Hypothesis

ON THE ORIGIN OF THE CYANOLYSABLE SULPHUR IN MOLYBDENUM IRON/SULPHUR FLAVIN HYDROXYLASES

Michael P. COUGHLAN
Department of Biochemistry, University College, Galway, Ireland

Received 3 June 1977

1. Introduction

Cyanide treatment of xanthine oxidase [1], xanthine dehydrogenase [2] and of aldehyde oxidase [3] results in the removal of sulphur, as free thiocyanate, from the active centres of these enzymes. The sulphur atoms in question may also be lost spontaneously during purification or as a result of 'aging'. Many, though not all, of the properties of each enzyme are affected as a result [1–7]. Treatment of the resulting 'desulphoenzyme' (terminology of Bray [8]) with sulphide effects partial restoration of activity [1,2,4]. Under the reactivation conditions used, the incorporation of $^{35}S^{2-}$ has been demonstrated as has the release of $^{35}SCN^-$ on subsequent treatment with cyanide [1].

What is the source of the cyanolysable sulphur? It could originate, a priori, from the iron–sulphur prosthetic groups, or from cysteine residues or from persulphide groups present in these enzymes. The iron–sulphur chromophores have been ruled out for two reasons. In the first place, the 'desulphoenzyme' has the full compliment of acid-labile sulphur [1]. Secondly, the change in the visible absorption spectra of these enzymes on treatment with cyanide is much less than would be expected had these chromophores been disrupted [1,2,9]. On the basis of these findings, Massey and Edmonson [1] proposed that the sulphur atoms released following cyanide treatment originate from a persulphide group (R–S–S$^-$) that is present at both active centres of fully functional enzyme.

While the 'persulphide theory' is compatible with the available evidence, an alternative interpretation is also possible. As outlined in this paper, an active centre cysteine residue could equally well be considered as the source of the cyanolysable sulphur. Such a hypothesis also provides one explanation for the finding [2] of one mole of dehydroalanine per mole of enzyme, whether cyanide-inactivated or untreated (cf. later).

2. Hypothesis

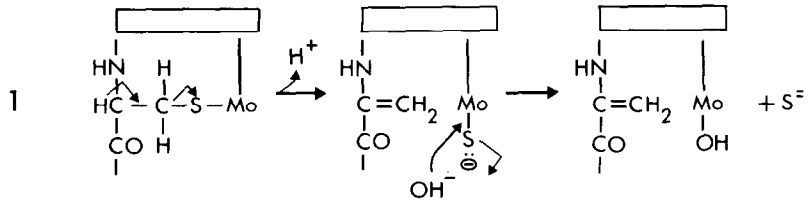
A considerable body of evidence suggests that the cyanolysable sulphur atoms are at or near the molybdenum centres (cf. [4] and reviews [8,10,11]). Substrates and competitive inhibitors that bind to these loci protect against cyanide inactivation [12,13]. Moreover, loss of sulphur alters the shape and other parameters of the molybdenum electron paramagnetic resonance (EPR) signals [9,11,14]. I therefore propose that at both active centres of fully functional enzyme a cysteine residue is linked via its sulphur to molybdenum (cf. [10]). This would explain, inter alia, why the cyanolysable sulphur is acid-stable.

3. Discussion

3.1. *'Spontaneous' loss of active centre sulphur*

During purification or 'aging' the α-hydrogen of the active centre cysteine residue may be lost (e.g., as in racemization). This would lead to a break in the carbon–sulphur bond thereby giving rise to a dehydroalanine residue while the sulphur remains bound to molybdenum (see Scheme 1). The latter complex is unstable as such with the result that the

Scheme 1
Spontaneous inactivation



A cysteine residue is shown linked via its sulphur to molybdenum at the active site. The rest of the enzyme molecule is represented by the bar. Spontaneous inactivation could result from loss of the α-hydrogen as a proton followed by transfer of the electron pair. The resulting cleavage of the carbon–sulphur bond produces a dehydroalanine residue. The sulphur is subsequently lost as sulphide perhaps being displaced from the molybdenum by a hydroxyl group.

sulphur is readily lost, perhaps being displaced by a hydroxyl group (cf. [8,10]). Thus, as spontaneous inactivation proceeds one would expect to find an increase in the amount of dehydroalanine formed (max. 2 mol/mol non-functional enzyme, fig.1) and a decrease in the amount of cyanolysable sulphur present (from max. 2 mol/mol functional enzyme originally present, fig.1). Acid hydrolysis of such enzyme samples would convert the dehydroalanine present to pyruvate which may then be measured using lactate dehydrogenase [2,15].

