An Expanded Mechanism for Rhodanese Catalysis*

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SUMMARY

The kinetic behavior of rhodanese (thiosulfate: cyanide sulfurtransferase, EC 2.8.1.1) was investigated at pH values from 5.0 to 10.8. The binding of thiosulfate was shown to be dependent upon a pK' of 9.9, with the protonated enzyme binding the substrate more strongly than the deprotonated form. An enzymic nucleophile displaces sulfite to form the sulfur-substituted enzyme. This reaction was shown to be dependent upon a pK' of 6.5, with the protonated form being unreactive. The sulfur-substituted enzyme has three forms which are related by pK' values of 5.9 and 9.4. The second order rate constants for the reaction of these enzymic forms with cyanide ion are 8.9 × 10^10 M⁻¹ s⁻¹, 2 × 10^7 M⁻¹ s⁻¹, and ≤ 10^5 M⁻¹ s⁻¹, as one goes from the most protonated to the least protonated form. The activation parameters for this reaction were studied at pH 8.7. The activation entropy was very small while the activation entropy was large and negative, probably indicating that there is only a transition state between the sulfur-substituted enzyme and the products and that the transition state involves considerable charge neutralization. The free enzyme and the sulfur-substituted enzyme are known to bind numerous anionic species, including substrates, which inhibit the reaction. The pH dependence of this inhibition indicated the importance of the same enzymic forms as the studies of the catalyzed reaction itself. This formal mechanism, combined with previous results, suggests a chemical interpretation on the following basis. (a) The enzyme contains a divalent cationic site which has at least one catalytically active water ligand. (b) Thiosulfate binding displaces one water ligand; the loss of a proton from this water ligand prevents both thiosulfate binding and the reaction with cyanide anion. (c) The enzymic nucleophile which displaces sulfite is a cysteine mercaptolate anion that is activated by protonation; this sulfhydryl may be either a direct ligand to the cationic site or hydrogen bonded to it, possibly through a second water ligand. (d) The sulfur-substituted enzyme has the cysteine persulfide structure as a ligand with the cationic site and this rapidly reacts with an outer sphere complex that the cationic site forms with cyanide. (e) Inhibitory anions displace a water ligand at the cationic site and decrease the enzyme-substrate interaction by mechanisms similar to those of the high pH ionizations of the free enzyme and the sulfur-substituted enzyme. The proposed chemical mechanism is consistent both with the results presented here and with the previous information concerning the enzyme.

Rhodanese (thiosulfate: cyanide sulfurtransferase, EC 2.8.1.1) catalyzes the substitution reaction between thiosulfate and cyanide to form the products thiocyanate and sulfite (1, 2). A double displacement mechanism for rhodanese catalysis has been established (3-6). Thiosulfate forms a complex with rhodanese that produces charge neutralization and polarization of the sulfur-sulfur bond of the thiosulfate (7-9). This complex appears to involve a divalent cationic group (8-11). The sulfite is then displaced by a nucleophilic group on the enzyme in the rate-limiting step of the catalytic cycle (7, 9). Correlation of inactivation and reactivation by reaction with group-specific reagents and isolation of the appropriate derivative amino acids has demonstrated that there is a cysteine essential for enzyme activity (12). The sulphydryl of the essential cysteine is assumed to be the attacking nucleophile in the displacement of the sulfite group. The isolated sulfur-substituted enzyme appears not to be a typical persulfide and there must be a fast unimolecular step to form the stable sulfur-substituted enzyme (13). The sulfur-substituted enzyme reacts rapidly with cyanide ion in a bimolecular step to form thiocyanate and regenerate active enzyme (3, 6). Alternatively the sulfur-substituted enzyme can combine with thiosulfate to form a dead-end complex which will not react with cyanide (5, 6). These data have led to the formal mechanism in Scheme 1. This formal mechanism and the consideration that k₄ is much smaller than k⁻ can permit derivation of the following steady state rate equation for the catalyzed reaction.

![Scheme 1](http://www.jbc.org/)

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Virtually all of the steps in rhodanese catalysis involve ionic species, implying an important role for electrostatic interactions

\[
\frac{E_0}{k} = \frac{k_{-1} - (S_2O_3^{-})^{2-} + \frac{K_4 (S_2O_3^{-})}{k_{+3} (CN^{-})^{2-} + \frac{1}{k_{+2}}}
\]  

in rhodanese catalysis. Changes in the ionization states of the reactants might be expected to produce a marked pH dependence of this catalytic cycle (8. 9). Thiosulfate itself has two ionizations but they should be of no importance in the pH range studied since their pK values are 0.16 and 1.3 (14). This leaves the hydrogen cyanide and enzymic ionizations to produce the pH dependence of the catalysis.

