Enzyme I (to label specifically the fast reacting -SH group, Cys-575) and seven consecutive decay curves were analyzed simultaneously, with the aid of a global procedure. Two native components, 3.7 ($\tau_i$) and 7.3 ns ($\tau_d$), were fixed since the PM-labeled Enzyme I exhibits two major native components. Two other components were linked (not fixed), and the lifetimes recovered were 0.5 ($\tau_v$) and 1.9 ns ($\tau_v$). The global reduced $\chi^2$ was 1.15.

Under certain conditions, the initial adduct formed by coupling pyrene maleimide to protein -SH groups may be unstable. The imide ring can open and rearrangement can occur (22-25). It is evident that the succinimido ring on the slow -SH groups open much more rapidly than maleimide adduct of the fast -SH group. Moreover, the derivatized “slow” -SH groups exhibit a small but not insignificant pyrene emission peak at 470 nm, indicating excimer formation. Such a peak at 470 nm has never been detected with Enzyme I selectively labeled at pH 7.5 as a function of time. A, PM-labeled only on the fast -SH group of Enzyme I. B, PM-labeled on the three slow -SH groups at pH 7.5. Emission spectra were recorded with excitation at 540 nm at 24-h intervals (top to bottom) at room temperature. Enzyme concentration was 0.1 mg/ml in 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA.

The kinetic data shown in Fig. 2 independently support the suggestion that only a 7.3-ns component is quenched upon modification of the “fast reacting” -SH group. The peak counts were less than 1000 in each curve. The seven decay curves were analyzed simultaneously, with the aid of a global procedure. Two native components, 3.7 ($\tau_i$) and 7.3 ns ($\tau_d$), were fixed since the PM-labeled Enzyme I exhibits two major native components. Two other components were linked (not fixed), and the lifetimes recovered were 0.5 ($\tau_v$) and 1.9 ns ($\tau_v$). The global reduced $\chi^2$ was 1.15.

Characterization of pyrene fluorescence in Enzyme I—Fig. 3 shows emission spectra of pyrene maleimide conjugated mainly to the fast (A) and also mainly to the slow reacting (B) -SH groups of Enzyme I at pH 7.5. Emmission spectra were recorded at 24-h intervals. When Enzyme I is labeled with less than 1 mol of PM/monomer at pH 7.5, preferential labeling takes place at the C-terminal cysteine residue (10). The spectra for this case (Fig. 3A) show that the emission peak at 376 nm is higher than the peak at 396 nm. In contrast, when slow -SH groups are labeled at pH 7.5 (with the fast -SH group protected by reaction with DTNB), the ratio of these two emission peak heights is quite different (Fig. 3B).
provides a unique labeling site. The C-terminal cysteine residue was therefore labeled with pyrene maleimide, and the monomer/dimer association of the labeled enzyme was characterized by fluorescence emission anisotropy experiments.

The temperature-dependent subunit interactions were examined, and a Perrin plot of the selectively labeled sample is shown in Fig. 4. Labeled Enzyme I exhibits a transition between 6 and 16 °C. In this range, steady-state anisotropy increased as the temperature increased, in accord with the expected monomer/dimer transition. This transition has also been observed by time-resolved emission anisotropy of tryptophan fluorescence (9) and previously by ultracentrifugation (5) and column chromatography (6). Moreover, the DTNB reaction with -SH groups of Enzyme I indicated changes in reactivity of the slow-reacting -SH groups in this temperature range (10). These results are consistent with previous studies showing that modification of Cys-575 yields both a catalytically active and a dimeric enzyme.

Since the monomer-dimer transition is a function of protein concentration, the emission anisotropy of labeled Enzyme I was studied at various concentrations at 23 °C (Fig. 5A) and 14 °C (Fig. 5B). At 23 °C, where native Enzyme I is predominantly a dimer, the pyrene maleimide-labeled enzyme likewise is dimeric down to 0.08 mg/ml; as the concentration is decreased at 14 °C. It should be noted that the pyrene fluorescence yield is not significantly dependent on the monomer/dimer transition. This transition has also been observed by time-resolved emission anisotropy of tryptophan fluorescence (9) and previously by ultracentrifugation (5) and column chromatography (6). Moreover, the DTNB reaction with -SH groups of Enzyme I indicated changes in reactivity of the slow-reacting -SH groups in this temperature range (10). These results are consistent with previous studies showing that modification of Cys-575 yields both a catalytically active and a dimeric enzyme.