3.2. *Cyanolysis of the active centre sulphur*

In theory cyanide might attack the proposed molybdenum cysteine complex in one or other of two ways. Thus, it could bind to the molybdenum atom thereby displacing the existing bond with sulphur. This possibility may be ruled out however since the EPR studies of Komai et al. [16] showed no isotope effect on the molybdenum signals whether $^{12}CN^-$ or $^{13}CN^-$ was used. Accordingly, cyanide is shown (Scheme 2) as attacking the carbon–sulphur bond, thereby producing a cyanoalanine residue and leaving the sulphur attached to molybdenum. Again the sulphur is readily lost but it now reacts with the excess of cyanide present to form the free thiocyanate observed experimentally [1,2]. Moreover, the 2 electrons liberated on the formation of thiocyanate would explain the observed [1] two-electron reduction of xanthine oxidase when cyanide-inactivation is carried out under anaerobic conditions.

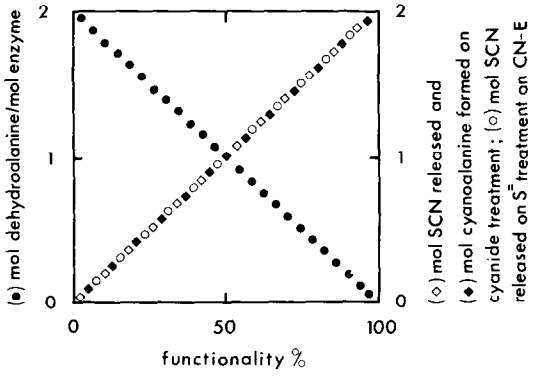
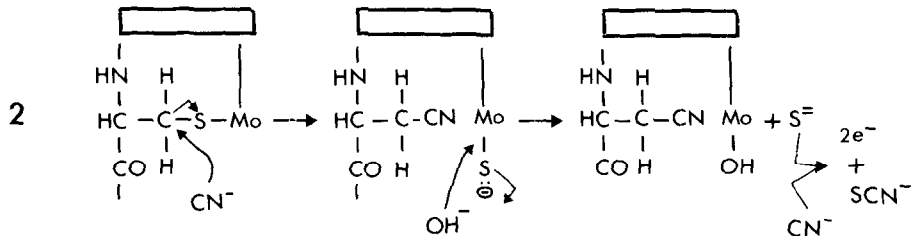


Fig.1. Properties of the enzyme dependent on the content of functional active sites. Dehydroalanine and cyanoalanine contents of enzyme samples would be measured following acid hydrolysis as pyruvate and as aspartate respectively (see text for details).

The amounts of thiocyanate and of cyanoalanine formed as above would be proportional to the degree of integrity of the active centres in the original enzyme samples used (in each case max. 2 mol/mol functional enzyme, fig.1). Detection of cyanoalanine residues should in theory be possible using infrared spectroscopy but may not be so in practice. However, using $^{14}CN^-$ and assuming the hypothesis to be correct, this difficulty could be obviated. The radioactively-labelled cyanoalanine residues formed would, following acid hydrolysis of the enzyme, be converted to aspartic acid. The isolation and identification of radioactive aspartate (2 mol/mol functional enzyme originally used) should be a relatively simple matter.

The formation of cyanoalanine is central to the theory being put forward here. Thus, using $^{14}CN^-$, and assuming the cyanoalanine to be stable, one

Scheme 2
Cyanide inactivation



Cyanide attacks the carbon–sulphur bond of the molybdenum–cysteine complex displacing the sulphur thereby producing a cyanoalanine residue. The sulphur which is subsequently released from the molybdenum reacts with the excess of cyanide present to give thiocyanate. This latter process is facilitated by the enzyme picking up the two electrons.

should observe the fixation of 1 mol $^{14}CN^-$ and release of 1 atom sulphur, as $^{14}CNS^-$, per functional active site (cf. Scheme 2).

The evidence in the literature with respect to this proposal is somewhat divided. Massey and Edmonson [1] have stated that the bulk of the $^{14}CN^-$ bound to enzyme is due to cyanolysis of disulphide bonds and occurs only when incubation with cyanide is carried out for periods (e.g. 17–19 h [1]) long in excess of that required for inactivation (2 h or less). However, in one of their experiments, using forty-five percent functional xanthine oxidase and 1 h incubation with cyanide, they found (as Scheme 2 predicts) the incorporation of 0.43 mol $^{14}CN^-$ and the release of 0.48 mol $^{14}CNS^-$ per mole of enzyme.

