Studies on the Interaction between *Escherichia coli* Pyruvate Oxidase and a Detergent Activator by Utilization of the Fluorescence Probe Bis(8-*p*-toluidino-1-naphthalenesulfonate)†

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ABSTRACT: Pyruvate oxidase is a peripheral membrane flavoenzyme isolated from *Escherichia coli*. The in vitro specific activity of the pure enzyme is enhanced 25-fold in the presence of certain lipids and detergents. In addition, the affinity of the protein for both phospholipids and detergents is significantly enhanced in the presence of the enzyme substrate and cofactor, pyruvate and thiamin pyrophosphate (Mg^{2+}). In this paper a novel fluorescent probe is used to examine the protein conformational changes concomitant with substrate reduction of the flavin, activation of the oxidase, and binding of the detergent activator, dodecyl sulfate. In the presence of dodecyl sulfate, the probe bis(8-*p*-toluidino-1-naphthalenesulfonate) (bis-Tns) binds to pyruvate oxidase with a resultant large increase in probe fluorescence at dye concentrations below 1 μM. No binding at low dye concentration is observed in the absence of the activator. The enzyme binds about 16 molecules of bis-Tns, in what appear to be hydrophobic binding sites. The substrate-reduced flavoprotein binds to bis-Tns not only at these high affinity sites in the presence of dodecyl sulfate but also binds to more dye at low affinity sites. The binding of the dye to the reduced flavoprotein at the weak binding sites is coincident with the inhibition of enzymatic activity by the dye and with irreversible denaturation of the oxidase. The fact that dodecyl sulfate appears to make accessible the high affinity binding sites for the hydrophobic probe may be related to the lipid binding properties of this enzyme. The resulting enhanced fluorescence upon addition of dodecyl sulfate to a solution containing pyruvate oxidase and bis-Tns was used to monitor detergent binding. The results are consistent with earlier reported results obtained at much higher protein concentrations using equilibrium dialysis with dodecyl [^{35}S]sulfate. The detergent activator binds at concentrations well below its critical micelle concentration, and the affinity of the protein for dodecyl sulfate is significantly greater in the presence of the substrate plus cofactor.

Pyruvate oxidase is a peripheral membrane flavoenzyme isolated from *Escherichia coli* (Hager, 1957; Williams & Hager, 1966; O'Brien et al., 1976). The enzyme catalyzes the oxidative decarboxylation of pyruvate to yield acetate plus CO_2. Pyruvate oxidase has been characterized as being an α_4 tetramer with a subunit molecular weight of 60 000 (O'Brien et al., 1976; Raj et al., 1977). In vivo, the oxidase is located on the inner surface of the *E. coli* cytoplasmic membrane and is coupled to the electron transport chain (Shaw-Goldstein, Gennis, & Walsh, 1978). In vitro, the enzyme activity is measured using a ferricyanide reductase assay (Blake et al., 1978). The specific activity of this membrane enzyme is stimulated about 25-fold when certain lipids or detergents are present in the assay cuvette. This lipid activation has been the focus of previous studies (Cunningham & Hager, 1971a,b; Blake et al., 1978). The binding of the detergent activator, dodecyl sulfate, to pyruvate oxidase has also been examined (Schrock & Gennis, 1977) and high affinity detergent binding sites were revealed. The study with dodecyl sulfate and a subsequent study of the interaction between pyruvate oxidase and dipalmitoylphosphatidylcholine vesicles (Schrock & Gennis, submitted) demonstrated a dramatic enhancement of the affinity between the protein and these lipid activators in the presence of the substrate, pyruvate, and cofactor, thiamin pyrophosphate (Mg^{2+}). A key feature appears to be the involvement of the oxidation–reduction state of the protein-bound flavin (FAD) in modulating the lipid binding properties of the membrane enzyme.

The protein conformational change accompanying flavin reduction has been characterized using proteolysis as a probe (Hager, 1957; Russell et al., 1977a,b). It appears that, upon flavin reduction by pyruvate, the lipid binding region on the enzyme becomes accessible and is highly susceptible to proteolytic cleavage by trypsin, α-chymotrypsin, or other endopeptidases. The proteolytically modified enzyme is activated in an identical manner as if it were bound to dodecyl sulfate, and, furthermore, the lipid binding properties are destroyed by this proteolysis.

