3-Mercaptopyruvate Sulfurtransferase: Rapid Equilibrium-Ordered Mechanism with Cyanide as the Acceptor Substrate†

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ABSTRACT: A steady-state kinetic analysis of 3-mercaptopyruvate sulfurtransferase (EC 2.8.1.2) using cyanide as the sulfur-acceptor substrate was performed. Measurement of pyruvate production gave initial velocity patterns and secondary plots characteristic of a rapid equilibrium-ordered sequential mechanism. Initial velocity data obtained by measuring the formation of thiocyanate, which is the other reaction product, revealed a discrepancy between the rates of pyruvate and thiocyanate production; the yield of thiocyanate per unit time was smaller than that of pyruvate for each reaction mixture. This velocity discrepancy, which diminished with approach to cyanide saturation, suggests that sulfur is not discharged from the enzyme as thiocyanate, but as elemental sulfur, and that thiocyanate is formed in a subsequent nonenzymic step. A formal mechanism which has a rapid equilibrium-ordered catalytic cycle and elemental sulfur as one of the initial reaction products is proposed. Computer simulation is used to show that this model is in agreement with all of the kinetic data.

3-Mercaptopyruvate arises in vivo by transamination of cysteine. Subsequent transfer of the sulfur atom to any of various thiophiles, including thiols and inorganic sulfite, yields physiological sulfur at the sulfane level and permits further metabolism of the carbon chain as pyruvate.

Until recently, the reactions catalyzed by 3-mercaptopyruvate sulfurtransferase (EC 2.8.1.2) were thought to proceed as double-displacement mechanisms. This literature has been reviewed by Sörbo (1975) and by Westley (1977). Recently, however, when the bovine kidney enzyme was highly purified and subjected to steady-state kinetic analysis, it was found that the formal mechanism of the reaction with thiols as acceptor substrates is of the sequential type (Jarabak & Westley, 1978).

The present study was undertaken to examine the formal mechanism of sulfur transfer by this enzyme with cyanide as the sulfur-acceptor substrate. In this system the formation of both reaction products, pyruvate and thiocyanate, can be followed.

Experimental Procedures

Materials. Sodium 3-mercaptopyruvate was purchased from ICN Pharmaceuticals. Distilled water was deionized before use. All materials used were of the best grades commercially available.

The 3-mercaptopyruvate sulfurtransferase used for these studies was purified from bovine kidney by the method of Jarabak & Westley (1978). The specific activity of the material obtained by this procedure was 807 μmol of pyruvate per min per mg of protein. A sample of the enzyme solution gave two prominent bands and several faint ones on disc gel electrophoresis at pH 8.3. Clearly, the preparation was not homogeneous; however, in kinetic assays production of thiocyanate and pyruvate was linear with time and with enzyme concentration and there was no evidence for the presence of activities which might interfere with kinetic studies. Moreover, critical features of the kinetic behavior (see Figures 2–6) have been rechecked with an enzyme preparation lacking the major contaminant band; the behavior was unchanged.

Enzyme Assays. 3-Mercaptopyruvate sulfurtransferase activity was assayed routinely by measuring thiocyanate production in a reaction mixture containing 3-mercaptopyruvate and potassium cyanide as substrates or, alternatively, by measuring pyruvate production in a mixture containing 3-mercaptopyruvate and 2-mercaptoethanol. Detailed procedures for these assays have been described previously (Vachek & Wood, 1972; Jarabak & Westley, 1978).

Determination of K_D for the Dissociation of 3-Mercaptopyruvate Cyanohydrin. The equilibrium constant for cyanohydrin dissociation was determined polarographically, using an apparatus assembled in this laboratory. A solution of 3-mercaptopyruvate in 0.225 M 2-methyl-2-amino-1,3-propanediol buffer, pH 9.55, gave a polarogram with a half-wave potential of −1.40 V vs. the saturated calomel electrode. In preliminary experiments the diffusion current, measured at −1.70 V, was shown to be proportional to the 3-mercaptopyruvate concentration.

