The Mechanism of the Rhodanese-catalyzed Thiosulfate-Lipoate Reaction

KINETIC ANALYSIS*

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SUMMARY

The individual reactions comprising the rhodanese-catalyzed thiosulfate-lipoate reaction were demonstrated kinetically. The mechanism was shown to be a reaction sequence in which the enzyme forms kinetically significant binary complexes with both thiosulfate and dihydrolipoate substrates. Moreover, the first product (sulfite) is discharged, forming a sulfur-substituted enzyme intermediate, before the second substrate (dihydrolipoate) is attached. It was further shown that the mechanism includes mutually competitive inhibition reactions with both substrates.

Extension of the analysis to several pH values permitted determination of the variation in kinetic coefficients with pH. The changes in these values over the pH range of 8 to 11 suggested that the enzyme exists in an association equilibrium between monomeric and dimeric species and that an enzymic sulfhydryl group is involved in lipoate binding.

It was previously shown that rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1) catalyzes the transfer of the outer sulfur atom of thiosulfate to dihydrolipoate by way of an enzyme-sulfur intermediate (2-5).

Enzyme + SSOS⁻ ⇌ enzyme−S + SO₄²⁻

Enzyme−S + dihydrolipoate ⇌ enzyme + lipoate persulfide

Lipoate persulfide → oxidized lipoate + HS⁻

The present work was undertaken to examine this sequence of reactions more closely. The investigation was a kinetic study based on recent developments in the kinetic theory of two-substrate reactions. The results established the existence of six of the seven individual reactions involved in rhodanese catalysis and provided independent evidence that the sequence proceeds by way of an enzyme-sulfur intermediate. The results of this analysis also suggested the participation of a sulfhydryl group in binding the lipoate substrate and led to further experimentation which showed that rhodanese with a molecular weight of 37,000 (6) is a dimer (7).

EXPERIMENTAL PROCEDURE

Enzyme Preparation—Crystalline bovine liver rhodanese was prepared as previously described (6, 8).

Kinetic Assay of Rhodanese-catalyzed Thiosulfate-Lipoate Reaction—Oxidized lipoate has an absorption maximum at 335 nm which is not present in the spectrum of the reduced form. As a result, rates in this reaction system could be measured spectrophotometrically by recording the appearance of oxidized lipoate with time. The measurements were made at 335 nm with a Perkin-Elmer model 202 ultraviolet-visible recording spectrophotometer equipped with a variable scale recorder (9). Fig. 1 shows recordings typical of those from which initial rates were calculated.

Solutions of oxidized lipoate were reduced with sodium borohydride. In a typical experiment, 1.2 g of DL-lipoic acid (obtained from the Aldrich Chemical Company) were dissolved in 25 ml of 0.2 M sodium potassium phosphate, and 400 mg of NaBH₄ were added. After reduction (15 to 30 min), the solution was acidified to the point of precipitation of dihydrolipoic acid (about pH 5.0). The acidification resulted in destruction of the excess BH₄⁻ with consequent formation of borate.¹ The dihydrolipoate solution, now in phosphate-borate buffer, was adjusted to the appropriate pH by addition of either phosphoric acid or NaOH. The solution was diluted to 30 ml and the final pH measured. All operations were carried out with the solution blanketed with nitrogen. Other solutions used in the assay were deaerated by nitrogen flow before addition to the assay mixture. A diluent buffer was made under conditions identical with those used for preparation of the dihydrolipoate stock solution and adjusted to the same final pH. The substrate sodium thiosulfate was dissolved in water.

The final buffer concentrations in all assay mixtures were: phosphate, 0.40 M; borate, 0.24 M; and glycine, 0.033 M. The

¹ Since BH₄⁻ is itself a substrate for rhodanese (2), the lipoate solutions were routinely examined in a polarograph before use to ensure the absence of residual BH₄⁻.
FIG. 1. Typical records obtained in the rhodanese-catalyzed thiosulfate-lipoate assay. Enzyme was added at zero time and the shutter was opened at S. The distance between the heavier lines on the abscissa represents a span of 30 sec.

total reaction volume was 3.0 ml. In all experiments, the reaction volume consisted of 2.0 ml of lipoate solution (or lipoate solution plus diluent buffer), 0.9 ml of thiosulfate solution (or thiosulfate solution plus water), and 0.1 ml of enzyme solution containing 90 μg of crystalline rhodanese dissolved in 1.0 M glycine. Where enzyme concentrations differed slightly, rates have been corrected to this concentration to facilitate comparison. Initial velocities were proportional to enzyme concentration over the range utilized in this work. Stock enzyme solutions were stable during the time periods required for individual experiments. When tested at seven intervals over 6 hours, one preparation gave the same value in the lipoate system with a standard deviation of 2.5%.

