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# A STUDY OF THE APOLAR INTERACTION OF THE ENZYME RHODANESE WITH OCTYL SUBSTITUTED AGAROSE GEL

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SUMMARY: The accessibilities of sites on the surface of the enzyme rhodanese for binding to macromolecular apolarity have been measured for the two forms of the enzyme related to obligatory catalytic intermediates: the free enzyme, E and the sulfur substituted enzyme, ES. This study was done using a micromethod developed for this purpose which allows facile assessment of the apolar binding of proteins to commercially available beads of cross-linked agarose on which hydrophobic groups have been immobilized. The results indicate that the enzyme rhodanese can bind to macromolecular apolarity and that there is considerably more binding of the E form than the ES form. The fact that the binding is relatively slow implicates a protein conformational change in the rate limiting binding step. In fact, there is a large increase in the binding when the temperature is raised from 23° to 40° which correlates with previous results showing a conformational change in rhodanese over the same temperature range. These results in comparison with other solution studies and with x-ray studies are consistent with a model for rhodanese which has an apolar active site and a mechanism for catalysis that includes a conformational change.

The enzyme rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1.) can transfer sulfur to a variety of acceptors such as cyanide ($CN^-$) and apo iron sulfur proteins. In the latter case, this provides the labile sulfur that constitutes the characteristic prosthetic group in these proteins (1,2). In performing this function, rhodanese cycles through two stable catalytic intermediates: the free enzyme (E), and the sulfur substituted enzyme (ES) (3). Rhodanese is monomeric (mw = 33,000) but can be induced to aggregate in the E form by modest increases in temperature (>30°) (4). During isolation, at least a portion of the rhodanese can be found noncovalently associated in a multiprotein complex with iron-sulfur proteins (2).

ABBREVIATIONS: TNS, 2-(p-toluidinyl)naphthalene-6-sulfonate; E, the sulfur-free form of the enzyme rhodanese; ES, the sulfur containing form of the enzyme rhodanese.

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A number of kinetic and spectral studies of rhodanese in solution have been interpreted as indicating that the protein has structural flexibility and that a reversible conformational change accompanies catalysis (4,5,6,7). X-ray analysis of the crystalline enzyme shows that the single polypeptide chain of rhodanese is folded into two domains with the active site in the inter-domain region. Although in broad outline the structural and chemical requirements deduced from solution behavior are verified, the crystal studies do not show the expected conformational changes (8,9).

Functional and x-ray studies have suggested that the active site is apolar (9,10,11,12). This apolar region may play a role in stabilizing the double domain structure and provide an appropriate environment for catalysis which is known to involve a stable persulfide intermediate that is normally labile in aqueous solution (3).

We have been focussing on the solute accessibility of the apolar region to investigate differences between the E and ES forms of rhodanese. Recent observations show that the active site can bind the fluorescent apolar probe 2-(p-toluidinyl)naphthalene-6-sulfonate (TNS) and that the binding to the E and ES forms are different (12).

In the present paper, we have investigated the ability of rhodanese to bind to macromolecular apolarity using a bead binding assay developed for this purpose.

The objective was to determine if binding occurred and whether this binding would be different for E and ES. In addition, these results would relate to whether the interdomain apolarity could become accessible and contribute to the macromolecular interactions that have been proposed for rhodanese. The results presented here show that there are differential interactions involving the E and ES forms in a way that is consistent with flexible models of this enzyme.

MATERIALS AND METHODS: Octyl-Sepharose was from Pharmacia, Inc. (Piscataway, New Jersey, USA). Other chemicals were reagent grade.

Rhodanese was purified from bovine liver and assayed as previously described. (13) Protein concentration was determined either by using a value of $E^{0.1}_{280}$=1.75 for purified rhodanese (14) or by measuring the quantitative binding

to the protein of the dye coomassie brilliant blue using the optical density at 595 nm (15). Standard curves were generated with both rhodanese and bovine serum albumin.

The sulfur-free form of rhodanese (E form) was obtained as previously described (4) by adding a 10-fold molar excess of cyanide over protein to a rhodanese solution in a buffer consisting of 50mM $Na_2HPO$ and 200 mM NaCl at pH 7.4 (buffer A). This buffer was used for all the studies reported here. The sulfur-substituted enzyme (ES form) was obtained similarly by using the sulfur-donor, sodium thiosulfate.

