Sensitive Monitoring of the Dynamics of a Membrane-Bound Transport Protein by Tryptophan Phosphorescence Spectroscopy†

Jaap Broos,*,‡ Giovanni B. Strambini,*,§ Margherita Gonnelli,§ Erwin P. P. Vos,‡ Michel Koolhof,‡ and George T. Robillard‡

Department of Biochemistry and Groningen Biomolecular Science and Biotechnology Institute (GBB), University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands, and CNR, Instituto di Biofisica, Area della Ricerca di Pisa, via Alfieri 1, 56010, Pisa, Italy

Received April 10, 2000; Revised Manuscript Received June 1, 2000

ABSTRACT: This paper presents a tryptophan phosphorescence spectroscopy study on the membrane-bound mannitol transporter, EII^{mtl}, from *E. coli*. The protein contains four tryptophans at positions 30, 42, 109, and 117. Phosphorescence decays in buffer at 1 °C revealed large variations of the triplet lifetimes of the wild-type protein and four single-tryptophan-containing mutants. They ranged from <70 μs for the tryptophan at position 109 to 55 ms for the residue at position 30, attesting to widely different flexibilities of the tryptophan microenvironments. The decay of all tryptophans is multiexponential, reflecting multiple stable conformations of the protein. Both mannitol binding and enzyme phosphorylation had large effects on the triplet lifetimes. Mannitol binding induces a more ordered structure near the mannitol binding site, and the decay becomes significantly more homogeneous. In contrast, enzyme phosphorylation induces a large relaxation of the protein structure at the reporter sites. The implications of these structural changes on the coupling mechanism between the transport and the phosphorylation activity of EII^{mtl} are discussed. Taken as a whole, our data show that tryptophan phosphorescence spectroscopy is a very sensitive technique to explore conformational dynamics in membrane proteins.

The mannitol permease of *E. coli*, EII^{mtl},[1,2] is responsible for the transport of mannitol across the inner membrane into the cytoplasmic space and its concomitant phosphorylation to mannitol 1-phosphate (*1, 2*). The enzyme is part of the phosphoenolpyruvate-dependent phosphotransferase system (PTS) (*3*) in which the phosphoryl group is transferred from phosphoenolpyruvate (PEP) to EII^{mtl} via the cytoplasmic proteins EI and HPr. EII^{mtl} is a single-chain protein with three domains: the membrane-embedded C domain harboring the sugar translocation channel, and the cytoplasmic A and B domains (Figure 1). Phosphoryl transfer within EII^{mtl}, from HPr-P to mannitol bound at the C domain, proceeds by sequential phosphorylation of H554 in the A domain and C384 in the B domain, followed by phosphoryl transfer to mannitol. Although the mechanism of coupling between phosphoryl transfer and sugar transport is still speculative, kinetic and calorimetric data showed that phosphorylation at the A and B domains of EII^{mtl} induces strong B/C interdomain interactions resulting in a 1000-fold faster mannitol translocation over the membrane (*4*−*6*).

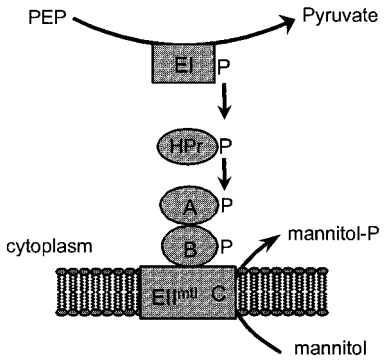

FIGURE 1: Schematic representation of the mannitol-specific PTS system as found in *E. coli*.

Structural information on the 36 kDa C domain is limited to a 5 Å projection map solved very recently (*7*). Tryptophan (Trp) fluorescence spectroscopy is being used to obtain site-specific structural and dynamic information on the C domain (*8*−*11*). EII^{mtl} contains four tryptophans all located in the C domain at positions 30, 42, 109, and 117. Single-Trp-containing mutants were constructed by replacing three of the four tryptophans by phenylalanines; the mutants were

† This work has been financially supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Scientific Research (NWO) and the Italian National Research Council.

* Corresponding authors. J.B.: Telephone +31 50 3634277, Telefax +31 50 3634165, E-mail J.Broos@chem.rug.nl. G.B.S.: Telephone +39 050 3153046, Telefax +39 50 3152760, E-mail strambin@ib.pi.cnr.it.

‡ University of Groningen.

§ CNR, Instituto di Biofisica.

[1] Abbreviations: PTS, phosphoenolpyruvate-dependent phosphotransferase system; EII^{mtl}, mannitol-specific transporting and phosphorylating enzyme from *E. coli*; EI, EI from the PTS system; HPr, histidine-containing protein; ISO, inside-out; DOC, sodium deoxycholate; $C_{10}E_5$, decylpenta(ethylene glycol); Trp, tryptophan; PEP, phosphoenolpyruvate; PG, 1,2-propylene glycol; BW, bandwidth in nanometers of the 0,0 vibronic band at two-thirds peak height; $\lambda_{0,0}$, peak wavelength of the 0,0 vibrational band.

[2] Nomenclature of the enzymes: EII^{mtl}, wild-type enzyme containing tryptophans at positions 30, 42, 109, and 117; W30, W42, W109, W117, single-Trp-containing EII^{mtl} mutants with tryptophans present at positions 30, 42, 109, 117, respectively, while the other three tryptophans have been replaced with phenylalanine.

10.1021/bi000803z CCC: $19.00
Published on Web 08/08/2000

characterized by steady-state and time-resolved fluorescence techniques.