Assays were carried out in quartz cuvettes with a 1.0-cm light path. The concentration of oxidized lipoate was calculated with a molar absorptivity coefficient of $1.2 \times 10^5$ M$^{-1}$ cm$^{-1}$ as determined in the course of this work.

The experimental cuvette was blanketed with nitrogen during the addition of the assay mixture. The zero slope of the records (Fig. 1) prior to the addition of enzyme showed that the rate of autoxidation of dihydrolipoate during assay was negligible. The small amount of autoxidation which resulted from the addition of the enzyme solution (which could not be deaerated) was measured by adding the same volume of 1.0 M glycine to the test mixture. In most cases, as in Fig. 1, the increase in absorbance due to autoxidation (0.005 absorbance unit in Fig. 1) was equal to the decrease in absorbance resulting from dilution of the oxidized lipoate already present.

The presence of a small amount of oxidized lipoate in all assay mixtures was unavoidable. The amount in most cases was less than 2% of the total lipoate concentration and never exceeded 7%. Substrate concentrations of dihydrolipoate were calculated by subtracting the amount of oxidized lipoate present at zero time from the total lipoate concentration added. Time course studies showed that this small amount of oxidized lipoate had no effect on the rates obtained. This observation was not surprising in view of evidence presented previously that lipoate persulfide is an intermediate product in the reaction (3). The same studies also showed that the stereospecificity for lipoate previously suggested (2) does not hold. Under suitable conditions, complete utilization of the racemic lipoate substrate was observed. More generally, the reaction velocity was directly proportional to total d,l-lipoate concentration at least to the point of 45% utilization. Under the conditions of the earlier studies, the reaction was limited by competitive inhibition by thiosulfate with respect to lipoate, a feature of the rhodanese reaction not known at that time.

Kinetic Assay of Rhodanese-catalyzed Thiosulfate-Cyanide Reaction—Cyanide inhibition was demonstrated by a spectrophotometric method which measures thiosulfate disappearance at 260.5 nm (10). For this assay, the reaction volume was 3.0 ml containing a 10-fold range of cyanide concentrations from 0.033 M to 0.0033 M, 0.0067 M sodium thiosulfate, and 0.010 mg of rhodanese. The reaction was carried out in a cell with a 1.0-cm light path in the Perkin-Elmer spectrophotometer described previously.

Protein Determination—Protein concentrations were measured by a modified biuret method (11).

Determination of $pK'$ of First Sulfhydryl Ionization in Dihydrolipoate—Two samples, the first containing 0.28 mmole of dihydrolipoate and 0.51 mmole of borate in a 2.7-ml volume and the second containing 0.51 mmole of borate alone in a 2.9-ml volume, were titrated with 2.50 M sodium hydroxide with the use of a Leeds-Northrup model 7401 pH meter equipped with micro-
The samples were back-titrated with 2.92 M hydrochloric acid. Titration of the sample containing dihydrolipoate was carried out in a cuvette with a 1.0-cm light path blanketed with nitrogen. This procedure allowed the sample to be examined before and after titration for the presence of oxidized lipoate. The change in oxidized lipoate concentration in the course of titration was negligible. The pK' value determined after subtracting the borate control was 9.9. This value is necessarily approximate since ionization of the second sulfhydryl group of dihydrolipoate begins before the first is completely ionized. The pK' of the second sulfhydryl group was too high to measure in this system but it was estimated to be near 11.6.

RESULTS

Mechanism of Rhodanese-catalyzed Thiosulfate-Lipoate Reaction—The mechanism was deduced primarily by analysis of initial rate measurements made at a series of dihydrolipoate concentrations for each of several thiosulfate concentrations. Experiments of this type were performed at six pH values between 8 and 11. The data obtained were diagnostic for the mechanism of the lipoate reaction; they also permitted calculation of the kinetic coefficients.