For determination of K_D in the enzyme assay buffer, increments of 0.25 M KCN solution were added to a 1.0×10^{-3} M solution of 3-mercaptopyruvate in the pH 9.55 buffer at 25 °C. The current–voltage curve of the solution was determined in the polarograph initially and after each addition of KCN, and the concentration of free 3-mercaptopyruvate was determined from the curve. Data obtained in this way were used to make the plot shown in Figure 1. The value of K_D obtained as the slope of this plot was 5.3×10^{-4} M.

Cyanohydrin formation in this system is rapid. All attempts to observe a time course for the establishment of this equilibrium failed because the dead time of the polarographic method is of the order of 10 s.

Kinetic Studies. The simple equilibrium expression for the dissociation of 3-mercaptopyruvate cyanohydrin and the K_D determined polarographically were used to calculate the amounts of 3-mercaptopyruvate and potassium cyanide to be added to achieve the desired concentrations of free 3-mercaptopyruvate and free CN^- in the reaction mixture for each kinetic assay. All assays were done at 30 °C. Each assay tube contained 450 μmol of 2-methyl-2-amino-1,3-propanediol, pH 9.55, and varying amounts of 3-mercaptopyruvate and potassium cyanide in a total volume of 2 mL. The reaction was initiated by the addition of enzyme and quenched with 1.0 mL of 0.5 M $CdCl_2$. After 10 min the sample was centrifuged for 1 min at 3000*g*. One-half milliliter of the su-

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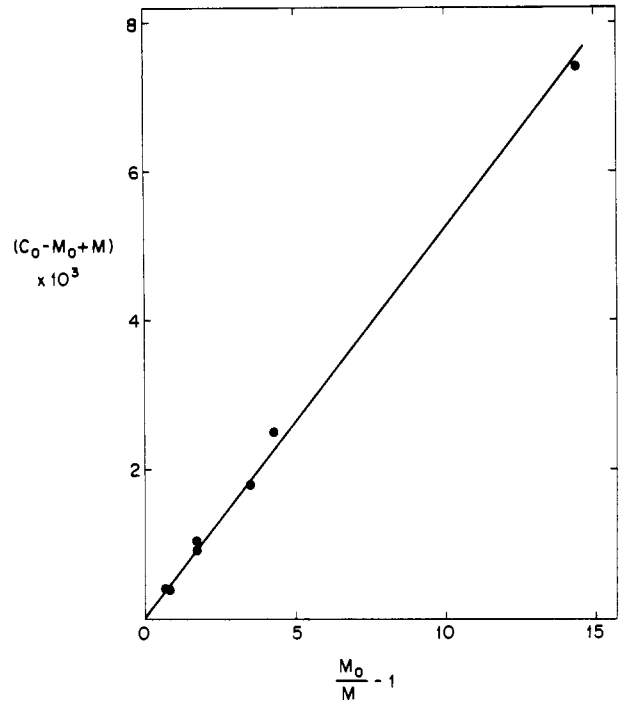


FIGURE 1: Determination of K_D for the cyanohydrin equilibrium. Data are plotted according to the equation $K_D = (C_0 - M_0 + M)/[(M_0/M) - 1]$, where C_0 = initial cyanide concentration, M_0 = initial 3-mercaptopyruvate concentration, and M = concentration of free 3-mercaptopyruvate at equilibrium.

pernatant was used for pyruvate determination and 1.0 mL for thiocyanate determination by the methods described previously (Vachek & Wood, 1972; Jarabak & Westley, 1978). Use of appropriate extinction coefficients and correction for aliquot volumes gave two sets of initial velocity data; this made it possible to compare directly the yields of pyruvate and thiocyanate for each substrate mixture. Velocities, measured by either pyruvate or thiocyanate production, were linear with time and with enzyme concentration over the range of substrate concentrations used.