The formal rate equation for rhodanese catalysis (Equation 1) lends itself to a careful steady state investigation. The Michaelis constant for thiosulfate is known to be a true equilibrium constant since \( k_{-1} \) is much larger than \( k_{+3} \) (7, 9). The limiting slope of the cyanide plot equals the inverse second order rate constant for the reaction between the sulfur-substituted enzyme and cyanide since this reaction involves no detectable intermediate at initial velocities (6, 8). Therefore, data reflecting the pH dependence of rhodanese catalysis can be interpreted with reference to specific equilibrium and rate constants in the catalytic mechanism. These data plus what was previously known concerning rhodanese catalysis allow the construction of a chemical mechanism for the action of this enzyme.

METHODS

The chemicals used as buffer components and substrates were all reagent grade materials from J. T. Baker Chemical Co. and were not further purified. Rhodanese was prepared from frozen bovine liver as described by Horowitz and DeToma (15). In all preparations the crystalline suspension of rhodanese had an activity and a protein concentration consistent with purity greater than 95%. These crystals were dissolved to make a stock enzyme solution, the titer of which was determined as described by Wang and Volini (19). Stock solutions were stable for at least a week with less than 10% loss of activity if they were frozen when not being used.

Buffer Preparation for Studying pH Dependence of Initial Velocities—The pH range studied, 5.0 to 10.8, required the use of several buffer systems to insure sufficient buffer capacity at all pH values. The acid component used was acetic acid, phosphoric acid, or potassium dihydrogen phosphate, and the basic component was Tris, ethanolamine, or ethylamine. The buffers were prepared so that all reaction mixtures had the same ionic strength (0.2 M). The buffers were used in measuring the activation parameters of the enzyme-catalyzed reaction were corrected for the temperature dependence of the amine ionization constants. The ionization constants of hydrogen cyanide under the reaction conditions used were determined by the method of Ang (16).

Initial Velocity Measurements—Immersion of the reaction tubes in a thermostated water bath gave temperature control to better than ±0.1°C. The pH of the reaction mixture was tested prior to starting the reaction and occasionally while the reaction was proceeding. The reaction was usually started by the rapid addition of one of the substrates. After a period of time 1.0 ml aliquots of the reaction mixtures were removed and quickly quenched with 0.5 ml of 15% formamidobalde solution. Thio- cyanate accumulation was determined as previously described (9, 11, 17). The initial velocity data are expressed as molar thiocyanate formed per s.

The activation parameters were determined by plotting logarithms of rate constants against the inverse values of absolute temperature and then using the standard equations for the Arrhenius activation energy, entropy of activation, and free energy of activation (18).
Fig. 2. Double reciprocal plots of initial velocities with cyanide as the varied substrate. These are the same data as in Fig. 1 plotted in the form most useful for interpretation when there was significant thiosulfate inhibition. Both types of plots were made for all the reaction conditions studied. The ordinate and abscissa units are $10^{-3}$ s and $M^{-1}$, respectively. The inset is a secondary plot of the intercepts against the reciprocals of the constant thiosulfate concentrations. The thiosulfate concentrations used were: $\bullet$, 0.05 M; $\bigcirc$, 0.01 M; $\triangle$, 0.005 M; and $\square$, 0.0005 M.

Fig. 3. The pH dependence of the rate-determining step at maximal velocity. The intercepts of secondary intercept plots (e.g. inset plots in Figs. 1 and 2) are plotted as their reciprocals in this figure. Those from thiosulfate plots are indicated as solid triangles and those from cyanide plots as closed circles. The data shown are for $25^\circ$ in phosphate buffer of ionic strength 0.2 M. The apparent rate constant $k_{+2}^{\text{app}}$ can be seen to depend upon a $pK'$ of 6.5.