The over-all reaction of thiosulfate with dihydrolipoate is shown in Fig. 2a. The detailed mechanism (Fig. 2b) and its corresponding initial rate equation, derived by the steady state treatment (Fig. 2c), are also presented. It will be shown that the experimental results correlate with the characteristic features of this equation and, as a consequence, with its corresponding mechanism. The question as to the uniqueness of this assignment will be dealt with under “Discussion.”

The mechanism shows that the enzyme combines with thiosulfate to form a binary complex (E—SSO3) which breaks down to sulfite and a sulfur-substituted enzyme (ES). The substituted enzyme combines with dihydrolipoate to form a second binary complex (ES—lipoate) which breaks down to oxidized lipoate, hydrosulfide, and free enzyme by way of a lipoate persulfide intermediate (lipoate—S). Evidence for the persulfide intermediate has been presented previously (3). However, time course analyses showed that, even at the highest rates obtained in the present experiments, breakdown of the persulfide was never rate-limiting in this system; for purposes of the present discussion, breakdown of the ES—lipoate complex to oxidized lipoate, hydrosulfide, and free enzyme can be considered as a single step.

The mechanism also includes inhibition reactions with both substrates. This type of inhibition differs from conventional substrate inhibition in that it does not result from mutual interference between 2 molecules of the same substrate but from competitive interference between molecules of the two different substrates.

At pH values between 8 and 9.3, thiosulfate inhibition predominated. At higher pH values dihydrolipoate inhibition was dominant. These observations were explained on the basis that the active species of dihydrolipoate is that which has one sulfhydryl group ionized. Titration studies gave a value of 9.9 for this pK'. Thus, at pH values between 8 and 9.3, the active species was present in low concentration and thiosulfate inhibition predominated. At higher pH values, at which the concentration of the active species was high, dihydrolipoate inhibition was dominant. Supporting evidence for this interpretation comes from the variation of kinetic coefficients with pH (see below).

In Fig. 3 are shown double reciprocal plots which illustrate the inhibition observed with thiosulfate at the lower pH values and that observed with dihydrolipoate at higher pH values. The series of plots of 1/v against 1/[SSO3] was derived from initial rate measurements made at pH 8.65. Each plot is for a different

2 It was also found that cyanide inhibits rhodanese at pH 10 in the thiosulfate-cyanide assay. The initial rate observed at 0.033 M cyanide was two-thirds of that at 0.007 M cyanide. No such inhibition has been observed for thiosulfate in this system.
Fig. 3. Double reciprocal plots showing the substrate inhibition observed with thiosulfate and dihydrolipoate. Left, at pH 8.65 each thiosulfate plot is for a different lipoate concentration; the concentration increases to its highest value in the lowest plot. □, 18 mM; ○, 31 mM; △, 62 mM; ×, 93 mM; ●, 125 mM. Right, at pH 10.1, each dihydrolipoate plot is for a different thiosulfate concentration; the concentration increases to its highest value in the lowest plot. 1/[lipoate], reciprocal of the dihydrolipoate concentration. □, 2.5 mM; ○, 5.0 mM; △, 7.5 mM; ×, 12.5 mM; ●, 25 mM.

Fig. 4. Double reciprocal plots of 1/v against 1/[lipoate] at pH 8.05 and pH 8.65. Each plot is for a different thiosulfate concentration; the concentration increases as the slopes of the plots increase. 1/[lipoate], reciprocal of the dihydrolipoate concentration. At pH 8.05: ○, 5.6 mM; □, 8.3 mM; ●, 12.5 mM; △, 37.5 mM. At pH 8.65: ○, 2.8 mM; □, 5.6 mM; ○, 8.3 mM; ×, 12.5 mM; ●, 25 mM. The initial rate equation corresponding to these plots includes only the thiosulfate inhibition term.

\[
\frac{1}{v} = \frac{1}{E_0} \left[ \frac{\left( k_{-4} + k_{+4} \right)}{k_{-3}k_{+4}} \right] \left[ \frac{1}{(lipoate)} + \frac{1}{\left( \frac{1}{k_{+3}} + \frac{1}{k_{-3}} \right)} \left( \frac{k_{-4} + k_{+4}}{k_{-3}k_{+4}} \right) \right] (SSO_3^-)
\]

dihydrolipoate concentration. As expected for competitive inhibition, the inhibitory effect decreased as the dihydrolipoate concentration increased. The series of plots of 1/v against 1/[lipoate] was derived from data obtained at pH 10.1. In this case, each plot is for a different thiosulfate concentration. The inhibitory effect decreased as the thiosulfate concentration increased. This observation is, again, the expected result for competitive inhibition.