In other experiments it is true that they found less cyanide bound than sulphur released but not always significantly less (e.g. [3]).

In support of Scheme 2, Fridovich and Handler [12] using xanthine oxidase and Rajagopalan and Handler [13] using xanthine dehydrogenase and incubation times with cyanide of 2 h and 30 min, respectively (i.e., times consistent with complete inactivation) found the incorporation of 1 mol $^{14}CN^-$ per equivalent of molybdenum. Moreover, both groups of investigators found that the presence of substrate or of competitive inhibitor reduced the amount of $^{14}CN^-$ bound. This is consistent with the binding of cyanide to the active site (cf. Scheme 2).

3.3. *Reactivation of desulphoenzyme*

Partial reactivation of cyanide-treated enzyme has been effected by incubation with sulphide ions [1,2,4]. This may be explained as in Scheme 3a, as a simple displacement of cyanide from cyanoalanine with regeneration of cysteine and ultimately of the molybdenum–cysteine complex. The cyanide so displaced could react with the excess of sulphide present to form free thiocyanate (2 mol/mol functional enzyme originally used, fig.1).

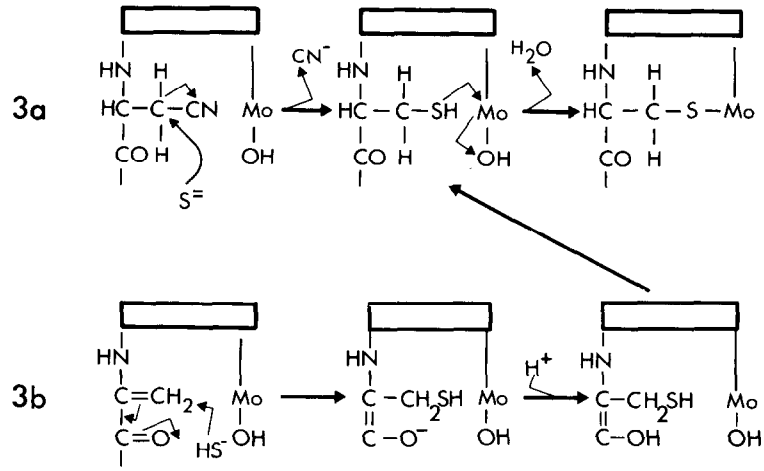
Naturally-occurring desulpho-xanthine oxidase has also been partially reactivated by treatment with sulphide ions [4]. Scheme 3b shows that this may result from the reaction of sulphide with the dehydroalanine residues at the active centre to regenerate cysteine followed in turn by reformation of the molybdenum–cysteine complex. In view of the earlier discussion (cf. also Scheme 1), however, one would expect the formation from dehydroalanine of equal amounts of L- and D-cysteine. Whether this would hinder reactivation is debatable. However, it may be noted that desulpho-xanthine oxidase prepared by cyanolysis is more fully reactivated by sulphide than is the spontaneously generated form (compare ref. [1] and [4]). It should also bé noted that the reaction of sulphide with native enzyme, a mixture of functional and non-functional (spontaneously-inactivated) molecules, is a complex process [2,17].

Schemes 3a and 3b also show, as has been demonstrated experimentally [1], that using $^{35}S^{2-}$ one would expect the incorporation of radioactivity into the enzyme and the release of sulphur as $^{35}SCN^-$ on subsequent treatment with cyanide (Scheme 2).

3.4. *Inactivation by arsenite*

It has been shown that the sites of action of

Scheme 3
Reactivation of desulphoenzyme



(a) Sulphide restores activity to cyanide treated enzyme by displacing cyanide from the cyanoalanine residue thereby regenerating cysteine and ultimately the molybdenum–cysteine complex. The released cyanide should react with the excess of sulphide present to give thiocyanate, the enzyme again picking up the two excess electrons (cf. Scheme 2).