There is clearly a complex conformational and energetic coupling between the catalytic active site and the lipid binding site. It was hoped that noncovalent fluorescent probes could be utilized to obtain further information about these phenomena. Specifically, it was desired to obtain a sensitive assay for lipid binding to the oxidase at sufficiently low protein concentrations where protein self-aggregation is not a complicating feature. For these reasons, numerous dyes were examined, and this paper reports the results obtained using one probe, bis(8-*p*-toluidino-1-naphthalenesulfonate) (bis-Tns)[1] (Farris et al., 1978). This is the first report of this probe being used to examine a protein.

Experimental Procedures

Materials. The following reagents were obtained from the indicated sources and used without further purification:

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[1] Abbreviations used: FAD, flavin adenine dinucleotide; BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate; 2,6-Tns, 6-*p*-toluidino-2-naphthalenesulfonate; 1,8-Tns, 8-*p*-toluidino-1-naphthalenesulfonate; Ans, 8-anilino-1-naphthalenesulfonate; bis-Ans, bis(8-anilino-1-naphthalenesulfonate); bis-Tns, bis(8-*p*-toluidino-1-naphthalenesulfonate); TPP, thiamin pyrophosphate.

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sodium dodecyl sulfate (Schwarz/Mann); bovine serum albumin (Armour); 6-*p*-toluidino-2-naphthalenesulfonate (2,6-Tns) (Sigma); 8-*p*-toluidino-1-naphthalenesulfonate (1,8-Tns) and 8-anilino-1-naphthalenesulfonate (Ans) (Eastman). Bis(8-anilino-1-naphthalenesulfonate) (bis-Ans) and bis(8-*p*-toluidino-1-naphthalenesulfonate) (bis-Tns) were obtained from the laboratory of Dr. G. Weber of this department. All other reagents were of the highest purity commercially available. The purity of the fluorescent dyes was confirmed by thin-layer chromatography and comparison between observed and reported fluorescence spectra. Glass-distilled, deionized water was used for all solutions.

Pyruvate oxidase was purified according to the procedure of O'Brien et al. (1976). The enzyme was assayed using a ferricyanide reductase assay (Blake et al., 1978).

Protease Activation. Protease-activated enzyme was prepared according to the procedure of Russell et al. (1977a). For the fluorescence experiments, pyruvate oxidase was activated at a concentration of 0.5 mg/mL and then diluted 100-fold into 0.1 M sodium phosphate, 10 mM $MgCl_2$, 100 mM pyruvate, 100 μM thiamin pyrophosphate, 0.1 μg/mL phenylmethanesulfonyl fluoride, pH 5.7. As a control native pyruvate oxidase was also diluted into the same buffer system. Fluorescence experiments were carried out in exactly the same manner for both native and protease-activated pyruvate oxidase.

Fluorescence Measurements. Fluorescence spectra and intensities were obtained with a Perkin-Elmer MPF-44A fluorimeter equipped with a thermostated cell holder.

Fluorescence polarization and lifetimes were measured using instruments in the laboratory of Dr. Gregorio Weber. The polarization of the emitted light was measured with the instrument described by Jameson (1978).

The excitation wavelength for polarization was 390 nm. The emission was defined with a Corning 3-71 cutoff filter. Parallel and perpendicular components of the emitted light were measured simultaneously. Corrections were made for scattering and for the differences in phototube efficiency. Fluorescence lifetimes were measured using the phase-modulation technique (Spencer, 1970) with an instrument which has been previously described (Spencer & Weber, 1969).

Bis-Tns Fluorescence Titrations. When bis-Tns binds to pyruvate oxidase, the fluorescence of the dye is greatly enhanced. This fluorescence enhancement was used as a technique for monitoring dye binding. Titrations were performed in 0.1 M sodium phosphate buffer, pH 5.7, at room temperature, unless specified otherwise. The enzyme subunit concentration was usually between 0.1 and 1.0 μM. Fluorescence titrations were done with excitation at 360 nm, unless specified otherwise. Fluorescence intensity was followed at 500 nm using excitation and emission band-passes of 10 nm. The Raman scattering intensity was negligible.