**pH Dependence of Maximal Velocity**—The maximal velocity was obtained from the secondary plots of both the thiosulfate and cyanide intercepts. A comparison of the maximal velocities from these two sources shows complete agreement at pH values greater than 7.50. Because of thiosulfate inhibition in the Tris-phosphate buffer, the thiosulfate intercepts could not be determined below pH 7.50, and the maximal velocities in this pH range were obtained by extrapolation of only the cyanide intercepts to their limiting values. From Equation 1 it is apparent that these extrapolated values are, in fact, $(k_{+2}^{\text{app}})^{-1}$. Fig. 3 is a plot of the logarithm of $k_{+2}^{\text{app}}$ versus pH.

**pH Dependence of Thiosulfate Binding**—Equation 1 indicates that the slopes of the thiosulfate plots are not expected to vary with the cyanide concentration. From pH 8 to 9 this was found to be true, and $k_{-1}/k_{+1}k_{+3}$ was determined directly from the primary plots. At pH values below 8.0 thiosulfate inhibition made it impossible to determine the thiosulfate slopes accurately and the secondary plots of the cyanide intercepts were used to determine $k_{-1}/k_{+1}k_{+3}$, as shown in Fig. 4. At pH values greater than 9.0 cyanide anion inhibition was prominent and the thiosulfate slopes had to be extrapolated to limiting values. By use of these three methods the thiosulfate slopes spanning the pH range 5 to 10.8 were determined.

By dividing the thiosulfate slope terms by the maximal velocities at the same pH values, one can separate pH effects upon $k_{+2}^{\text{app}}$ from the pH effects upon the dissociation constant of the enzyme thiosulfate complex $K_{\text{thp}}$. The pH dependence of the logarithm of $K_{thp}$ is plotted in Fig. 5.

Thiosulfate is also a dead-end inhibitor of the reaction of the sulfur-substituted enzyme with cyanide (Fig. 1). The association constant for thiosulfate with the sulfur-substituted enzyme, $K_4$, was obtained from the secondary plot of the cyanide slope versus the thiosulfate concentration. The pH dependence of thiosulfate binding to the sulfur-substituted enzyme was determined over the range 3 to 10.8. A plot of log $K_4$ against pH was a complex curve qualitatively similar to those obtained for log $k_{+3}$ (Fig. 6), with $pK'$ values of 5.4 and 9.9.

**pH Dependence of Cyanide Reaction with Sulfur-substituted Enzyme**—At pH values above 7.50 it was possible to use substrate concentrations that gave no thiosulfate inhibition to deter-
The pH dependence of $k_{+gP}$. Upper curve, phosphate buffer; lower curve, Tris-acetate buffer. The results were corrected to the concentration of cyanide anion before plotting. $K_{ES}$ and $K_{ESn}$ are the first and second ionizations of the sulfur-substituted enzyme. $k_{+1}, k_{+2}$, and $k_{-1}$, are, respectively, the second order rate constants for the reaction of the doubly protonated, singly protonated, and unprotonated forms of the sulfur-substituted enzyme with cyanide anion.

Fig. 6. The pH dependence of $k_{+gP}$. Upper curve, phosphate buffer; lower curve, Tris-acetate buffer. The results were corrected to the concentration of cyanide anion before plotting. $K_{ES}$ and $K_{ESn}$ are the first and second ionizations of the sulfur-substituted enzyme. $k_{+1}, k_{+2}$, and $k_{-1}$, are, respectively, the second order rate constants for the reaction of the doubly protonated, singly protonated, and unprotonated forms of the sulfur-substituted enzyme with cyanide anion.

mine the cyanide slopes directly. At pH values below this, it was necessary to obtain the limiting cyanide slopes by extrapolating the secondary plots of the cyanide slopes against thiosulfate to the ordinate intercept. From Equation 1 it is evident that the cyanide slope is the reciprocal of $k_{+gP}$, the second order rate constant for the reaction between cyanide and the sulfur-substituted enzyme. Before the pH dependence of $k_{+gP}$ was plotted, the data were corrected on the assumption that the cyanide anion was the only active species of cyanide (see "Discussion"). Plots of $k_{+gP}$ versus pH in phosphate buffer and in Tris-acetate buffer are given in Fig. 6. Where necessary the experimental cyanide slopes determined in Tris-acetate buffer were corrected for thiosulfate inhibition using the $K_{gP}$ determined in the same buffer.

**Temperature Dependence of Some Reaction Constants**—Initial velocities were measured at five temperatures and a family of plots similar to Figs. 1 and 2 was constructed at each temperature. On the basis of Equation 1 as the formal rate equation, $k_{+gP}$ and $k_{+P}$ were computed for each temperature. Fig. 7 contains Arrhenius plots giving the activation parameters for $k_{+2}$ and $k_{+}$. It was necessary to correct the $k_{+2}$ data for the ionization of hydrogen cyanide; the cyanide anion concentrations were computed using the temperature dependence of this pK value determined previously (20, 21).