Analysis of the Data. The concentrations of *free* 3-mercaptopyruvate and *free* cyanide in the various reaction mixtures were used as the respective substrate concentrations for all analyses of the kinetic data.

A Hewlett-Packard 2000 C digital computer and BASIC computer programs were used in the analysis. Initial velocity data were fitted to the best rectangular hyperbola whenever the corresponding double-reciprocal plot was linear. Secondary plots of intercepts and slopes were treated similarly to obtain initial estimates of the kinetic constants to be used in simulation studies.

Finally, the data were matched with the theoretical simulations based on eq 1 and 2 (Discussion), which correspond to the formal mechanism shown in Scheme II (Discussion). The lines presented in Figures 2 and 3 (Results) are the theoretical lines generated by using these equations and a single set of constants adjusted for best overall fit.

Results

Initial velocity patterns obtained for 3-mercaptopyruvate sulfurtransferase when 3-mercaptopyruvate and cyanide were the substrates and when velocity was measured by pyruvate production are shown in Figure 2. The double-reciprocal plots for 3-mercaptopyruvate (Figure 2A) form a linear, intersecting pattern with the point of intersection to the left of the ordinate. The secondary intercept plot is linear with a nonzero intercept;

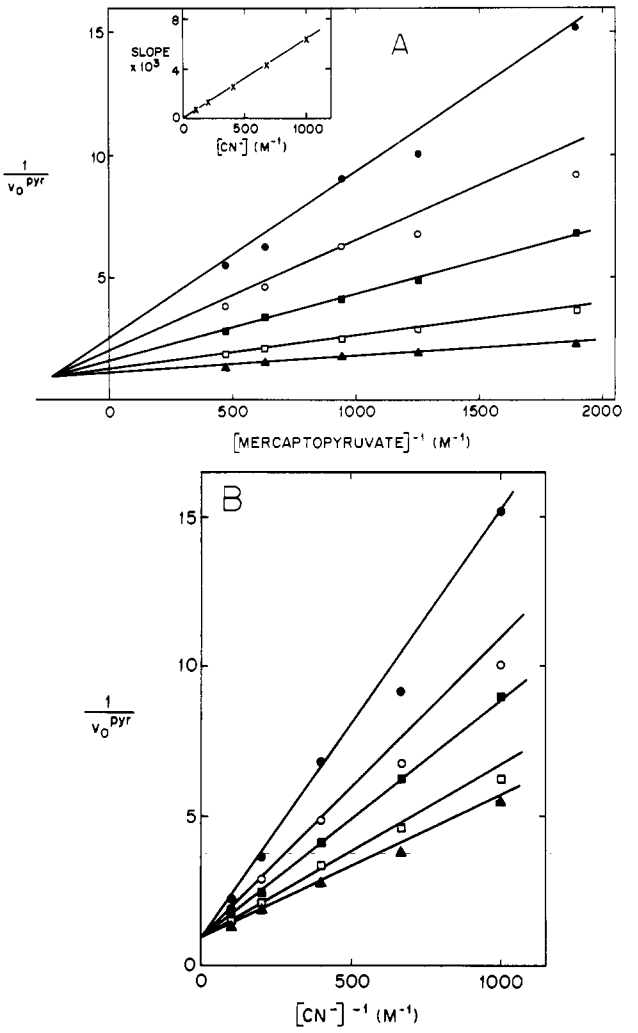


FIGURE 2: Kinetics of 3-mercaptopyruvate sulfurtransferase with 3-mercaptopyruvate and cyanide as the substrates. Initial velocity data were obtained by measuring pyruvate production. The lines were generated by computer simulation using eq 1 and the constants listed under Discussion. (A) Cyanide concentrations: (●) 1.0×10^{-3}; (○) 1.5×10^{-3}; (■) 2.5×10^{-3}; (□) 5.0×10^{-3}; (▲) 1.0×10^{-2} M. Each point on the inset plot represents the slope of one of the lines in the primary plot, where the set of data for that line has been analyzed individually by statistical procedures. (B) 3-Mercaptopyruvate concentrations: (●) 5.3×10^{-4}; (○) 7.95×10^{-4}; (■) 1.06×10^{-3}; (□) 1.59×10^{-3}; (▲) 2.12×10^{-3} M.