Under conditions in which there was inhibition by only one substrate, the general rate equation reduced to simpler forms. In Fig. 4, the rate equation which includes only the thiosulfate inhibition term is presented with two series of 1/v against 1/[lipoate] plots obtained at pH 8.05 and 8.65, respectively.

1/ [lipoate] refers to the reciprocal of the dihydrolipoate concentration.

4 The plots in this series have not been corrected to the standard
FIG. 5. Secondary plots of the intercept and slope values obtained from the double reciprocal plots shown in Fig. 4. pH 8.05; pH 8.65. As indicated by the equation accompanying Fig. 4, the intercept values are

\[
\frac{1}{E_0} \left( \frac{1}{k_{-3} + 1/k_{+3}} + \frac{1}{k_{-5} + 1/k_{+5}} \right) \left( \frac{1}{SSO_3^-} \right)
\]

The slope values are

\[
\frac{1}{E_0} \left( \frac{k_{-4} + 1/k_{+4}}{k_{-2}k_{+4}} + \frac{1}{E_0} \left( \frac{k_{-6} + 1/k_{+6}}{k_{-4}k_{+6}} \right) \right) \left( \frac{1}{SSO_3^-} \right)
\]

The rate equation, arranged in slope-intercept form, has three characteristic features. It specifies that (a) primary plots of \(1/v\) against \(1/\text{lipoate}\) should be linear; (b) the slope of the primary plots should be a linear function of the thiosulfate concentration, approaching a minimum value other than zero as the thiosulfate concentration approaches zero; and (c) the intercept of the primary plots should be a linear function of the reciprocal thiosulfate concentration, approaching a minimum value other than zero as the reciprocal thiosulfate concentration approaches zero. Thus, the data satisfy all three features of the rate equation for the case in which thiosulfate inhibition was observed.

A similar analysis for the case in which lipoate inhibition was observed is shown in Figs. 6 and 7. In Fig. 6, the rate equation which includes only the lipoate inhibition term is presented with two series of plots of \(1/v\) against \(1/\text{SSO}_3^-\) obtained at pH 10.13 and 11.0, respectively.

The rate equation specifies that (a) the primary plots of \(1/v\) against \(1/\text{SSO}_3^-\) should be linear; (b) the slope of the primary plots should be a linear function of the dihydrolipoate concentration, approaching a minimum value other than zero as the dihydrolipoate concentration approaches zero; and (c) the intercept of the primary plots should be a linear function of the reciprocal dihydrolipoate concentration, approaching a minimum value other than zero as the reciprocal dihydrolipoate concentration approaches zero. The primary plots (Fig. 6), as well as the secondary plots of the slope and intercept values obtained from these primary plots (Fig. 7), are linear. The slope and intercept functions approach minimum values other than zero as the dihydrolipoate or reciprocal dihydrolipoate concentration approaches zero. Thus, the data also satisfy the three features of the rate equation for the case in which lipoate inhibition was dominant.

The correlation of the experimental data with these features of the rate equation classifies the mechanism as a linear reaction sequence, belonging to the substituted enzyme class, in which there is competitive inhibition by both substrates. It further allows the conclusion that there is at least one binary enzyme-substrate complex formed. According to the theoretical treatments of Wong and Hanes (12) and of Dalziel (13), this excludes all but two kinds of mechanisms from final consideration. These mechanisms differ only in whether they contain one or two binary complexes.

One means of demonstrating the existence of an enzyme-substrate complex is the study of the effect of substrate analogues on the maximum velocity (\(V\)) of the reaction. \(V\) reflects only the rate constants of breakdown of the enzyme-substrate complexes; i.e., \(V\) must reflect a monomolecular event. If the value of \(V\) differs for alternate substrates (the second substrate in a two-substrate reaction being held constant), then it can be concluded that the alternate substrate which gives the lower value of \(V\) must form a complex and that breakdown of this complex is not zero as the reciprocal thiosulfate concentration approaches zero.

