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Human Liver Rhodanese. Nonlinear Kinetic Behavior in a Double Displacement Mechanism†

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ABSTRACT: Rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1) has been purified from human liver tissue to apparent monodispersity. Comparison of this enzyme with that isolated from bovine liver showed differences in specific activity, ultraviolet absorption, and kinetic behavior which indicate that the two proteins are not identical. The substrate activation behavior shown by the human but not by the bovine rhodanese with cyanide as sulfur-acceptor substrate has been analyzed in terms of the formation of an isomerizing substituted enzyme.

Many (Wong and Hanes, 1962; Fisher and Hoagland, 1968) but not all (Cleland, 1970) branched single displacement formal mechanisms[1] yield nonlinear double reciprocal plots. In contrast, the only nonlinearity usual in plots for double displacement formal mechanisms has been that resulting from the competitive substrate inhibition characteristic of this form. The rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1) of human liver, however, is a double displacement enzyme that shows substrate activation behavior with cyanide as sulfur-acceptor substrate. Purification of the human enzyme now has provided an opportunity to compare it with the better known bovine rhodanese and to examine the causes of its nonlinear kinetic behavior.

Experimental Procedures

Materials. Normal human liver tissue was obtained from the morgue of Billings Hospital at the University of Chicago and then cubed and frozen. Sodium ethanethiosulfonate was synthesized as described previously (Westley and Heyse, 1971); calcium phosphate for chromatography was prepared by the method of Anacker and Stoy (1958). Sephadex G-100 and DEAE-Sephadex A-50 were purchased from Pharmacia. Distilled water was deionized before use. All other materials used were of the best analytical grades commercially available.

Enzyme Assay. Rhodanese activity was measured as the rate of thiocyanate production in the colorimetric system of Wang and Volini (1968). The units of activity used in this report are defined as micromoles of thiocyanate produced per minute in this assay system at 25°.

Protein Determinations. Biuret methods for macro (Gornall *et al.,* 1949) and micro (Zamenhof, 1957) scale work and absorbance measurements at 280 nm were all used in estimating protein concentrations, as indicated in the Results section.

Purification of Human Liver Rhodanese. Rhodanese was prepared from extracts of human liver tissue by fractionation with ammonium sulfate followed by chromatography on successive columns of DEAE-Sephadex A-50, Sephadex G-100, and calcium phosphate. Table I is a summary of the data from a purification by this procedure. For additional information describing this experiment see the Supplementary Material Available paragraph at the end of the paper.

Steady-State Kinetics. Kinetic assays were carried out at 0° in Tris-acetate buffer (pH 8.5) containing glycine at 1.4 M, ionic strength 0.5, as described previously (Westley and Heyse, 1971). Initial rate data were plotted in double reciprocal form and also processed with a Hewlett-Packard 2000 C digital computer using a BASIC program[2] for least-squares fitting to a hyperbolic function assuming equal variance for the velocities. The same computer was used to generate theoretical curves corresponding to rate equations for postulated mechanisms.

Results

Characterization of Purified Human Liver Rhodanese. All of the protein in final preparations migrated as a single band on polyacrylamide disc gel electrophoresis at pH 8.3. The ultraviolet absorbance at 280 nm of solutions containing 1 mg of protein (biuret method) per ml in cells with a 1.00-cm light path was 1.5 ± 0.1. In contrast, bovine liver rhodanese had a

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[1] Also known as "sequential" mechanisms; double displacement mechanisms are sometimes referred to as "ping-pong."

[2] Written in this laboratory by Dr. S. R. Burstein.

TABLE I: Purification of Human Liver Rhodanese.