The pH-dependent anisotropy experiments were repeated further as a function of pH, in the range pH 6.0-8.0, in 0.1 M potassium phosphate buffer containing 1 mM EDTA, and the results are shown in Fig. 6. The labeled enzyme remains dimeric between pH 6.8 and 8.0. At lower pH values the anisotropy decreased, indicating dissociation of dimers. Anisotropy values between pH 6.0 and 6.2 were around 0.12, which may indicate that the enzyme is not completely monomeric at these conditions.

The pH-dependent anisotropy experiments were repeated with nanosecond time-resolved emission anisotropy. In order to study the time-dependent decay of the emission anisotropy of the labeled enzyme, two fluorescence decay curves Icv(t) and Inv(t) were obtained under dimer conditions (25 °C). The data were analyzed in terms of a single rotational correlation time, which should reflect some average of the monomer and dimer values (Table II).

At pH 7.0, the best single rotational correlation time extracted from the data was 105 ns. The values decreased at lower pH (94 ns at pH 6.65 and 74 ns at pH 6.2). Therefore, the effect of pH on the rotational correlation times is consistent with the steady-state emission anisotropy results shown in Fig. 6. In contrast, the rotational correlation time of a dansylated monomer at pH 7.5 and 20.5 °C is 45 ns (10).
Therefore, the rotational correlation time of 74 ns at 25 °C indicates that Enzyme I is not totally monomeric at pH 6.2 at the concentration used.

**Kinetic Measurement of Monomer/dimer Association—Fluorescence emission anisotropy is not only a useful tool for hydrodynamic measurements of proteins under equilibrium conditions, but the technique can be used to obtain kinetic information during protein-protein or subunit interactions.** For kinetic studies of subunit association, it is desirable for the initial studies to start with sample containing only monomeric Enzyme I. Temperature can be used to shift the equilibrium, but temperature also changes solvent viscosity and fluorescence properties, thus complicating hydrodynamic measurements. An alternative method was therefore developed.

The modification of all four -SH groups results in inactivation of Enzyme I with subsequent monomerization (10). When the -SH groups are derivatized with DTNB, the TNB moieties linked to the protein can be removed by reduction with DTT, thereby reactivating the enzyme with concomitant dimerization (10, 26).

The reversibility of TNB inactivation of Enzyme I was therefore used in steady-state emission anisotropy experiments to measure the kinetics of Enzyme I dimerization. Enzyme I was selectively and irreversibly labeled with pyrene maleimide at the C-terminal cysteine, Cys-575, which does not inactivate the enzyme. The other three -SH groups were reversibly labeled with DTNB, removed with DTT, and the dimerization process was followed by measuring changes in the emission anisotropy.

The time course of dimerization was measured at 3 and 25 °C after addition of dithiothreitol, and the results are shown in Fig. 7. At both 3 and 25 °C, the addition of DTT resulted in an initial drop in emission anisotropy for about 2 min. The TNB chromophore covalently linked to the protein has reasonable spectral overlap with pyrene and is expected to decrease the decay time of pyrene by resonance energy transfer. When the TNB anion is released from the protein, the expected results is an increase in the pyrene decay time or a decrease in the steady-state emission anisotropy (since steady-state anisotropy is inversely related to the lifetime of the probe). The difference in the initial steady-state emission anisotropy (prior to addition of DTT) at 3 °C versus 25 °C results from temperature effects on anisotropy; higher anisotropy (at time = 0) is expected at lower temperatures mostly because of the increase in the viscosity of water.

Following the initial rapid change (2 min) at 25 °C, the anisotropy slowly increased from about 0.10 to 0.145 with the rate constant of 7.7 × 10⁻⁴/s (τ_{an} = 15 min). By contrast, only small anisotropy changes are seen at 3 °C, a condition in which the native enzyme is predominantly monomeric. This confirms the absence of large volume changes upon TNB release. Nevertheless, small changes in anisotropy may indicate either a conformational change without dimer formation or partial dimer formation.