The linearity of the primary plots in Fig. 4 satisfies the first feature of the rate equation. Secondary plots of the slope and intercept values obtained from these same primary plots are shown in Fig. 5. For both pH values the slope is a linear function of the thiosulfate concentration and the intercept is a linear function of the reciprocal thiosulfate concentration. Both the slope and intercept functions approach minimum values other than zero as the thiosulfate, or reciprocal thiosulfate, concentration approaches zero. Thus, the data satisfy all three features of the rate equation for the case in which thiosulfate inhibition was observed.

The series at pH 8.65 was derived from the same data as those plots shown for this pH in Fig. 3.

The ordinate intercept of each primary plot of \(1/v\) against \(1/\text{substrate}\) is equal to the reciprocal of the velocity at saturation in the variable substrate for a particular concentration of the constant substrate. Extrapolation of the secondary plot of these intercept values to infinite concentration of the constant substrate (Figs. 7 and 9) gives the reciprocal velocity at saturation in both substrates (\(1/V\)). If no binary complex were formed, the value of \(1/V\) would be zero.

\[4\] The series at pH 10.1 was derived from the same data as those plots shown for this pH in Fig. 3.

\[7\] The ordinate intercept of each primary plot of \(1/v\) against \(1/\text{substrate}\) is equal to the reciprocal of the velocity at saturation in the variable substrate for a particular concentration of the constant substrate. Extrapolation of the secondary plot of these intercept values to infinite concentration of the constant substrate (Figs. 7 and 9) gives the reciprocal velocity at saturation in both substrates (\(1/V\)). If no binary complex were formed, the value of \(1/V\) would be zero.
FIG. 6. Double reciprocal plots of $1/v$ against $1/(SSO_3^-)$ at pH 10.1 and pH 11.0. Each plot is for a different dihydrolipoate concentration; the concentration increases as the slopes of the plots increase. In both plots: O, 31 mM; A, 62 mM; X, 94 mM; ●, 125 mM. The initial rate equation corresponding to those plots includes only the dihydrolipoate inhibition term,

$$
\frac{1}{v} = \frac{1}{E_0} \left[ \frac{k_{-1} + k_{+2}}{k_{-1} k_{+2}} + \frac{k_{-1} + k_{+2}}{k_{-1} k_{+2}} \frac{k_{+3}}{k_{-3}} \text{(lipoate)} \right]
$$

rate-limiting at maximum velocity. The substrate which gives the higher value for $V$ may or may not form a complex. These conclusions hold even in the presence of competitive inhibition by substrate, since competitive inhibition does not alter the maximum velocity.

Evidence that the rhodanese-catalyzed thiosulfate-lipoate reaction contains two binary complexes comes, in part, from studies with the thiosulfate-cyanide reaction. The existence of an enzyme-thiosulfate complex was previously shown kinetically by the use of alternate substrates for thiosulfate, with cyanide as the constant second substrate (14). It was shown that breakdown of this complex is rate-limiting at pH 8.6. At this same pH, a lower value of $V$ is observed with dihydrolipoate than with cyanide (in this case, with thiosulfate as the constant substrate). This observation permits the conclusion that a binary complex is also formed with dihydrolipoate.

This analysis constitutes a final identification of the form of the mechanism of the rhodanese-catalyzed thiosulfate-lipoate reaction as that shown in Fig. 2b.

Calculation of Kinetic Coefficients—The kinetic coefficients of the reaction were calculated from the slopes and intercepts of the secondary plots. Since unequivocal identification of the form of the mechanism was made, the coefficients could be defined in terms of actual rate constants. Fig. 8 shows how the coefficients relate to the rate constants for the rate equation which includes the thiosulfate inhibition term.