the secondary slope plot is linear and intersects the origin. The cyanide double-reciprocal plots (Figure 2B) form a linear, intersecting pattern with the point of intersection on the ordinate. Here the secondary slope plot is linear with a nonzero intercept. The plots shown in Figure 2 suggest a rapid equilibrium-ordered formal mechanism with 3-mercaptopyruvate the first substrate on the enzyme (Frieden, 1976).

When initial velocities for the same substrate mixtures were measured as the rate of thiocyanate production, the primary double-reciprocal plots were not identical with those in Figure 2; these are shown in Figure 3. The 3-mercaptopyruvate plots form a linear intersecting pattern; the cyanide data form a pattern of parabolic curves intersecting on the ordinate.[1]

Comparison of the two sets of double-reciprocal plots reveals a discrepancy between the rates of production of SCN^- and

[1] The conclusion that the plots in Figure 3B are parabolic in form was not reached solely on the basis of the data which appear in this figure. A substantial amount of further experience with this reaction system (some of it included in Figure 4) has shown that the data do describe a parabolic function.

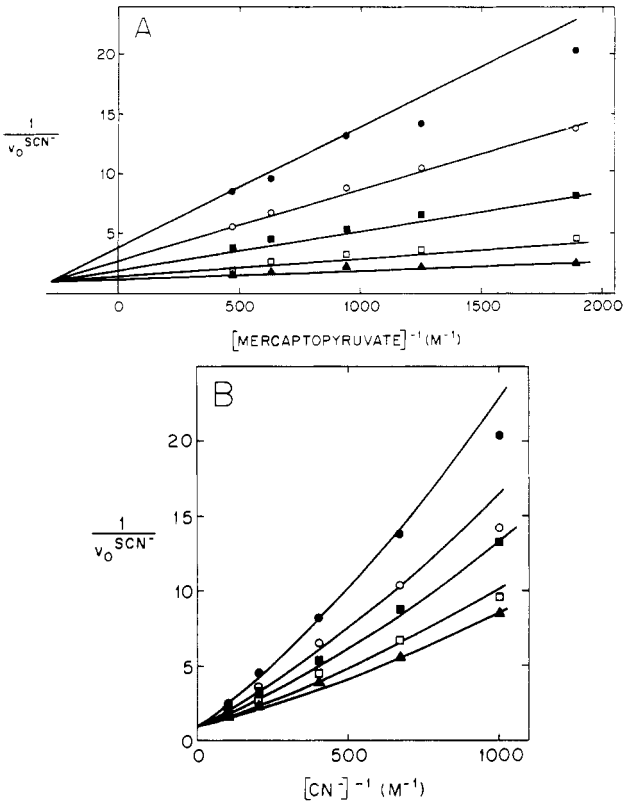


FIGURE 3: (A and B) Kinetics of 3-mercaptopyruvate sulfurtransferase when 3-mercaptopyruvate and cyanide are the substrates. Here initial velocities were measured as the rate of thiocyanate production in aliquots from the same 25 reaction mixtures used for the pyruvate determinations (see Figure 2, parts A and B). The lines were generated by computer simulation using eq 2 and the same set of constants listed under Discussion. The concentrations of 3-mercaptopyruvate and cyanide are, of course, the same as those in Figure 2.