Octyl Sepharose was washed in a column with 20 bed volumes of buffer A. The resin was resuspended in a sufficient volume of buffer A such that 500 µl of the resulting slurry would give 100 µl of packed gel when the slurry was centrifuged in an Eppendorf microcentrifuge for 2 min. Quantities of resin were measured in terms of this slurry.

The time course of the binding of the E form of rhodanese was measured at both room temperature and 40°. At each temperature, individual 100 µg samples of the E form of rhodanese were added to multiple 200 µl aliquots of Octyl-Sepharose slurry and incubated in a water bath for 0 - 30 min. The aliquots were centrifuged at intervals and the protein concentrations in the supernatants were determined by quantitative dye binding.

Fixed 100 µg samples of the E and ES forms of rhodanese were titrated with octyl sepharose as follows. Increasing volumes of octyl sepharose slurry (0 - 500 µl) were mixed with appropriate volumes of buffer A to give a series of samples each with a total volume of 500 µl. The rhodanese was added to each tube and incubated at either 23° or 40° for 30 min. The samples were centrifugated and the supernatants were assayed for protein by quantitative dye binding.

## RESULTS AND DISCUSSION

Apolar groups covalently coupled to insoluble supports are used for chromato graphic separtions that depend on apolar interactions between proteins and the immobilized ligand. These procedures are valuable for preparative purposes but are unwieldy for making quantitative comparisons of differential binding and not useful for measuring the kinetics of these hydrophobic interactions.

The binding assay reported here using beads of octyl sepharose can potentially provide information about both the extent of apolar interactions (Figure 2) and the rate of binding (Figure 1).

The results presented here in figures 1 and 2 indicate that rhodanese can bind to octyl sepharose and that the binding of the E form is different from the binding of the ES form (Figure 2).

Figure 1 shows that the rate of binding of the E form is greatly increased as the temperature is raised to 40°. At this temperature, the enzyme is still active (Data not shown) and the binding result is consistent with previous data showing that there is a conformational change in the protein over this same

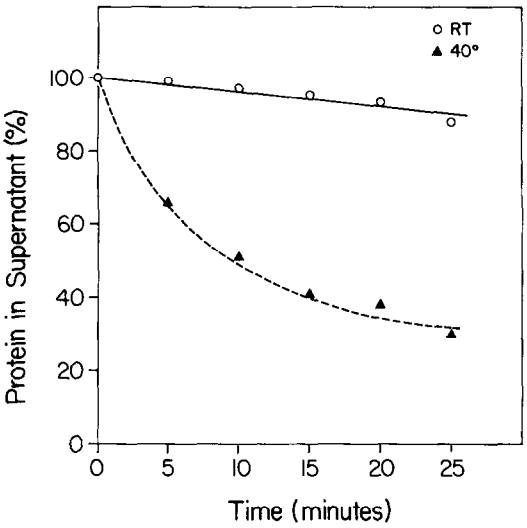


**Fig. 1.** **Time course of binding of the E form of rhodanese to octyl sepharose. The ordinate represents the percentage of the total protein remaining in the supernatant as a function of time assessed by quantitative dye binding. For each time point 100 µg of the E form of rhodanese were added to 200 µl of octyl sepharose slurry (40 µl of packed gel). The upper curve represents samples incubated at 23° and the lower curve represents incubations at 40°. Sample preparation and other experimental details are described in Experimental Procedures.**

temperature range (4). The rate of binding of the small molecule apolar probe TNS also shows a large increase when the temperature is raised from 23° to 40° (12). The rate of binding to the octyl sepharose shown here is relatively slow, just as is the case for binding of the probe TNS and in both cases, it takes several minutes for the binding to be complete. The slowness of the rate in both these cases makes it appear likely that a conformational change of the protein must contribute to the rate limiting step for the observed apolar interactions.