Step		Rhodanese Act. (IU)	Protein (mg)	Sp Act. (IU/mg)	Deg of Purification	Yield (Overall) (%)
1. Combined extracts from 750 g of tissue		242,820	68,400[a]	3.55		100%
2. 2.5 M ammonium sulfate precipitate (redissolved and dialyzed for chromatography)		209,450	36,875[a]	5.68	1.6-fold	86
3. Eluate from DEAE-Sephadex A-50 chromatography[c]	Form A	65,320	1,260[b]	51.8	14.6-fold	27
	Mixture	14,910	477			6
	Form B	50,410	504	100	17.6-fold	21
		130,640	2,241			54
4. Eluate from Sephadex G-100 chromatography (form A)		41,180	44.7[d]	921	259-fold	17[e]
5. Eluate from calcium phosphate chromatography (form A)		24,253	19.5[d]	1243	350-fold	10[e]

[a] Determined by macrobiuret method. [b] Calculated from A_{280} measurement, assuming A_{280}/mg = 1. [c] Activity is eluted in two peaks, designated A and B. Only the rhodanese in peak A has been purified beyond this step. [d] Calculated from A_{280} measurement, using A_{280}/mg = 1.5 for human rhodanese. [e] Yield of form A only.

corresponding absorbance of 1.75 ± 0.04, in agreement with the value (1.75) reported by Sörbo (1953).

The specific activity of the homogeneous human enzyme is 1.24 international units (IU)/μg of biuret protein; the corresponding value for the bovine enzyme is 0.71 IU/μg.

Human liver rhodanese and bovine liver rhodanese were found to have identical elution volumes on Sephadex G-100 chromatography. It is likely, therefore, that the two proteins are very similar in molecular weight, although extensive physical studies on the human protein have not yet been carried out.

Kinetic Studies. Figure 1 shows double reciprocal plots and appropriate secondary plots for the steady-state behavior of human liver rhodanese with thiosulfate as sulfur-donor substrate and cyanide ion as sulfur acceptor. Figure 2 illustrates the cyanide activation nonlinearity obtained with the human enzyme when thiosulfate is the sulfur donor, in contrast to the familiar cyanide inhibition nonlinearity obtained with the same rhodanese when ethanethiosulfonate is the sulfur donor. The bovine liver rhodanese gives no cyanide activation nonlinearities with either sulfur-donor substrate under identical experimental conditions (Westley and Heyse, 1971; Westley, 1972).

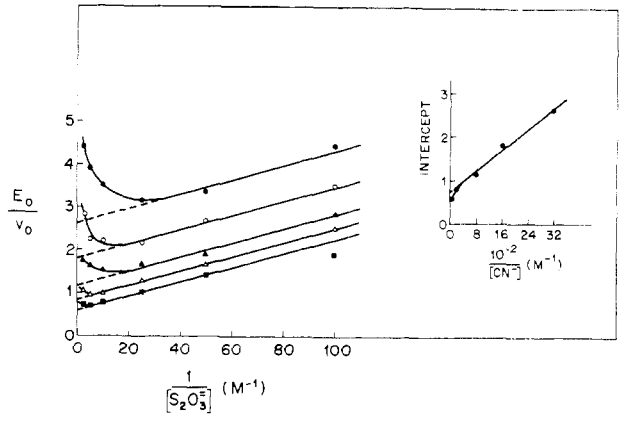


FIGURE 1: Kinetics of the thiosulfate-cyanide reaction catalyzed by human liver rhodanese. Cyanide concentrations: (●) 3.125 × 10^{-4} M; (○) 6.25 × 10^{-4} M; (▲) 1.25 × 10^{-3} M; (△) 5.0 × 10^{-3} M; (■) 2.0 × 10^{-2} M.

The rate constants in Table II were evaluated by analysis of the steady-state data from directly comparable experiments with the human and bovine enzymes. All experimentally derived values were computed from appropriately extrapolated secondary plots of the slopes or intercepts of primary data plots.

Discussion

The data reported here show that the human and bovine rhodaneses differ considerably. The lower ultraviolet absorbance and higher specific catalytic activity of the enzyme isolated from human liver suggest differences in primary structure, although the molecular weights appear to be the same. In addition, steady-state kinetic studies have demonstrated some differences in formal mechanism.

