Mixed Alternate Substrate Kinetics. A Technique for Distinguishing Independent-Site from Same-Site Catalytic Activity†

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ABSTRACT: In enzymes that function by double displacement mechanisms, it can be determined unambiguously whether different group-donor or group-acceptor substrates act at the same enzymic site or at independent sites, by the use of mixed substrates in steady-state kinetic experiments. At the same time it can be established whether two donor substrates form identical or kinetically distinguishable substituted enzymes. The method is applied to reactions catalyzed by rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1). The sulfur-donor substrates thiosulfate and ethanethiosulfonate utilize the same site in this enzyme but produce different sulfur-substituted enzymes.

Unusual kinetic effects involving alternate substrates in enzymic reactions often have several possible interpretations. Even in mechanisms of the double displacement form, the basis for such effects cannot be unambiguous kinetically unless it can be shown whether or not the alternate substrates utilize the same active site. Steady-state kinetic experiments with a mixture of the alternate substrates can be used to establish this point.

The mechanism of action of rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1) provides an application for this type of analysis. Recent studies with rhodanese have indicated that the sulfur-substituted enzymes obtained with thiosulfate and ethanethiosulfonate as donor substrates are kinetically distinct for both the human and bovine liver enzymes (Jarabak and Westley, 1974b). Assignment of a detailed mechanism, however, depends on showing whether the alternate sulfur-donor substrates act at the same active site.

Experimental Procedure

Bovine liver rhodanese was prepared by the method of Horowitz and DeToma (1970). Human liver rhodanese was isolated as described previously (Jarabak and Westley, 1974a). Preparation of the sodium ethanethiosulfonate used also was described previously (Westley and Heyse, 1971). The other materials used were the best commercial grades, and solutions were made with deionized water.

Kinetic assays were conducted at 0° in a Tris-glycine buffer system as described previously (Jarabak and Westley, 1974a,b; Westley and Heyse, 1971). Measured velocities were linear with time and with enzyme concentration. Data were plotted in double reciprocal form and subjected to statistical analysis with a digital computer program providing an iterative fit to the best hyperbola, assuming the error function to yield a homogeneous envelope, as described previously (Jarabak and Westley, 1974a,b). Rate equations for the formal mechanisms considered were derived by the procedure of King and Altman (1956).

Results and Discussion

Theoretical Analysis. A comparison of different group-donor substrates in a double displacement mechanism[1] must be based on one of the three general forms given in Figures 1–3. Inclusion of some or all of the possible binary enzyme–substrate complexes in these mechanisms does not change the forms of rate equations that apply in the presence of both group-donor substrates (A and B) and a single acceptor substrate (C). The symmetry of these forms implies the applicability of an exactly similar set of considerations for alternate acceptor substrates.

Figure 1 gives the formal mechanism usually assumed when two alternate donor substrates are used. The catalytic action is at a common active site and the same substituted enzyme is formed from both donor substrates. If initial velocity is followed by measuring the rate of appearance of product Z, inverse velocity in the presence of A, B, and C will be nonlinear in $[A]^{-1}$ or $[B]^{-1}$, since these substrates are mutually competitive, but linear in $[C]^{-1}$.

Figure 2 shows the formal mechanism in which reaction of the two donors is at the same site, but kinetically distinguishable substituted enzymes are formed. Here, as in the mechanism of Figure 1, the donor substrates are hyperbolic competitive and the plots of inverse initial velocity against $[C]^{-1}$ are linear. Unlike the Figure 1 mechanism, however, this form requires that the slopes of double reciprocal plots with C as varied substrate show a hyperbolic dependence on the percentage composition of the donor-substrate mixture.

Figure 3 illustrates the formal mechanism in which reaction of the two donor substrates is at independent sites. Substrates A and B are no longer competitive, and all double reciprocal plots of data from systems containing all three substrates are nonlinear. In separate experiments with single donors, the slopes of the double reciprocal acceptor plots depend on the donor used. The fourth permutation of these possibilities, in which donor substrates react at independent sites but produce kinetically indistinguishable substituted enzymes, would exhibit qualitatively identical behavior. That is, there would be no competition between donors, and double reciprocal plots for all substrates would be hyperbolically curved. In this case, however, the slopes of double reciprocal acceptor plots from kinetic

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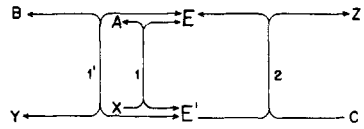
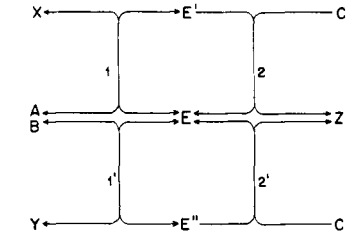
[1] Also called a "ping-pong" mechanism.