At the end of a titration, the fluorescence intensity values were corrected for small volume changes (always less than 5%) and for the small amount of fluorescence from the free dye at the highest bis-Tns concentrations. Intensities of fluorescence were corrected for self-absorption of incident light using the relationship

$$I_{corr} = I_{obsd}[2.303\delta_{360}F_0/(1 - 10^{-\epsilon\ 360^{F_0}})]$$

where F_0 refers to the total bis-Tns concentration, ϵ_{360} is the molar absorbance of bis-Tns at 360 nm, and I_{corr} and I_{obsd} refer to the corrected and observed intensities, respectively (McClure & Edelman, 1967). An ϵ_{360} value of 13 270 M^{-1} cm^{-1} was used for bis-Tns in aqueous solutions. The concentration of bis-Tns was determined spectrophotometrically from the molar absorbance of 16 540 M^{-1} cm^{-1} at 390 nm (Farris et al., 1978).

Table I: Fluorescence Enhancement of Various Fluorescent Probes[a]

dye[b]	(∓) 25 μM $NaDodSO_4$	fluorescence enhancement (ratio)[c]
2,6-Tns	−	1.4
	+	1.9
Ans	−	2.1
	+	2.4
1,8-Tns	−	4.0
	+	11.7
bis-Ans	−	29.7
	+	42.8
bis-Tns	−	176.3
	+	302.0

[a] The experiments were done in 0.1 M sodium phosphate buffer at pH 5.7 and 25 °C. Dye concentrations were 10 μM, while the pyruvate oxidase subunit concentration was 0.25 μM. [b] The fluorescent dyes were excited at the following wavelengths: 2,6-Tns, 360 nm; Ans, 360 nm; 1,8-Tns, 380 nm; bis-Ans, 400 nm, and bis-Tns, 400 nm. [c] The value was obtained by comparing the fluorescence intensity in the presence and absence of enzyme under identical conditions.

The number of bis-Tns binding sites per pyruvate oxidase subunit was determined in the following manner. The fluorescence intensity of completely bound bis-Tns was determined by titrating the dye with pyruvate oxidase. This value was then used to convert the maximal fluorescence intensity in a bis-Tns titration of pyruvate oxidase into the number of moles of bound dye. Since the concentration of enzyme is known, the number of bis-Tns binding sites per pyruvate oxidase subunit can be calculated. Implicit in this determination is the assumption that the binding affinity and the quantum yield of fluorescence of all the bis-Tns binding sites are the same. Thus the values of bis-Tns stoichiometry determined by this method are meaningful only as long as this assumption is valid.

Results

A large number of noncovalent probes were systematically examined in the effort to obtain fluorescent reporter groups for pyruvate oxidase. These included electrically neutral probes such as *N*-phenyl-1-naphthylamine and diphenylhexatriene, cationic probes such as rose bengal, and anionic probes such as the 1,8 and 2,6 isomers of both anilinonaphthalenesulfonate (Ans) and toluidinonaphthalenesulfonate (Tns). It was hoped that the anionic probes might bind specifically at the thiamin pyrophosphate binding site of pyruvate oxidase, since Tns has been successfully used to probe such sites previously with pyruvate decarboxylase (Ullrich & Donner, 1970; Ostrovskii et al., 1971). The various dyes were initially screened by examining the fluorescence enhancement of a 10 μM dye solution upon addition of 15 μg/mL pyruvate oxidase both in the absence and presence of 25 μM dodecyl sulfate. Representative results comparing several anionic probes are given in Table I. The most dramatic results obtained were with bis-Tns (Figure 1), which binds to the enzyme at very low concentrations and also responds to dodecyl sulfate binding to the oxidase. The interaction between this dye and pyruvate oxidase was further characterized. The monomeric form of this probe, 1,8-Tns (Figure 1), also interacts with the oxidase and this was also examined in detail. All the results obtained using bis-Tns can be duplicated using 1,8-Tns, the only difference being that the interaction between 1,8-Tns and the protein is more than an order of magnitude weaker than the

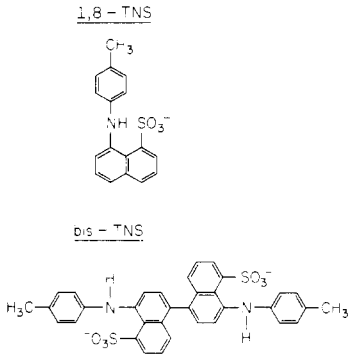

FIGURE 1: The structures of 1,8-Tns and bis-Tns.