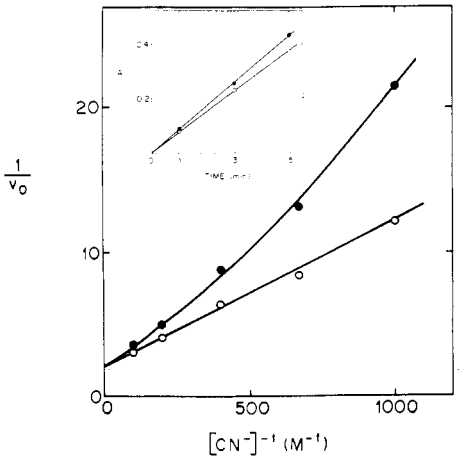


FIGURE 4: Direct comparison of double-reciprocal cyanide plots obtained by measuring pyruvate production (○) and thiocyanate production (●) in aliquots from the same five reaction mixtures. The fixed concentration of 3-mercaptopyruvate was 1.59×10^{-3} M. The inset plot is a time course analysis of the 3-mercaptopyruvate sulfurtransferase reaction. Substrate concentrations were (3-mercaptopyruvate) = 2.12×10^{-3} M and (CN^-) = 5.0×10^{-3} M. (●) Pyruvate assays and (○) thiocyanate assays from the same reaction mixtures.

pyruvate for each reaction mixture. The thiocyanate yield per unit time is smaller than that for pyruvate in all cases.

Several other kinetic plots are useful in describing the nature of the velocity discrepancy. Figure 4 is a double-reciprocal plot comparing the rates of production of pyruvate and thiocyanate directly at several cyanide concentrations. When

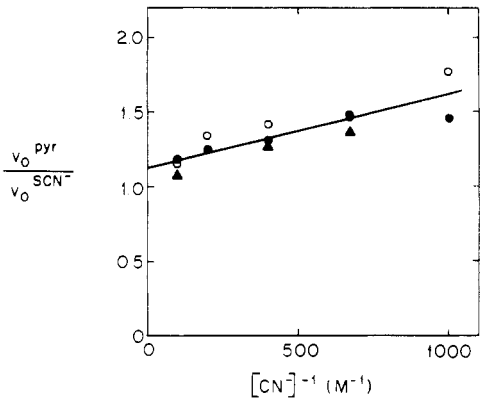
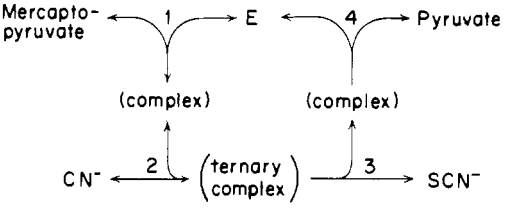


FIGURE 5: Plot of the ratio $v_0^{pyr}/v_0^{SCN^-}$ vs. the reciprocal cyanide concentration. Three different symbols have been used in plotting data from three independent experiments.

Scheme I



initial velocities were measured as the rate of pyruvate production, the plot was linear; when thiocyanate production was measured, the plot was parabolic. The inset figure is a time course plot for the enzyme-catalyzed 3-mercaptopyruvate–cyanide reaction. Measurement of either pyruvate or thiocyanate production gave a straight line through zero time. No time lag could be detected. A third plot (Figure 5) shows the dependence of the velocity discrepancy on cyanide concentration. The ratio $v_0^{pyruvate}/v_0^{SCN^-}$ has been plotted vs. the reciprocal cyanide concentration. The plot is a straight line with the intercept near 1.0; thus, the velocity discrepancy decreases to zero (i.e., $v_0^{pyruvate} = v_0^{SCN^-}$) as the cyanide concentration is increased without limit.

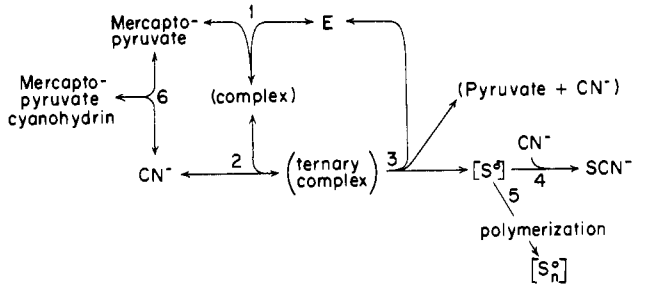
Discussion

A formal mechanism for the reaction catalyzed by 3-mercaptopyruvate sulfurtransferase with 3-mercaptopyruvate and cyanide as the substrates must be consistent with two sets of initial velocity data, one obtained by measuring pyruvate production and the other by following thiocyanate formation. In addition, the formal mechanism must take into proper account the rapid, spontaneous formation of a cyanohydrin equilibrium mixture involving the substrates. Finally, the mechanism should bear an understandable relationship to the formal mechanism previously shown for this enzyme with 3-mercaptopyruvate and 2-mercaptoethanol as the substrate pair (Jarabak & Westley, 1978).