The value $1/V$ (Fig. 8, 3a) and the ratio $K_{m(SSO_3^-)/k_{+2}}$ (Fig. 8, 3b) were calculated from the intercept and slope, respectively, of the secondary plot of the intercept function (Fig. 5). The ratio $K_{mliposte}/k_{+4}$ (Fig. 8, 3c) was calculated from the intercept of the secondary plot of the slope function (Fig. 5). The inhibitor constant $k_{+3}/h_{-4}$ (Fig. 8, 3d) could also be calculated from a ratio of the slope and intercept values obtained from the secondary plot of the slope function (Fig. 5). Calculation of the constants at high pH values, at which the rate equation including the lipoate inhibition term applied, was made by a similar analysis. In this case, the inhibitor constant $k_{+3}/k_{-3}$ for lipoate inhibition could be obtained.

As shown in Fig. 8, 3a

$$
\left[ \frac{1}{k_{+2}} + \frac{1}{k_{+4}} \right] = \frac{1}{E_0} = \frac{1}{V}
$$

The rate constant of breakdown of the enzyme-thiosulfate complex, $k_{+3}$, was calculated from the maximum velocity of the thiosulfate-cyanide reaction. This value, in turn, permitted calculation of $k_{+4}$, the rate constant of breakdown of the ES-dihydrolipoate complex. The ratios $K_{m(SSO_3^-)/k_{+2}}$ and $K_{mliposte}/k_{+4}$ (Fig. 8, 3b, 3c), when multiplied by the appropriate rate constants, yielded the two $K_m$ values.

Change in Kinetic Coefficients with pH—Since experiments were performed at six pH values between 8 and 11, it was possible to follow the change in the coefficients with pH. Log values of the coefficients were plotted as a function of pH according to the method of Dixon and Webb (15) in Fig. 9. According to Dixon and Webb, the intersection of lines of integral slope indicate approximate $pK'$ values of functional groups important for enzymic activity. The log $k$ plots reflect groups important for breakdown of the enzyme-substrate complexes. The log $k/K_m$ plots reflect groups important for the binding of substrate. The log $k/K_m$ plots reflect both with the exception that if there is an inflection in the same place and in the same direction in both the log $k$ plot and log $k/K_m$ plot, it will cancel in the $K_m$ plot.

It was shown previously that $k_{+2}$ remains constant over the pH range of interest (10). The $k_{+4}$ plot indicates that a group with a $pK'$ of 10.1 is important for the breakdown of the ES-dihydrolipoate complex. (The small wave occurring at about pH 9.0 is discussed below with the $k_{+4}/K_{mliposte}$ plot.) This $pK'$ most likely relates to the ionization of dihydrolipoate in the complex. As mentioned previously, the $pK'$ of the first sulfhydryl group in dihydrolipoate is approximately 9.9. These
FIG. 7. Secondary plots of the intercept and slope values obtained from the double reciprocal plots shown in Fig. 6. •, pH 10.1; O, pH 11.0. As indicated by the equation accompanying Fig. 6, the intercept values are

\[ \frac{1}{E_0} \left( \frac{1}{k_{+4} + k_{-4}} + \frac{k_{-4}}{k_{+4}(k_{+4} + k_{-4})} \right) \]

The slope values are

\[ \frac{1}{E_0} \left( \frac{k_{-3} + k_{+2}}{k_{+2}k_{-2}k_{+4}} + \frac{k_{-3} + k_{+2}}{k_{+2}k_{-2}k_{+4}} \right) \]

The observations are consistent with the fact that lipoate inhibition is observed only at high pH values if the mutually competitive behavior of thiosulfate and lipoate has an ionic basis and only the presence of the sulfhydryl ionized form of the lipoate on the enzyme is significant for exclusion of thiosulfate. The values of \( k_{+4}/k_{-4} \) were 70 at pH 10.1 and 140 at pH 11.0.

The \( k_{+4}/K_{\text{m lipoate}} \) plot indicates that a group with a pK' of 8.9 is important for the binding of lipoate. Since this value is far below the lipoate sulfhydryl pK, it was postulated that this ionization indicates the participation of an enzymic sulfhydryl group. If the ionization of an enzymic sulfhydryl group is important for the binding of lipoate, then the close proximity of this group would be expected to affect the ionization of dihydrolipoate in the complex and would account for the small wave around pH 9.0 observed in the log \( k_{+4} \) plot. Essentially, there would be two forms of the enzyme-sulfur-lipoate complex, with different limiting velocities for breakdown to products. Ionization of this sulfhydryl group may also account for the variation of the inhibitor constant for \( \text{SSO}_3^-/(k_{+4}/k_{-4}) \) with pH. The values of \( k_{+4}/k_{-4} \) were 430 at pH 8.05 and 190 at pH 8.65. In the \( pK_{\text{m lipoate}} \) plot, the small wave in the \( k_{+4} \) plot and the ionization at pH 8.9 in the \( k_{+4}/K_{\text{m lipoate}} \) plot tend to cancel. The inflection at pH 10.1 also probably relates to the ionization of dihydrolipoate in the complex. The proposal that an enzymic sulfhydryl group is involved in lipoate binding is supported by the fact that rhodanese which has been inactivated by exposure to air oxidation can be totally reactivated by dihydrolipoate.

