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Oxidation–Reduction Potentials of Molybdenum and Iron–Sulphur Centres in Nitrate Reductase from *Escherichia coli*

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The potentials of the couples Mo(IV)–(Mo(V) and Mo(V)–Mo(VI) in nitrate reductase from *Escherichia coli* K12 were measured as +180mV and +220mV respectively at pH 7.14. The potentials associated with two other e.p.r. signals, believed to be due to iron–sulphur centres, were measured as +50mV and +80mV.

Oxidation–reduction potentials have been measured for some of the metal components of the dissimilatory nitrate reductase from *Escherichia coli* K12. Values were measured for the Mo(VI)–Mo(V) and Mo(V)–Mo(IV) mid-point potentials by potentiometric titration and quantitative measurement of [Mo(V)] by e.p.r. spectroscopy. Values were also obtained for other e.p.r. signals in the enzyme, believed to arise from iron–sulphur centres.

This work was undertaken mainly to compare the properties of molybdenum in nitrate reductase with molybdenum in other enzymes. Corresponding molybdenum potentials have been measured for xanthine oxidase (Cammack *et al.*, 1976) and xanthine dehydrogenase (Barber *et al.*, 1977). It was thought that the potentials of molybdenum in nitrate reductase might be much more positive than the corresponding potentials in xanthine oxidase.

Firstly, the Mo(V) signal from nitrate reductase is elicited on oxidation by nitrate, and is abolished on reduction (Vincent & Bray, 1978). The various Mo(V) signals seen in xanthine oxidase are seen only on reduction of the enzyme, as are signals from sulphite oxidase (Bray, 1975). These observations, though suggestive, are only relevant when the oxidation–reduction potentials of the substrates are taken into consideration, as discussed below.

Secondly, nitrate reductase in *E. coli* occupies a position in intermediary metabolism similar to mammalian cytochrome oxidase, insofar as both enzymes are the terminal components of the major electron-transport chains in *E. coli* and mitochondria respectively. The oxidation–reduction potentials of the prosthetic groups of cytochrome oxidase are known to be highly positive. By analogy, and also by general considerations of bioenergetics, it seems not unreasonable to suppose that the prosthetic groups of nitrate reductase, particularly the molybdenum atom, which is involved in nitrate reduction, will likewise exhibit high oxidation–reduction potentials.

Thirdly, Table 1 shows that nitrate reductase is anomalous among molybdenum-containing enzymes. With all molybdenum-containing enzymes other than nitrate reductase the oxidation–reduction potential of the half-reaction catalysed by the molybdenum atom is extremely low, whereas for nitrate reductase it is extremely high. Although it might be possible to explain this anomaly on the basis of nitrate reduction by a one-electron rather than a two-electron process, it is nevertheless reasonable to speculate that the high potential of the nitrate–nitrite couple might be reflected in correspondingly high potentials of the molybdenum of nitrate reductase.

Table 1. *Reactions catalysed by molybdenum-containing enzymes*

Values (apart from xanthine–uric acid) are taken from *Data for Biochemical Research*, selected 2nd edition (1970), published by the Chemical Rubber Co., Cleveland. The value for xanthine–uric acid was taken from Cammack *et al.* (1976).

Enzyme	Half-reaction catalysed by molybdenum	Potential of reactant–product couple E_0 (mV)
Xanthine oxidase	Xanthine–uric acid	−440
Sulphite oxidase	Sulphite–sulphate	−454
Formate dehydrogenase	Formate–CO_2*	−420
Aldehyde oxidase	Acetaldehyde–acetate	−581
Nitrogenase	Nitrogen–ammonia	Low
Nitrate reductase	Nitrate–nitrite	+421

* It is presumed that this reaction takes place at the molybdenum centre.

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Materials and Methods

Nitrate reductase was purified from *E. coli* K12 A1002 by a procedure previously described (Vincent & Bray, 1978).