A detailed description of the transport mechanism requires the characterization of the conformations of the protein involved in the different stages of solute translocation. However, because these changes are in general subtle, they either are not detected by conventional biophysical methods or, as in the case of fluorescence, are not readily ascribed to specific structural alterations. In the past decade, the phosphorescence emission of Trp residues was shown to provide an exquisitely sensitive monitor of the local protein structure (*12*−*14*). Spectral energies give information about the polarity of the chromophore's environment (*15*) while the excited-state lifetime monitors the rate of electron/energy transfer to reactive centers from which distance relationships may be derived. Most importantly, in the absence of van der Waals contact quenching by cysteine, tyrosine, or histidine, the Trp phosphorescence lifetimes of proteins in fluid solutions provide a remarkably sensitive probe of the local fluidity of the protein matrix. Thus, depending on the microenvironment, the lifetime ranges from submilliseconds, when the Trp side chain is in unstructured fluid regions of the polypeptide, to seconds for completely buried and immobilized sites (*16, 17*). Last, information on structural flexibility can also be derived from the rate of diffusion of phosphorescence-quenching solutes such as oxygen and acrylamide (*18, 19*). These parameters have been instrumental in uncovering subtle changes in the globular structure of enzymes brought about by the binding of substrates and allosteric effectors (*20*−*22*) or by varying the medium and experimental conditions (*13, 23, 24*).

This paper examines the phosphorescence characteristics of the wild-type and four single-Trp mutants of EII^{mtl} in low temperature (−130 °C) glasses and in buffer at 1 °C. The results show that the four tryptophans are embedded in protein environments quite distinct in micropolarity and local flexibility. Relatively broad spectra and nonuniform decays in single-Trp mutants point to intrinsic heterogeneity of the C domain structure. Interestingly, both mannitol binding and especially enzyme phosphorylation induce large changes in C domain dynamics and heterogeneity. The involvement of the A and B domains in these conformational changes is discussed.

MATERIALS AND METHODS

Plasmids and Bacterial Strains. The *E. coli* bacterial strain LGS-322 (*25*), containing a chromosomal deletion in the wild-type mtlA gene, was used for the expression of EII^{mtl}. Plasmids bearing the genes of the single-Trp mutants of EII^{mtl} (W30, W42, W109, W117) (*9*) were used; the 5′ regions of the genes were engineered to obtain EII^{mtl} with an N-terminal thrombin-cleavable his-tag. Construction of the plasmid specifying the N-terminal his-tagged EII^{mtl} will be published elsewhere.

Protein Purification. The EII^{mtl} purification was performed with purified DOC and $C_{10}E_5$ detergents (*8*), and guidelines were followed to prevent the introduction of UV-absorbing impurities during the isolation procedure (*8*). ISO vesicles were prepared as described (*11*). The extraction of EII^{mtl} by DOC (*11*) was performed in the presence of 100 μM mannitol. The DOC extract was loaded on a Ni−NTA resin (Qiagen Inc.) column (1.5 mL/ mL vesicle solution), equilibrated with 20 mM Tris-HCl, pH 8.4, 0.5% DOC, 300 mM NaCl, 100 μM mannitol, 2 mM reduced glutathione, and 7.5 mM imidazole. After being loaded, the column was washed with 5 column volumes of the equilibration buffer, followed by 5 column volumes of 20 mM Tris-HCl, pH 7.6, 0.25% $C_{10}E_5$, 150 mM NaCl, 100 μM mannitol, and 2 mM reduced glutathione. The column was eluted with 50 mM L-histidine in this buffer, and the NaCl was omitted. Then 150 mM NaCl and 2 mM $CaCl_2$ were added to the pooled enzyme fraction, and the N-terminal his-tag was cleaved off overnight at 10 °C by thrombin (from bovine plasma, ICN; 10% w/w compared to EII^{mtl}). The EII^{mtl} sample was diluted 4-fold with 20 mM Tris-HCl, pH 8.4, 0.25% $C_{10}E_5$, and 2 mM reduced glutathione and loaded on a MonoQ HR 5/5 column (Phamarcia Biotech), equilibrated with 20 mM Tris-HCl, pH 8.4, 0.25% $C_{10}E_5$, and 2 mM reduced glutathione. The column was washed with the equilibration buffer and 100 mM NaCl until a low and stable fluorescence signal was achieved. The enzyme was eluted with a NaCl gradient from 100 to 400 mM (2 × 20 mL, 1 mL/min). The isolated proteins were not contaminated by fluorescent or phosphorescent impurities, and the same fluorescence emission spectra as reported previously for these enzyme were obtained (*9*). The purification procedures were performed in the cold room. The PEP-dependent mannitol phosphorylation activity of EII^{mtl} was determined as described (*26*). EII^{mtl} concentrations of the purified enzymes were determined by a pyruvate burst assay (*27*). EI and HPr were purified as described previously (*28, 29*).

