Sulfite and Complex-bound Cyanide as Sulfur Acceptors for Rhodanese

BO SÖRBO

Research Institute of National Defence, Dept. 1, Sundbyberg 4,
and Medicinska Nobelinstitutet, Biokemiska avdelningen, Stockholm, Sweden

The catalytic effect of rhodanese on the reaction between thio-sulfonates and sulfite has been demonstrated. Rhodanese, combined with thiosulfate, can in part reactivate cyanide inhibited cytochrome oxidase. A similar effect on the cyanide compounds of catalase and methemoglobin was also demonstrated. Vitamin B_{12} (cyanocobalamin) was not converted to the corresponding thiocyanate compound by the action of rhodanese and thiosulfate. A possible function of cobalamins in thiocyanate metabolism is suggested.

Previous studies of the substrate specificity of rhodanese [1,2] have demonstrated that thiosulfonates could replace thiosulfate as sulfur donor in the enzyme catalyzed reaction. Up to this time cyanide was the only known sulfur acceptor in the system. The present paper reports that sulfite can replace cyanide as acceptor; the reaction products being in this case thiosulfate and the corresponding sulfinate. The effect of rhodanese and thiosulfate on certain cyanide complexes of biological importance have also been investigated.

EXPERIMENTAL

Materials. Rhodanese was a twice crystallized preparation from beef liver [3]. The thiosulfonates were prepared as previously described [2]. Catalase was a recrystallized preparation from beef liver and methemoglobin was prepared by oxidizing a rabbit blood hemolysate with ferricyanide, followed by dialysis in order to remove excess ferricyanide. Cytochrome oxidase was a 10 % rabbit brain homogenate in 0.01 M phosphate buffer of pH 7.4. Ferrocytochrome c was a commercial beef heart preparation, reduced with hydrogen in the presence of platinum asbestos.

Methods. The formation of thiosulfate from a mixture of a thiosulfonate and sulfite was followed with the iodometric titration method given by Foss [4]. A correction for "induced oxidation" was obtained by titrating standard amounts of thiosulfate in the presence of the thiosulfonate and sulfite. The disappearance of the cyanide complexes of catalase and methemoglobin was followed spectrophotometrically at 425 and 630 mμ, respectively. Cytochrome oxidase activity was assayed spectrophotometrically by following the oxidation of ferrocytochrome c at 550 mμ[5]. A test for the formation of thiocyanate from vitamin B_{12} was developed on the base of the copper-pyridine-reaction [6]. For this
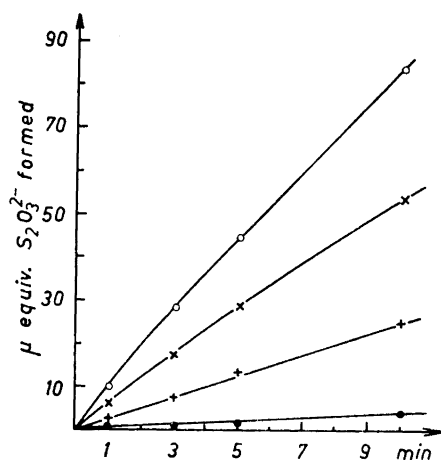
Fig. 1. Reaction between thiosulfonates and sulfite.
● ● ● Ethanethiosulfonate, spontaneous reaction.
○ ○ ○ In the presence of 6.8 μg rhodanese.
+ + + Toluenethiosulfonate, spontaneous reaction.
× × × In the presence of 13.5 μg rhodanese.
  Test conditions: Final volume 5 ml, pH 7.4, phosphate concentration 0.1 M, thiosulfonate and sulfite 0.05 M, bovine serum albumin 0.005 %, temp. 20 °C. Reaction stopped at indicated time by the addition of 0.5 ml formaldehyde followed by 10 ml 10 % KI and 2.5 ml 10 % acetic acid. Titrated with 0.01 N iodine with starch as indicator.

test, 0.5 ml of reaction mixture, containing 0.005 M vitamin B_{12} and 0.01 M thiosulfate in 0.1 M phosphate buffer pH 7.4 was incubated with rhodanese and the enzyme, then destroyed by heating the sample in boiling water for 2 min. The sample was cooled and 0.05 ml 0.1 M KCN was added, in order to liberate thiocyanate from any thiocyanate-cobalamin formed, followed by 4.5 ml water, 1 ml pyridine and 1 ml 10 % $CuSO_4$, $5H_2O$. Any copper-pyridinethiocyanate complex formed was then extracted with 2 + 1 + 1 ml bromobenzene and the optical density of the latter determined at 410 mμ. Vitamin B_{12} is not extracted by bromobenzene, but appropriate blank determinations had to be carried out, as both cyanide and thiosulfate interfered with the determinations. Recovery experiments demonstrated that a conversion of 5 % of the cyanide of vitamin B_{12} could be safely detected by this method.