Figure 2 shows the binding of rhodanese to increasing quantities of resin after incubation for a fixed time of 30 min.
At both 23° (open symbols) and 40° (closed symbols) more enzyme is bound when it is in the E form than when it is in the ES form and the binding behavior can clearly distinguish between the enzyme forms. Again, in this method of assessing binding, there is a dramatic increase in binding as the temperature is raised to 40°.

Binding studies with the apolar probe TNS show that the binding to both enzyme species is weak, the stoichiometry is low and that TNS is a competitive

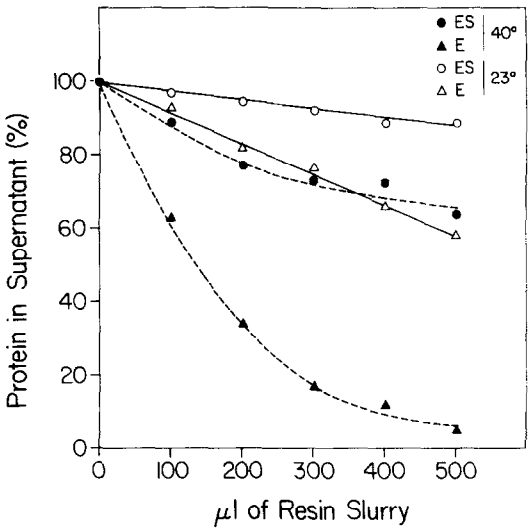


Fig. 2. Titration of the binding of rhodanese to increasing quantities of octyl sepharose. Each curve was determined by treating 100 μg samples of rhodanese with increasing quantities of octyl sepharose in a constant final volume of 500 μl. The ordinate represents the percentage of the initial protein present in the supernatant after incubating for 30 min. as assessed by quantitative dye binding. The open symbols represent data at 23° and the closed symbols represent data at 40°. The individual curves are designated as follows: ○, the ES form at 23°; △, the E form at 23°; ●, the ES form at 40°; ▲, the E form at 40°. The resin has been calibrated such that 500 μl of slurry contains 100 μl of packed resin. Sample preparation and other experimental details are described in Experimental Procedures.

inhibitor of the catalyzed reaction (12). These results, together with structural similarity between TNS and substrates for the catalyzed reaction, support the suggestion that TNS reports on the active site environment. Therefore, the TNS results extend the conclusion that the active site of rhodanese is apolar and that the E and ES forms are different. This is particularly interesting since TNS is an amphiphilic probe while the octyl sepharose is more purely apolar and change interactions have been implicated in modulating access to the active site (7,16).

X-ray crystallographic studies of rhodanese show regions which can be responsible for apolar interactions (8,9). The single polypeptide chain which constitutes rhodanese is folded into two domains connected by a flexible strand of polypeptide chain. The active site is in the interdomain region. Cysteine 247 which provides the active site sulfhydryl group is located at the bottom of a pocket in a turn between a β strand and a helix. Side chains from both domains form the walls of the pocket and these in turn are largely segregated

into apolar and hydrophilic regions. These regions apparently provide the cationic and hydrophobic areas suggested from solution studies (3). In fact, there is a continuum of hydrophobic interactions in going from the first domain to the second and ~ 900Å$^2$ of contact area are buried when the two domains are brought together, thus making apolar interactions major stabilizing influences in maintaining the interdomain contacts. Since these regions constitute the major areas of potentially accessible apolarity, it is tempting to speculate that the slow rates of binding are related to changes in the interactions between the domains and that the details of ligand association are modulated by their charge character. This in fact would correlate with the observation that in catalysis rhodanese must utilize a strongly ionic substrate and transfer a sulfur atom to form a persulfide intermediate that is inherently unstable in aqueous solution (3).

In sum, then, rhodanese can bind to macromolecular apolarity and this binding is different for the E and ES forms of the enzyme. This result supports other solution studies that indicate a conformational change occurs in the catalytic event. Changes in the apolar accessibility may also relate to the fact that rhodanese can be found associated in non-covalent complexes with iron-sulfur proteins in crude homogenates and can associate with iron-sulfur proteins in in vitro studies. The fact that both the exposure of apolarity and the ability of rhodanese to self associate depend in the same way on the form of the enzyme tested may indicate that apolar interactions can contribute to the observed macromolecular associations. Studies are underway to directly test the implications of these possibilities.

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