The methodology of potentiometric titrations has been described elsewhere (Cammack *et al.*, 1976). Titrations were performed at 25°C at pH7.14 by using 200mM-$K_2Fe(CN)_6$ as oxidant and 100mM-$Na_2S_2O_4$ in 10mM-NaOH as reductant. Mediators were used at a concentration of 30μM: Toluidine Blue (+34mV), 1,4-naphthoquinone (+60mV), 1,2-naphthoquinone (+143mV), 2,5-dimethyl-*p*-benzoquinone (+185mV), 2,6-dichlorophenol-indophenol (+217mV), diphenylbenzidine (+254mV) and quinhydrone (+286mV). A quinhydrone electrode was used as a standard.

Samples were withdrawn and frozen for e.p.r. spectroscopy, which was carried out as described previously (Lowe *et al.*, 1972). Quinquivalent molybdenum was measured by double integration, with a Cu^{2+}–EDTA solution as standard. Appropriate corrections were made for unseen hyperfine structure from ^{95}Mo and ^{97}Mo, for microwave power saturation and for *g*-value corrections.

The activity of the enzyme decreased substantially during some experiments, by as much as 30%. Since inactivation of the enzyme during storage or manipulation has been found to be accompanied by loss of the molybdenum signal, it was assumed that this inactivation might be due to either a change in the chemistry of the molybdenum centre or loss of molybdenum from the enzyme. In either case an appropriate correction must be made to the integrated intensity of the Mo(V) signal. This was done for all the points on the titration curve, bar the first one. First-order decay kinetics were assumed.

Molybdenum and iron analyses were carried out colorimetrically as described previously (Hart *et al.*, 1970).

The best-fit semiquinone and other curves were determined by using a computer program based on least-squares analysis.

Results

A typical result of such an experiment is shown in Fig. 1. The Mo(V) concentration varies as a function of potential, and behaves, as expected, as a semiquinone type of signal. The titration is fully reversible. It is possible to obtain the molybdenum potentials from a knowledge of the maximum proportion of molybdenum in the quinquivalent state and the potential at which this occurs. This experiment was repeated several times and the overall pattern of behaviour was reproducible.

The results, together with the known oxidation–reduction potentials of molybdenum in xanthine oxidase and xanthine dehydrogenase, are shown in Table 2.

It is by no means certain that all the molybdenum, as revealed by metal analysis, is responsible for the signal-giving species. This has been discussed elsewhere (Vincent & Bray, 1978), but it should be noted that the shape of the semiquinone curves is such that quite a considerable variation in total molybdenum content will have only a minor effect on the values of the two potentials calculated from the data.

The enzyme also exhibits a multiplicity of signals that can be seen at low temperature (about 20K) and that are presumed to arise from iron–sulphur

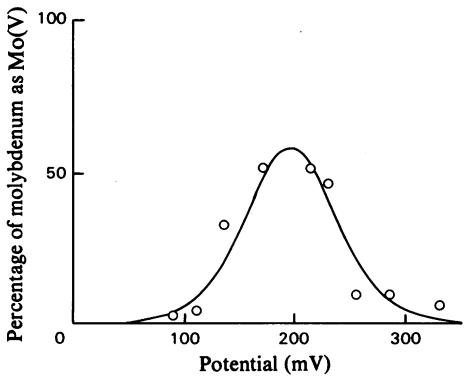

Fig. 1. *Proportion of molybdenum in the quinquivalent state as a function of redox potential*

The curve is a 'best-fit' line. The enzyme was 5.6μM with respect to molybdenum, and was in 100mM-sodium Mops (4-morpholinepropanesulphonic acid) buffer, pH7.14, containing 0.4M-NaCl, 0.1mM-EDTA and 0.1% Triton X-100. At this pH the molybdenum e.p.r. signal is in the 'low-pH' form, which is the signal from the active catalytic enzyme (Vincent & Bray, 1978).

