RESULTS AND DISCUSSION

When α-DNS-Thr^8-(1–16) was mixed with pepsin, a very rapid reaction ($t_{1/2} < 3$ s) led to a species with greatly enhanced dansyl fluorescence arising from an additional excitation peak at 290 nm. This may be attributed to energy transfer from Trp residues of pepsin. Since R_0 is known to be 22 Å for the Trp-dansyl pair, we can calculate the distance between the two chromophores of 20.5 Å for this initial complex.

This initial complex is short-lived, however, since the fluorescence decays to a second value with a half life of 2 min. The final level of fluorescence enhancement may be used to calculate a new distance of 31.7 Å. This shift in position of 11.2 Å is of profound significance since the appearance of inhibition of pepsin activity occurs with the same time dependence as the fluorescence decay as shown in Fig. 2.

Received for publication 19 December 1979.

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STUDIES OF FUNCTIONALLY IMPORTANT STRUCTURAL FLEXIBILITY OF THIOSULFATE SULFURTRANSFERASE

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The enzyme thiosulfate sulfurtransferase (TST; rhodanese; EC 2.8.1.1) catalyzes the transfer of the outer sulfur of thiosulfate ($S_2O_3^-$) to a variety of nucleophilic acceptors such as cyanide (CN^-). During the course of this reaction the enzyme cycles through two stable isolatable catalytic intermediates: the free enzyme, (E), and the sulfur substituted enzyme, (ES). The study of TST is of interest because it displays a number of nonartifactual discrepancies between the x-ray structure (1) and numerous solution studies. The x-ray structure shows that the enzyme is a single polypeptide chain that is folded into two distinct domains with the active site in the interdomain region. Addition of CN^- to crystals of ES is reported not to produce the large conformational changes that would be expected from solution studies (2, 3). In addition, the active site sulfhydryl group has characteristic reactivities in solution (4, 5) that are not possible based on the crystal structure of the ES form. It appears that fluctuations in the structure of TST are in part responsible for these observations, and thus this enzyme will be valuable in developing diagnostics and techniques for the recognition and study of flexibility and domain interactions in proteins.

In the present studies various solution and fluorometric approaches have been used to examine structural fluctuations in TST. These include studies of the solubilities of crystals of

the enzyme; the reactivity of the active site sulfhydryl group; controlled tryptic digestion; the fluorescence depolarization of labeled enzyme, and the fluoresence of apolar probes.

TST activity and crystal stability were studied as a function of ionic strength. At 2 M ammonium sulfate (where the x-ray studies were done), soluble enzyme has low activity (<20% of the enzyme's activity at an ionic strength of 0.1) and crystals of the enzyme are stable when substrates are added. However, at 1.7 M ammonium sulfate, crystals of TST rapidly dissolve in 1 mM CN^- but are relatively stable in 1 mM $S_2O_3^=$. This is consistent with a conformational change on converting ES to E and explains why this change was not observed in the crystallographic studies.

Iodoacetic acid (IAA) rapidly inactivates E but ES is unaffected. Analysis of the kinetics and stoichiometry of TST inactivation indicates that IAA is an affinity analog for the substrate thiosulfate. In contrast, iodoacetamide (IAM) has no effect on the activity of either E or ES. These results indicate that the differential reactivity of the active site sulfhydryl group cannot be explained simply on the basis of steric constraints imposed by the introduction of the transferred sulfur, but is consistent with a model involving a flexible protein.

TST (mol wt ≃ 33,000) can be clipped by trypsin (20% wt/wt) to give two species (mol wt ≃ 28,000 and mol wt ≃ 4,000) and an increase in activity. At 25°C the 28,000 species is further cleaved to give two species (mol wt ≃ 18,000 and mol wt ≃ 10,000), again with no loss of activity. The kinetics of trypsin cleavage have been analyzed and are consistent with a product precursor relationship in which TST is first clipped near one end and the resulting structure sufficiently altered so that a subsequent clip can be made in the tether connecting the TST domains. Gel filtration results indicate that the clipped peptides can remain associated as in the parent structure.