RESULTS

The rhodanese catalyzed reactions between two thiosulfonates and sulfite and the corresponding spontaneous reactions are shown in Fig. 1. In agreement with Foss [4] it was found that the spontaneous reaction between toluenethiosulfonate and sulfite was more rapid than the corresponding reaction with ethanethiosulfonate. The rhodanese catalyzed reaction was on the contrary more rapid with ethanethiosulfonate. A similar behaviour of the thiosulfonates was previously [2] noticed in their reactions with cyanide. A comparison between the two sets of data demonstrates that sulfite is inferior to cyanide as sulfur acceptor in the rhodanese reaction. With ethanethiosulfonate as
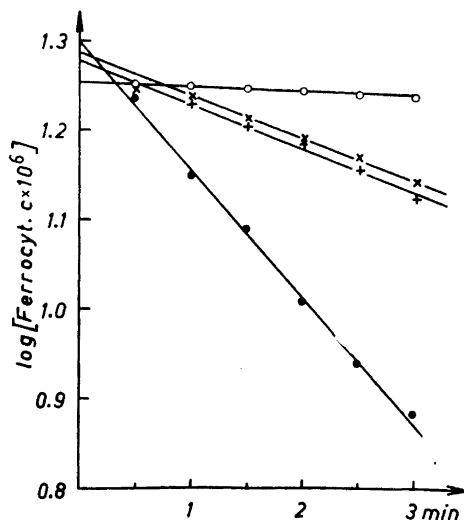
Fig. 2. Reactivation of cyanide inhibited cytochrome oxidase by rhodanese.

●●● Cytochrome oxidase alone ($k = 5.4 \times 10^{-3}$ sec^{-1})

○○○ Cytochrome oxidase + 0.001 M cyanide ($k = 0.18 \times 10^{-3}$sec^{-1}).

× × × Cytochrome oxidase + 0.001 M cyanide + 0.05 M thiosulfate + 36 μg rhodanese/ml added in the indicated order. Tested immediately after the rhodanese addition ($k = 1.8 \times 10^{3-}$ sec^{1-}).

+ + + The same tested 15 min. later ($k = 1.8 \times 10^{-3}$sec^{-1}).

The oxidase activity assayed by adding 0.04 ml of the reaction mixtures to 4.0 ml of ferrocytochrome c (appr. 2 $\times$ 10^{-5} M) and following the reaction at 550 mμ. Figures in brackets indicate the first order reaction constant.

donor and sulfite as acceptor, 1 μg of rhodanese gave 6.4 μ-equiv. of thiosulfate in 5 min, whereas with cyanide as acceptor 10 μ-equiv. of thiocyanate were formed. With *p*-toluenethiosulfonate as donor and sulfite as acceptor 1 μg of rhodanese formed in 5 min 1.1 μ-equiv. of thiosulfate and the corresponding value with cyanide was 3.8 μ-equiv. of thiocyanate. (These values have been corrected for the effect of the spontaneous reaction.)

In connection with a study of the antidote effect of rhodanese in cyanide poisoning it became of interest to study the activity of rhodanese to transform complex-bound cyanide into thiocyanate. As the toxic properties of cyanide is attributed to its strong inhibitory action on cytochrome oxidase, the established antidote effect of rhodanese combined with thiosulfate [7] suggests, that this combination can reactivate cyanide inhibited cytochrome oxidase. Albaum *et al.*[8] have previously demonstrated that another cyanide antidote, methemoglobin, in part reactivates cytochrome oxidase. When rhodanese + thiosulfate was added to cyanide inhibited cytochrome oxidase (Fig. 2), a rapid reactivation was in fact obtained, but it was incomplete. As thiosulfate, thiocyanate and sulfite at the actual concentrations did not affect the oxidase, it must be concluded also that the conversion of cyanide to thiocyanate was
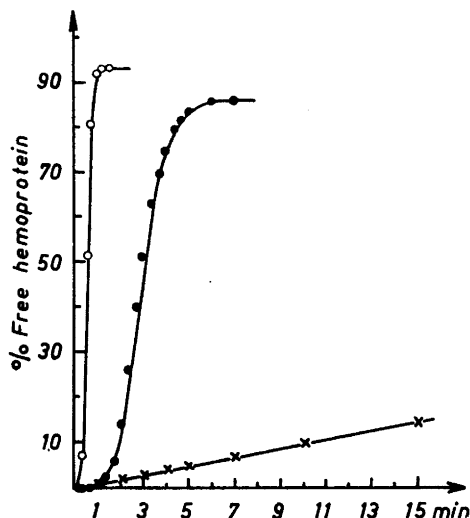
Fig. 3. Action of rhodanese on cyanide compounds of catalase and methemoglobin.

●●● 2.8×10^{-6} M catalase $+ 3.10^{-4}$ M cyanide $+ 0.05$ M thiosulfate in 0.025 M phosphate buffer pH 7.4. At zero time rhodanese (8.6 μg/ml) added.

○○○ As the preceding with 39 μg rhodanese/ml.