Sample Preparation for Phosphorescence Measurements. In phosphorescence measurements, protein concentrations of 2−4.4 μM in 20 mM Tris-HCl, pH 8.4, 0.275 mM NaCl, 0.25% $C_{10}E_5$, and 2 mM reduced glutathione were used. The latter compound was added to prevent irreversible EII^{mtl} inactivation caused by oxidation of the very reactive C384. Control experiments with wild-type and W117 samples showed that the decays were not affected by the presence of reduced glutathione. For experiments in low-temperature (140 K) glasses, solutions were diluted by 1 volume of 1,2-propylene glycol (Merck). The effect of mannitol or perseitol binding was determined at a substrate concentration of 50 μM. EII^{mtl} was phosphorylated by incubating the protein in 5 mM $MgCl_2$, 50 μM PEP, 1 μM HPr, and 25 nM EI for 10 min at room temperature. The phosphorylated enzyme is stable under the experimental conditions (*30*). Prior to phosphorescence decay measurements, the samples were placed in 5 × 5 mm^2 quartz cuvettes especially designed for allowing a thorough removal of oxygen by the alternating application of moderate vacuum and purging with ultrapure N_2 (*16*).

Phosphorescence Measurements. A conventional home-made instrument was employed for all phosphorescence measurements in low-temperature glasses. The excitation provided by a Cermax xenon lamp (LX 150 UV; ILC Technology, Sunnyvale, CA) was selected by a 0.25 m grating monochromator (Jobin-Yvon, H25) (5 nm band-pass), and the emission, dispersed by a 0.25 m grating monochromator (Jobin-Yvon, H25) (1.5 nm band-pass), was detected with an EMI 9635QB photomultiplier. Phosphorescence decays in fluid solutions were measured as described before (*16*). Briefly, pulsed excitation was provided by a frequency-

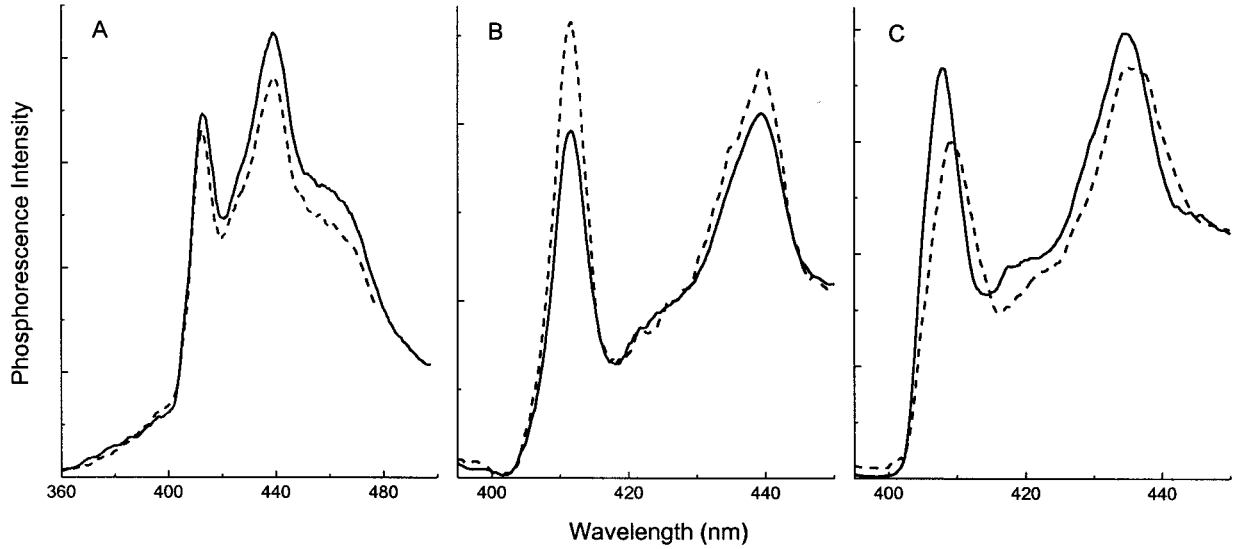

FIGURE 2: Phosphorescence spectra of wild-type EII^{mtl} (A), W30 (B), and W42 (C) in PG/buffer glass at 140 K. Dashed spectra refer to phosphorylated samples. The tyrosine contribution (Trp-less EII^{mtl}) was subtracted from the spectra in (B) and (C). The protein concentration is 1.2 μM for wild-type, 1.1 μM for W30, and 2.2 μM for W42. The excitation wavelength was 295 nm.

doubled flash-pumped dye laser (UV 500 M-Candela) (λ_{ex} = 292 nm) with a pulse duration of 1 μs and an energy per pulse of typically 1−10 mJ. The emitted light was collected at 90° relative to the excitation direction and selected by a filter combination with a transmission window between 420 and 460 nm. The photomultiplier was protected from the intense excitation and fluorescence light pulse by a high-speed chopper blade that closed the slits during laser excitation. The minimum dead time of the apparatus is about 10 μs. The photocurrent was amplified by a current to voltage converter (SR570, Stanford Research Systems) and digitized by a computerscope system (EGAA; RC Electronics) capable of averaging multiple sweeps. Subsequent analysis of decay curves in terms of discrete exponential components was carried out by a nonlinear least-squares fitting algorithm, implemented by the program Global Analysis. The reproducibility of τ was typically about 5% for samples taken from the same protein batch and about 15% among different batches. All data reported refer to the same protein preparation.

