Biochemistry

 Volume 24, Number 11 May 21, 1985

Articles

Differential Binding of the Fluorescent Probe 8-Anilinonaphthalene-2-sulfonic Acid to Rhodanese Catalytic Intermediates†

Paul M. Horowitz* and Nick L. Criscimagna

Department of Biochemistry, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284

Received September 7, 1984

ABSTRACT: Studies have been performed to quantitate the binding of the fluorescent probe 8-anilinonaphthalene-2-sulfonic acid (2,8-ANS) to catalytic intermediates of the enzyme rhodanese: the sulfur-substituted form (ES) and the sulfur-free form (E). The molecule 2,8-ANS has not been extensively used for protein studies, and some characterization is presented to demonstrate its usefulness as a probe for apolar binding sites. The molecule 2,8-ANS binds to at least two classes of sites on rhodanese. One class (class 1) is present in the ES form and has a K_d of 1.7 mM. The E form of rhodanese appears to have a second class of sites (class 2) in addition to the class 1 sites. Two independent fluorometric methods of analyzing the class 2 binding of 2,8-ANS to the E form gave an average value for $K_d \simeq 179$ μM. These fluorometric titrations, together with a Job plot, clearly indicate that 2,8-ANS binds to more than one site on the E form of rhodanese. The apparent apolarity is slightly higher for class 2 sites than for the class 1 sites, but both give Z factors of >85. The substrate thiosulfate is able to displace the probe that is bound to the class 2 sites on the E form of the enzyme. Further, 2,8-ANS is found to be a competitive inhibitor of the catalyzed reaction with an apparent K_d of 170 μM. Circular dichroism measurements detect no significant changes in the average conformation of rhodanese that can be ascribed to the presence of 2,8-ANS. Ultracentrifugation studies show that there is no aggregation of the normally monomeric rhodanese attributable to 2,8-ANS binding. The results are interpreted in terms of a model in which the two-domain structure, into which rhodanese is folded, participates in a catalytically important conformational change that is associated with alterations in solute-accessible apparent apolarity.

The enzyme rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1) catalyzes the transfer of the outer sulfur of thiosulfate ($S_2O_3^{2-}$) to a variety of nucleophilic acceptors such as cyanide (CN^-). In addition, rhodanese can transfer sulfur to apo iron-sulfur proteins, thereby providing the labile sulfur in their characteristic prosthetic group (Pagani et al., 1982; Volini et al., 1977). In these reactions, the enzyme cycles through two stable catalytic intermediates: the free enzyme (E)[1] and the sulfur-substituted enzyme (ES) (Westley, 1973).

Kinetic and chemical studies of rhodanese in solution have implicated several structural requirements for catalysis: a sulfhydryl group, a cationic site, and, somewhat indirectly, a hydrophobic region. X-ray crystal studies of the ES complex of rhodanese have shown (Ploegman et al., 1978; Lijk, 1981) that the 293-residue single polypeptide chain is folded into two nearly equal size domains that are connected by a single strand of polypeptide chain. The active site is in a region formed by the juxtaposition of the two domains, and the sulfur atom transferred during catalysis is bound in a persulfide linkage to cysteine-247 in the active site. The interdomain surfaces contain a number of hydrophobic residues, and apolar interactions involving this region may, along with a few electrostatic interactions, play an important role in stabilizing the double-domain structure as well as providing an appropriate environment for catalysis.

Although in broad outline the structural requirements deduced from solution behavior are verified in the crystal studies, several significant discrepancies remain. A number of solution studies have indicated that rhodanese has structural flexibility

†Supported by Grant GM25177 from the National Institutes of Health and Grant AQ723 from the Robert A. Welch Foundation.

[1] Abbreviations: E, the free enzyme form of rhodanese; ES, the sulfur-substituted form of rhodanese; 2,8-ANS, 8-anilinonaphthalene-2-sulfonate; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/85/0424-2587$01.50/0

and that a reversible conformational change accompanies catalysis as the enzyme cycles between E and ES (Wasylewski & Horowitz, 1982; Volini & Wang, 1973; Guido et al., 1976; Horowitz & Criscimagna, 1982, 1983a; Horowitz & Falksen, 1983). However, addition of CN^- to crystals of ES removes the sulfur but only produces small conformational changes (Ploegman et al., 1978; Lijk, 1981). On the other hand, recent observations of the stability of crystals of rhodanese have been interpreted as indicating that since the energy between the E and ES conformations is likely to be small, lattice forces in the crystal may be strong enough to prevent the ES to E conformational conversion when the bound sulfur is removed with CN^- (Horowitz & Patel, 1980). To the extent that these conclusions about the small conformational energy differences can be supported, they make rhodanese an important model for studying domain interactions and functional protein flexibility.

In this paper, we have studied the interaction of rhodanese with the apolar fluorescent probe 8-anilinonaphthalene-2-sulfonate (2,8-ANS) to extend previous indirect and preliminary investigations (Horowitz & Westley, 1970). We report here that the 2,8-ANS binding studies support the presence of apolar sites on rhodanese that are differentially accessible in the E and ES forms in a manner consistent with a model for this enzyme that includes a catalytically coupled conformational change.