The slow changes in apparent volume during TNB release were also studied by time-resolved techniques. Anisotropy decay was measured before the addition of dithiothreitol and 40 min after. Following the initial rapid change (2 min) at 25 °C, the correlation time prior to DTT addition was 64 ns compared to 90 ns measured 40 min after DTT addition (data not shown). These results, taken together with the steady-state data, suggest that anisotropy changes observed in these experiments represent slow dimer formation.

**Kinetic Studies of TNB Release from the DTNB-modified Enzyme I of PTS—**Because the absorption spectrum of TNB bound to the protein overlaps the tryptophan emission spectrum, quenching of tryptophan fluorescence was expected and observed upon reaction of Enzyme I with DTNB. In addition, tryptophan fluorescence was sensitive to temperature changes, an effect which can be ascribed to monomer/dimer equilibrium. As Fig. 8 shows, the intensity of tryptophan fluorescence at 23 °C (as a dimer) was higher than at 1 °C (as a monomer). This intensity change also accompanies a slight spectral shift, suggesting that conformational changes accompany subunit association. Moreover, the difference spectrum is blue-shifted from the total emission spectrum.

Neyroz et al. (9) reported that the 2 tryptophan residues of Enzyme I exhibit two distinctive decay-associated emission profiles, one of which (that associated with the shorter decay component) is more sensitive to temperature. This may indicate that each of the two tryptophans exhibit unique decay times. This, in turn, suggests that one of the 2 tryptophan residues is relatively more sensitive to monomer/dimer association than the other tryptophan residue.
Thus, derivatization of the sulfhydryl groups of Enzyme I with DTTNB may alter tryptophan fluorescence by both resonance energy transfer (tryptophan to TNB) and monomer/dimer-associated conformational changes. It is important to examine closely the changes in tryptophanyl fluorescence to determine if these two processes can be distinguished.

One way to approach this problem is to study the release of TNB from the modified enzyme. Specifically, one can compare the rate of TNB release to the rate of fluorescence recovery. If only quencher release was involved in tryptophan fluorescence, the rate of fluorescence recovery should be the same as the rate of TNB release. More generally, fluorescence changes may also arise from any conformational changes associated with subunit-subunit interactions and the recovery rate might not be the same.

For this experiment, TNB-bound Enzyme I was first prepared by incubating native Enzyme I at room temperature with excess DTNB in 100 mM potassium phosphate, pH 7.5, and 1 mM EDTA. Modified Enzyme I was separated from excess DTNB by chromatography on a Sephadex G-25 column, equilibrated with 100 mM potassium phosphate, pH 7.5, and 1 mM EDTA at 23 °C. The degree of labeling was estimated by treating TNB-bound enzyme with a 20-fold excess of DTT followed by dialysis and Sephadex chromatography, and by measuring the amount of TNB anion released. The enzyme released 3.9 mol of TNB anion/mol, indicating that all four -SH groups per monomer were fully occupied by TNB.

The rate of TNB release can easily be measured by monitoring absorbance at 412 nm (27), and the result obtained at room temperature is shown in Fig. 9A. Fig. 9B shows the concomitant increase in steady-state tryptophan fluorescence. It is interesting to note that the kinetics of fluorescence recovery are biphasic with the rate constants of 8.3 × 10⁻⁷/s and 6.6 × 10⁻⁴/s. In contrast, the release of TNB (measured by absorbance) is essentially complete within a minute (with the rate constant of 8.45 × 10⁻³/s).

Thus, the rapid increase in the tryptophan fluorescence intensity is most likely associated with the loss of resonance energy transfer due to the fast release of TNB anion, since these two rates are the same (within experimental error). The slow phase of fluorescence recovery is intriguing because it indicates conformational changes accompanying dimerization.

**DISCUSSION**

Enzyme I is a key protein in the bacterial PTS since it is the first protein in the phosphotransfer chain and therefore may regulate the PTS and associated systems, such as adenylate cyclase. Previous studies (5-7) based on ultracentrifugation and molecular sieve chromatography presented evidence that Enzyme I exhibits a temperature dependent monomer/dimer transition and that the monomers are identical. It is likely (8, 28) that the dimer form of the enzyme is the active species.