RESULTS

Phosphorescence Spectra in Low-Temperature Glasses. The spectral energy and its distribution are usually indicated by the peak wavelength ($\lambda_{0,0}$) and the bandwidth (BW) of the 0,0 vibronic band which, in turn, are related to the polarity and to the structural homogeneity of the Trp environment, respectively. The high-resolution phosphorescence spectrum of wild-type EII^{mtl} in PG/buffer (50/50, v/v) glasses, at 140 K, is shown in Figure 2A. The spectrum is broad, typical of proteins with multiple tryptophans. $\lambda_{0,0}$ is 412.7 nm, but the 0,0 vibronic band exhibits a clear shoulder at 408 nm, indicative of Trp residues with distinct spectral energies. The spectral resolution increases with all single-Trp mutants of EII^{mtl} as shown for W30 and W42 in Figure 2B,C. $\lambda_{0,0}$ and the BW of the single-Trp mutants are collected in Table 1. $\lambda_{0,0}$ ranges from 407.7 nm for W42 to 413.2 nm for W117. The spectral energies of the mutants indicate that the aromatic ring of each Trp is effectively shielded from the aqueous interface. $\lambda_{0,0}$ is typically 406 nm for Trp exposed to PG/buffer and 411−411.5 nm for the indole ring in a nonpolar environment (*15*). Among the four mutants, only W30 has a $\lambda_{0,0}$ consistent with a fully nonpolar surrounding. The BW is smallest for W30 (4.5 nm) and widest for W117 (7.5 nm). These values are considerably larger than the 2.6 nm typically found for Trp in a homogeneous protein site like W84 in the single-Trp mutant of glyceraldehyde-3-phosphate dehydrogenase (GADPH) (*20*). Because the mutants are single-Trp proteins, such a relatively wide distribution of spectral energies can only be a consequence of environmental heterogeneity. This implies that the protein in these micellar solutions adopts a multiplicity of conformations. Since the specific phosphorylation activity of the enzymes was not affected by purifying the enzymes from ISO vesicles, we can rule out the presence of inactive enzyme, causing the BW broadening.

Table 1: Peak Wavelength ($\lambda_{0,0}$) and Bandwidth (BW) of the 0,0 Vibronic Band at 2/3 Height in the Phosphorescence Spectrum of EII^{mtl} Wild-Type and Single-Trp Mutants[a]

sample	$\lambda_{0,0}$ (nm)	BW (nm)	$\Delta\lambda_{0,0}$ (nm)	ΔBW (nm)
W30	411.4	4.5	−	−0.8
W42	407.7	5.3	+1.5	+1.6
W109	410.2	6.0	−	−0.4
W117	413.2	7.5	−	+0.4
WT	412.7[b]	8.6	−0.5	−1.1
NATA[c]	406.0	6.3		
W84 GAPDH[d]	406.0	2.6		

[a] Obtained in PG/buffer glass at 140 K. $\Delta\lambda_{0,0}$ and ΔBW refer to the spectral changes induced by enzyme phosphorylation. [b] Shoulder at 408 nm. [c] *N*-Acetyl-L-tryptophanamide. [d] From ref *20*.

Effect of Enzyme Phosphorylation on Phosphorescence Spectra in Low-Temperature Glasses. The phosphorescence spectrum of every Trp in the C domain is affected by phosphorylation of C384 in the B domain and H554 in the A domain (see Figure 2 and Table 1). Among the single-Trp mutants, variations in $\lambda_{0,0}$ are detected only for W42 which shifts 1.5 nm to the red. The BW increases for W42 (+1.6 nm) and W117 (+0.4 nm) but decreases for W30 (−0.8 nm) and W109 (−0.4 nm). According to these alterations, the structure of the C domain responds to phos-

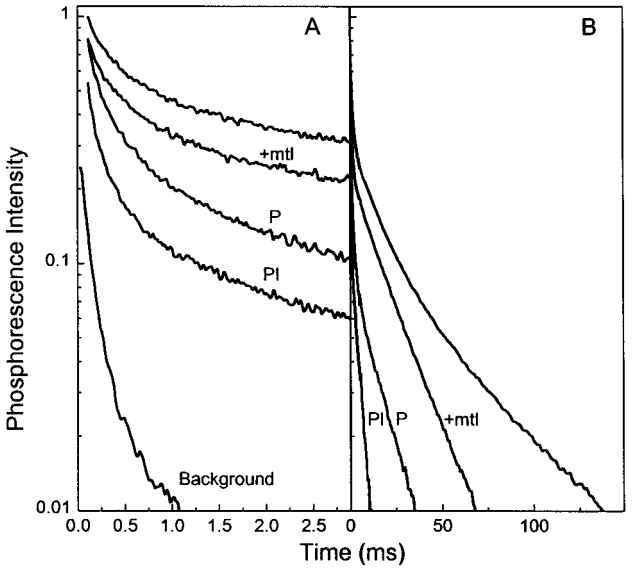

FIGURE 3: Representative phosphorescence decays of wild-type EII^{mtl} showing the effect of mannitol binding (+mtl), enzyme phosphorylation (P), and perseitol binding to the phosphorylated enzyme (PI). The protein concentration is 2.4 μM in buffer at 1 °C. The background signal, provided by a Trp-less mutant, was subtracted from each decay and is also shown separately in panel A. The excitation wavelength is 292 nm. Panel A zooms in on the initial part of the decay shown in panel B.