On the basis of the pyruvate data, with the concentration of free 3-mercaptopyruvate and free cyanide used for plotting (Figure 2), the formal mechanism appears to be sequential and of the rapid equilibrium-ordered type, with 3-mercaptopyruvate as the first substrate on the enzyme (Scheme I).[2] The diagnostic features of this form are linear initial velocity patterns intersecting to the left of the ordinate for the first substrate (Figure 2A) and on the ordinate for the second

[2] The order of product release is dictated by the previous demonstration that pyruvate inhibits this enzyme competitively with respect to 3-mercaptopyruvate (Jarabak & Westley, 1978).

Scheme II



(Figure 2B) and linear secondary slope plots, with the one derived from primary plots for the first substrate extrapolating through the origin (Figure 2A inset). None of the other simple formal mechanisms that have been considered in the biochemical literature yields the same combination of features, and the pyruvate data fit this pattern well.[3]

On the other hand, the data obtained by following thiocyanate formation in the same experiments show a somewhat different pattern (Figure 3). Point by point comparison of the data of Figure 2 with those of Figure 3 shows that the rate of production of thiocyanate is smaller than that of pyruvate for each reaction mixture and that the discrepancy is least where the cyanide concentration is highest. Careful reexamination of all analytical procedures involved, including the evaluation of the equilibrium constant for cyanohydrin formation, has shown that they contain no systematic errors of a magnitude or kind that could explain this behavior. These findings make necessary the expansion of the formal mechanism shown in Scheme I.

A number of alternative mechanisms were considered as possible explanations for the kinetic data. Included were various forms involving competing acceptor substrates, the cyanohydrin as substrate or inhibitor, and either a persulfide or free sulfur as the primary sulfur product of the enzymic reaction. The form shown in Scheme II is the only one of these found to be capable of generating all of the kinetic behavior observed. This mechanism retains the rapid equilibrium-ordered catalytic cycle but has elemental sulfur rather than thiocyanate as the primary sulfur product (see below). In this model, partitioning the product sulfur between alternative nonenzymic reactions to form thiocyanate or unreactive colloidal sulfur produces a discrepancy between pyruvate formation and thiocyanate production that diminishes as cyanide concentration is increased, in qualitative accord with the experimental observations.

Equations for v_0^{pyruvate} and $v_0^{\text{SCN}^-}$ for the formal mechanism in Scheme II are[4]

$$\frac{E_0}{v_0^{\text{pyr}}} = \frac{1}{k_{+3}K_2(\text{CN}^-)} + \frac{1}{k_{+3}K_1K_2(\text{3-mercaptopyruvate})(\text{CN}^-)} + \frac{1}{k_{+3}} \quad (1)$$

$$\frac{E_0}{v_0^{\text{SCN}^-}} = \frac{k_{+4}(\text{CN}^-) + k_{+5}}{k_{+4}(\text{CN}^-)}\left[\frac{1}{k_{+3}K_2(\text{CN}^-)} + \frac{1}{k_{+3}K_1K_2(\text{3-mercaptopyruvate})(\text{CN}^-)} + \frac{1}{k_{+3}}\right] \quad (2)$$

[3] The diagnostic features of the rapid equilibrium-ordered form all result from the fact that the binding of the first substrate is at equilibrium practically undisturbed by the steady-state flux; the binding of the second substrate need not be similarly at equilibrium to generate this behavior.