Materials and Methods

The fluorescent probe 8-anilinonaphthalene-2-sulfonic acid (2,8-ANS) was from Molecular Probes (Junction City, OR). Other chemicals were reagent grade.

Rhodanese was purified from bovine liver and assayed as previously described (Horowitz, 1978). The enzyme was stored as a frozen ammonium sulfate pellet at −40 °C. Protein concentration was determined by using a value of $E_{280}^{0.1\%}$ 1.75 (Sörbo, 1953). The molecular weight of the protein was taken to be 33 000 (Trumpower et al., 1974; Ploegman et al., 1978). All determinations and experiments were performed by using a buffer consisting of 0.01 M Tris–SO_4 at pH 8.6 unless otherwise indicated. Unless otherwise noted, sulfur-free enzyme (E form) was obtained as previously described (Wasylewski & Horowitz, 1982; Horowitz & Criscimagna, 1983b) by adding a 10-fold molar excess of cyanide to a rhodanese solution containing no added sulfur donor, thiosulfate. Kinetic studies to assess the effect of 2,8-ANS on the catalysis by rhodanese were performed using the rhodanese assay previously described which depends on quantitating the complex of the product, thiocyanate, with ferric ions from the absorbance at 460 nm (Wang & Volini, 1968). Each assay contained 50 mM KCN, 50 mM KH_2PO_4, and $K_2S_2O_3$ between 1.67 and 10 mM. The assay pH was 7.6. Replicate reaction mixtures were initiated by additions of 1-μg aliquots of rhodanese and terminated at 15-s intervals. Initial velocities were calculated from the best straight line fits to the initial portions of these progress curves. Similar assays were performed at the following concentrations of 2,8-ANS: 110, 330, and 500 μM. In these cases, 2,8-ANS was included in the blank.

Fluorescence Measurements. Fluorescence measurements were made on an SLM Model 4800 spectrophotofluorometer operated in the ratio mode. The temperature was controlled at 23 °C by circulating water through the cell holder. Time-dependent fluorescence changes were measured by using a strip chart recorder that monitored the instrumental response. Corrected spectra were obtained by using a set of correction factors supplied by the manufacturer. Unless otherwise indicated, all measurements were performed by using a 4-nm band-pass for both excitation and emission. Fluorescence intensities were corrected for inner filter effects due to the absorbance of 2,8-ANS either by using the optical density of the sample at the exciting wavelength (McClure and Edelman, 1967) or, because 2,8-ANS has a measurable fluorescence in the absence of protein, by reference to a standard curve obtained by measuring the fluorescence of increasing concentrations of 2,8-ANS in buffer. Control samples showed that the addition of rhodanese substrates had no significant effect on the fluorescence of 2,8-ANS in buffer or in ethanol.

The concentrations of 2,8-ANS in stock solutions were quantitated by using $\epsilon_M = 5.6 \times 10^3$ M^{-1} cm^{-1} at 360 nm.

Binding Site Polarity. The frequency of the fluorescence maximum of 10 μM 2,8-ANS was measured as a function of the effective solvent polarity expressed by the Z factor (Kosower, 1958). The Z factors were varied by using a series of aqueous ethanol solutions as previously described (Turner & Brand, 1968). To assess binding site polarity on rhodanese, corrected spectra were run for solutions containing 10 μM 2,8-ANS and increasing concentrations of either the E or ES form of rhodanese (0–10 mg/mL). To account for weak binding, double-reciprocal plots were constructed for each enzyme form by using data for the frequency of the fluorescence maximum from corrected spectra as a function of the protein concentration. The extrapolated values for the peak frequencies at infinite protein concentration were used to estimate the empirical solvent polarity characterizing the 2,8-ANS binding sites on rhodanese.

Binding Studies. Several methods were used to measure binding of 2,8-ANS to the E and ES forms of rhodanese:

Method 1. For the first method of binding analysis, the fluorescence intensities of 2,8-ANS bound to the E and ES forms of rhodanese were determined by addition of increasing quantities of the appropriate rhodanese species (0–300 μM) to a solution of 9.52 μM 2,8-ANS in buffer at 23 °C. The cuvette was designed to hold 100 μL and had a path length of 2 mm. The excitation was at 400 nm, and the emission was monitored at 480 nm. The reciprocals of the corrected relative intensities were plotted as a function of the reciprocals of the enzyme concentrations and extrapolated to infinite protein concentration. These intercept values were used as the fluorescence of totally bound 2,8-ANS and were used to calculate the concentrations of bound 2,8-ANS when fixed concentrations of rhodanese were titrated with increasing amounts of 2,8-ANS. The concentration of unbound 2,8-ANS was calculated by difference. In the experiments reported under Results, rhodanese at 0.2 mg/mL was titrated with 0–1000 μM 2,8-ANS. A 25-fold molar excess of cyanide was used to produce the E form. This titration was performed also by using 360-nm excitation and 2,8-ANS concentration to 400 μM and gave essentially identical results. Binding data with this procedure were analyzed by a Scatchard plot for the E form (see Results) or by a double-reciprocal plot for the ES form where the binding is weak. All derived binding parameters were from least-squares fits to the data.

