On the Mechanism of Inactivation of Xanthine Oxidase by Cyanide*

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SUMMARY

The inactivation of xanthine oxidase by cyanide is accompanied by the extraction of sulfur from the protein which is eliminated as thiocyanate. When the cyanide inactivation is carried out under anaerobic conditions, partial reduction of the enzyme occurs, equivalent to approximately a 2-electron uptake per eq of thiocyanate released. Cyanide-inactivated enzyme can be largely reactivated by incubation with NaS. Experiments with 35S-labeled NaS reveal that the reactivation is accompanied by the reincorporation of sulfur into the protein. Treatment of such 35S-labeled enzyme with cyanide results in inactivation again and the elimination of 35S-labeled thiocyanate.

METHODS AND MATERIALS

Milk xanthine oxidase was prepared as described previously (6). Xanthine-oxygen reductase activity was measured spectrophotometrically at 295 nm and at a temperature of 25° (6). The catalytic activity is expressed as AFR25° values (7). This value is obtained by dividing the change in absorbance per min at 285 nm by the absorbance at 450 nm of the xanthine oxidase used in the assay.

K14CN and Na235S were obtained from Amersham/Searle (Arlington Heights, Illinois) and had respective specific activities of 1.01 and 4.5 mCi per mmole.

RESULTS AND DISCUSSION

Our interest in the mechanism of cyanide inactivation arose from the observation that when the inactivation is carried out under anaerobic conditions, substantial reduction of the flavin and iron-sulfur chromophores is obtained. This phenomenon is illustrated in Fig. 1. The time course of the reduction is found to be very similar to that of the aerobic reaction as monitored either by difference spectral changes or by enzyme activity. On admitting air to the anaerobic cuvette there is an immediate reoxidation to the spectrum characteristic of cyanide-inactivated enzyme. The inset of Fig. 1 shows the difference spectrum between cyanide-inactivated enzyme before and after reoxidation (Curve A) and that between untreated enzyme and cyanide-inactivated enzyme after reoxidation (Curve B). It can be seen that the latter difference spectrum is very similar to that reported previously by Coughlan et al. (4). The magnitude of the extinction changes on anaerobic treatment with cyanide indicate considerable reduction. By reference to the spectra obtained previously by anaerobic titration with dithionite and substrates (6), the extent of reduction obtained can be estimated to be of the order of 1.5 electron eq per eq of enzyme-bound flavin. As the AFR25° value of the enzyme used in these experiments was 158, the enzyme contained 73% of its active sites in a functional form (8, 9). If the reaction with cyanide was only with functional enzyme, the observed 1.5 electron reduction obtained on cyanide inactivation would thus correspond to a 2-electron reduction per functional active site.

It is shown in Fig. 2 that the reaction of cyanide giving rise to the characteristic difference spectrum between active and inactive enzyme (cf. Fig. 1, inset, Curve B) is proportional to the content of functional sites. In these experiments enzyme preparations of differing functional active site content were employed. It can

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Fig. 1. The effect of incubation of xanthine oxidase with cyanide under anaerobic conditions. Enzyme, in 0.1 M Pi, pH 8.5, was reacted with 0.0033 M KCN at 25°, and the reaction followed until no further spectral changes occurred. The results are expressed as molar extinction coefficient per molecule of enzyme flavin. Curve 1, untreated enzyme; Curve 2, immediately after admitting air. The inset shows difference spectra. Curve A, the difference between Curves 3 and 2. The different symbols represent results from two separate experiments. Curve B, the difference between Curves 1 and 2.

Fig. 2. The extent of the extinction coefficient change at 320 μm (cf. Fig. 1, inset, Curve B) on reaction of xanthine oxidase with cyanide, correlated with the AFR26° value of the enzyme. The symbol (●) shows the results obtained with enzyme initially of AFR26° of 21 which had been activated with Na2S to yield enzyme of AFR26° of 90.

It can be seen that there is a close correlation between the AFR26° value and the decrease in extinction at 320 μm characteristic of cyanide inactivation. From these results it can be concluded that the xanthine oxidase employed by Coughlan et al. (4) contained approximately 60% of its active sites in a functional form.