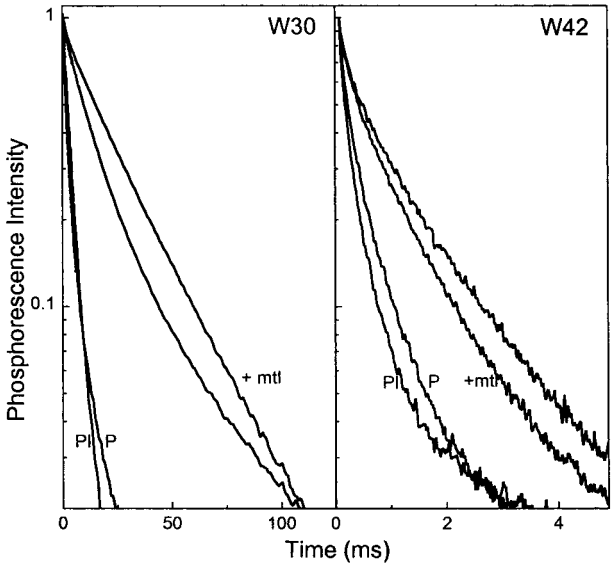

FIGURE 4: Representative phosphorescence decays of mutants W42 and W30 showing the effect of mannitol binding (+mtl), enzyme phosphorylation (P), and perseitol binding to the phosphorylated enzyme (PI). The background signal was subtracted from each decay. The protein concentration is 2.2 μM for W30 and 4.4 μM for W42. Other conditions are as in Figure 3.

phorylation with enhanced conformational order in the region of residues 30 and 109 as opposed to greater conformational disorder in the region of residues 117 and 42, the nature of the environment of residue 42 being considerably affected.

Trp Phosphorescence of Wild-Type and Single-Trp Mutants in Fluid Solution. Whereas in rigid matrixes spectral energies report on the polarity of the chromophore's environment, the intrinsic phosphorescence lifetime (τ) in fluid media reports on the motional freedom of the structure surrounding the chromophore. The phosphorescence decay kinetics were monitored in buffer at 1 °C for wild-type EII^{mtl}, each single-Trp mutant, and a Trp-less mutant. Typical raw data are shown in Figures 3 and 4. Inspection of these figures shows that the decay of wild-type EII^{mtl} is highly inhomogeneous with contributions by lifetime components that range from submilliseconds to 60 ms (Table 2). For simplicity, the data were fitted as a sum of three exponential components, but as the fit improved with four components, it means that more than three distinct classes of chromophore environments are necessary to account for the heterogeneity. Note that the detection of very short lifetimes (<70 μs) is hampered by a nonnegligible short-lived emission of an impurity, observed both with control and with Trp-less samples (Figure 3A).

Among the four single-Trp mutants, phosphorescence is detected from W30, W42, and W117, but not from W109. For the latter, $\tau \leq 70$ μs and is masked by the background. Because its lifetime is significantly smaller than the 200−500 μs typically observed in short, structureless peptides, it is likely that, in fluid solution, the emission of residue 109 is effectively quenched by the side chain of a nearby (<2 Å) cysteine, histidine, or tyrosine (*17*). W30 exhibits the longest-lived phosphorescence. Its decay (Figure 4) is multiexponential and requires three discrete components to adequately fit the data. Amplitudes (α) and lifetimes (τ), as obtained from the analysis of the decays, are collected in Table 2. According to the amplitude, over 70% of the population has a lifetime of 14.2 ms. The remaining 13% has a lifetime of 2.3 ms while 14% has a lifetime of 55 ms. Judging by τ, the dynamic structure at the site of W30 is not uniform, with a small portion of the population adopting either a looser or a tighter configuration relative to the dominant form. Note that these are rather stable conformations of the C domain in that they do not interconvert rapidly on the time scale of several milliseconds. Further, the magnitude of τ_{av} is characteristic of relatively rigid protein cores found within folded globular structures (*17*). The similarity in lifetime between W30 and the long-lived components of the wild-type phosphorescence makes the assignment of the latter to W30 straightforward.

Table 2: Lifetimes (τ_i) and Amplitudes (α_i) Derived from Fitting the Phosphorescence Decay of EII^{mtl} Wild-Type and Single-Trp Mutants with 3 Discrete Exponential Components

sample	α_1	α_2	α_3	τ_1 (ms)	τ_2 (ms)	τ_3 (ms)	τ_{av}[a] (ms)
W30	0.13	0.73	0.14	2.3	14.2	55.1	18.4
W30 + mtl	0.14	0.75	0.11	3.1	24.5	48.2	24.2
W30 phosph	0.74	0.22	0.04	1.7	5.7	26.0	3.5
W30 phosph + perseitol	0.47	0.51	0.02	1.8	4.2	21.0	3.4
W42	0.41	0.55	0.04	0.20	1.2	5.3	0.95
W42 + mtl	0.41	0.56	0.03	0.20	1.0	8.3	0.86
W42 phosph	0.63	0.35	0.02	0.11	0.54	6.1	0.38
W42 phosph + perseitol	0.69	0.30	0.01	0.10	0.41	5.6	0.25
W117	0.67	0.31	0.02	0.13	0.65	9.2	0.52
WT	0.60	0.26	0.14	0.64	8.7	54.1	10.3
WT + mtl	0.67	0.20	0.13	0.61	13.3	29.5	6.9
WT phosph	0.69	0.22	0.09	0.25	1.64	17.2	2.08
WT phosph + perseitol	0.77	0.19	0.04	0.24	3.0	9.0	1.14

[a] $\tau_{av} = \sum \alpha_i \tau_i$.

The phosphorescences of both W42 and W117 are shorter-lived and multiexponential (Table 2), again reflecting a multiplicity of environments. For W42, the average lifetime

(τ_{av}) is 1 ms, and that of W117 is 0.5 ms, indicating that these Trp residues are in a flexible protein environment. From a comparison of the decay kinetics of these mutants with those of the wild-type EII^{mtl}, it is evident that most of the submillisecond/ millisecond components in the wild-type protein decay are due to residues 42 and 117.