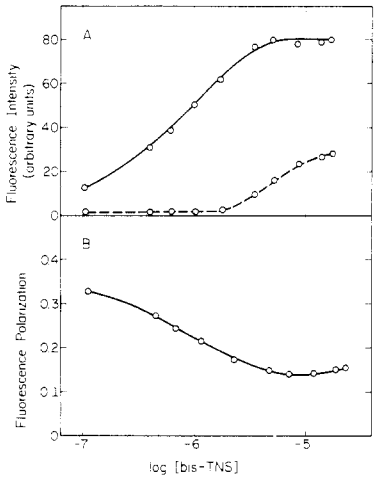

FIGURE 2: (A) Fluorescence titration of pyruvate oxidase with bis-Tns in the presence and absence of dodecyl sulfate. Pyruvate oxidase (5 μg/mL) in the presence (—) and absence (- - -) of 25 μM dodecyl sulfate was titrated with bis-Tns as described in the text. The excitation and emission wavelengths used were 360 and 500 nm, respectively. (B) Titration of pyruvate oxidase in the presence of dodecyl sulfate as monitored by the fluorescence polarization of the bis-TNS emission. Pyruvate oxidase (7 μg/mL) in the presence of 25 μM dodecyl sulfate was titrated with bis-Tns and monitored by fluorescence polarization as described in the text. Excitation was at 390 nm and emission was defined by a Corning 3-71 cutoff filter.

interaction between bis-Tns and pyruvate oxidase.

Figure 2A demonstrates the dependence of the high affinity interaction between bis-Tns and pyruvate oxidase on the presence of the detergent activator, dodecyl sulfate. The bis-Tns concentration was increased incrementally in samples containing 5 μg/mL pyruvate oxidase (0.02 μM) both with and without 25 μM dodecyl sulfate. In the presence of dodecyl sulfate there is substantial binding below 1 μM bis-Tns ($K_d \approx 0.8$ μM) which reaches a constant level, apparently saturating at about 3 μM dye. By contrast virtually no fluorescence enhancement is observed below 3 μM bis-Tns in the absence of the activator. Presumably the fluorescence enhancement is proportional to the amount of dye bound to the protein. Figure 2B shows that the fluorescence polarization decreases substantially. As the amount of dye bound to the oxidase–dodecyl sulfate complex increases, virtually all the fluorescence intensity is from the bound probe since the emission from bis-Tns in water is very slight. This decrease in polarization is likely due to self-energy transfer between bis-Tns molecules bound in close proximity on the protein surface.

The effect of bis-Tns on the enzymatic activity of pyruvate oxidase is shown in Figure 3. At very low concentrations of bis-Tns (ca. 10^{-7} M) the dye has no effect on enzymatic activity and the large stimulatory effect of dodecyl sulfate is

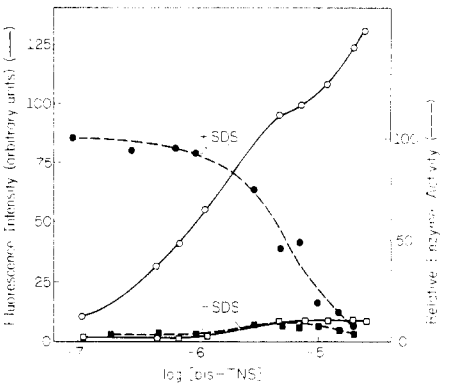

FIGURE 3: Titration of substrate-reduced pyruvate oxidase with bis-Tns in the presence and absence of dodecyl sulfate (SDS). Fluorescence titration: Reduced pyruvate oxidase (5 μg/mL) in 0.1 M sodium phosphate, 200 mM pyruvate, 100 μM TPP, 10 mM $MgCl_2$ at pH 5.7 was titrated with bis-Tns in the presence (○) and absence (□) of 25 μM dodecyl sulfate as described in the text. Excitation was at 390 nm and emission was monitored at 500 nm, with excitation and emission band-passes of 10 nm. Enzyme inhibition: The binding of bis-Tns to substrate-reduced pyruvate oxidase was also monitored by measuring the effect of bis-Tns on pyruvate oxidase specific activity. Enzyme activity was measured using a ferricyanide reductase assay in the presence (●) and absence (■) of 20 μM dodecyl sulfate.