✕✕✕ 1.3×10^{-4} M methemoglobin $+ 3 \times 10^{-4}$ M cyanide in 0.025 M phosphate buffer pH 7.4. After 5 minutes incubation thiosulfate (to 0.05 M concentration) and rhodanese (36 μg/ml) were added.

The lag period observed in the two curves with catalase corresponds to the time necessary for converting the excess of cyanide into thiocyanate.

incomplete, although rather high concentrations of rhodanese and thiosulfate were used. Thiosulfate alone had no reactivating effect on the inhibited cytochrome oxidase, as the rhodanese activity of brain homogenates is low. The behaviour of the cyanide complexes of two other heme proteins was then investigated. Catalase cyanide was rapidly reactivated by the action of rhodanese and thiosulfate, whereas methemoglobin cyanide reacted much more slowly (Fig. 3). These results are explained, if rhodanese does not react with the heme-bound cyanide but only with the free cyanide (hydrocyanic acid), which is in equilibrium with the heme-cyanide complex. The dissociation velocity constant for catalase cyanide is about 3 000-fold greater than that for methemoglobin cyanide [9,10], which explains the much more rapid reaction with the catalase compound. The reaction with catalase cyanide like that with cytochrome oxidase cyanide was incomplete even in the presence of high concentrations of rhodanese. However, 93 % of the catalase cyanide was converted to free catalase as compared with 33 % of cytochrome oxidase cyanide under similar conditions *. When the rhodanese concentration was reduced

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* The maximum conversion, obtainable with methemoglobin cyanide, could not be determined, as the reaction was so slow, that about 24 h were required for equilibrium, and free methemoglobin was not completely stable under these conditions.

5-fold, the maximum conversion of catalase cyanide was reduced to 86 %, indicating that the concentration of rhodanese also influences the extent of conversion. From the known values for the dissociation constants of catalase cyanide [9] (K: 4×10^{-6}) and cytochrome oxidase cyanide [5] (K: 7×10^{-7}) it was possible to calculate the concentration of free cyanide (hydrocyanic acid), which would give the final value of inhibited heme-enzyme. This calculation gives 1.2×10^{-6} M cyanide in the cytochrome oxidase experiment and 3×10^{-7} M cyanide in the corresponding catalase experiment. This discrepancy, observed in repeated experiments, is yet unexplained.

Vitamin B_{12} (cyanocobalamin) is a cyanide complex of biological importance. Furthermore, it was of interest to test cyanocobalamin as a substrate for rhodanese. When cyanocobalamin (0.004 M) and thiosulfate (0.01 M) were incubated for 30 min at pH 7.4 and 20 °C in the presence of high concentrations of rhodanese (39 μg/ml) no conversion of cyanocobalamin to thiocyanatocobalamin was detected. In the light of the previous results this was not unexpected, as the cyanide is very tightly bound to the cobalt in vitamin B_{12} and thus no free cyanide is accessible to rhodanese.

DISCUSSION

The formation of thiosulfate from a thiosulfonate and sulfite by the action of rhodanese may occur in the body as both thiosulfonates [11] and sulfite can be formed by enzymatic mechanisms. The precursors to thiosulfonates are, however, a sulfinic acid and β-mercaptopyruvic acid, and as the latter gives thiosulfate directly with sulfite in an enzymatic reaction [11], this latter reaction is probably of greater importance.

As cyanide, vitamin B_{12} and thiocyanate are in a dynamic equilibrium *in vivo* (demonstrated by isotope experiments [13]) the failure of vitamin B_{12} to react with rhodanese is of certain interest. It is possible that the cyanide of vitamin B_{12} is first released from the cobalt atom by specific mechanisms before being converted to thiocyanate by the rhodanese system. It is also possible that this conversion occurs through other enzyme systems. In this connection an interesting metabolic function of vitamin B_{12} may be considered. Vitamin B_{12} is in part present in liver not only as cyanocobalamin but also as hydroxycobalamin (vitamin B_{12}a) [13]. The latter has a very high affinity for cyanide and it is possible that the presence of hydroxycobalamin may change the overall equilibrium of certain enzyme systems, so that thiocyanate may be converted to cyanide (bound in vitamin B_{12}) and a sulfur compound, even though the equilibrium constant of these enzyme systems are in favour of the formation of thiocyanate from cyanide. Thiocyanate could thus be converted to thiosulfate by rhodanese, or more interesting, thiocyanate and pyruvic acid may yield mercaptopyruvic acid by the action of an enzyme, which catalyses the formation of thiocyanate from mercaptopyruvic acid and cyanide [14]. A subsequent transamination of mercaptopyruvic acid would then give cysteine and the net result would be a synthesis of cysteine with thiocyanate as the sulfur source. Wokes and Picard [15] have also recently proposed a mechanism, although different from that outlined above, by which the sulfur of thiocyanate could be converted into the sulfur containing amino acids by the action of

vitamin B_{12}. It must be added that no experimental evidence for the formation of cysteine from thiocyanate has as yet been obtained, but experiments designed to test this hypothesis, using sulfur labeled thiocyanate, are now planned in this laboratory.

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