**Discussion**

**pH Dependence of Rate and Equilibrium Constants**—Inspection of Equation 1 shows that the pH dependence of the thiosulfate slope depends upon the pH dependencies of $K_1$ and $k_{+2}$. The experimental results show that the value of $k_{+2}$ depends upon a single ionization, $pK_{ESSO_3} = 6.5$, which was determined graphically (Fig. 3). This ionization defines two catalytically important forms of the enzyme-thiosulfate complex, an inactive protonated form, $EHSSO_3^-$, and the active unprotonated form, $ESSO_3^-$ (Fig. 8). The value of $K_1$ obtained by multiplying the experimental thiosulfate slopes by $k_{+2}$ also depends on a single ionization, $pK_E = 0.9$ (Fig. 5). This pH dependence requires three forms of the free enzyme, $EH_2$, $EH_1$, and $E$ related by ionization constants as shown in Fig. 8. The flatness of the plot in Fig. 5 in the pH region 5.5 to 8.5 indicates that the pK of 6.5 which affects sulfur-sulfur bond cleavage in the thiosulfate substrate has no effect upon the thiosulfate binding.

The sulfur-substituted enzyme reacts in a bimolecular step ($k_{+gP}$) with cyanide to regenerate the free enzyme and produce thiocyanate. The enzyme could react with hydrogen cyanide, cyanide anion, or both. There is some evidence that it is solely the cyanide anion which reacts. First, the pH dependence of this reaction has a pK that is the same as that for hydrogen cyanide and it shows a decreased rate of reaction at lower pH values. Second, the cyanide anion is well known to be a much stronger nucleophile than the protonated hydrogen cyanide (22). Third, in the uncatalyzed reaction with thiosulfate or with sulfane sulfur in general the reaction has been shown to be exclusively with the cyanide anion (23). Fourth, the sulfur-substituted enzyme binds many anionic species whose uncharged forms show no affinity for the sulfur-substituted enzyme (8, 11). For these reasons cyanide anion was assumed to be the second substrate for the rhodanese-catalyzed reaction, and all rate constants were then corrected for the temperature dependence of the hydrogen cyanide ionization, and the activation parameters were computed.
were calculated on the basis of the cyanide anion concentration.

The pH dependence of $k_{+3}^{app}$ (Fig. 6) is composed of two sigmoid curves that are manifestations of a high pH and a low pH of the sulfur-substituted enzyme. The resulting three ionization states of the sulfur-substituted enzyme, $ESH_2$, $ESH$, and $ES$, must react at different rates with cyanide for the pH values to be detected. The rate of reaction with cyanide increases with the protonation of the sulfur-substituted enzyme so that the reaction of $ESH_2$ approaches rates expected for diffusion-limited reactions (24), while the reaction of $ES$ was not detectable. This model requires a function of rate and ionization constants for the pH dependence of $k_{+3}^{app}$ that has the form of Equation 2. If the experimental data are sufficient, all the constants in Equation 2 can be evaluated.

$$k_{+3}^{app} = \frac{k_{+3} + \frac{k_{+3}}{K_{ESH}} (H^+)}{1 + \frac{(H^+)}{K_{ESH}}}$$  \hspace{1cm} (2)

At low pH values where $k_{+3}^{app}$ becomes large, the high pH ionization is negligible and Equation 2 is simplified (Equation 3). Rearranging some terms and taking the logarithm of both sides yields Equation 4. This form has two limiting values.

$$\log k_{+3}^{app} = \log k_{+3} + \log (1 + \frac{K_{ESH}}{k_{+3}} (H^+))$$  \hspace{1cm} (4)

1. As $H^+$ becomes much larger than $K_{E,SH}$, Equation 4 approaches a low pH limit.

$$\log k_{+3}^{app} = \log k_{+3}$$  \hspace{1cm} (5)

2. As $H^+$ becomes much smaller than $K_{E,SH}$, Equation 4 approaches its high pH limit.

$$\log k_{+3}^{app} = \log k_{+3} + \log (1 + \frac{K_{ESH}}{k_{+3}} (H^+))$$  \hspace{1cm} (6)