Effects of Mannitol Binding on the Phosphorescence Lifetime in Fluid Solution. Among the single-Trp mutants, mannitol binding affects principally the phosphorescence decay of W30, which becomes slower and more homogeneous, as can be appreciated by visual inspection of Figure 4. Thus, complex formation results in a significantly more rigid structure and in a narrower distribution of conformers about the dominant form. Increased uniformity in the decay is found also for the long-lived components of the wild-type protein (Figure 3) that we ascribe to the contribution of W30. Note, however, that the response of W30 to mannitol binding differs somewhat between the mutant and the wild-type protein. If components 2 and 3 (Table 2) of the WT decay are taken to represent the contribution of residue 30, then their combined change leads to a shorter τ_{av} for the complex and, therefore, to the conclusion that in the wild-type protein the structure of this region is somewhat looser in the complex. The difficulty of singling out the individual contributions of the various Trp residues from the composite emission of the wild-type and/or slight modifications of the protein structure introduced with the mutation may be responsible for this difference. For W42, mannitol binding decreases τ_{av} by less than 10% while the lifetime distribution is practically invariant. The effects are negligible on W117.

Effects of C384 and H554 Phosphorylation on the Phosphorescence Lifetime. Phosphorylation of C384 and H554 causes a remarkable shortening of τ_{av} with each mutant and wild-type protein alike. Control experiments with excess substrate (50 μM PEP) or product (10 μM pyruvate) of the phosphorylation reaction ruled out that either compound acts as a quencher of Trp phosphorescence. Consequently, the change in lifetimes monitors alterations of the C domain conformation that apparently render its structure significantly more flexible. For W30, the lifetime of the dominant fraction changes from 14.2 to 1.7 ms. A few percent of the molecules retain a long lifetime of 26 ms, and it is likely that these molecules have not been phosphorylated. Without the 26 ms component, τ_{av} would become 2.6 ms, a value that is consistent with a mobile environment but excludes full exposure of W30 to the surface of the molecule because the lifetime in short unstructured peptides does not exceed 0.5 ms (*17*). Another effect of phosphorylation is to narrow the lifetime distribution, again implying that the probe now experiences a more uniform structure. This finding is in agreement with the increased resolution of the phosphorescence spectrum at 140 K (see above). The effect of perseitol binding to the phosphorylated enzyme has also been investigated. This inhibitor binds with high affinity at the mannitol binding site but is not phosphorylated or transported by EII^{mtl} (*31*). When perseitol is added to the phosphorylated protein, the main effect on the decay of W30 is a further increase in homogeneity, without appreciable changes in τ_{av} (Figure 4).

Phosphorylation of the W42 mutant decreases its τ_{av} from 1 to 0.38 ms, the major fraction of the intensity having a lifetime of about 110 μs. This lifetime is in the range found for Trp in unfolded peptides (*17*), and from it, one infers that the protein structure about W42 has become loose and completely mobile with phosphorylation. The addition of perseitol does not change appreciably the emission of the phosphorylated W42 mutant except for a modest 20% reduction of τ_{av}. The short-lived phosphorescence of W117 is no longer detectable in the phosphorylated mutant, implying that its lifetime is less than 100 μs.

The influence of mannitol and of phosphorylation on the single-Trp mutants can, with some variations, also be recognized on the overall phosphorescence decay of wild-type enzyme. As can be seen in Figure 3A, a portion of the intensity decays during the dead-time of the apparatus, and this could represent Trp117 quenching. In the time window of the W42 decay, there is a distinct reduction in lifetime with both phosphorylation and perseitol binding. Further, upon phosphorylation, the slow part of the wild-type protein decay becomes distinctly more homogeneous and the lifetime is considerably reduced (Figure 3B).

DISCUSSION

This paper presents the first systematic study of Trp phosphorescence spectroscopy on single-Trp-containing membrane proteins. Emission signals with good signal-to-noise ratios were obtained by using only micromolar enzyme concentrations. Interference from impurity emission, as verified by control experiments with the Trp-less EII^{mtl} mutant, is negligible for spectral measurements in glasses and limited to the 100 μs time range for phosphorescence decays in fluid solutions. Due to high background levels, Trp spectroscopy is rarely used to study the structure and dynamics of membrane proteins (*32*−*35*). In general, because the phosphorescence of free chromophores in fluid solutions is drastically quenched, the issue of impurity emission is bound to be less severe with phosphorescence than with fluorescence (*8*).

In the detergent buffer system used in this study, purified EII^{mtl} is very stable and shows a specific mannitol phosphorylation activity comparable with the activity found in *E. coli* vesicles (*1*). The phosphorescence spectra of the wild-type enzyme (in glasses) and the decay kinetics in buffer show that its overall emission can largely be accounted for by the various contributions of the single-tryptophan mutants. This indicates that the environment of the four tryptophans is not considerably changed by replacing three tryptophans by phenylalanines in each mutant. The individual Trp residues are characterized by distinct spectral energies and by a particularly wide range of phosphorescence lifetimes. Only the phosphorescence of the W109 mutant was not detected in solution at 1 °C. This implies that its lifetime is shorter than 70 μs, and it is plausible that internal quenching by the sulfhydryl group of the adjacent cysteine at position 110 is responsible for it. The phosphorescence of W109 could only be recorded in the glass state where cysteine quenching is known to be practically absent (*17, 36*). Residue 30 exhibits a rather long τ and a value of $\lambda_{0,0}$ typical of residues buried in a relatively well-structured nonpolar site. This finding is in accord with previous fluorescence data which showed a blue-shifted emission maximum and limited rotational mobility of the Trp side chain on the nanosecond time range (*9, 10*). The phosphorescence of residue 42 is also characteristic of a buried residue, but its environment

is more polar and flexible than that of residue 30. The spectrum of residue 117 indicates that it is shielded from the aqueous phase but its τ is short, typical of unstructured loops of the polypeptide. It should be remarked that the nature of the Trp environments inferred from phosphorescence data is consistent with the proposed hydropathy model of the C domain as it places residues 30 and 42 within a transmembrane α-helix, while residues 109 and 117 are predicted to be located in a cytoplasmic loop (*37*). It is also consistent with fluorescence data (*9*).