Initial velocity patterns for human rhodanese with thiosulfate and cyanide as substrates (Figure 1) are generally characteristic of the double displacement type of formal mechanism. The secondary plots of intercepts have nonzero intercepts, indi-

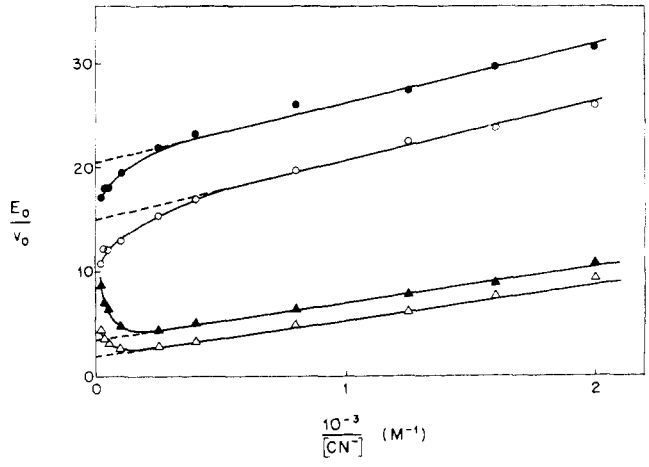


FIGURE 2: Double reciprocal cyanide plots for human liver rhodanese, showing substrate activation when thiosulfate is the sulfur donor and substrate inhibition when ethanethiosulfonate is the donor. Donor concentrations: (●) 0.01 M thiosulfate; (○) 0.02 M thiosulfate; (▲) 5 × 10^{-4} M ethanethiosulfonate; (△) 1 × 10^{-3} M ethanethiosulfonate.

TABLE II: Comparison of Kinetic Constants for Thiosulfate–Cyanide Reactions of Human and Bovine Liver Rhodaneses.

Constant[a]	Human Rhodanese[b]	Bovine Rhodanese[b]
k_{+2}	167 ± 5 sec^{-1}	50 ± 1 sec^{-1}
k_{+2}'	333 ± 15 sec^{-1}	---
k_{+3}	(9.5 ± 1) × 10^4 M^{-1} sec^{-1}	(17 ± 1) × 10^4 M^{-1} sec^{-1}
$K_m^{SSO_3^{2-}}$	(4.5 ± 1) × 10^{-2} M	(2.8 ± 0.15) × 10^{-2} M

[a] Constants are numbered according to the formal mechanisms in Figures 3 and 4. [b] The confidence limits given are standard error spans.

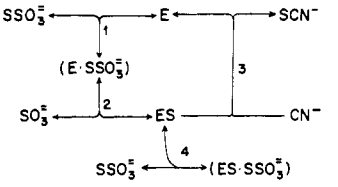
cating that, as in the case of the bovine enzyme, there is at least one kinetically significant unimolecular step in the mechanism. Typical competitive substrate inhibition is observed for thiosulfate, causing curvature of the primary thiosulfate plots near the ordinate and slope change in the primary cyanide plots.

However, there is also present cyanide activation behavior (Figure 2), which has not been seen with the bovine enzyme for any combination of substrates and is not seen with the human enzyme where the sulfur donor is a thiosulfonate rather than thiosulfate. No substrate activation is detectable with either thiosulfate or a thiosulfonate as the varied substrate.

The formal mechanism for the action of bovine liver rhodanese with thiosulfate as sulfur donor was established by direct studies of the sulfur-substituted enzyme (Green and Westley, 1961; Westley and Nakamoto, 1962) and by kinetic studies (Westley and Heyse, 1971; Volini and Westley, 1966; Schlesinger and Westley, 1974). It is shown in Figure 3, with the corresponding initial velocity equation. This formal mechanism appears to be an adequate description of the behavior of both bovine (Westley and Heyse, 1971; Westley, 1972) and human (Figure 2; Jarabak and Westley, 1974) liver rhodaneses with a thiosulfonate as the sulfur donor, with the minor modifications that no kinetically significant binary complexes have been established for the bovine enzyme with thiosulfonate as donor (Westley and Heyse, 1971) and none has been eliminated for the human enzyme with thiosulfonate as donor.[3] What requires explanation is the cyanide activation behavior shown by the human enzyme, but not by the bovine enzyme, in the presence of thiosulfate, but not thiosulfonate, as sulfur donor.