Table 2. *Oxidation–reduction potentials of molybdenum in enzymes*

Enzyme	Type of e.p.r. signal	Mo(VI)–Mo(V) potential (mV)	Mo(V)–Mo(IV) potential (mV)	pH	Reference
Xanthine oxidase	Rapid	−355±20	−355±20	8.2	Cammack *et al.* (1976)
Xanthine oxidase	Slow	−440±25	−480±25	8.2	Cammack *et al.* (1976)
Xanthine dehydrogenase	Rapid	−350±20	−362±20	8.2	Barber *et al.* (1977)
Nitrate reductase	Low pH	+220±20	+180±20	7.14	Present work

Table 3. *Potentials of iron–sulphur signals in nitrate reductase at pH 7.14*

g value(s) of signal	Form of enzyme in which signal is seen	Potential (mV)
$g_1 = 2.003$ $g_2 = 1.888$ $g_3 = 1.870$	Reduced	$+50 \pm 20$
$g \simeq 2.02$	Oxidized	$+80 \pm 20$

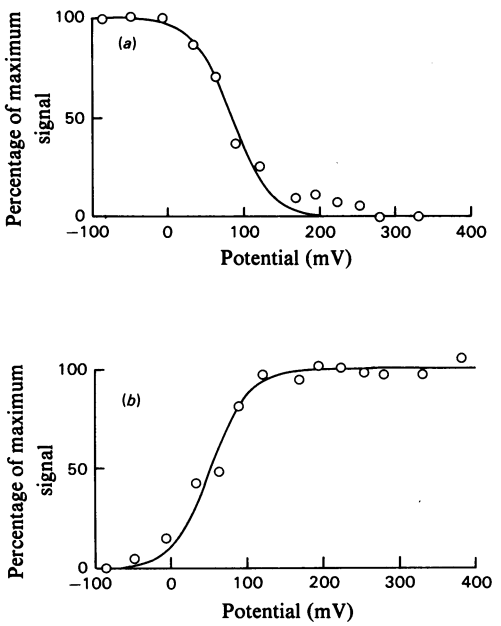

Fig. 2. *Intensity of low-temperature iron–sulphur signals as a function of potential*

The enzyme was in the same buffer as in Fig. 1. E.p.r. spectra were run at 20K with a microwave power of 10mW. (*a*) Reduced iron–sulphur signal ($g_1 = 2.003$, $g_2 = 1.888$, $g_3 = 1.870$). Signal strength was measured from the g_3 peak. (*b*) Oxidized signal ($g \simeq 2.02$). The curves are 'best-fit' lines, assuming a one-electron oxidation–reduction process.

centres (Vincent & Bray, 1978). Although the complexity of the signals has so far precluded full analysis of potentiometric data, potentials have been obtained for one of the reduced iron–sulphur centres and the oxidized signal. The data are shown in Fig. 2 and the potentials presented in Table 3.

Discussion

Not unexpectedly, the molybdenum potentials turn out to be high relative to those of xanthine oxidase. It is remarkable that the potentials of the same metal in the same oxidation state vary by over half a volt in different biological systems, although the potentials of iron also vary considerably (cf. cytochrome a_3/ferredoxin). This will no doubt be reflected in the different chemical environment of molybdenum in the two enzymes, despite the similarities of the Mo(V) e.p.r. spectra of the two enzymes. The precise significance of the finding (Pienkos *et al.*, 1977) that xanthine oxidase and nitrate reductase (albeit from *Neurospora crassa*) share a 'common cofactor' thus remains to be elucidated.

The potentials of two of the iron–sulphur signals seen are both considerably lower than those of the molybdenum signals, an observation that may be of significance in the proton-translocation activity associated with nitrate reductase activity. Possibly these iron–sulphur centres are used as an 'electron sink' for the rapid supply of electrons to molybdenum for nitrate reduction.

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