apparent. The dye is a potent inhibitor of dodecyl sulfate activated pyruvate oxidase at concentrations greater than 1 μM bis-Tns. In the absence of the detergent activator, the dye has a slight stimulatory effect above 1 μM bis-Tns. The inhibition of the detergent-activated oxidase occurs at dye concentrations where dye binding has reached at plateau (e.g., Figure 2A). However, the titration with the oxidase reported in Figure 2 is performed with the oxidized form of the flavoprotein. When the titration with bis-Tns is repeated with substrate-reduced pyruvate oxidase, considerable dye binding is observed above 3 μM bis-Tns. Titrations were performed (Figure 3) with pyruvate oxidase under steady-state assay conditions, including saturating quantities of the substrate, pyruvate, and cofactor, thiamin pyrophosphate (Mg^{2+}). Only the electron acceptor was omitted, so the enzyme flavin is held in the reduced state and cannot turn over. Very little dye binding occurs to the reduced form of pyruvate oxidase in the absence of dodecyl sulfate. However, this is apparently sufficient to result in a several-fold increase in the specific activity of the enzyme at about 5 μM bis-Tns. In the presence of dodecyl sulfate, the bis-Tns binding to the reduced and oxidized forms of pyruvate oxidase appears very similar below 3 μM dye. Above this concentration, there is additional dye binding only to the reduced flavoprotein. It is apparently this additional binding of the dye which is responsible for the potent inhibition of the enzyme activity by bis-Tns.

The observed inhibition is irreversible. The inhibitory effect due to high concentrations of bis-Tns (e.g., 20 μM) could not be reversed by dilution, even after long incubation times (up to 30 min) to allow for dye dissociation. Also, it should be noted from Figure 3 that bis-Tns at sufficiently high concentrations is also effective at inhibiting unactivated pyruvate oxidase activity, suggesting the inhibition of dodecyl sulfate activated enzyme is not simply a matter of displacement of the detergent activator.

The inhibitory effect of bis-Tns was further examined by varying the substrate and cofactor concentrations with different amounts of the dye. The results are shown in Figure 4 in the form of double-reciprocal plots. The inhibitory pattern with thiamin pyrophosphate is mixed type (Segel, 1975) and complex. The inhibition with pyruvate is pure noncompetitive, with the intercept occurring on the x axis. The suggestion is

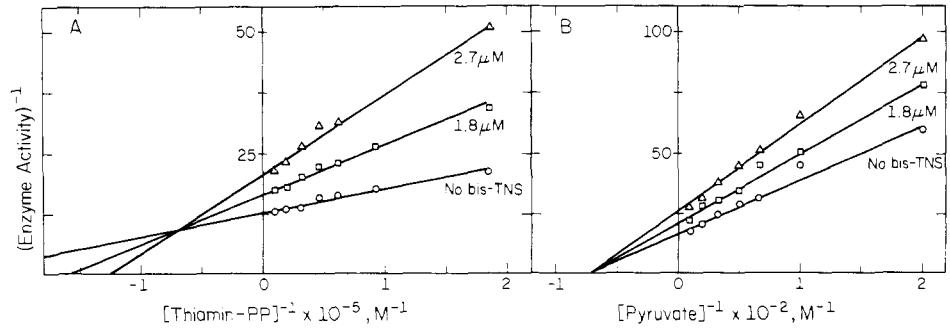

FIGURE 4: Inhibition of dodecyl sulfate activated pyruvate oxidase by bis-TNS. Enzyme activity was measured using a ferricyanide reductase assay. The data are in the form of double-reciprocal plots of enzyme activity vs. TPP concentration (A) and pyruvate concentration (B) at different bis-Tns concentrations. The TPP concentrations ranged from 5 to 100 μM, and the pyruvate concentrations ranged from 5 to 100 mM. The bis-Tns concentrations were none (○); 1.8 μM (□); and 2.7 μM (△).

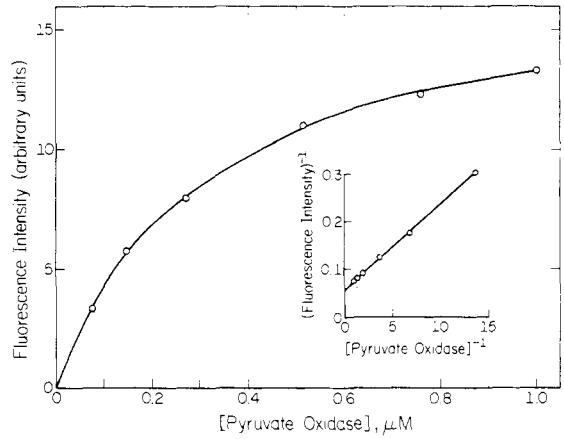

FIGURE 5: The effect of pyruvate oxidase on the fluorescence of bis-Tns in the presence of dodecyl sulfate. Bis-Tns (0.11 μM) in the presence of 25 μM dodecyl sulfate was titrated with pyruvate oxidase, concentration ranging from 0.07 to 1.0 μM. The insert in the figure is a double-reciprocal plot of the data. The y intercept of this figure is the reciprocal of the fluorescence intensity from completely bound bis-Tns.