Nonlinear kinetic behavior has not been observed frequently with formal mechanisms of the double displacement type. Only three kinds appear to have been examined explicitly. One of these is the "bridged" double displacement mechanism established by Folk (1969) and Chung and Folk (1972) for transglutaminase, in which the substituted enzyme is subject to spontaneous hydrolysis as well as reaction with the group-acceptor substrate. This mechanism provides an alternate way to regenerate free enzyme without involving reaction of the second substrate. Here, initial velocities obtained by measuring the rate of disappearance of the second substrate (or the rate of appearance of the second product) yield linear, intersecting patterns (Folk, 1969). In contrast, initial velocities obtained by measuring the rate of disappearance of the first substrate (or the rate of appearance of the first product) yield only parallel patterns.

[3] That is, there could be two kinetically significant binary complexes in this case.



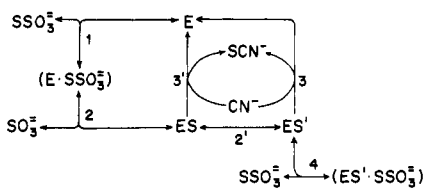
$$\frac{E_0}{v_0} = \frac{k_{-1}+k_{+2}}{k_{+1}k_{+2}[SSO_3^=]} + \frac{1+K_4[SSO_3^=]}{k_{+3}[CN^-]} + \frac{1}{k_{+2}}$$

FIGURE 3: Formal mechanism and corresponding rate equation for the thiosulfate–cyanide reaction catalyzed by bovine liver rhodanese: (E) enzyme; (ES) sulfur-substituted enzyme; (E_0) total enzyme concentration; (v_0) initial velocity.

In this case double reciprocal plots of velocity against concentration of the first substrate are linear; those against concentration of the second substrate are curved, inflecting downward near the ordinate. While the behavior seen in the present work thus superficially resembles that of the bridged double displacement formal mechanism, the latter cannot be applicable here, since initial velocities in this case were evaluated as rates of production of the second product, for which the bridged mechanism yields only linear plots.

A second kind of double displacement formal mechanism known to be capable of yielding nonlinearities of the substrate activation variety is a mixed (or branched) single–double displacement (Westley, 1972; Krenitsky and Papaioannou, 1969). In this form, the binary complex of enzyme with the first substrate can react in two ways: in a unimolecular reaction to yield the substituted enzyme which reacts with the second substrate, and in a bimolecular reaction directly with the second substrate. This case has been analyzed in the context of the rhodanese reaction (Westley, 1972) and it is clear that such mixed single–double displacement character would be expected to be more pronounced with monoanionic than with dianionic sulfur-donor substrates. The present data, however, show cyanide activation behavior only with the doubly charged donor substrate, so that this mechanism also fails to explain the observations.

The remaining kind of double displacement formal mechanism that could yield substrate activation behavior involves conformation-mediated negative interactions between sites on the same enzyme molecule. Such a mechanism has been proposed for the substrate activation behavior observed with alkaline phosphatase at low ionic strength, where there is independent evidence that the enzyme contains two phosphate-binding sites displaying different apparent dissociation constants (Simpson and Vallee, 1970). In the present case, the simpler model proposed below appears to fit all of the evidence now available without invoking multiple sites, but relies instead on



$$\frac{E_0}{v_0} = \frac{k_{-1}+k_{+2}}{k_{+1}k_{+2}[SSO_3^=]} + \frac{k_{-2'}+k_{+2'}(1+K_4[SSO_3^=])+k_{+3}[CN^-]}{(k_{+3'}k_{-2'}+k_{+2'}k_{+3})[CN^-]+k_{+3'}k_{+3}[CN^-]^2} + \frac{1}{k_{+2}}$$

FIGURE 4: Formal mechanism proposed for the thiosulfate–cyanide reaction catalyzed by human liver rhodanese: (ES′) isomerized sulfur-substituted enzyme. Other symbols are as in Figure 3.