When xanthine oxidase was incubated at 25° with 0.009 M KClCN the inactivation was found to proceed in a pseudo first order fashion, with a half-time of inactivation of 5.7 min. When the reaction was carried out for 60 min and the products chromatographed on a column of Sephadex G-25, it was found to our surprise that the amount of 14CN fixated to the completely inactivated protein was only 0.28 eq per eq of enzyme flavin, even though the enzyme used initially contained 73% of its active sites in a functional form. Analysis of the column fractions showed that unreacted K14CN emerged as a fairly sharp peak centered around 34 ml, while a third peak of radioactivity emerged at a position centered around 52 ml. This latter peak was found to correspond in elution volume to that of ammonium thiocyanate run separately through the column and assayed colorimetrically by its absorbance at 460 μm as ferric thiocyanate when mixed with Sörbo's reagent (10). The same technique was used to identify the third peak of radioactivity as thiocyanate. By comparison of the spectrophotometric and radio assay it was found that the specific activity was 1.03 mole of 14CN per mole of CNS−, and that the amount of thiocyanate liberated corresponded to 0.70 eq per eq of enzyme flavin applied to the column. The reason for the discrepancy between these results and those reported earlier (2, 3, 4), which claimed the binding of 1 eq of 14CN per eq of flavin, is due apparently to reaction of CN− at sites other than that responsible for inactivation. For example, incubation with 14CN− for periods long in excess of that required for inactivation results in the fixation of more 14CN to the protein (Table I). The fixation reaction may well be due to the well known cyanolysis of disulfide bridges.

\[
\text{Protein} + \text{CN}^- \rightarrow \text{Protein} + \text{SCN}^- \quad (1)
\]

We were able to confirm the finding of Fridovich and Handler...
Release of $^{14}$CN$^{-}$ from xanthine oxidase on reaction with $^{14}$CN$^{-}$

$^{14}$CN$^{-}$ was incubated with milk xanthine oxidase in 0.1 M PPi, buffer, pH 8.5. After reaction for periods stated, the enzyme was cooled in ice and passed through a column of Sephadex G-25 (1.2 X 30 cm) and eluted with 0.1 M PPi, pH 8.5. Enzyme was eluted at a peak volume of 15 ml, cyanide at a volume of 34 ml, and thiocyanate at a volume of 52 ml. Thiocyanate was measured quantitatively as the ferric salt in acid solution. For such estimations 0.5-ml aliquots were mixed with 0.4 ml of water and 0.6 ml of Söbo's reagent (10). The pink solutions were clarified by centrifuging and the absorbance at 460 nm determined. The amount of enzyme taken for each experiment varied from 820 to 880 mpmoles with respect to bound FAD.

Table I

| Experiment | AFRAH after treatment | $^{14}$CN bound to protein | $^{14}$CN$^{-}$ released |
|------------|-----------------------|---------------------------|-------------------------|
| Native enzyme (AFR 153) incubated at 25° with 0.009 M $^{14}$CN$^{-}$ | 1 hour | 0.28 | 0.70 |
| | 17 hours | 0.90 | 0.88 |
| Native enzyme (AFR 93) incubated at 25° with 0.009 M $^{14}$CN$^{-}$ for 1 hour | | 0.43 | 0.48 |
| Native enzyme (AFR 153) incubated anaerobically at 25° with 0.009 M $^{14}$CN$^{-}$ for 10 hours in presence of Na$_2$S$_2$O$_4$ | 150 | 1.16 | <0.05 |

Table II

Recombination reaction with Na$_2$S$^{35}$S

Na$_2$S$^{35}$S (used within 2 weeks of preparation) was reacted with native enzyme or with cyanide-inactivated enzyme at a final concentration of 0.01 M; mixed with 0.1 M PPi, pH 8.5, the final pH was found to be 9.0. It was reacted in screw capped tubes at 45° for the times shown, cooled in ice, and separated from reactants and products on a column of Sephadex G-25 as detailed in Table I. Unreacted Na$_2$S was found to emerge at a peak volume of 34 ml; CN$^{35}$S$^{-}$ was quantitated by radioassay of the CN$^{-}$ peak emerging at a peak volume of 52 ml. Amounts of enzyme, quoted in respect to FAD content were 370 mpmoles for Experiment 1, 100 mpmoles each for Experiments 2 and 4, 114 mpmoles each for Experiments 5, 6, and 7, and 43 mpmoles for Experiment 8. Approximately 60% of the enzyme remained soluble in Experiment 7 due to precipitation during the long period of heating. The analytical figures shown are for the soluble fraction. A separate minor radioactive fraction emerging at a peak volume of 32 ml was observed in the elution patterns in Experiments 3, 4, and 8. The identity of this fraction is presently unknown.