Table II: The Effects of Solvent Polarity, Detergent Micelles, and Protein Binding on the Fluorescence Properties of Bis-Tns

conditions	emission max (nm)	rel fluorescence intensity	fluorescence lifetimes (ns)
water	552		0.09[a]
methanol	505	0.46	3.4
ethanol	498	0.96	4.6
1-propanol	498	1.00	4.6
$NaDodSO_4$ micelles[b]	552		1.2
BSA complex[c]	505		6.6
pyruvate oxidase[d] complex	510		5.6
pyruvate oxidase[e] plus $NaDodSO_4$ complex	505		6.1

[a] From Farris et al., 1978. The following samples contained 3.6 μM bis-Tris in 0.1 M sodium phosphate buffer at pH 5.7: [b] 35 mM sodium dodecyl sulfate; [c] 10 mg/mL of bovine serum albumin; [d] 0.9 μg/mL of pyruvate oxidase; [e] 0.9 μg/mL of pyruvate oxidase plus 25 μM $NaDodSO_4$.

that bis-Tns is not binding at the substrate binding site, but the situation with thiamin pyrophosphate may be more complicated. However, any such conclusions must be considered highly speculative. It should also be emphasized at this point that the inhibition is not reversible and, hence, all the species may not be at true equilibrium under the conditions of the assays.

The effects of pyruvate and thiamin pyrophosphate (Mg^{2+}) on the bis-Tns fluorescence titrations were investigated (results not shown). In the presence of either ligand alone the flavoprotein remains in the oxidized state. In the presence of 200 mM pyruvate and 25 μM dodecyl sulfate, the titration curve is similar to that pictured in Figure 2A, except the fluorescence intensity is larger by about 20%. It is not known whether this is due to enhanced binding or to an increased average quantum yield of the bound probe. Thiamin pyrophosphate (100 μM) has the opposite effect and decreases the fluorescence intensity, perhaps by competing for some of the dye binding sites. This was not further characterized.

The dye is apparently not binding to a single specific site on the oxidized form of pyruvate oxidase. This is confirmed by the stoichiometry of dye binding which was determined by titrating a fixed amount of bis-Tns with increasing amounts of pyruvate oxidase (Figure 5). A double-reciprocal plot was constructed (insert, Figure 5) to obtain the fluorescence intensity when all the dye is bound. The number of moles of bis-Tns bound per mole of pyruvate oxidase can then be easily determined (Daniel & Weber, 1966). At the plateau (Figure 2A) 16 mol of bis-Tns are bound per mol of oxidase, or about 4 per protein subunit. This measurement was not repeated with the substrate or cofactor present, and the stoichiometry was determined only in the presence of dodecyl sulfate.

To further characterize the nature of the dye binding sites on the oxidase, the emission characteristics were compared with those from the dye in different environments (Table II). The emission intensity and fluorescence lifetime increase very substantially when the dye is transferred from water to an alcoholic nonpolar solvent. The emission maximum of the dye bound to the oxidase (510 nm) or the oxidase–dodecyl sulfate complex (505 nm) is similar to that of the dye in methanol. The emission characteristics are also very similar to those of bis-Tns bound to bovine serum albumin. The greatly enhanced binding of the dye to the oxidase in the presence of dodecyl sulfate suggests the possibility that the bis-Tns is dissolved in a detergent micelle on the surface of the protein. Previous studies with dodecyl sulfate, however, indicate only a few detergent molecules bound per mole of oxidized enzyme at 25 μM dodecyl sulfate (Schrock & Gennis, 1977), but the presence of the bis-Tns might alter this. The emission characteristics of bis-Tns in the presence of 35 mM dodecyl sulfate (Table II), however, do not support the view that the dye is complexed with a form of detergent micelle in the presence of pyruvate oxidase. It appears that the dye is bound to protein binding sites and that these sites are made accessible due to dodecyl sulfate binding to the oxidase.