Studies of intrinsic protein fluorescence and the fluorescence of covalently bound probes have revealed low temperature thermal transitions in TST. For example, the intrinsic fluorescence intensity decreases sharply at 27°C and the magnitude of this quenching is smaller for ES than for E. The effect is fully reversible with ES and partially so with E. This thermal transition is not observed when either the wavelength of maximum fluorescence or the intrinsic fluorescence polarization is monitored. TST can be labeled with the fluorophore dimethylamino naphthalene in three regions: (*a*) amino groups; (*b*) the active site sulfhydryl group; and (*c*) nonactive site sulfhydryl groups as well as the active site sulfhydryl group. Fluorescence depolarization studies of these derivatives all show a sharp increase in polarization at 27°C when the temperature/viscosity ratio is varied with temperature and no transition when the ratio is varied with glycerol. Perrin plots of the data below 27°C or with glycerol give the following rotational relaxation times (ρh): (*a*) ρh = 58 ns; (*b*) ρh = 38 ns; (*c*) ρh = 47 ns. TST activity shows no sharp transition at 27°C but falls abruptly above 49°C as the protein denatures. Long incubations (>1 h) at 27°C lead to turbidity which is especially pronounced at protein concentrations >0.3 mg/ml.

Studies have been performed to quantitate the binding of the apolar probe 2-*p*-toluidinonaphthalene-6-sulfonate (2,6-TNS) to both the free E and sulfur-substituted ES forms of TST. TNS binding shows a rapid fluorescence increase followed by a slower increase in fluorescence intensity. By preincubation, the enzymatic activity is maintained and the fluorescence intensities are constant. This finding has enabled us to perform a Scatchard analysis of the TNS binding. At 25°C two molecules of TNS are bound to E with a dissociation constant (K_{diss}) of 10^{-3} M, while for ES only one molecule of TNS is bound with $K_{diss} = 2.5 \times 10^{-4}$ M. The slow phase of TNS binding was studied as a function of

temperature and shows there is a sharp structural transition in the protein that occurs at 27°C. When the TNS binding is repeated at 30°C, $K_{diss} = 1.4 \times 10^{-4}$ M for E while all other binding parameters are the same as at 25°C.

These results are consistent with a picture of a mobile bilobed protein with an apolar active site region between two flexibly connected domains. Further, it is suggested that TST participates in catalytically obligatory conformational changes which include an "open-close" equilibrium between the two domains.

It is further proposed that the comparison between x-ray structural predictions and solution behavior can serve as a diagnostic for structural flexibility, when these comparisons are based on a number of experimental approaches.

Received for publication 4 February 1980.

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TIME DIFFERENTIAL PERTURBED ANGULAR CORRELATION MEASUREMENTS IN METAL ION COMPLEXED ATP

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INTRODUCTION

The perturbed angular correlation technique is being increasingly used to study the motion, conformation, or binding properties of biologically relevant molecules (1–3). It does, however, suffer because, except in a few cases, the P.A.C. nuclides are not directly involved in biological processes and the results therefore must be treated with caution. Nevertheless, it has been argued, for example, that a study of the binding of In ATP to 3-phosphoglycerate kinase (4) is not adversely affected by the replacement of Fe^{3+} by In^{3+}. In this work we wish to point out that whether or not a metal ion replacement is valid from a biological viewpoint, the interpretation of the results can be frustrated by the particular radiochemical properties of the probe nuclide. Our work has used ^{181}Hf and ^{57}Co bound to ATP in an attempt to separate effects due on the one hand to the rotational diffusion of the metal ion-ATP unit as a whole, and on the other to the aftereffects of the nuclear decay and any possible static quadrupole effects at the metal ion binding site. To do this successfully, complementary data also has to be obtained. Because of the difficulties associated with the interpretation of viscosity data on large molecules, i.e., the use of Debye theory to give the rotational correlation time and the measurement of viscosity itself in nonnewtonian fluids, we have used dielectric relaxation measurements to provide additional information on molecular rotation.