Between these limiting values when $k_{+3}^{app} (H^+)/k_{+3} K_{ESH} > 1$ and $(H^+)/K_{ESH} < 1$, Equation 4 assumes a linear form with a unit slope. Extrapolating Equation 7 to its intersection with the limits defined by Equations 5 and 6 yields numerical values for $K_{ESH}$ and $(k_{+3}/k_{-3}) K_{ESH}$, respectively. The remaining rate and equilibrium constants can be estimated by an analogous procedure when $(H^+)/K_{ESH}$. This interpretation was used to estimate the constants of Equation 2 graphically and the values are listed in Fig. 6. These numerical values were used to compute a pH dependence curve for $k_{+3}^{app}$ based upon Equation 2. This curve is plotted as the solid lines in Fig. 6 and is within experimental error an accurate representation of the data.

$$k_{+3}^{app} = \frac{k_{+3} + \frac{k_{+3}}{K_{ESH}} (H^+)}{1 + \frac{(H^+)}{K_{ESH}}}$$  \hspace{1cm} (7)

The pH dependence of $k_{+3}^{app}$ in acetate buffer was thus calculated on the basis of the cyanide anion concentration.

Formal Mechanism—Fig. 8 is the formal mechanism for rhodanese catalysis including all the enzymic species that the pH dependence of the catalysis requires. The derivation of a complete steady state rate equation for such a mechanism would be a forbidding task. By assuming that all proton transfer steps are at equilibrium during steady state catalysis, however, the task becomes manageable. This assumption is justified even though the reaction of the sulfur-substituted enzyme approaches diffusion control at low pH values. The rates of proton transfer reactions for oxygen, nitrogen, and sulfur acids are known to be faster than the rates of other diffusion-controlled reactions by a factor that would make an error due to the equilibrium assumption insignificant (25). In addition, the observed linearity of the cyanide primary plots in the presence of alternate pathways of reaction is likely to occur only if the pathways are connected by equilibrium steps. Fig. 8 can be simplified as shown in Scheme 2 and its rate equation derived using the method of King and Altman as modified by Cha (26). $X$ and $Y$ represent the rapid equilibrium segments of Fig. 8 and the resulting rate law is Equation 8. The slope and intercept terms in Equation 8 correspond to the pH-dependent functions determined experimentally in the preceding sections.

Inhibition by Anions—Although phosphate has not been shown to alter the rhodanese catalysis, many anionic species do inhibit (8, 11). Therefore, the pH dependence of catalysis was also determined in acetate buffers having a constant concentration of acetate anion. It has been shown that acetate and other anionic species complex with the sulfur-substituted enzyme and this complex reacts with cyanide at a reduced rate (11). These anions also inhibit the formation of the enzyme thiosulfate complex competitively (11). Therefore, the formal mechanism must be expanded to include the three forms of the sulfur-substituted enzyme-anion complex and reactions of these complexes to the free enzyme-anion complex plus thiocyanate. These additional steps are shown in Scheme 3.

The rate equation for this expanded formal mechanism also can be derived by Cha's method (26) if the proton transfers and the anion-binding steps are at equilibrium during steady state catalysis. This assumption may not be strictly true for the enzyme forms $ESH_2$ and $ESH_4$, but it should be noted that the experimental values of $k_{+3}^{app}$ in acetate buffer were never very close to the diffusion limit in the pH range studied (Fig. 6) and the role of these enzymic forms must be minor in this study. On the basis of the equilibrium assumption, the complete rate equation in the presence of inhibition anion ($A^-$) is given in Equation 9.

$$k_{+3}^{app} = \frac{k_{+3} + \frac{k_{+3}}{K_{ESH}} (H^+)}{1 + \frac{(H^+)}{K_{ESH}}}$$  \hspace{1cm} (8)

Although the pH dependence of $k_{+3}^{app}$ in acetate buffer is thus
much more complex, it should be qualitatively similar to the results in phosphate buffer and a comparison of the curves in Fig. 6 shows this to be the case. Inspection of the coefficient of (CN\(^-\))\(^2\) in Equation 9 indicates that a geometrical evaluation of the acetate buffer similar to that used for the phosphate buffer data in Fig. 6 should yield slightly smaller numerical values for the pK' values, which indeed it does.