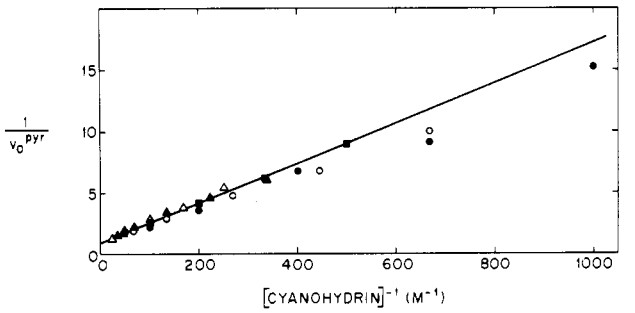


FIGURE 6: Initial velocity data shown in Figure 2 have been replotted here to show the relationship between cyanohydrin concentration and initial velocity as measured by pyruvate production. Concentrations of 3-mercaptopyruvate were (●) 5.3×10^{-4}, (○) 7.95×10^{-4}, (■) 1.06×10^{-3}, (▲) 1.59×10^{-3}, and (△) 2.12×10^{-3} M. As with the other double-reciprocal plots presented here, the line drawn is based on the rectangular hyperbola that best fits the data in uninverted form.

When velocity is defined as the rate of pyruvate production, eq 1 expresses the dependence of v_0^{pyruvate} on substrate concentrations; when velocity is defined as the rate of thiocyanate production, eq 2 applies.

Equations 1 and 2 have been used with the following set of constants to generate the lines shown on all the primary data patterns (Figures 2 and 3): $K_1 = 233$; $K_2 = 605$; $k_{+3} = 1.05$; $k_{+4}/k_{+5} = 2000$. The goodness of fit to the data for the entire set of patterns strongly supports a mechanism of the form shown in Scheme II.

The same conclusion follows from a consideration of the ratio of the experimental velocities. Division of eq 1 by eq 2 yields the following relationship:

$$\frac{v_0^{\text{pyr}}}{v_0^{\text{SCN}^-}} = \frac{k_{+4}(\text{CN}^-) + k_{+5}}{k_{+4}(\text{CN}^-)} = 1 + \frac{k_{+5}}{k_{+4}}\frac{1}{(\text{CN}^-)} \quad (3)$$

That the data are in substantial agreement with this quantitative expression related to the velocity "discrepancy" is shown in Figure 5.

Similarly, the model in Scheme II is in accord with the data on the basis of the relationship between initial velocity and cyanohydrin concentration. The following expression is readily derived from eq 1:

$$\frac{E_0}{v_0^{\text{pyr}}} = \frac{1}{k_{+3}K_1K_2K_D(\text{cyanohydrin})} + \frac{1}{k_{+3}K_2(\text{CN}^-)} + \frac{1}{k_{+3}} \quad (4)$$

K_D is the dissociation constant for reaction 6 of Scheme II; its value was determined polarographically. A double-reciprocal plot of initial velocity data according to this equation is given in Figure 6. As the equation predicts, the plot is linear (coefficient of correlation = 0.992) and independent of the concentration of free 3-mercaptopyruvate.

Other formal mechanisms were considered as possible explanations for the kinetic data, but all failed to be satisfactory. The simplest of these was a sequential mechanism having 3-mercaptopyruvate as sulfur donor and 3-mercaptopyruvate and cyanide ion as competing sulfur acceptors. This mechanism provides for a discrepancy between v_0^{pyruvate} and $v_0^{\text{SCN}^-}$ and reflects the known fact that 3-mercaptopyruvate can serve as an acceptor at sufficiently high concentrations (Jarabak & Westley, 1978). However, the initial velocity patterns for this mechanism do not have the form observed in Figures 2 and 3. Furthermore, the range of 3-mercaptopyruvate concen-

[4] It has been assumed that the enzyme forms and also elemental sulfur are present at steady-state concentration.

trations used in these studies was below that in which the reaction pathway involving two molecules of 3-mercaptopyruvate contributes significantly to the overall velocity.