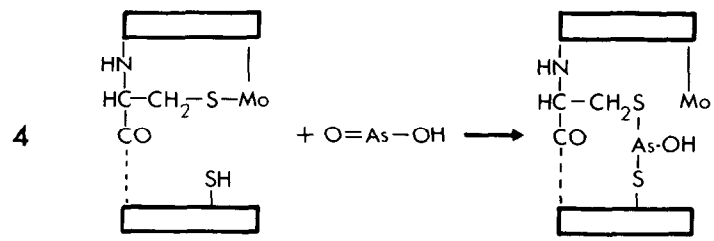
(b) Sulphide restores activity to spontaneously-inactivated enzyme by nucleophilic addition to the carbon–carbon double bond of the dehydroalanine residue activated by the adjacent carbonyl. This results in the regeneration of the cysteine residue and thus of the molybdenum–cysteine complex.

cyanide and of arsenite are mutually exclusive [9] and that the presence of the active centre sulphur is essential to arsenite binding [4,6]. One may visualize that on incubation with enzyme the bidentate arsenite binds to the sulphur of the active centre cysteine, thereby displacing the bond with molybdenum, and to a vicinal thiol or hydroxyl group (cf. Scheme 4). Thus, loss of the active centre sulphur, whether spontaneous or as a result of cyanide action, would destroy the arsenite binding site.

3.5. *Reaction mechanism*

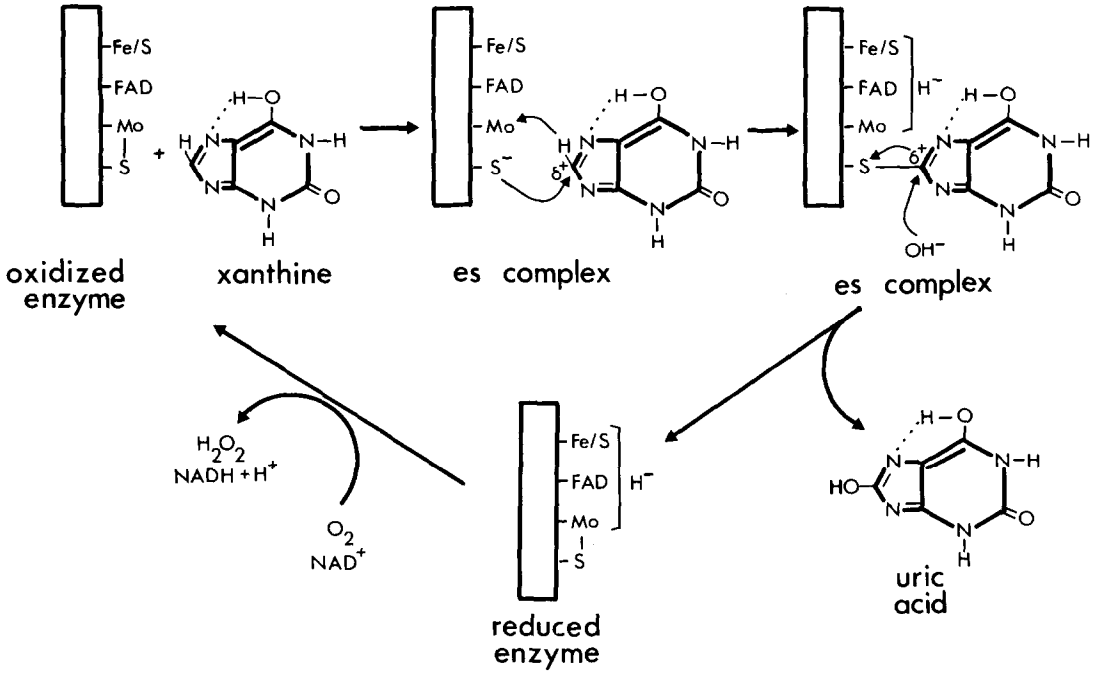
Edmonson et al. [4] have proposed a reaction mechanism for xanthine oxidase whereby the initial step is the transfer of a hydride ion from the substrate. This transfer is induced by the proposed per-

Scheme 4
Arsenite inactivation



Inactivation of these enzymes by arsenite results from its binding to the cyanolysable sulphur of the molybdenum–cysteine complex and to a vicinal thiol (as shown) or hydroxyl group.

Scheme 5
Mechanism of action



The mechanism of action involving an active centre cysteine residue is intentionally analogous to that proposed by Edmonson et al. [4] which involves a persulphide group. Note that cleavage of the molybdenum sulphur bond which takes place on substrate binding (cf. ref. [22]) generates the ionized sulphur needed for attack on the substrate. The various steps are described in the text.

sulphide group which then stabilizes the resulting carbonium ion product. Electrons from the hydride ion are subsequently distributed among the various redox components of the enzyme. Attack by a hydroxyl ion (from water) would then release uric acid and regenerate the persulphide group. An active centre cysteine, rather than a persulphide group, can also fit such a reaction mechanism if one makes one assumption; namely, that on binding substrate the proposed bond between the cysteine sulphur and molybdenum is broken (cf. Scheme 5). The ionized thiol group so liberated could then act as the 'acceptor' of the substrate carbonium ion. There is evidence consistent with the above assumption in that the reduction of enzyme with substrate exposes a mercurial-reactive sulphydryl group that is not readily accessible in the oxidized enzyme [12,18,19]. Indeed Bray et al. [20] have suggested that the exposure of a sensitive thiol group on reduction is due to the breakage of a molybdenum–mercaptide linkage.