Method 2. A double titration method was developed to increase the precision of the binding data obtained with the E form and remove the necessity to reference all binding data to a determination of the intensity of bound 2,8-ANS as a single intercept on a double-reciprocal plot as above. An alternative description of the binding process is based on a derivation similar to that presented previously (Wang & Edelman, 1971). For a protein with n independent sites, the binding of a fluorescent probe whose fluorescence when bound is much greater than it is when free (as is the case for 2,8-

ANS) can be described by one of two equations depending on the amount of probe relative to protein:

$$\frac{1}{I} = \frac{1}{nE_0\psi} + \frac{K}{nE_0\psi}\frac{1}{F} \quad (1)$$

when $F_0 > E_0$ and

$$\frac{1}{I} = \frac{1}{\psi F_0} + \frac{K}{\psi F_0 n}\frac{1}{E_0} \quad (2)$$

when $nE_0 > F_0$ where I = observed fluorescence intensity, F = concentration of free probe, F_0 = total concentration of probe, E_0 = total concentration of protein, n = the total number of sites on the protein, K = dissociation constant for a dye-site complex, and ψ = proportionality constant connecting the fluorescence intensity to the concentration of the probe–site complex.

When the concentration of probe is large relative to that of protein ($F_0 > E_0$), eq 1 shows that a plot of $1/I$ vs. $1/F$ will be linear for fixed protein concentration. If the titration is repeated for a number of protein concentrations, a family of lines will result whose ordinate intercepts will be inversely proportional to the protein concentrations used and will have a common abscissa intercept of $-1/K$. When the concentration of probe–site complexes is small relative to the total number of sites ($nE_0 > F_0$), eq 2 shows that a family of lines will result when the enzyme concentration is varied at a series of fixed small concentrations of probe. In this case, the ordinate intercept is inversely proportional to the probe concentration used, and the common intercept on the abscissa is given by $-n/K$. Thus, by a combination of eq 1 and 2 it is possible to extract a value for both n and K. The use of this method and details of solution composition are presented under Results and in Figure 5.

Job plots were constructed by using the fluorescence induced when 2,8-ANS binds to rhodanese for both the E and ES forms, keeping the total concentration of 2,8-ANS plus enzyme at 30 μM. The fluorescence was excited at 360 nm and viewed at 480 nm (Cantor & Schimmel, 1980).

Details of other fluorescence procedures and the preparation of samples are presented under Results.

Ultracentrifugation. Ultracentrifugation was performed on a Beckman Model E analytical ultracentrifuge equipped with schlieren optics. Samples of rhodanese were sedimented in a double sector cell at 47 251 rpm for 90 min at a temperature of 25 °C and a protein concentration of 1.3 mg/mL. The E form was prepared by the addition of a 10-fold molar excess of cyanide over enzyme. The probe 2,8-ANS was included at 91 μM to assess its effect on the sedimentation behavior of the enzyme. Photographs were taken every 8 min during sedimentation, and the rate of boundary movement was used to calculate $s_{20,w}$ values.

Circular Dichroism. CD measurements were performed on a Jasco J500C spectropolarimeter equipped with a Model DP 500 data processor. Spectra were run at 23 °C in a cell with 0.05-cm path length. Spectra were scanned from 260 to 200 nm and corrected by using a blank containing all components except the enzyme. CD spectra were run on both the E and ES forms of rhodanese in the presence and absence of 50 μM 2,8-ANS.

Results

Figure 1 shows the time course for the binding of 2,8-ANS to the E and ES forms of rhodanese. The probe is present at $t = 0$ and the ES form was added at (a). At (b) CN^- was added to produce the E form. At (c) SSO_3^{2-} was added to 100 μM to regenerate ES. In a survey, data similar to that shown here were generated with a number of isomeric ANS derivatives (data not shown) including 1,8-ANS, 2,6-ANS, and 1,5-ANS. These other probes gave time-dependent fluorescence changes to varying degrees rather than the stable readings shown here. For the studies reported here, therefore, the 2,8-ANS derivative was used exclusively.