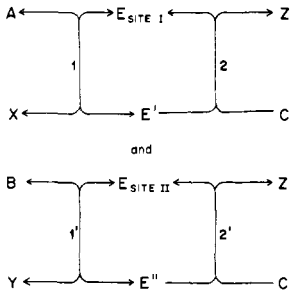
$$\frac{E_0}{v_0} = \frac{1}{k_{+1}A + k_{+1'}B} + \frac{1}{k_{+2}C}$$

FIGURE 1: Formal mechanism and rate equation of alternate donor substrates at the same site to produce the same substituted enzyme: (E) enzyme; (E′) substituted enzyme; (A and B) alternate donor substrates; (X and Y) alternate first products; (C) acceptor substrate; (Z) second product; (v_0) initial velocity based on rate of formation of Z; (E_0) total enzyme concentration. The symbols for the substrates used in the rate equation represent concentrations.



$$\frac{E_0}{v_0} = \frac{1}{k_{+1}A + k_{+1'}B} + \frac{k_{+1}k_{+2'}A + k_{+1'}k_{+2}B}{k_{+2}k_{+2'}(k_{+1}A + k_{+1'}B)C}$$

FIGURE 2: Formal mechanism and rate equation for reaction of alternate donor substrates at the same site to produce kinetically distinct substituted enzymes: (E′) substituted enzyme produced from A; (E″) substituted enzyme produced from B; other symbols as in Figure 1.



$$\frac{E_0}{v_0} = \frac{1}{k_{+1}A} + \frac{1}{k_{+2}C}$$

$$\frac{E_0}{v_{0'}} = \frac{1}{k_{+1'}B} + \frac{1}{k_{+2'}C}$$

$$\frac{E_0}{v_0 + v_{0'}} = \frac{(k_{+1}A + k_{+2}C)(k_{+1'}B + k_{+2'}C)}{\left[k_{+1}k_{+2}A(k_{+1'}B + k_{+2'}C) + k_{+1'}k_{+2'}B(k_{+1}A + k_{+2}C)\right]C}$$

FIGURE 3: Formal mechanism and rate equation for reaction of alternate donor substrates at independent sites. Symbols are as in Figures 1 and 2.

experiments with the two donors separately would be the same.

In addition to their qualitative use in suggesting a formal mechanism, data obtained in mixed donor substrate experiments can be compared with computer-generated curves to test whether the rate equation for the suggested mechanism is capable of yielding the observed behavior quantitatively. Values for the Michaelis and rate constants, approximating those determined in separate experiments with the single donor substrates, are inserted into the rate equation for computational simulation of the mixed-substrate data. The applications that follow illustrate this usage.[2]

[2] Besides the uses noted here, other applications of kinetics done with mixed alternate substrates have provided useful distinctions, primarily in single displacement (also called "sequential") mechanisms (Wong and Hanes, 1962; Rudolph and Fromm, 1970; Bradbury and Jakoby, 1971; Olive *et al.*, 1971).

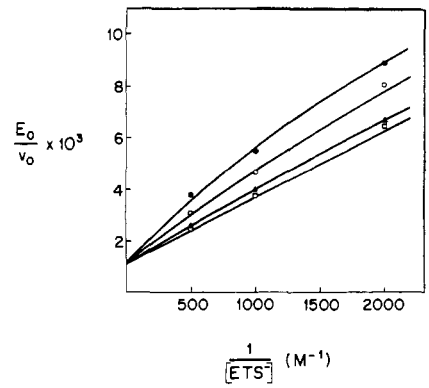
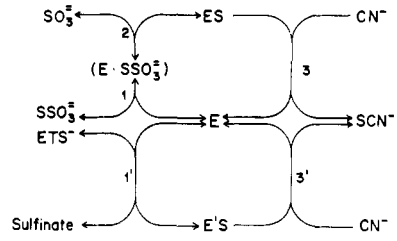


FIGURE 4: Initial velocity pattern for ethanethiosulfonate (ETS^-) as sulfur-donor substrate for bovine liver rhodanese in the presence of thiosulfate as an alternative donor substrate. Thiosulfate concentrations: (●) 0.04 M; (○) 0.02 M; (▲) 0.005 M; (□) no thiosulfate. Cyanide was present as acceptor substrate at 2.5×10^{-3} M in all reactions. The curves are computer-generated lines based on the rate equation given in Figure 5, with assignment of the following values for constants: $K_m^{SSO_3^{2-}} = (k_{-1} + k_{+2})/k_{+1} = 0.04$ M; $k_{+2} = 70$ sec^{-1}; $k_{+3} = 1.7 \times 10^5$ M^{-1} sec^{-1}; $k+1' = 3.85 \times 10^5$ M^{-1} sec^{-1}; $k_{+3'} = 3.6 \times 10^5$ M^{-1} sec^{-1}.