4. Conclusion

Cyanide treatment of xanthine oxidase and related enzymes effects a decrease in absorbance, maximal at 320 nm, that is proportional to the content of cyanolysable sulphur in the enzyme samples used [1,3,6]. For functional enzyme $\Delta\epsilon$ is around 6000 litres $\times$ mol^{-1} $\times$ cm^{-1}. This may be explained by a conformational change in the enzyme following on the breakage of the carbon–sulphur bond of the active centre molybdenum–cysteine complex and subsequent loss of sulphur (Schemes 1,2). It may be noted that while protein persulphide groups do absorb between 320–350 nm (e.g. [21]), the

molar absorption coefficient of such groups is only 400 litres $\times$ mol^{-1} $\times$ cm^{-1}.

In a previous study [2] we found that turkey liver xanthine dehydrogenase, approx. 50% functional, contained 1.02 mol dehydroalanine/mol enzyme. Cyanide treatment of this material effected the release of 1.13 mol thiocyanate/mol enzyme while the product contained 1.04 mol dehydroalanine/mol enzyme. These results are consistent with Schemes 1, 2 and fig.1.

Acknowledgements

I thank Drs A. Finan and P. O'Carra for much helpful discussion.

References

[1] Massey, W. and Edmonson, D. (1970) J. Biol. Chem. 245, 6595–6598.
[2] Cleere, W. F. and Coughlan, M. P. (1974) Biochem. J. 143, 331–340.
[3] Branzoli, U. and Massey, V. (1974) J. Biol. Chem. 249, 4346–4349.
[4] Edmonson, D., Massey, V., Palmer, G., Beacham, L. M. III and Elion, G. B. (1972) J. Biol. Chem. 247, 1597–1604.
[5] McGartoll, M. A., Pick, F. M., Swann, J. C. and Bray, R. C. (1970) Biochim. Biophys. Acta 212, 523–526.
[6] Cleere, W. F., O'Regan, C. and Coughlan, M. P. (1974) Biochem. J. 143, 465–468.
[7] Ní Fhaoláin, I., and Coughlan, M. P. (1976) Biochem. J. 157, 283–285.
[8] Bray, R. C. (1975) The Enzymes 12B, 299–419.
[9] Coughlan, M. P., Rajagopalan, K. V. and Handler, P. (1969) J. Biol. Chem. 244, 2658–2663.
[10] Bray, R. C. and Swann, J. C. (1972) Struct. Bond. 11, 107–144.
[11] Massey, V. (1973) in: Iron–Sulphur Proteins (Lovenberg, W. ed) pp. 301–360, Academic Press, New York.
[12] Fridovich, I. and Handler, P. (1958) J. Biol. Chem. 231, 899–911.
[13] Rajagopalan, K. V. and Handler, P. (1967) J. Biol. Chem. 242, 4097–4107.
[14] Bray, R. C., Barber, M. J., Coughlan, M. P., Dalton, H., Fielden, E. M. and Lowe, D. J. (1976) in: Flavins and Flavoproteins (Singer, T. P. ed) pp. 553–565, Elsevier, Amsterdam.
[15] Malkin, R. and Rabinowitz, J. C. (1966) Biochemistry 5, 1262–1268.
[16] Komai, H., Massey, V. and Palmer, G. (1969) J. Biol. Chem. 244, 1692–1700.
[17] Coughlan, M. P. and Cleere, W. F. (1976) in: Flavins and Flavoproteins (Singer, T. P. ed) pp. 576–581, Elsevier, Amsterdam.
[18] Harris, J. and Hillerman, L. (1956) in: Inorganic Nitrogen Metabolism (McElroy, W. D. and Glass, B. eds) pp. 565–574, Johns Hopkins Press, Baltimore.
[19] Massey, V., Brumby, P. E., Komai, H. and Palmer, G. (1969) J. Biol. Chem. 244, 1682–1691.
[20] Bray, R. C., Malmström, B. G. and Vänngård, T. (1959) Biochem. J. 73, 193–197.
[21] Cavallini, D., Federici, G. and Barboni, E. (1970) Eur. J. Biochem. 14, 169–174.
[22] Bray, R. C. (1974) J. Less-Common Metals 36, 413–427.