An important feature of the phosphorescence decay of each single-Trp mutant is the pronounced heterogeneity in that, in each case, the data could be properly fitted only by using at least three lifetime components. This indicates the presence of three or more different, long-lived conformational states of the protein that do not interconvert rapidly during τ_{av}. The protein heterogeneity is also reflected in relatively broad spectra at low temperature (Figure 2). A multiplicity of conformations is not unusual for proteins in fluid media. By investigating the phosphorescence decay of 15 proteins, Cioni et al. found only 3 cases where the phosphorescence decayed with a single lifetime (*38*). Multiple conformations have also been reported in small well-characterized monomeric systems (*39*). EII^{mtl} is dimeric under the conditions used in this study (*1*), and conformational heterogeneity could in part arise from subunit asymmetry. However, the 2D projection map of the C domain at 5 Å suggests a dimer consisting of two identical subunits (*7*). Certainly, the fact that two lifetimes are not sufficient to account for the decay heterogeneity suggests a multiplicity of conformations within a given EII^{mtl} subunit.

EII^{mtl} has a high-affinity mannitol binding site ($K_D = 50$ nM) located in the C domain (*1*) whose exact position is not yet known. Kinetic and fluorescence experiments revealed the sensitivity of three regions to mannitol binding. These include residues 254−257, forming the highly conserved GIXE element among EII proteins (*2*), residues near position 196 (*11, 40*), and residue 30 (*9*). Fluorescence resonance energy transfer experiments between residue 30 and mannitol, containing a diazirine moiety, revealed a distance of 10 Å (to be published). Phosphorescence data show that of the four locations studied, only the microenvironment of W30 is significantly changed upon mannitol binding. In this mutant, the lifetime of the major component increases by 73%, and the phosphorescence decay becomes much more homogeneous (Figure 3). It may be significant that well-ordered 2D crystals of the C domain could only be obtained in the presence of mannitol; this might reflect a more conformationally homogeneous protein structure (*7*).

B/C domain coupling is crucial for the catalytic activity of the EIIs of the PTS system (*1, 2, 41*). It is regulated by enzyme phosphorylation of the B domain and possibly also by phosphorylation of the A domain. The coupling has been best characterized for EII^{mtl}. The rate of mannitol transport increases 1000-fold upon phosphorylation of C384 in the B domain (*4, 5*). How the C domain structure is affected by enzyme phosphorylation was not clarified in kinetic studies or in fluorescence studies using single-Trp mutants (*9, 10*).

Time-resolved phosphorescence reveals that enzyme phosphorylation causes a drastic reduction in the phosphorescence lifetime of W30, W42, and W117, showing that the C domain structure is remarkably relaxed in this state. Further, while the region of residue 30 becomes more uniform in structure, that of residue 42 undergoes a considerable change in polarity (Figure 2C). Binding of the inhibitor perseitol does not affect the τ_{av} of phosphorylated W30 but decreases slightly τ_{av} of phosphorylated W42 and wild-type enzymes. Isothermal titration calorimetry measurements on perseitol binding to phosphorylated EII^{mtl} gave a small $\Delta C^{o}_{p,obs}$ value, also indicative of limited conformational changes (*6*).

The above phosphorescence findings shed more light on the mechanism of coupling between mannitol transport and phosphorylation activity in EII^{mtl}. The data suggest that mannitol binding energy may be used to create a homogeneously strained C domain structure in the region of the mannitol binding site. The strained EII^{mtl}−mannitol complex relaxes upon enzyme phosphorylation, possibly acting as a trigger for the release of mannitol into the cytoplasmic space. Indeed, comparison of the decays of W30 + mannitol with that of phosphorylated W30 + perseitol shows that the relaxation of the protein structure induced by phosphorylation overrules the increased rigidity induced by substrate binding. Time-resolved phosphorescence decay data of more single-Trp mutants are being gathered to refine this model.

In summary, this work demonstrates that Trp phosphorescence can be readily detected from micellar solutions of EII^{mtl}. Because the background emission caused by using detergent solutions is modest and does not interfere with Trp phosphorescence, this approach is potentially useful for investigating membrane proteins in general. The results obtained with EII^{mtl} demonstrate that Trp phosphorescence can probe changes in protein structure and dynamics and shed new light on transport and signal transduction mechanisms. Moreover, by identifying conditions that result in higher structural homogeneity, Trp phosphorescence might facilitate the difficult task of finding proper conditions for membrane protein crystallization.