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

$$+ \left[\frac{k_{+1}k_{+2}k_{+3'}[SSO_3^=] + k_{+1'}k_{+3}(k_{-1} + k_{+2})[ETS^-]}{k_{+1}k_{+2}k_{+3}k_{+3'}[SSO_3^=] + k_{1'}k_{+3}k_{+3'}(k_{-1} + k_{+2})[ETS^-]}\right]\frac{1}{[CN^-]}$$

FIGURE 5: Formal mechanism and rate equation for bovine rhodanese with thiosulfate and ethanethiosulfonate (ETS^-) as mixed donor substrates: (ES) sulfur-substituted enzyme obtained with thiosulfate as donor; (E′S) sulfur-substituted enzyme obtained with ETS^- as donor.

A Simple Case: Bovine Rhodanese. The sulfur-donor substrates inorganic thiosulfate and ethanethiosulfonate may be used with cyanide as acceptor substrate for rhodanese, which functions by a double displacement mechanism (Westley and Heyse, 1971; Green and Westley, 1961; Westley and Nakamoto, 1962). Figure 4 shows steady-state kinetic data obtained by varying the ethanethiosulfonate concentration at several concentrations of thiosulfate in the presence of fixed concentrations of cyanide and bovine liver rhodanese. The data show clearly that thiosulfate, the "slower" donor substrate, competes with ethanthiosulfonate for the catalytic site. The lines in Figure 4 are computer-generated curves based on the rate equation for a "same-site" double displacement formal mechanism with a kinetically significant donor complex for thiosulfate but none for the thiosulfonate or for cyanide, in accord with what is known about these rhodanese-catalyzed reactions (Figure 5). Numerical values assigned to the various rate and Michaelis constants for the computer simulation approximate the values obtained experimentally for the reactions with these donor substrates separately (Jarabak and Westley, 1974a,b).

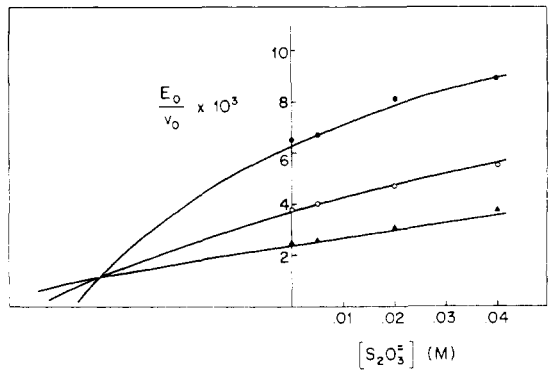


FIGURE 6: Dixon plot of mixed donor substrate data for bovine rhodanese with thiosulfate as donor inhibitor. Ethanethiosulfonate concentrations: (●) 5×10^{-4} M; (○) 10^{-3} M; (▲) 2×10^{-3} M. The curves are computer-generated lines based on the rate equation given in Figure 5 and assignment of values for constants as in Figure 4.

The results given in Figure 4 conform to those expected for a mechanism in which the two donor substrates react at the same enzymic site; all models involving independent sites for the two substrates are eliminated from consideration.

Studies of this kind can also be treated in terms of Dixon plots (Dixon, 1953), where the "slower" substrate is treated as a nonlinear competitive inhibitor. Figure 6 presents the bovine rhodanese data plotted in this way. The lines are again computer-generated curves based on the equation given in Figure 5, with the same numerical values for constants as before. The point of intersection of the theoretical curves is near the negative K_m value for thiosulfate as a substrate in this system, as would be expected.

The further distinction that can be made relating to the formal possibilities presented is illustrated with data for bovine rhodanese in Figure 7. The hyperbolic dependence of the inverse cyanide slopes on the percentage composition of the donor substrate mixture shows that the mechanism belongs to the category defined by Figures 2 and 5. A mechanism involving formation of kinetically indistinguishable substituted enzymes would yield only a straight horizontal line in such a plot. The observation of different cyanide slopes with these two donor substrates in separate experiments was the basis for the previous proposal of the enzymic memory phenomenon (Jarabak and Westley, 1974b).