The aim of this study was to use both intrinsic and extrinsic fluorescence probes to characterize the monomer/dimer equilibrium of Enzyme I with fluorescence emission anisotropy, (b) to examine conformational changes of the enzyme associated with the monomer/dimer transition, and (c) to characterize the kinetics of dimer formation from chemically induced monomers. Extrinsic fluorescence probes offer many advantages. For instance, probes with suitable excited state lifetimes, and advantageous excitation and emission spectral characteristics may be selected so that custom tailored experiments can be performed. Extrinsic probes also have advantages. Covalent coupling of the probe at specific and unique sites is often difficult to achieve and the modified protein may have altered functions as compared to the native molecule.

In this study, site-specific labeling was achieved by reacting pyrene maleimide to the fast reacting –SH group, Cys-575, of Enzyme I. The resulting enzyme was fully active and formed a dimer. Confirmation of the site-specific labeling was presented in the accompanying paper (10). Pyrene was chosen as an extrinsic probe because it has a long decay time (29-33) and is thus useful for time-resolved emission anisotropy studies as well as for simpler steady-state fluorescence anisotropy studies, which may be extended to stopped-flow speeds.

A potential difficulty with the use of pyrene maleimide as a probe is that ring opening can occur after conjugation (22-25). It has been suggested that 5-[N-(1-pyrene)-succinimido] cysteine, a product of the reaction of the sulfhydryl group of cysteine with the olefinic double bond of the maleimide moiety of N-(1-pyrene)-maleimide, undergoes a slow cleavage of the succinimido ring by either hydrolysis or aminolysis. Aminolysis accompanies cyclization of the succinimido ring to form thiazine derivatives as a result of subsequent nucleophilic attack by the amino group on a carbonyl carbon (22, 23).

The stability of N-ethylmaleimide adducts with thiol groups has been described (22-24). The adduct is reasonably stable at pH 7.0 in potassium phosphate buffer but is much less stable under more alkaline conditions. Smyth and Tuppy (23) concluded that intramolecular cross-linking of amino and thiol groups by maleimide reagents occurs only when the groups are located in sterically favorable positions. They showed further that under optimal conditions, the competing reactions of intermolecular aminolysis and hydrolysis are minor.

Wu et al. (25) extended the studies of intramolecular aminolysis with pyrene maleimide (PM). They reported that cleavage of the succinimido ring causes a spectral shift in both the excitation and emission spectra of pyrene and to changes in decay times. The nature of the rearrangements often depends on the environment of the cysteine residue (25), and we find this to be the case with Enzyme I.

In the present study it has been shown that, while pyrene maleimide conjugated to the slow –SH residues is susceptible to rapid ring opening, the derivative at Cys-575 is relatively stable to the ring opening reactions. Spectral changes taking place over a 48-h period at pH 7.5 with pyrene specifically conjugated to the fast –SH (Cys-575) and also with pyrene reacted only with the slow cysteine residues are shown in Fig. 3. The derivative of the fast –SH is reasonably stable for 7 days at pH 7.5 and even more stable at more acidic pH. The pyrene maleimide derivatives of the three slow –SH groups

![Fig. 9. The release of TNB from the DTNB-modified Enzyme I was measured by A, absorbance (A₄₁₂) and B, tryptophan fluorescence with excitation and emission wavelengths of 295 and 350 nm, respectively, at pH 7.5 (room temperature). A₄₁₂ of the sample was 0.1.](image-url)
gave different results. Fig. 3B shows that the imide ring opened much more rapidly, so that at 48 h (pH 7.5), the derivative had lost about 50% of the initial emission. Thus, the fast and slow sulphhydryl residues are distinguishable by: (a) their relative reactivity to -SH reagents, (b) a spectrally observed ring opening reaction which depends on the microenvironment, and (c) excimer formation between closely located pyrene probes on the protein. Because of these results, the remaining experiments were conducted with freshly prepared conjugates of Enzyme I, labeled with pyrene maleimide specifically at Cys-575, in which negligible ring opening had taken place.