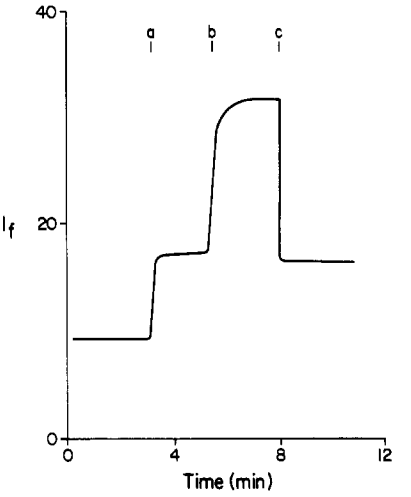

FIGURE 1: Strip chart tracing of the time course of fluorescence intensity for 2,8-ANS in the presence of E and ES forms of rhodanese. The sample contained 0.2 mg/mL rhodanese and 50 μM 2,8-ANS in 0.01 M Tris–SO_4, pH 8.6. The probe was present at $t = 0$, and the ES form of rhodanese was added at (a). At (b) CN^- was added to 16 μM to produce the E form. At (c) $S_2O_3^{2-}$ was added to 100 μM to regenerate ES. For this particular experiment, the excitation wavelength was 350 nm, and the emission was viewed at 450 nm.

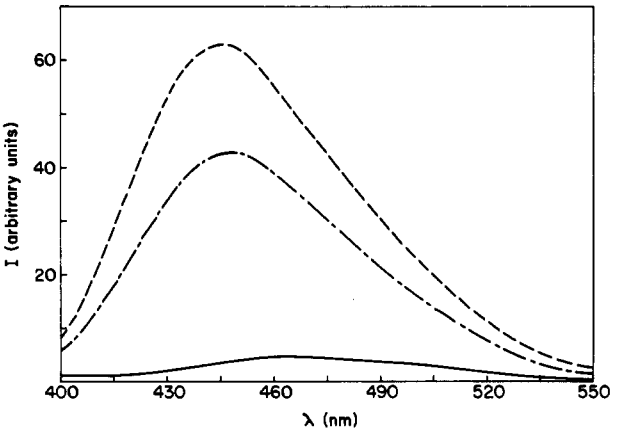

FIGURE 2: Spectra for 2,8-ANS in the presence of the E and ES forms of rhodanese. Spectra for 2,8-ANS with E (upper spectrum), ES (middle spectrum), and a control with no protein (lowest spectrum). Each sample contained 10 μM 2,8-ANS. For the rhodanese spectra the protein was present at 10 mg/mL. All spectra were run at 23 °C with 360-nm excitation. Other details are presented under Materials and Methods.

Figure 2 shows spectra of the E and ES forms of rhodanese in the presence of 2,8-ANS compared with the probe in buffer. There is a pronounced increase in the intensity as well as a shift of the fluorescence maximum to shorter wavelengths. The molecule 2,8-ANS has not been widely used as a fluorescence probe for apolar sites, but by analogy with other closely related isomeric anilinonaphthalenesulfonates, it is expected that the fluorescence of 2,8-ANS would be responsive to changes in the polarity of its binding site. Figure 3 shows the response of the frequency of the fluorescence maximum of 2,8-ANS to changes in the effective dielectric constant (Z factor) achieved by varying the ethanol concentration of the solution. The data follow the behavior seen with other ANS derivatives

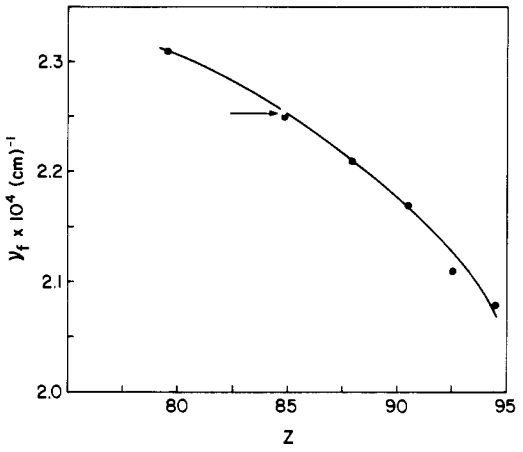

FIGURE 3: Frequency of the fluorescence maximum for 2,8-ANS vs. the solution Z factor. The probe 2,8-ANS was present at 10 μM, and the effective polarity of the solvent (Z factor) was varied by the addition of ethanol. The maxima for both E and ES give an extrapolated frequency for the emission maximum of bound ANS shown by the arrow. Experimental details are presented under Materials and Methods.

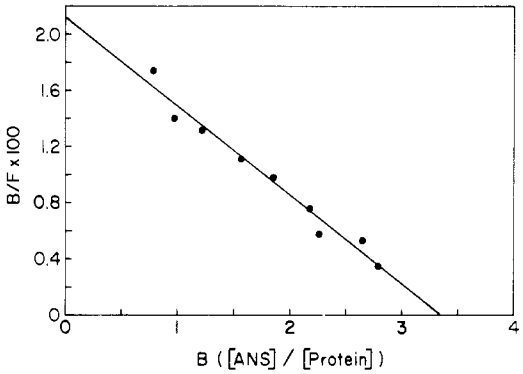

FIGURE 4: Scatchard plot for the binding of 2,8-ANS to the E form of rhodanese. The E form was present at 0.2 mg/mL, and the data shown were derived by using excitation at 400 nm and emission at 480 nm. B denotes the moles of bound 2,8-ANS per mole of protein, and F denotes the concentration of free 2,8-ANS. All other experimental details are presented under Materials and Methods.

such as 1,5-ANS, 1,7-ANS, etc. The Z factor for 2,8-ANS bound to rhodanese was estimated by measuring the frequency of the maximum in the fluorescence spectra of solutions containing a fixed concentration of 2,8-ANS and increasing concentrations of either the E or ES forms of rhodanese, and extrapolating these frequencies to infinite protein concentration. The extrapolated value was 85 for each enzyme form (arrow, Figure 3). The details of the extrapolation procedure are presented under Materials and Methods.