REFERENCES

1. Robillard, G. T., and Broos, J. (1999) *Biochim. Biophys. Acta 1422*, 73−104.
2. Lengeler, J. W., Jahreis, K., and Wehmeier, U. F. (1994) *Biochim. Biophys. Acta 1188*, 1−28.
3. Kundig, W., Ghosh, S., and Roseman, S. (1964) *Proc. Natl. Acad. Sci. U.S.A. 52*, 1067−1074.
4. Elferink, M. G., Driessen, A. J., and Robillard, G. T. (1990) *J. Bacteriol. 172*, 7119−7125.
5. Lolkema, J. S., ten Hoeve Duurkens, R. H., Swaving Dijkstra, D., and Robillard, G. T. (1991) *Biochemistry 30*, 6716−6721.
6. Meijberg, W., Schuurman Wolters, G. K., and Robillard, G. T. (1998) *J. Biol. Chem. 273*, 7949−7956.
7. Koning, R. I., Keegstra, W., Oostergetel, G. T., Schuurman Wolters, G., Robillard, G. T., and Brisson, A. (1999) *J. Mol. Biol. 287*, 845−851.
8. Swaving Dijkstra, D., Broos, J., and Robillard, G. T. (1996) *Anal. Biochem. 240*, 142−147.
9. Swaving Dijkstra, D., Broos, J., Lolkema, J. S., Enequist, H., Minke, W., and Robillard, G. T. (1996) *Biochemistry 35*, 6628−6634.
10. Swaving Dijkstra, D., Broos, J., Visser, A. J., van Hoek, A., and Robillard, G. T. (1997) *Biochemistry 36*, 4860−4866.
11. Broos, J., ter Veld, F., and Robillard, G. T. (1999) *Biochemistry 38*, 9798−9803.
12. Vanderkooi, J. M. (1992) in *Topics in fluorescence spectroscopy* (Lakowicz, J. R., Ed.) Vol. 3, pp 113−136, Plenum Press, New York.
13. Schauerte, J. A., Steel, D. G., and Gafni, A. (1999) *Methods Enzymol. 278*, 49−71.
14. Strambini, G. B. (1989) *J. Mol. Liquids 42*, 155−165.

15. Hershberger, M. V., Maki, A. H., and Galley, W. C. (1980) *Biochemistry 19*, 2204−2209.
16. Strambini, G. B., and Gonnelli, M. (1995) *J. Am. Chem. Soc. 117*, 7646−7651.
17. Gonnelli, M., and Strambini, G. B. (1995) *Biochemistry 34*, 13847−13857.
18. Cioni, P., and Strambini, G. B. (1998) *J. Am. Chem. Soc. 120*, 11749−11757.
19. Strambini, G. B., and Cioni, P. (1999) *J. Am. Chem. Soc. 121*, 8337−8344.
20. Gabellieri, E., Rahuel Clermont, S., Branlant, G., and Strambini, G. B. (1996) *Biochemistry 35*, 12549−12559.
21. Strambini, G. B., Cioni, P., Peracchi, A., and Mozzarelli, A. (1992) *Biochemistry 31*, 7535−7542.
22. Strambini, G. B., and Gonnelli, M. (1990) *Biochemistry 29*, 196−203.
23. Cioni, P., and Strambini, G. B. (1994) *J. Mol. Biol. 242*, 291−301.
24. Strambini, G. B., and Gabellieri, E. (1996) *Biophys. J. 70*, 971−976.
25. Grisafi, P. L., Scholle, A., Sugiyama, J., Briggs, C., Jacobson, G. R., and Lengeler, J. W. (1989) *J. Bacteriol. 171*, 2719−2727.
26. Robillard, G. T., and Blaauw, M. (1987) *Biochemistry 26*, 5796−5803.
27. Brouwer, M., Elferink, M. G., and Robillard, G. T. (1982) *Biochemistry 21*, 82−88.
28. Robillard, G. T., Dooijewaard, G., and Lolkema, J. S. (1979) *Biochemistry 18*, 2984−2989.
29. van Dijk, A. A., de Lange, L. C., Bachovchin, W. W., and Robillard, G. T. (1990) *Biochemistry 29*, 8164−8171.
30. Pas, H. H., ten Hoeve Duurkens, R. H., and Robillard, G. T. (1988) *Biochemistry 27*, 5520−5525.
31. Lolkema, J. S., Wartna, E. S., and Robillard, G. T. (1993) *Biochemistry 32*, 5848−5854.
32. Gorga, F. R., and Lienhard, G. E. (1982) *Biochemistry 21*, 1905−1908.
33. Ladokhin, A. S., Wang, L., Steggles, A. W., and Holloway, P. W. (1991) *Biochemistry 30*, 10200−10206.
34. Menezes, M. E., Roepe, P. D., and Kaback, H. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1638−1642.
35. Vanderkooi, J. M., Papp, S., Pikula, S., and Martonosi, A. (1988) *Biochim. Biophys. Acta 957*, 230−236.
36. Li, Z., Lee, W. E., and Galley, W. C. (1989) *Biophys. J. 56*, 361−367.
37. Sugiyama, J. E., Mahmoodian, S., and Jacobson, G. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9603−9607.
38. Cioni, P., Gabellieri, E., Gonnelli, M., and Strambini, G. B. (1994) *Biophys. Chem. 52*, 25−34.
39. Schlyer, B. D., Schauerte, J. A., Steel, D. G., and Gafni, A. (1994) *Biophys. J. 67*, 1192−1202.
40. Boer, H., ten Hoeve Duurkens, R. H., and Robillard, G. T. (1996) *Biochemistry 35*, 12901−12908.
41. Mao, Q., Schunk, T., Flukiger, K., and Erni, B. (1995) *J. Biol. Chem. 270*, 5258−5265.

BI000803Z