Since \( k_{+4} \) remains constant, the \( pK_{\text{m SSO}_3^-} \) and \( k_{+4}/K_{\text{m SSO}_3^-} \) plots are similar. In both plots, the inflection gives a line with a slope of 2, indicating that two groups with pK' values close to 9 are important in the binding of thiosulfate. These data suggested sulfhydryl groups. However, it seemed highly unlikely that the appearance of two negatively charged groups could increase the affinity for the doubly negatively charged thiosulfate ion in the ordinary sense. It was postulated, instead, that each of these groups is on a different subunit of the enzyme and that ionic repulsion between the groups could result in a dissociation of the enzyme into subunits, each subunit in turn having a greater affinity for the substrate, thiosulfate. This postulate led to the experiments reported previously (7), which showed that rhodanese does exist in a mobile equilibrium between monomeric and dimeric species and that a stable dimer is formed under conditions favorable for sulfhydryl oxidation.

### DISCUSSION

To identify the form of an enzymatic mechanism by kinetic analysis, it is necessary to show that the rate behavior is correlated with a single rate equation, excluding all other possibilities. During the past 10 years, largely through the efforts of Alberty (17), Dalziel (13), Wong and Hanes (12), and Cleland (18), a theoretical treatment of the kinetics of two-substrate reactions has been developed that makes such unambiguous analysis possible. In particular, Wong and Hanes (12) have shown that two-substrate enzymatic mechanisms can be treated exhaustively, with the result that formal mechanisms can be assigned unequivocally in some cases by the evaluation of certain kinetic features.

All two-substrate mechanisms which have substituted enzyme intermediates (also known as double displacement or "ping-pong" mechanisms) yield parallel double reciprocal plots at different concentrations of the constant substrate. It has been shown that rhodanese functions by such a mechanism (2-5) and the kinetic data confirm this observation. Kinetic data also indicate that binary enzyme-substrate complexes are formed with both thiosulfate and lipoate. Furthermore, the kinetic data establish the occurrence of binary "dead end" complexes between the enzyme and both substrates at high concentrations.

It is readily shown that inclusion of competitive inhibition terms describing the formation of the dead-end complexes does not interfere with differentiation between substituted enzyme mechanisms and other linear mechanisms. The following are general double reciprocal equations relating to these two groups of mechanisms when there is competitive inhibition by the substrate \( Y \).
FIG. 8. The relation of the kinetic coefficients (3) to the initial rate equation for thiosulfate inhibition (1) and to its slope and intercept functions (2).

\[
\frac{1}{v} = \frac{1}{E_0} \left( \frac{k_{-3} + k_{+4}}{k_{+3}k_{+4}} \right) \left[ \frac{k_{+2} + k_{+4}}{k_{-2} + k_{+4}} \right] \frac{1}{(SSO_3^2)^{1/4}} + \frac{1}{E_0} \left( \frac{k_{-1} + k_{+2}}{k_{+1}k_{+2}} \right) \frac{1}{(SSO_3)^{1/4}}
\]

FIG. 9. The variation of the kinetic coefficients with pH. The units of \(k_{+2}\) and \(k_{+4}\) are sec\(^{-1}\), of \(K_{mSO_3^-}\) and \(K_{mLipoate}\) are moles per liter, and of \(k_{+2}/K_{mSO_3^-}\) and \(k_{+4}/K_{mLipoate}\) are moles liter\(^{-1}\) sec\(^{-1}\). The invariance of \(K_{mLipoate}\) over the pH range 7.0 to 10.2 was shown previously (10).