A Scatchard plot for the binding of 2,8-ANS to the E form is shown in Figure 4. Analysis gives K_d = 158 μM and n = 3 (Table I). The finding of more than one binding site for 2,8-ANS was interesting, and it was necessary to confirm this value. Figure 5 shows an independent binding assessment of the interaction of 2,8-ANS with E by the double titration method described under Materials and Methods (method 2). In Figure 5A the ordinate intercepts are inversely proportional to the concentrations of 2,8-ANS, and in Figure 5B the ordinate intercepts are inversely proportional to the concentrations of protein. The families of lines in Figure 5 define common abscissa intercepts that, when used together as described above, give K_d = 200 μM and n = 2.98.

The binding of 2,8-ANS to the ES form was very weak and not able to be treated as that for the E form. The data for the ES form were analyzed by using a double-reciprocal plot as described under Materials and Methods and gave K_d = 1670 μM.

As a further test of the stoichiometry, fluorescence data were collected by the method of continuous variation (Job plot) for

Table I: Summary of Parameters for Binding of 2,8-ANS to Rhodanese Intermediates

rhodanese intermediate	binding		kinetics K_I (μM)
	K_{dis} (μM)	n	
E	158[a]	~3[a,b]	170
	200[b]		
ES	1670	~1[c]	

[a] Scatchard plot binding method 1 (Materials and Methods). [b] Binding method 2 (Materials and Methods). [c] Based on method of continuous variation (Materials and Methods).

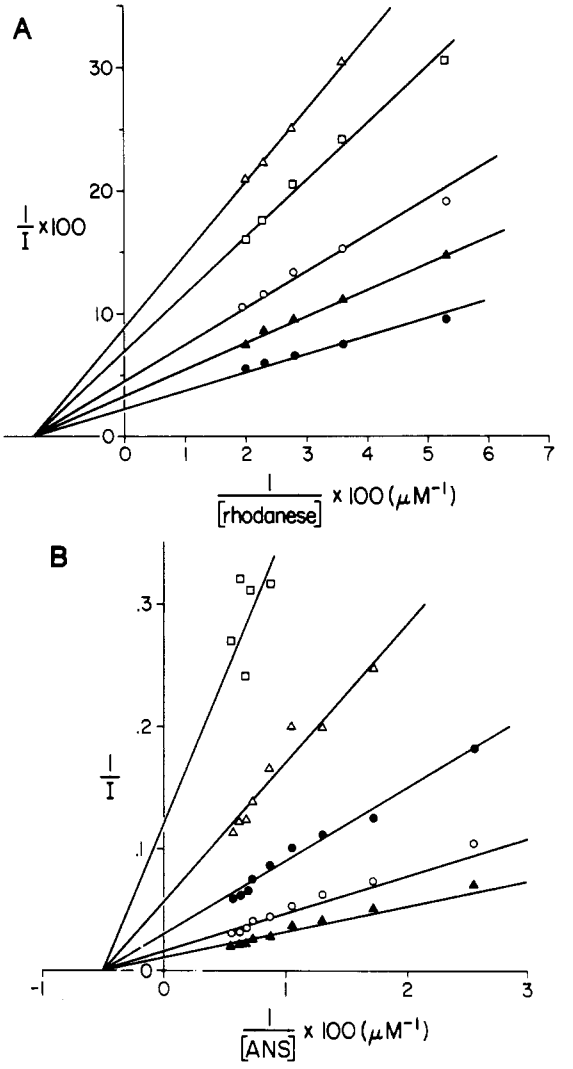

FIGURE 5: Family of lines method for characterizing the binding of 2,8-ANS to the E form of rhodanese (method 2, Materials and Methods). (A) Plot of the inverse of the fluorescence intensity as a function of the inverse of the protein concentration at various fixed concentrations of 2,8-ANS. The protein concentration was varied from 10 to 51 μM of the E form. Each line corresponds to a different fixed concentration of 2,8-ANS which were (from the upper line) 2.2, 3.3, 5.5, 7.7, and 11 μM. Excitation was at 360 nm, and emission was at 480 nm. (B) Plot of the inverse of the fluorescence intensity as a function of the inverse of the 2,8-ANS concentration at various fixed concentrations of the E form of rhodanese. The 2,8-ANS concentration was varied from 20 to 182 μM. Each line corresponds to different fixed concentrations of the E form which were (from the upper line) 0.025, 0.05, 0.1, and 0.4 mg/mL. Excitation was at 400 nm, and the emission was viewed at 480 nm. All other experimental details are presented under Materials and Methods.