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

Fig. 3. The reactivation of cyanide-inactivated xanthine oxidase by Na$_2$S. Enzyme, initially with an AFRAH value of 153, was inactivated by treatment with 0.01 M KCN for 4 hours at 25°, followed by extensive dialysis against 0.1 M PPi, pH 8.5, containing 3 X 10$^{-4}$ M EDTA. This enzyme, at a concentration of 1.85 X 10$^{-4}$ with respect to flavin content, was incubated at 45° in a capped vial with 0.01 M Na$_2$S. The figure shows duplicate experiments; •, reacted with unlabeled Na$_2$S; ○, reacted with Na$_2$S$^{35}$S.

(2) that when the cyanide reaction is carried out anaerobically in the presence of dithionite, no inactivation occurs. However, our results do not support their claim that these conditions prevent fixation of $^{14}$CN$^{-}$ to the protein. As shown in Table I, in our hands fixation of more than 1 eq of $^{14}$CN$^{-}$ to the reduced enzyme can be obtained without any loss of catalytic activity, presumably through Reaction 1 above.

In confirmation of the conclusion that only functional enzyme reacts with cyanide to liberate thiocyanate, Table I also shows that enzyme of AFRAH 93 (45% functional) yielded 0.48 mole of CN$^{-}$ per eq of enzyme flavin.

As it was evident from the above results that cyanide inactivation was due to elimination of sulfur from the protein as thiocyanate, it was of obvious interest to determine if the sulfur could be replaced with return of activity. It was found that incubation with Na$_2$S would accomplish this under suitable conditions. The degree and speed of reactivation depends significantly on pH and temperature. Maximal reactivation was obtained with good reproducibility by incubation with 0.01 M Na$_2$S at 45°, pH 9.0. Fig. 3 shows the results of two such reactivation experiments, one carried out with Na$_2$S$^{35}$S. The enzyme from the
latter experiment was found at the end of 4 hours of incubation to have incorporated 3 moles of $^{35}$S per eq of enzyme flavin. When this labeled enzyme was subjected to ammonium sulfate precipitation and dialysis, it was found to have retained 2.76 moles of $^{35}$S per eq of enzyme flavin. Further treatment with unlabeled KCN resulted in the usual cyanide inactivation and the loss of $^{35}$S as CN$_3$S$^-$ (Table II). It is evident from these results that in addition to the reincorporation of sulfur to the active site, extraneous sulfur had been introduced into the protein, presumably by formation of persulfide by reaction with disulfides.

\[
\text{Protein} - S-S- + \text{CN}^- \rightarrow \text{Protein} - S^- + \text{CN}_3S^- \quad (3)
\]

Such reactions have been documented previously with model compounds by Rao and Gorin (11) and more recently with protein by Cavallini, Frederici, and Barbonti (12).

The results presented suggest very strongly that the active site of xanthine oxidase contains a persulfide grouping that reacts readily with cyanide to be liberated as thiocyanate.

\[
\text{Protein} - S-S- + \text{CN}^- \rightarrow \text{Protein} - S^- + \text{CN}_3S^- \quad (3)
\]

That the thiocyanate sulfur does not originate from the acid-labile sulfur associated with the nonheme iron chromophore is substantiated by little difference in spectral properties in the visible absorption spectrum upon cyanide inactivation and by identical sulfide analysis for both native and cyanide-inactivated enzymes. If another sulfhydryl group were in the vicinity of the persulfide, an attractive possibility to account for the 2-electron reduction accompanying the cyanide inactivation would be available through the formation of a new disulfide bond.

Scheme I presents this hypothesis in diagrammatic form. In addition to explaining the elimination of thiocyanate accompanying cyanide inactivation, this scheme would account for the reduction of the enzyme found when the cyanide inactivation is carried out anaerobically, as well as for the reformation of the active center persulfide on incubation with Na$_2$S. In addition it could account for other facets of this enzyme. Arsine has been shown to be a powerful reversible inhibitor of the oxidized native enzyme and to interfere with cyanide inactivation (4). This could be due to arsine forming a complex with the persulfide and the neighboring sulfhydryl group. Finally, the possibility exists that the presence of nonfunctional active sites in all known preparations of xanthine oxidase (8, 9) is due to destruction (during preparation or storage) of the persulfide. The nature of the cyanide reaction seems more complex than a simple nucleophilic displacement as other nucleophiles such as sulfite and hypotaurine (a sulfinate which readily attacks persulfide (12)) do not mimic the cyanide reaction. This is indicative that a group(s) on the enzyme (possibly molybdenum) stabilizes the postulated persulfide as well as mediating its attack by cyanide. Work is now in progress to elucidate these points.

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