Enzyme I labeled specifically at Cys-575 showed a temperature-, concentration-, and pH-dependent monomer/dimer equilibrium. As indicated by a Perrin plot (Fig. 4), the emission anisotropy showed an inflection at the temperature region of 6–16 °C, conditions where the native enzyme undergoes the monomer/dimer transition (9, 10). These results, together with concentration dependent anisotropy data, indicate that the labeled Enzyme I behaves similar to the native enzyme. Moreover, emission anisotropy of the labeled enzyme was pH-dependent; optimum anisotropy was found between pH 7 and 7.5. Interestingly, Waygood and Steeves (34) reported that the activity of Enzyme I was also pH-dependent with maximum activity at pH 7. These results indicate a good correlation between enzyme activity and the amount of dimeric form present, suggesting that the dimer form of the enzyme is the active species.

As indicated by Neyroz et al. (9), tryptophan fluorescence of Enzyme I exhibits two major decay-associated spectra (35): the 3.7-ns component is blue-shifted compared to the 7.3-ns component. Moreover, the spectrum associated with the 3.7-ns component comprises 17% of total fluorescence intensity at 1 °C (monomeric condition), whereas this contributes 44% of total intensity under dimeric conditions, at 23 °C (9). This is also clearly evident in Fig. 8 which shows that fluorescein intensity at 23 °C is higher than at 1 °C, and that the difference emission spectrum is suggestive of the 3.7-ns decay-associated spectra. These data suggest that the two decay times originate from tryptophan residues in different unique environments.

The results obtained with the tryptophan to pyrene resonance energy transfer experiments provide valuable information for distinguishing the origins of the two major decay times. Enzyme I with pyrene specifically conjugated to the fast -SH (Cys-575) contains two fluorescence energy donors (Trp-355 and Trp-498) and a common acceptor (pyrene). Both titration and kinetic data indicate that the conjugation of pyrene maleimide to the protein results in quenching of the tryptophan fluorescence of Enzyme I and the emission anisotropy of pyrene conjugated to Cys-575. Although the release of TNB is essentially complete within a minute, the dimerization as measured by pyrene emission anisotropy takes place over a longer time range. There is a rapid increase in tryptophan fluorescence intensity almost certainly associated with abolishment of quenching due to energy transfer to enzyme-bound TNB. This is followed by a slower increase in tryptophan fluorescence intensity which occurs at a rate similar to the dimerization. This change in fluorescence intensity may represent a conformational change (most likely near Trp-498) which occurs upon dimerization. It is also possible that this conformational change precedes dimerization and actually represents the rate limiting process in the dimerization reaction. The requirement for the subunits to adopt the correct conformation for productive interaction may explain the relatively slow rates of dimerization observed in Enzyme I.

As indicated above it was previously established that the monomer/dimer transition of Enzyme I could be controlled by changing the temperature. The work described in the present paper provides a new way of "preparing" monomer species, namely derivatization of the slow -SH residues. It remains to be determined if the conformation of the monomer at low temperature and at room temperature but with blocked -SH residues is similar.

The fluorescence techniques described here including the kinetics of time-resolved fluorescence changes and decay-associated emission spectroscopy should prove useful in obtaining a better understanding of the structure-function relations of Enzyme I and other subunit proteins.