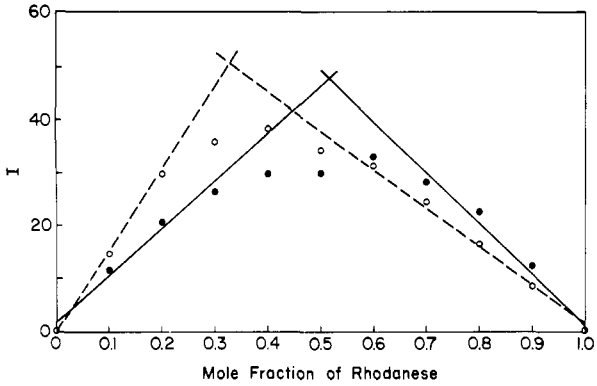

FIGURE 6: Job plot for binding of 2,8-ANS to the E and ES forms of rhodanese. The observed fluorescence is denoted I, and the total concentration of [rhodanese] + [2,8-ANS] was 30 μM. The samples were (○) E and (●) ES. Other experimental details are presented under Materials and Methods.

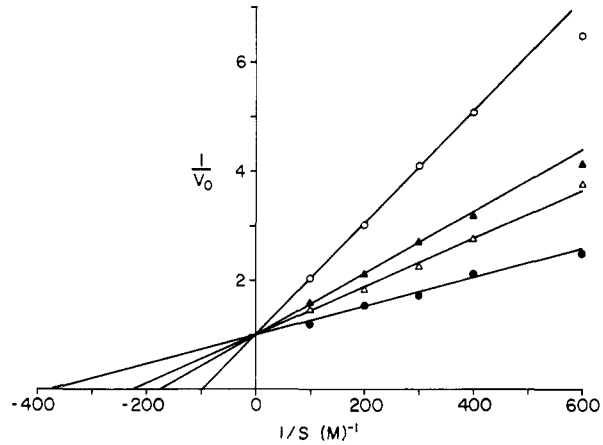

FIGURE 7: Effect of 2,8-ANS on the kinetics of the rhodanese-catalyzed reaction. Double-reciprocal plots of the inverse of the initial velocity of the rhodanese reaction vs. the inverse of the thiosulfate concentration. Kinetics were measured in the presence of the following concentrations of 2,8-ANS: 0 (●), 110 (△), 330 (▲), and 500 (○) μM. Each assay contained 1 μg of rhodanese/mL and was run at 23 °C.

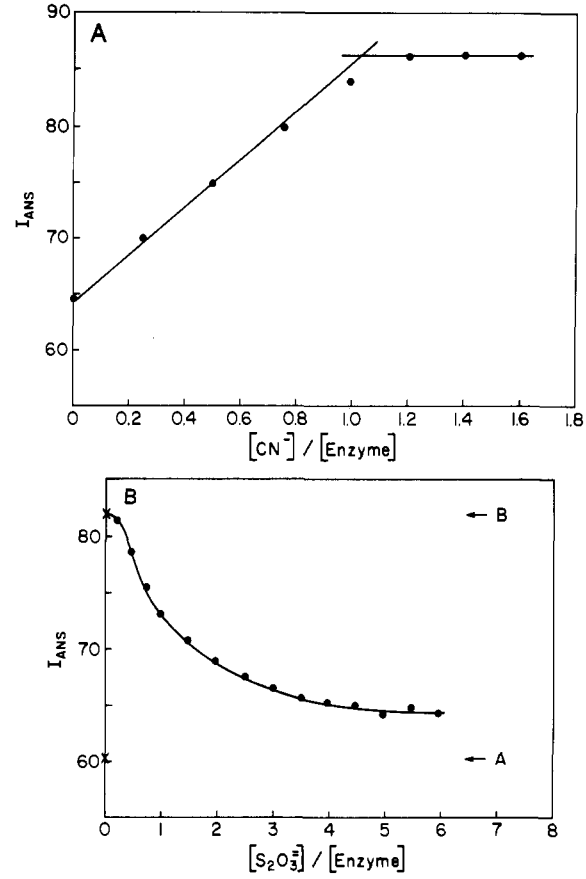

FIGURE 8: Interconversion of the E and ES forms of rhodanese in the presence of 2,8-ANS. (A) Fluorescence intensity of 2,8-ANS as a function of the ratio of [CN^-]/[rhodanese]. (B) Back-titration of E to ES by the addition of $S_2O_3^{2-}$ monitored by the fluorescence intensity of 2,8-ANS. In the presence of ES, 2,8-ANS originally gave the intensity denoted by the arrow at A. Addition of 1.3 mol of CN^-/mol of rhodanese gave an intensity increase for 2,8-ANS to B. Titration of this solution with $S_2O_3^{2-}$ gave the response shown. In these experiments the 2,8-ANS concentration was 100 μM, and rhodanese was present at 0.2 mg/mL. All other experimental conditions are given under Materials and Methods.

both E and ES. The data are shown in Figure 6. These plots are consistent with there being multiple sites on the E form and one site on the ES form for the binding of 2,8-ANS.