\[
\frac{1}{v} = \frac{1}{E_0} \left[ \frac{d}{a} \left( 1 + \frac{Y}{K_i} \right) \right] \frac{1}{X} + \frac{1}{E_0} \left[ \frac{b}{a} + \frac{e}{a} \right]
\]

(1)

\[
\frac{1}{v} = \frac{1}{E_0} \left[ \left( \frac{d}{u} + \frac{e}{a} \right) \left( 1 + \frac{Y}{K_i} \right) \right] \frac{1}{X} + \frac{1}{E_0} \left[ \frac{b}{a} + \frac{e}{a} \right]
\]

(2)

The concentrations of substrates are \(X\) and \(Y\), that of the enzyme \(E_0\); the terms \(a\) through \(e\) are constants in a given reaction system. Equation 1 represents substituted enzyme mechanisms; Equation 2, other linear mechanisms. The inhibitor term in each case is \(1 + Y/K_i\), appearing only in the slope term of the equation, as expected for competitive inhibition. Equation 1 describes a family of lines which, instead of having the same slope as in uncomplicated substituted enzyme mechanisms, have slopes that are an increasing function of \(Y\). For other linear mechanisms (Equation 2) the slopes are a more complex function of \(Y\) described by a curve that proceeds through a minimum value as \(Y\) increases. Thus, even in the presence of competitive inhibition by substrates, substituted enzyme mechanisms can be distinguished from other linear mechanisms.

There are three basic mechanisms of the substituted enzyme type which differ in whether they contain one, two, or no binary complexes. As shown for the rhodanese mechanism, these can be distinguished by the use of substrate analogues. This final distinction serves as the last step in the correlation of the rate behavior of such a system to a single rate equation in which the coefficients are defined in terms of actual rate constants. The complete analysis fulfills the essential requirements for final identification of the formal mechanism.

Completely detailed catalytic mechanisms for enzymes cannot,
of course, be assigned on the basis of kinetic evidence alone. Unequivocal identification of the forms of some mechanisms, however, can be made on the basis of the exhaustive treatment by Wong and Hanes of a model so general as to include all permutations of two-substrate mechanisms, including all those ever suggested in the literature (12). Thus, the present analysis permits the conclusion not only that the data obtained are consistent with the formal mechanism presented in Fig. 2b but also that all other reasonable forms at this level of detail have been eliminated.

The pH-activity data, acquired in this study from the $k/K_m$ plots, implicated the sulphydryl groups in rhodanese activity. The $pK$ values interpreted as belonging to enzymic sulphydryl groups were close to 9.0 in both the $k_{+2}/K_{mSSO_3}$ and $k_{+4}/K_{mlipoate}$ plots. Since borohydride was required for reduction of lipoate, borate, which has a $pK$ of 9.26, was necessarily a component in all reaction mixtures used in this study. The possibility that any of the $pK$ values observed was that of borate was discounted for the following reasons. By use of the thiosulfate-cyanide test system, it has been shown that borate is an innocuous ion for the thiosulfate part of the reaction. Furthermore, the fact that $K_{mSSO_3}$ decreases in the lipoate system with the pH variation that increases the concentration of borate ion excludes the possibility of a general ionic strength effect. It has been shown in the cyanide assay at pH 8.6 that increasing ionic strength increases $K_{mSSO_3}$. Moreover, ionic strength effects can be expected to reach a plateau at high ionic strength values such as those in the lipoate assay system (1.35 to 1.45) so that any effects resulting from the borate ionization would be minimized.

There is also other evidence which indicates the involvement of sulphydryl groups in the enzymic activity. The pH-activity data as well as the appearance of a stable dimer in rhodanese preparations exposed to air are consistent with the interpretation that ionization of sulphydryl groups lying close together in the reversible dimer causes dissociation of the dimer by ionic repulsion (7). The pH-activity data in combination with reactivation studies have also indicated the probable involvement of an enzymic sulhydryl group in the binding of dihydrolipoate. Furthermore, the recent findings (7) that rhodanese binds zinc ion in a stoichiometric amount (approximately 1 ion per monomer) and that reactivation of the enzyme with Zn$^{2+}$ under certain conditions requires a reducing agent also suggest a role for an enzymic sulhydryl group as the binding site for zinc ion.

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