Mechanisms involving direct participation of 3-mercaptopyruvate cyanohydrin were also considered. It seemed reasonable that the cyanohydrin might be a sulfur-acceptor substrate in this system, since other thiols such as 2-mercaptoethanol are acceptors. The possibility that the cyanohydrin might be the only acceptor substrate was considered. Thiocyanate could then be formed by an uncatalyzed reaction of cyanide ion with the cyanohydrin persulfide product. An alternative mechanism in which the cyanohydrin and cyanide ion were competing sulfur-acceptor substrates was also considered. Neither of these cases yielded rate equations compatible with the data.

All of these considerations indicate that the formal mechanism in Scheme II is the simplest one that is capable of generating the kinetic behavior observed. The form of the initial velocity patterns and also the characteristic features of the velocity discrepancy inherent in this mechanism are in complete qualitative agreement with the data. Finally, the quantitative fit of this mechanism to the data is very good.

The work reported here agrees with the earlier finding that 3-mercaptopyruvate sulfurtransferase functions by a sequential formal mechanism (Jarabak & Westley, 1978). The details of the sequential mechanism differ, however, depending on the nature of the acceptor substrate. When the acceptor is cyanide ion, the mechanism is rapid equilibrium-ordered; when it is 2-mercaptoethanol, the mechanism appears to be random. This difference simply reflects the fact that 2-mercaptoethanol can bind productively to the free enzyme whereas cyanide ion cannot.

That the particular type of sequential mechanism should depend on which acceptor substrate is present is not surprising. There are in the literature several examples of enzymes for which changing the substrate changes some feature(s) of the sequential formal mechanism: liver alcohol dehydrogenase (Dalziel & Dickinson, 1966), bovine liver fructokinase (Raushel & Cleland, 1977), and gentamicin acetyltransferase I (Williams & Northrop, 1978).

This study has provided kinetic evidence suggesting that sulfur is not discharged from the enzyme as thiocyanate but as elemental sulfur. Free sulfur has not been positively identified as the intermediate product, but cyanolysis to form thiocyanate (Foss, 1950; Bartlett & Davis, 1958) and the competing aggregation into colloidal particles largely inaccessible to aqueous reagents are well-known reactions of elemental sulfur. Because of the competing aggregation, the reaction of elemental sulfur with cyanide to give thiocyanate is not quantitative, especially at low cyanide concentrations. This causes the discrepancy between the rates of pyruvate and thiocyanate production first reported by Vachek & Wood (1972) for the enzyme from *Escherichia coli*. The identity of the other initial product(s) is also somewhat uncertain. Formally, these must be equivalent to pyruvate plus cyanide; possibly pyruvate cyanohydrin is the initial product.

The role of cyanide in this mechanism is unusual. Although the kinetic analysis shows clearly that cyanide is required for the production of both pyruvate and thiocyanate, it also rules out the direct enzymic transfer of sulfur from 3-mercaptopyruvate to cyanide. Instead, the evidence suggests that thiocyanate is formed by reaction of elemental sulfur with cyanide nonenzymically. Cyanide ion, in this mechanism, appears to fulfill the functions of both an essential cofactor and a scavenger. The parabolic character of the double-reciprocal plots for thiocyanate production as a function of cyanide concentration is in accord with this double role.

It was concluded previously on the basis of pyruvate measurements only that this enzyme catalyzes the direct transfer of sulfur from 3-mercaptopyruvate to 2-mercaptoethanol to form a persulfide in a conventional sequential mechanism (Jarabak & Westley, 1978). In light of the present study, the possibility must be considered that sulfur transfer to 2-mercaptoethanol might not occur directly on the enzyme. If this acceptor substrate functions as cyanide does, free elemental sulfur would be released as an intermediate in this process. However, it does not appear that this question can be approached experimentally for thiol acceptor substrates, since initial velocity measurements of persulfide product accumulation cannot be made.

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