The binding data are collected in Table I.

The effect of 2,8-ANS on the catalysis by rhodanese was tested. Figure 7 shows a double-reciprocal plot of rhodanese kinetics with the inclusion of various amounts of 2,8-ANS. This plot indicates that 2,8-ANS acts as a competitive inhibitor with respect to SSO_3^{2-}. The K_i values were derived from a number of determinations run at different concentrations of 2,8-ANS (10–80 μM) and give K_i = 170 μM independent of the 2,8-ANS concentrations used for its determination, thereby indicating that 2,8-ANS itself does not cause any large irreversible change in the enzyme. This K_i, because of the competitive inhibition and the mechanism of the rhodanese reaction, is entered in Table I as a binding parameter for the E form of the enzyme (see Discussion).

Equilibrium dialysis measurements were attempted, but because of limitations in the amounts of material available, the unavailability of a radiolabeled form of the probe, and the instability of the enzyme, the data, although consistent with the conclusions reached from the fluorescence experiments, were of insufficient quality to expand the conclusions that were reached on the basis of the fluorescence measurements.

Circular dichroism measurements were performed on the E and ES forms in the presence and absence of 2,8-ANS as described under Materials and Methods. Changes observed here were consistent with those previously reported for the transition between the E and ES forms (Volini & Wang, 1973), but no changes in the far-UV region of the CD spectra were observed that could be specifically attributed to the presence of 2,8-ANS.

Ultracentrifugation measurements were performed to test further for overall changes in protein conformation induced by the binding of 2,8-ANS. Sedimentation velocity experiments were performed as described under Materials and Methods for the E and ES forms in the presence and absence of 2,8-ANS and gave a value of 3.34 S for all samples. This value is consistent with previous reports for the native enzyme (Horowitz & Falksen, 1983).

The CD and ultracentrifugation measurements indicate that there are no gross changes in structure specifically attributable to the binding of 2,8-ANS.

The comparisons between the binding of 2,8-ANS and its effect on rhodanese function suggested that the E–ES interconversion during catalysis could be monitored by observing fluorescence changes in 2,8-ANS. Figure 8A shows a titration of the conversion of the ES to E forms induced by cyanide and monitored by observing the fluorescence of 2,8-ANS included in the reaction mixture. This is the behavior expected for the irreversible removal of sulfur from ES which is known to occur

with CN^- with an apparent equivalence at CN^-/enzyme = 1. Figure 8B shows the back-titration of E to ES by the addition of SSO_3^{2-} monitored as in Figure 8A. The form of this response is in keeping with the fact that the interaction of rhodanese with thiosulfate to form ES is a reversible equilibrium. The operation of this equilibrium explains why the fluorescence does not return to the base line with the concentration of SSO_3^{2-} used here. These results are in agreement with those previously reported for the interconversion of E and ES monitored by an independent procedure (Horowitz & Criscimagna, 1983b).

Discussion

The results here show that 2,8-ANS binds tighter to the E forms of rhodanese and is a competitive inhibitor of catalysis.

The fluorophore 2,8-ANS, as might be expected, has the relevant properties to be an apolar probe like closely related anilinonaphthalenesulfonates that have been discussed (Slavik, 1982). Bound 2,8-ANS gave stable fluorescence intensities with rhodanese as opposed to slowly changing fluorescence with other isomers. These kinetic differences may be related to previous results showing large differences in rates of inactivation of phosphoenolpyruvate carboxykinase by the various isomers of ANS (Nelson & Silverstein, 1983).

The Z value for the 2,8-ANS binding site was the same for E and ES and is similar to that for the binding of 1,5-ANS and 2,6-ANS to liver alcohol dehydrogenase (Turner & Brand, 1969). This is interesting since X-ray studies report a similarity between the folding of a portion of the rhodanese backbone and the nucleotide binding domain of liver alcohol dehydrogenase (Lijk, 1981).

The effect of 2,8-ANS on the catalyzed reaction correlates very well with the fluorescence studies, and 2,8-ANS appears to be a classical competitive inhibitor. This is in keeping with previous studies showing aromatic sulfonates are good competitive inhibitors of catalysis. The overall reaction catalyzed by rhodanese can be understood by using the following scheme (Westley, 1973):