A More Complex Case: Human Rhodanese. Figure 8 gives data from mixed donor substrate experiments with the human

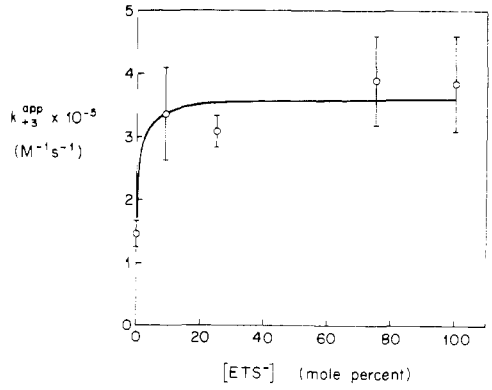


FIGURE 7: Dependence of inverse slopes from double reciprocal cyanide plots on the percentage composition of the mixture of sulfur donors in mixed donor substrate studies with bovine rhodanese. The curve is a computer-generated line based on the rate equation given in Figure 5 with constants as in Figure 4. Vertical bars represent standard error spans of individual slope determinations.

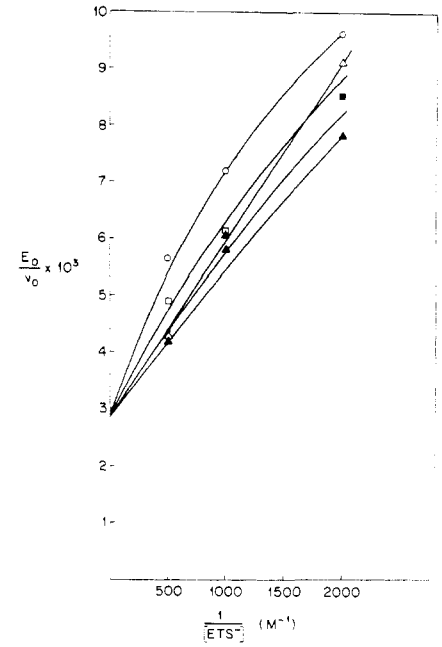


FIGURE 8: Initial velocity pattern for ethanethiosulfonate (ETS^-) as sulfur-donor substrate for human liver rhodanese in the presence of thiosulfate as second donor substrate. Thiosulfate concentrations: (○) 0.04 M; (□) 0.02 M; (●) 0.01 M; (▲) 0.005 M; (△) no thiosulfate. Cyanide was present as acceptor substrate at 2.5×10^{-3} M in all reactions. The curves are computer-generated lines based on a formal mechanism and rate equation like those in Figure 5, with insertion of a kinetically significant enzyme-thiosulfonate complex and corresponding 2′ reaction step and with assignment of the following values for constants: $K_m^{SSO_3^{2-}} = (k_{-1} + k_{+2})/k_{+1} = 0.036$ M; $k_{+2} = 160$ sec^{-1}; $k_{+3} = 6.5 \times 10^4$ M^{-1} sec^{-1}; $K_m^{ETS^-} = (k_{-1}' + k_{+2}')/k_{+1}' = 1.9 \times 10^{-3}$ M; $k_{+2}' = 850$ sec^{-1}; $k_{+3}' = 2.4 \times 10^5$ M^{-1} sec^{-1}.

liver rhodanese. Here again the data points are shown in relation to lines generated by a computer using a rate equation and kinetic constants based on previous experiments (Jarabak and Westley, 1974a,b) with the donor substrates singly.

These results differ in two ways from the results obtained with the bovine liver rhodanese. First, the rate equation differs because the human enzyme forms kinetically significant complexes with both donor substrates (Jarabak and Westley, 1974b), a change which does not alter the form of any of the plots. Second, the data do not fit all of the computer-generated curves unless it is assumed that the Michaelis constant for ethanethiosulfonate is decreased by about one-third in the presence of thiosulfate. When this alteration is included in the specifications, as it has been in Figure 8, the theoretical curves are an adequate fit to the data points. Although the cause of the change in K_m for one sulfur donor in the presence of the other cannot be assigned definitely on the basis of present information, this behavior, like the cyanide substrate activation and enzymic memory phenomena (Jarabak and Westley, 1974a,b), may relate to unusual conformational mobility in this enzyme. Specifically, these findings suggest that, for the human rhodanese only, some part of the enzymic memory of sulfur-donor substrate may persist all the way around the catalytic cycle to the free enzyme.