When dodecyl sulfate binds to the enzyme, the result is activation, presumably mediated through conformational

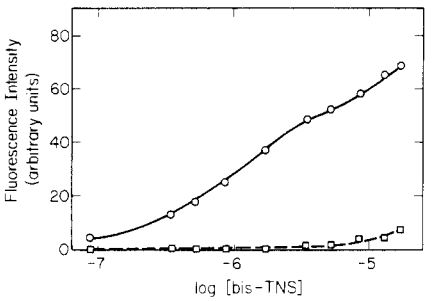

FIGURE 6: Fluorescence titrations of dodecyl sulfate activated and protease-activated forms of pyruvate oxidase with bis-Tns. Pyruvate oxidase (5 μg/mL) was activated either by 25 μM dodecyl sulfate (○) or by proteolytic cleavage (□) as described in the text. The samples were then titrated with bis-Tns. Both samples contained saturating concentrations of both pyruvate and thiamin pyrophosphate (Mg^{2+}).

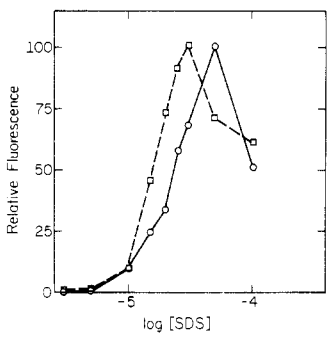

FIGURE 7: Sodium dodecyl sulfate binding to substrate-reduced and oxidized pyruvate oxidase as monitored by bis-Tns fluorescence. Oxidized pyruvate oxidase (5 μg/mL) in 0.1 M sodium phosphate buffer, pH 5.7 (○), and reduced enzyme (5 μg/mL) in 0.1 M sodium phosphate buffer, 100 mM pyruvate, 100 μM TPP, 10 mM $MgCl_2$, pH 5.7 (□), were both titrated with dodecyl sulfate in the presence of 4 μM bis-Tns. Binding of the detergent to the enzyme was monitored by the resulting increase in bis-Tns fluorescence intensity. The data in this figure are plotted as relative fluorescence intensities. The maximal fluorescence intensity for substrate-reduced enzyme is only 40–50% of the value for oxidized pyruvate oxidase. The excitation wavelength was 400 nm and emission was monitored at 500 nm; excitation and emission band-passes were 10 nm.

changes in the protein. The enzyme can also be activated proteolytically (Russell et al., 1977a,b) without the need for a lipid or detergent activator. Figure 6 compares the bis-Tns fluorescence titrations of protease-activated pyruvate oxidase and dodecyl sulfate activated pyruvate oxidase. In both cases, the enzyme flavin is substrate reduced. Protease activated pyruvate oxidase does not bind to bis-Tns substantially below 1 μM dye. It is clear that the high affinity interaction between pyruvate oxidase and bis-Tns responds directly to the detergent binding and is not dependent on the oxidase being in an activated state.

Figure 7 compares the effect of dodecyl sulfate on the fluorescence intensity from solutions containing a fixed amount of bis-Tns (4 μM) and either reduced or oxidized flavoprotein (5 μg/mL). At 25 μM dodecyl sulfate, the emission intensities are comparable, but at higher detergent concentrations the fluorescence intensity with the oxidized enzyme reaches a maximum more than twice that for the reduced oxidase. It is likely that at high detergent concentrations the probe is being displaced, resulting in a decrease in the emission intensity. When the data are scaled as in Figure 7, the results indicate detergent interaction with the reduced form of pyruvate oxidase occurs at lower concentrations of detergent than with the oxidized enzyme. The results are consistent with those obtained previously by equilibrium dialysis at much higher protein concentrations (Schrock & Gennis, 1977).