REFERENCES

1. Kundig, W., Gosh, S., and Roseman, S. (1964) Proc. Natl. Acad. Sci. U. S. A. 52, 1067–1074
2. Enotini, R. D., Levinthal, M., Kundig, F. D., Kundig, W., Anderson, B., Hartman, P. E., and Roseman, S. (1967) Proc. Natl. Acad. Sci. U. S. A. 58, 1963–1970
3. Roseman, S. (1968) J. Gen. Physiol. 54, 115–130
4. Weigel, N., Kukuruzinska, M. A., Nakazawa, A., Waygood, E. B., and Roseman, S. (1982) J. Biol. Chem. 267, 14477–14491
5. Kukuruzinska, M. A., Harrington, W. F., and Roseman, S. (1982) J. Biol. Chem. 287, 1470–1476
6. Kukuruzinska, M. A., Turner, B. W., Ackers, G. K., and Roseman, S. (1984) J. Biol. Chem. 259, 11679–11681
7. Meadow, N. D., Kukuruzinska, M. A., and Roseman, S. (1984) in Enzymes of Biological Membranes (Martonosi, A., ed) pp. 523–599, Plenum Publishing Corp., New York
8. Weigel, N., Waygood, E. B., Kukuruzinska, M. A., Nakazawa, A., and Roseman, S. (1982) J. Biol. Chem. 257, 14461–14469
9. Neyroz, P. N., Brand, L., and Roseman, S. (1987) J. Biol. Chem. 262, 15900–15907
10. Han, M. K., Roseman, S., and Brand, L. (1990) J. Biol. Chem. 265, 1985–1995
11. Safven, D. W. (1985) Ph.D. dissertation, Molecular Cloning Characterization and Sequencing of the E. coli leucine operon, The Johns Hopkins University
12. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
13. Spencer, R. D., and Weber, G. (1969) Ann. N. Y. Acad. Sci. 158, 361–376
14. Parker, C. A., and Rees, W. T. (1962) Analyst 87, 83–111
15. Badea, M. G., and Brand, L. (1979) Methods Enzymol. 61H, 378–394
16. Knutson, J. R., Beechem, J. M., and Brand, L. (1983) Chem. Phys. Lett. 102, 501–507
17. Beechem, J. M., Ameloot, M., and Brand, L. (1986) Anal. Instrum. 14, 379–402
18. Selinger, B. K., and Harris, C. M. (1983) in Time-resolved Fluorescence Spectroscopy in Biochemistry and Biology (Cundall, R. B., and Dale, R. E., eds) pp. 155–179, Plenum Publishing Corp., New York
19. Walbridge, D. G., Knutson, J. R., and Brand, L. (1987) Anal. Biochem. 161, 467–476
20. Knutson, J. R. (1987) Biophys. J. 51, 285a
Sugar Transport by the Bacterial Phosphotransferase System

21. Han, M. K., Wallbridge, D. G., Knutson, J. R., Brand, L., and Roseman, S. (1987) Anal. Biochem. 161, 473-486
22. Gregory, J. D. (1955) J. Am. Chem. Soc. 77, 3922-3933
23. Smyth, D. G., and Tuppy, H. (1968) Biochim. Biophys. Acta 168, 173-180
24. Heitz, J. R., Anderson, C. D., and Anderson, B. M. (1968) Arch. Biochem. Biophys. 127, 627-636
25. Wu, C. W., Yarbrough, L. R., and Wu, V. H. (1976) Biochemistry 15, 2863-2868
26. Kundig, W., and Roseman, S. (1971) J. Biol. Chem. 246, 1393-1400
27. Ellman, G. (1959) Arch. Biochem. Biophys. 82, 70-77
28. Waygood, E. B., Weigel, N., Nakazawa, A., Kukuruzinska, M., and Roseman, S. (1977) Proc. Can. Fed. Biol. Soc. 20, 54
29. Knopp, J. A., and Weber, G. (1969) J. Biol. Chem. 244, 6309-6315
30. Weltman, J. K., Szaro, R. P., Frackelton, A. R., Jr., Dowben, R., M., Bunting, J. R., and Cathou, R. E. (1973) J. Biol. Chem. 248, 3173-3177
31. Holowka, D. A., and Hammes, G. G. (1977) Biochemistry 16, 5538-5545
32. Rao, A., Martin, L., Reithmeier, R. A. F., and Cantly, L. C. Biochemistry 18, 4505-4516
33. Graceffa, P., and Lehrer, S. S. (1980) J. Biol. Chem. 255, 11296-11300
34. Waygood, E. B., and Steeves, T. (1980) Can. J. Biochem. 58, 40-48
35. Knutson, J. R., Wallbridge, D. W., and Brand, L. (1982) Biochemistry 21, 4671-4679
36. Han, M. K., Knutson, J. R., Roseman, S., and Brand, L. (1987) Biochem. J. 241, 278a
37. Han, M. K., Knutson, J. R., Roseman, S., and Brand, L. (1987) Proceedings of the International Biophysics Congress, Jerusalem, Israel
Sugar transport by the bacterial phosphotransferase system. Fluorescence studies of subunit interactions of enzyme I.
M K Han, J R Knutson, S Roseman and L Brand

J. Biol. Chem. 1990, 265:1996-2003.

Access the most updated version of this article at http://www.jbc.org/content/265/4/1996

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/265/4/1996.full.html#ref-list-1