$$E + SSO_3^{2-} \rightleftharpoons E{\cdot}SSO_3^{2-} \quad (3)$$

$$E + ANS \rightleftharpoons E{\cdot}ANS \quad (4)$$

$$E{\cdot}SSO_3^{2-} \rightleftharpoons ES + SO_3^{2-} \quad (5)$$

$$ES + CN^- \rightarrow E + SCN^- \quad (6)$$

Normal catalysis is represented by reactions 3, 5, and 6. Reaction 4, represented here as an equilibrium binding between the E form and 2,8-ANS, is required to explain the kinetics of a variety of competitive inhibitors, like the substituted sulfonates most relevant to understanding the 2,8-ANS interaction, e.g., benzenesulfonate and naphthalenesulfonate. The similarity between the binding constants measured for the interaction of 2,8-ANS with the E form and the kinetically determined inhibition constant is consistent with the idea that the same binding step is responsible for the fluorescence response and the inhibition. It then follows that thiosulfate binding can displace the probe, and it is not necessary to invoke steps leading to the formation of ES as in step 5. It is interesting that previous work (Volini & Wang, 1973) suggested the conformational change on forming the ES complex occurs in two steps—part on binding thiosulfate (reaction 3) and part after bond breaking and release of sulfite (reaction 5). Therefore, the present results indicate that the first part of the conformational change is associated with an alteration in the apolar accessibility on the surface of the rhodanese molecule sufficient to account for the binding of as many as three molecules of 2,8-ANS in competition with the binding of one molecule of thiosulfate. In addition, when the 2,8-ANS site becomes unavailable upon binding SSO_3^{2-}, it remains inaccessible in ES and only reappears when the S atom is removed from the enzyme. It seems unlikely, then, that a static steric effect could be responsible for the fact that binding of one molecule of thiosulfate or the presence of one atom of sulfur precludes the binding of more than one molecule of 2,8-ANS.

The major stabilizing interaction for the binding of thiosulfate to the enzyme has been suggested to be an interaction of the anionic thiosulfate with a cationic site on the enzyme (Ploegman et al., 1978). This ionic interaction also appears to control the interaction of a number of inhibitors with the active site region (Westley, 1973; Schlesinger & Westley, 1974). In fact, otherwise appropriate molecules are only substrates in their anionic form (Westley & Heyse, 1981). In addition, iodoacetamide will not react with the active site sulfhydryl group of rhodanese while the closely related anionic reagent iodoacetate reacts quickly (Horowitz & Criscimagna, 1983a). These results indicate that access to the active site region is controlled by intraprotein interactions that can be displaced by anionic ligands with the appropriate geometry. These facts, then, may help to explain the unusual effectiveness of 2,8-ANS at interacting with the enzyme and point out that simple steric exclusion is unlikely to be responsible for all the observations reported here. In fact, it is unlikely that ionic interactions alone could account for the binding of 2,8-ANS since the dianionic substrate thiosulfate has a K_s of 1.3 mM (Westley, 1973), while as shown here, the K_d for 2,8-ANS is only 150 μM.

The functional connection between the exposure of 2,8-ANS binding sites and the catalytic cycle is further supported by finding an exact correlation between 2,8-ANS binding and the interconversion of E and ES. This result is just like that reported for the interconversion of these catalytic intermediates followed through changes in the intrinsic fluorescence of the enzyme (Horowitz & Criscimagma, 1983b). This, then, further supports the idea that the catalytic cycle is correlated closely with cyclic changes in accessibility for the binding of 2,8-ANS.

The stoichiometry for 2,8-ANS binding is surprising in that conversion of ES to E is correlated with binding of more than one molecule of 2,8-ANS. This is more in keeping with the exposure of a surface than that of a single site. In fact, recent results from tritium exchange measurements showed rapid release of a large number of tritium ions from rhodanese on converting ES to E which was taken as consistent with increased surface exposure that could correspond to changes in the interactions between the two structural domains into which the enzyme is known to be folded (Horowitz & Falksen, 1983).

The results of the present study can be related to the X-ray structure for the ES form of rhodanese (Lijk, 1981). The active site SH group that holds the transferred sulfur atom is provided by Cys-247 in the carboxyl-terminal domain. The normally reactive persulfide is kept out of direct contact with solvent and stabilized by interactions with the protein. A cationic site for binding and orientation of the anionic substrate can be provided by Arg-186 and Lys-249. These interactions are supplemented by a strongly hydrophobic region formed by a cluster of side chains from both domains: Trp-35, Phe-106, Tyr-107, Phe-212, and Val-251. Stereo representations of the active site show that 2,8-ANS binding to the cationic site would enable the anilinonaphthalene moiety to interact with the hydrophobic cluster whose extended size might permit the binding of more than one molecule of 2,8-ANS and explain how changes in domain interactions could affect binding of

2,8-ANS. It is interesting that the planes of the aromatic rings of Trp-35, Phe-106, and Tyr-107 are approximately parallel and may contribute to stacking with respect to one or more 2,8-ANS molecules. This potential for geometric restriction of bound 2,8-ANS may relate to the differences in kinetics and strength of binding of the various ANS isomers, and this phenomenon is presently under investigation.

In conclusion, the present results indicate that interconversion of E and ES intermediates for rhodanese catalysis is correlated with differential binding of the apolar probe 2,8-ANS and the observed pattern of apolar accessibility can be accommodated within the X-ray structure by proposing that the catalytic cycle includes a conformational change that may involve alterations in interdomain interactions.

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