Thiosulfate substrate inhibition can be taken as a special case of an anion forming a complex with the sulfur-substituted enzyme which prevents reaction with cyanide (5, 6, 11). Comparing Equations 1 and 9 gives Equation 10 as an explanation for the pH-dependent thiosulfate inhibition constants. From the pH dependence of K\(_d\) the ionization constants in Equation 10 can be evaluated. The numerical values for K\(_{ES}\) and K\(_{ESH}\) determined in this manner are within experimental error the same as the values obtained from the pH dependence of the reaction of cyanide substrate with the sulfur-substituted enzyme in the same buffer (Fig. 6). This emphasizes the similarity of the electrostatic interactions of the substrates and the anionic inhibitors with enzyme.

Activation Parameters—Because \(k^{300}\) approaches the diffusion-controlled rate at low pH, the activation parameters of this step and of \(k^{300}\) were determined. The results for \(k^{300}\) (Table I) were in accord with previous work (9). The activation enthalpy for \(k^{300}\) was negative but since the data had to be corrected for the temperature dependence of the hydrogen cyanide ionization (20, 21) these values are zero within the probable error of this correction. At a pH of 8.7 it seems that the major portion of the free energy of activation is contributed by the large negative entropy term. As the pH is lowered and the reaction becomes faster, the free energy of activation must be

![Scheme 3](http://www.jbc.org/)

<table>
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<th>Source</th>
<th>(k) (kcal/mole)</th>
<th>(\Delta H^1_{298}) (kcal/mol)</th>
<th>(\Delta S^1_{298}) (cal mole(^{-1}) deg(^{-1}))</th>
<th>(\Delta G^1_{298}) (kcal/mol)</th>
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<td>(k_{E}) (Leininger and Westley (9))</td>
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<td>7.5</td>
<td>-35.3</td>
<td>15.1</td>
</tr>
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<td>(k_{E}) (this work)</td>
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<td>8.66</td>
<td>-20.0</td>
<td>14.7</td>
</tr>
<tr>
<td>(k_{E}) (this work)</td>
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<td>-1.90</td>
<td>-87</td>
<td>9.2</td>
</tr>
</tbody>
</table>
decreasing. Since it is unlikely that the enthalpy of activation becomes more negative at low pH, the entropic barrier to reaction must be decreasing. On the other hand, if on closer examination the activation enthalpy can be shown genuinely to have a small negative value, correction for the viscosity change with temperature will make this a somewhat larger negative number, and it will become necessary to invoke the protein conformational changes recently demonstrated by Volini and Wang (11) to explain these findings.

Chemical Mechanism—The expanded formal mechanism for rhodanese action indicated by the pH dependence of the catalysis suggests the following chemical mechanism for the enzyme-catalyzed reaction. While some parts of this chemical mechanism are frankly speculative, it serves to correlate most of the current information about rhodanese.

Rhodanese is known to bind Zn$^{2+}$ strongly, and it would seem reasonable to propose a transition metal ion as the divalent cationic site, although its functional significance to catalysis has not been shown (10, 11, 27). In Scheme 4 the cationic site is represented by $M^\oplus$ and is shown to have at least two catalytically active ligands. The enzyme-thiosulfate complex forms when thiosulfate displaces one of these ligands. If this ligand is a water molecule, the observed $pK'$ of 9.9 is consistent with ionization constants that have been determined for metals coordinated with water molecules (28). The hydroxyl group present at high pH would be more difficult to displace and thiosulfate binding would decrease as the pH increased. The anion inhibition of thiosulfate binding described by Wang and Volini (11) would be a competitive substitution of the anion for the water ligand. This is analogous to the anion inhibition observed...
with carbonic anhydrase (29). The pK' of 9.9 would disappear in the enzyme-thiosulfate complex since the water ligand is absent in this form.

The lower pK' that is observed in both the free enzyme and the enzyme thiocysteine complex is proposed to be that of the essential cysteine sulfhydryl. Its pK' has been shifted by interaction with the divalent cationic site. If the sulfhydryl group were directly liganded to the cationic site, the pK' probably would be displaced to a much lower value. Therefore in Scheme 4, the sulfhydryl group has been shown hydrogen bonded to a second water ligand of the cationic site. In the enzyme-thiocysteine complex, the mercaptolate group would be the active form of the cysteinyi residue and would displace sulfite from thiocysteine by an S2-like mechanism. The ionization of the cysteinyi sulfhydryl does not have any marked effect upon thiosulfate binding. This is an additional fact which is consistent with the formulation in which the sulfhydryl is not a direct ligand of the cationic site.