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Different Expressions of Cooperativity in the Kinetics of Two Forms of Cytoplasmic Malic Dehydrogenase†

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ABSTRACT: Initial rate kinetics were carried out on two forms of cytoplasmic malic dehydrogenase (S-MDH *a* and *b*). Nonhyperbolic kinetics were observed with NADH and oxaloacetate as substrates using S-MDH *b*, while the double-reciprocal plots for S-MDH *a* showed marked deviations from linearity with NAD^+ and L-malate, and only slight curvature with oxaloacetate as substrate. In all cases, the departure from linearity in the double reciprocal plots was dependent on the concentration of cosubstrate. The two forms of S-MDH had essentially identical values of V_{max} in the direction of NAD^+ reduction, but S-MDH *a* had a maximum rate of NADH oxidation approximately threefold higher than S-MDH *b*. The kinetic behavior of these enzymes cannot be uniquely defined in terms of a given molecular mechanism. However, the kinetics, together with previous physical and structural studies, were consistent with a physiological role for S-MDH in regulating the oxidation of glycolytic NADH.

Metabolic regulation on the enzyme level is generally indicated by the presence of allosteric interactions. The term "allosteric" was initially defined as indirect interactions between distinct binding sites (Monod *et al.*, 1965). Operationally, the phenomena which characterizes an allosteric protein are (1) a change in the apparent affinity, or in the Michaelis constant, with changing fractional saturation of a ligand ("cooperativity"); and (2) a change in the apparent affinity, or in the Michaelis constant, of one ligand by the presence or absence of a second, structurally unrelated, ligand ("allosteric modifier"). Although the nature of the molecular mechanism(s) which generate these phenomena is of considerable interest in understanding the relation of structure and function in proteins, the physiological significance of allosteric behavior is independent of its molecular genesis. The importance of allostery in metabolic regulation was further emphasized by the discovery that enzymes could exist in two chemically distinct, stable forms having different allosteric properties. In several cases, these forms could be shown to be interconvertible by cellular processes involving chemical modification of the proteins (Holzer and Duntze, 1971). In effect, this process of covalent modification adds another element to metabolic control mechanisms, allowing given enzymes to function with or without superimposed allosteric constraints.

In our earlier studies we had demonstrated that cytoplasmic malic dehydrogenase (S-MDH),[1] isolated from beef heart, could exist in two stable enzyme forms (S-MDH *a* and *b*) with different regulatory properties (Cassman and King, 1972; Cassman, 1973; Cassman and Vetterlein, 1974). S-MDH *b* showed cooperative binding and kinetics with respect to NADH, and exhibited allosteric inhibition of NADH binding by fructose 1,6-bisphosphate. S-MDH *a* was catalytically more active, and showed no allosteric interactions with NADH. S-MDH *a* contained 1.3–1.8 mol of trichloroacetic acid precipitable phosphate/mol of enzyme, while S-MDH *b* contained 0.3–0.6 mol of phosphate/mol of enzyme.

In this paper, detailed kinetic studies are reported which indicate that S-MDH *a* and *b* show striking differences in their overall kinetic behavior, and that both forms interact cooperatively with substrates other than NADH. The results are discussed in terms of a proposed role for the two enzyme forms in regulating the mode of reoxidation of glycolytic NADH.

Materials and Methods

Materials

NADH, NAD^+, oxaloacetate, and L-malate were all A grade, from Calbiochem. All other chemicals were standard reagent grade.

Both forms of cytoplasmic malic dehydrogenase were prepared from beef heart by the method of Guha *et al.* (1968). The enzyme preparations were characterized as S-MDH *a* and *b* using the criteria described previously (Cassman and Vetterlein, 1974). Phosphate content appears to be the best quantitative criterion for determining the amount of each enzyme form in a given preparation. Based on analysis of several different preparations, it was assumed that S-MDH *a* has 2 mol of phosphate/mol of enzyme, and S-MDH *b* 0 mol of phosphate/mol of enzyme (Cassman and Vetterlein, 1974). The enzymes used in the work reported here had 1.85 mol of phosphate/mol of S-MDH *a*, and 0.17 mol of phosphate/mol of S-MDH *b*. Be-

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[1] Abbreviation used is: S-MDH, cytoplasmic malic dehydrogenase.