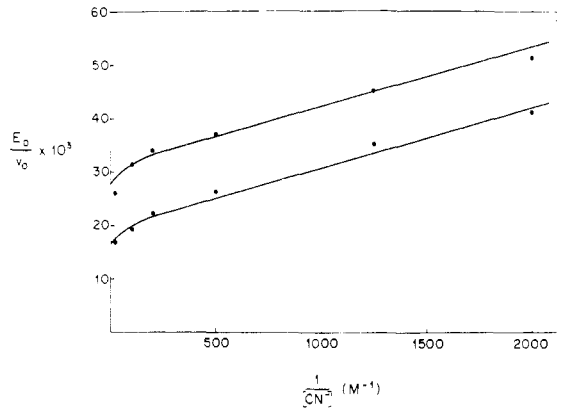


FIGURE 5: Kinetic plot showing experimental data (points) for the thiosulfate–cyanide reaction catalyzed by human liver rhodanese together with computer-generated curves (solid lines) for the rate equation in Figure 4. The constants used to achieve this fit were: $K_1 = 0.046$ M, $k_{+2} = 200$ sec^{-1}, $k_{+3} = 9 \times 10^4$ M^{-1} sec^{-1}, $k_{+2'} = 250$ sec^{-1}, $k_{-2'} = 0$ sec^{-1}, $k_{+3'} = 1.25 \times 10^4$ M^{-1} sec^{-1}. The curvature of the plots does not depend on irreversibility of reaction 2′. Thiosulfate concentrations: upper curve, 0.01 M; lower curve, 0.02 M.

the effects of conformation changes of a kind known to occur in bovine rhodanese (Leininger and Westley, 1968; Volini and Wang, 1973). A model involving negative homotropic interactions for human rhodanese would have to account for substrate activation behavior with cyanide as varied substrate only in the presence of thiosulfate as sulfur donor. Cyanide activation is not observed with other sulfur donors; donor-substrate activation is not observed at all. Generation of such a model would require elaborate assumptions that appear unnecessary in the present state of our knowledge. This is especially true since the model would have to involve sulfur-enzyme-cyanide complexes, and there is no evidence for the occurrence of kinetically significant complexes with cyanide for either the human or the bovine rhodanese.

The formal mechanism proposed here for the action of human liver rhodanese with thiosulfate and cyanide as substrates is shown in Figure 4. The feature of this mechanism yielding the nonlinearity in cyanide double reciprocal plots is the isomerizing sulfur-substituted enzyme, both forms of which can react with cyanide to yield free rhodanese and thiocyanate.

The rate equation corresponding to this formal mechanism is also given in Figure 4. Approximate values for the various rate constants obtained from the kinetic data were inserted into this equation in computer generation of theoretical curves corresponding to the proposed formal mechanism. The lines in Figure 5 are such theoretical curves; the points shown are corresponding experimental data.

It is clear from this analysis that an isomerizing substituted enzyme could be the cause of the kinetic nonlinearity in the double displacement mechanism of the human liver rhodanese. What is required is two forms of the substituted enzyme that can react with the second substrate at different rates and a rate constant for isomerization in the same order of magnitude as that for the rate-limiting step in the catalytic cycle at maximal velocity. Some additional evidence supports the view that this is a correct assignment. One relevant fact is the substantial conformational mobility of rhodanese suggested by earlier kinetic work (Leininger and Westley, 1968) and elegantly established by the recent direct studies of Volini and Wang (1973). Another, detailed in an accompanying paper (Jarabak and Westley, 1974), is the finding that the sulfur-substituted form of the human liver rhodanese, as well as that of the bovine enzyme, differs in reactivity depending on the nature of the donor substrate from which it received its sulfur.

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Supplementary Material Available

Supplementary material on the enzyme purification will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 24× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th St., N.W., Washington, D. C. 20036. Remit check or money order for $3.00 for photocopy or $2.00 for microfiche, referring to code number BIO-74-3233.

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