Following the displacement of the sulfite from the sulfinyl sulfur, a persulfide intermediate of the enzyme is left, as shown in Scheme 4. This intermediate undergoes two rapid and perhaps concerted reactions. The sulfite is displaced by water and the persulfide becomes the second ligand of M\(\square\) by displacing a water molecule. These reactions are much faster than the attack by the mercaptolate anion, and would not be observed under steady state conditions. However, the reverse reaction with sulfite to form thiocysteine shows a large entropy change associated with complex formation with a cationic group prior to reaction with the sulfinyl sulfur (11). Also, the sulfur-substituted enzyme does not appear to contain a typical persulfide bond (13).

There are known sulfur-rich metal compounds in which a sulfinyl sulfur is liganded to a metal and bonded to another sulfur (30). In Scheme 4 the sulfur-substituted intermediate is shown as having the sulfone sulfur formed in complex with the divalent cation, and the other ligand to the cationic site is water. This form of the enzyme has a pK' of 0.4, which is represented as an ionization of the water ligand similar to that in the free enzyme. The acid pK' of the sulfur-substituted enzyme, 5.9, is much higher than would be expected for a persulfide, but this is consistent with its occurrence as a sulfur-metal complex. Therefore, the structure and ionizations of the sulfur-substituted enzyme intermediate are proposed as in Scheme 4.

There are at least two possible mechanisms which could explain the pH dependence of the reaction of cyanide anion with the sulfur-substituted enzyme. The most obvious would have cyanide displacing the remaining water ligand at the cationic site to form an inner sphere complex much as thiosulfate does in \(k_{+1}\). Several experimental facts speak against this possibility. In \(k_{+1}\) ionizations other than that of the water being displaced had little effect upon thiosulfate binding, whereas both of the observed ionizations in the sulfur-substituted enzyme have strong effects upon \(k_{+1}\). The diffusion-limited nature of \(k_{+1}\) and the lack of experimental evidence for a kinetically significant enzyme-substrate intermediate under initial velocity conditions indicate that this ligand substitution must be very fast, and that further reaction of the complex must be even faster. It may be concluded that the reaction path crosses only one significant energy barrier in passing from reactants to free enzyme and thioate. In addition to this, the low activation enthalpy for \(k_{+1}\) indicates that the bond relationships in the transition state must be very similar to those in the reactants, and the formation of an inner sphere complex seems unlikely.

It appears more reasonable to suppose that the transition state resembles an outer sphere complex of cyanide anion with the enzyme cationic site. The substantial free energy of activation at high pH is mostly entropic (see Table I) and results from charge neutralization in the transition state requiring the rearrangement of the solvent structure plus the contribution from bringing 2 molecules together. The major portion of the activation entropy for \(k_{+1}\) may be caused by electrostatic effects upon solvent structure, and would depend upon the charge and consequently the ionization states of the reactants. Alberty and Hammes (31) have shown that electrostatic interaction can provide a large change in the rate constant under the conditions that are likely to prevail in the hydrophobic active site of rhodanese (11, 32). The loss of 2 protons from the active site between the pH values of 5 and 11 would easily account for the observed 100-fold change in the rate of this reaction. Therefore, Scheme 4 shows the reaction of the sulfur-substituted enzyme with cyanide as having no intermediate species but only a transition state with cyanide in the outer hydration sphere of the cationic site.

The anion inhibition affecting \(k_{+PP}\) can also be explained using the mechanism proposed above. If the water ligand of the sulfur-substituted enzyme was displaced by an acetate anion, this would be electrostatically similar to removing a proton from the active site. The addition of anion to the active site results in a 5- to 6-fold decrease in \(k_{+PP}\), which is significantly less than the 10-fold decrease attendant on the removal of a proton. The decrease in \(k_{+PP}\) should depend upon the extent to which a substitution at \(M\square\) alters its field strength. In such a case the charge density of the proton would dispose one to expect a greater effect from it than from acetate ion. The complete inhibition of \(k_{+PP}\) by the double charged thiosulfate is consistent with this mechanism (11).

Scheme 4 thus appears to represent a qualitatively correct way of interpreting the results of this study. This mechanism does not provide an explicit role for the essential tryptophyl residue (33) or for the conformational changes known to occur during the catalytic cycle (11). However, it does serve to correlate and rationalize the rest of what is known about rhodanese catalysis.

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