Discussion

The dimers of Ans and Tns were first prepared and reported by Weber and colleagues (Rosen & Weber, 1969; Farris et al., 1978). The structure of bis-Ans has been determined (Farris et al., 1978) and it has been shown to be a particularly useful probe of nucleotide binding sites on proteins. Those proteins examined using bis-Ans include glutamate dehydrogenase (Anderson, 1971), the myosin ATPase (Takashi et al., 1977), and RNA polymerase (Wu & Wu, 1978). This paper is the first reported use of bis-Tns as a probe of protein conformation. The dimeric probes generally have an advantage over the monomeric forms 1,8-Tns and Ans in that they bind with considerably greater affinity to protein binding sites, and since they have exceptionally low quantum yields in water, the extent of the fluorescence enhancement upon binding to a hydrophobic site is much larger. Although it is too soon to make a general statement, it appears that, upon binding, these large aromatic probes can change the protein conformation in such a way as to unfold or denature the protein, opening the way to further dye binding. Dye binding studies with pyruvate oxidase, glutamate dehydrogenase (Anderson, 1971), and RNA polymerase (Wu & Wu, 1978) indicate large quantities of the probe binding to these proteins. Despite the fact that the binding of bis-Tns to pyruvate oxidase is not to a well-defined site, such as the cofactor binding site, the interaction between the probe and the enzyme has been useful in exploring this complicated membrane enzyme.

In the absence of the detergent activator, pyruvate oxidase binds to bis-Tns only at dye concentrations greater than 1 μM. This dye binding results in the irreversible denaturation of the enzymatic activity and is not of particular interest. In the presence of low concentrations of dodecyl sulfate, however, pyruvate oxidase binds to the dye at much lower concentrations. It should be noted that detergent micelles are not present in solution at 25 μM dodecyl sulfate, and the detergent binding to the enzyme under these conditions has been previously quantitated (Schrock & Gennis, 1977). The dye bound to these high affinity sites (Figures 2A and 3) appears to have little or no effect on the enzymatic activity. Under steady-state assay conditions, the reduced form of pyruvate oxidase binds to bis-Tns at a second class of low affinity sites, resulting in irreversible inhibition of the enzymatic activity. It is likely that the dye is denaturing and unfolding the protein under these conditions. The oxidized enzyme, in the presence of dodecyl sulfate apparently does not bind this additional quantity of bis-Tns, as evidenced by the leveling of the fluorescence titration curve in Figure 2.

Hence, one result of these studies is to provide additional evidence concerning the differences between the oxidized and reduced forms of pyruvate oxidase. Previously, it was shown that reduction of the flavin resulted in exposure of a region of the polypeptide chain to proteolytic attack (Russell et al., 1977a). Furthermore, it seems likely that it is this region which is the lipid and detergent binding site on the protein (Russell et al., 1977b). The results with bis-Tns suggest that the reduced form of pyruvate oxidase has low affinity, hydrophobic binding sites available to the dye which are not available in the oxidized form in the presence of dodecyl sulfate. Perhaps the reduced flavoprotein is more disposed to unfold in response to the dye in the presence of dodecyl sulfate to expose a hydrophobic region. Whatever the detailed explanation, it is clear that the reduced and oxidized flavoproteins are responding quite distinctly to bis-Tns in the presence of dodecyl sulfate.

It is important that the binding of the activator appears to open up or make more accessible to the dye what must be a sizable hydrophobic region. Four dye molecules bind per subunit of oxidase without substantial effect on the enzyme activity. The bis-Tns under these conditions is clearly not causing extensive unfolding or damage. When the concentration of dodecyl sulfate is increased (Figure 7), the fluorescence intensity goes through a maximum and decreases. There seems to be a cooperative relationship between dodecyl sulfate and bis-Tns binding. At sufficiently high detergent concentrations, the dye is displaced. It is reasonable to assume that the high affinity bis-Tns binding sites are in part coincident with the lipid binding domain on pyruvate oxidase. The slight stimulation of oxidase activity observed upon bis-Tns binding in the absence of dodecyl sulfate further suggests direct involvement of this domain in dye binding. The fact that protease-activated oxidase does not bind to bis-Tns indicates that the conformational changes at the active site which result in the activation phenomenon are not related to dye binding. It is not likely that the bis-Tns is binding at the active site of detergent-activated pyruvate oxidase, although the steady-state kinetics suggests some possible competition between bis-Tns and thiamin pyrophosphate.

In conclusion, it is likely that the hydrophobic lipid binding domain is directly involved in the high affinity bis-Tns binding to pyruvate oxidase but that the cooperative effect of the detergent activator is required for this dye binding. The substrate-reduced oxidase binds additional dye molecules at higher bis-Tns concentrations at the same or different sites, with the resulting irreversible denaturation. Bis-Tns has been useful for examining detergent binding to pyruvate oxidase and the protein conformational changes which result from this binding. It is hoped that other techniques will further clarify the conformational changes and states of pyruvate oxidase which are being reported by this probe.

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