Synthesis of the active sites of molybdoenzymes: MoO_2(VI) and MoO(IV)-dithiolene complexes mimicking enzymatic reactions of sulphite oxidase with saturation kinetics

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Abstract. $[Mo^{VI}O_2(S_2C_2(CN)_2)_2]^{2-}$ (1) and $[Mo^{IV}O(S_2C_2(CN)_2)_2]^{2-}$ (2) mimick oxido-reductase enzymatic activities of sulphite oxidase with biological electron donor, SO_3^{2-}, and *in vitro* electron acceptor, $[Fe(CN)_6]^{3-}$, demonstrating proton coupled electron transfer reaction in water and inhibition of the oxidation of (2) in the presence of KCN. The sulphite oxidizing system is characterized by substrate saturation kinetics indicating the biological significance of the reactions.

Since 1891 molybdenum hydroxylases have been extensively studied by biochemical and genetic investigations (Horbaczewski 1891; Bowden 1975; Newton and Otsuka 1980; Coughlan 1980; Spiro 1985). EPR (Bray and Meriwether 1966; Cohen *et al* 1971; Kessler and Rajagopalan 1972; Bray 1975) and EXAFS (Tullius *et al* 1979; Cramer *et al* 1981) spectroscopy have provided much information to understand the structure–function relationship of molybdenum in this class of enzymes. Whereas there has been much work on the chemistry of the synthesized molybdenum complexes to simulate enzymatic reactions (Berg and Holm 1985; Holm 1990), comparatively little has been achieved by these complexes owing to the lack of (a) basic dithiolene coordination, a ligand with a pterin side chain for molybdenum cofactor (Pateman *et al* 1964; Kramer *et al* 1987); (b) mediating the transfer of protons and electrons in aqueous medium (Bray and Meriwether 1966; Stiefel 1973, 1980) (c) to make use of specific physiological or *in vitro* electron donor and acceptor responded by a specific enzyme to complete the oxidoreductase cycle. We present here functional analogue reactions of sulphite oxidase, the simplest enzyme of this class, addressing all the aspects stated above using synthesized complexes, $[(C_4H_9)_4P]_2[Mo^{VI}O_2(mnt)_2]$ and $[(C_4H_9)_4[Mo^{IV}O(mnt)_2]$ $(mnt = S_2C_2(CN)_2^{2-})$.

To demonstrate the similar reductive half-reaction of sulphite oxidase by sulphite, we synthesized the complex, $[(C_4H_9)_4P]_2[Mo^{VI}O_2(mnt)_2]$ (1), by reacting stoichiometric amounts of sodium molybdate and sodium 1,2-dicyanoethylene-1,2-dithiolate (Na_2mnt) in phosphate–citric acid buffer (pH, 6·0–6·6) at 5°C and
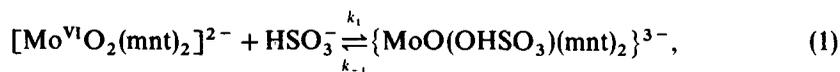
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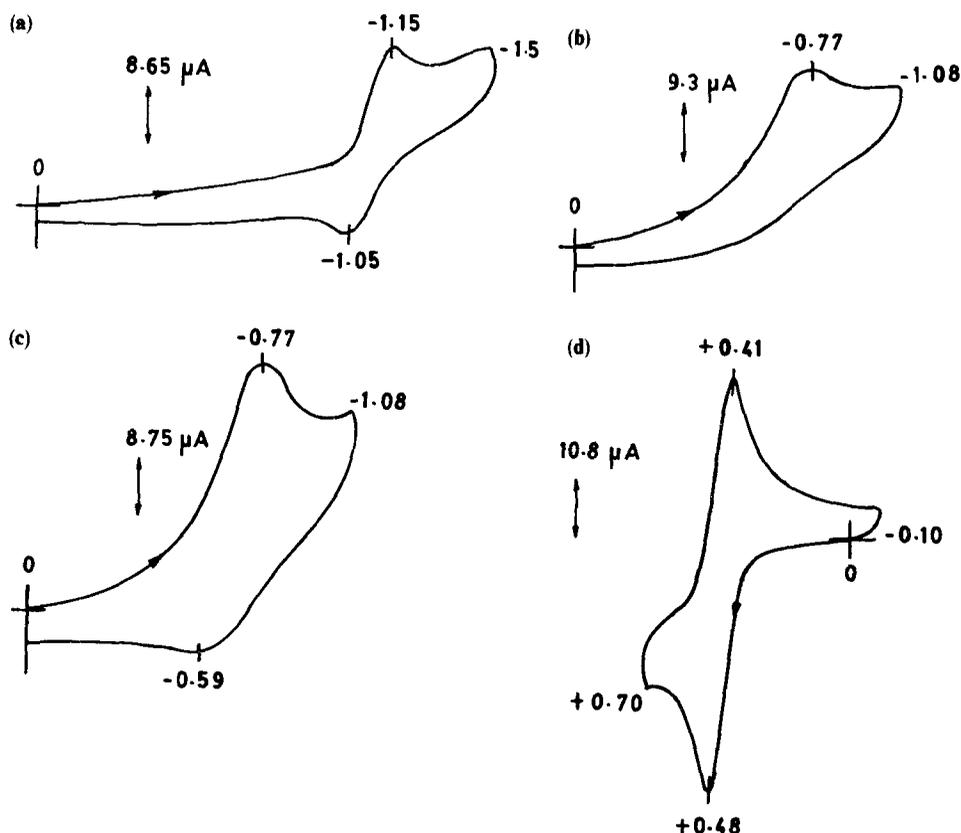
Figure 1. Cyclic voltammograms; $[(C_4H_9)_4P]_2$ $[MoO_2(mnt)_2]$ in (a) CH_3CN, (b) CH_3CN containing $0.13 M$ CH_3COOH, (c) CH_3CN with $0.13 M$ CH_3COOH and $3.5 M$ H_2O. (d) $[(C_4H_9)_4P]_2$ $[MoO(mnt)_2]$ in CH_3CN. Samples were 1 mM each containing $0.1 M$ $[Et_4N][ClO_4]$ and were measured as 100 mV/s at 25°C. Peak potentials *vs* Ag/AgCl are indicated.

precipitating it by adding $[(C_4H_9)_4P]Br$. Recrystallization in CH_3CN-isopropanol-ether yielded 60% of this in analytically pure form as a deep red crystalline solid. (Diamagnetic; negative ion FAB mass spectrum showing isotopic pattern and the molecular anion centred at $m/Z = 410$; IR (KBr) 880 *vs*, 850 *vs*, $v(Mo = 0)$ for *cis* $\{Mo^{VI}O_2\}$ moiety, 2200 *vs*, $v(CN)$ cm^{-1}; ^{13}C NMR 130·62 (C=C), 119 (CN) ppm; λ_{max} (MeCN) 365($\varepsilon = 6660$), 425(6680), 525(1580) nm). Its CV in CH_3CN showed a quasi-reversible reduction at $-1·1$ V *vs* Ag/AgCl (figure 1a). However, in CH_3CN containing $0.13 M$ CH_3COOH the cathodic peak potential dropped to $-0.77V$ (figure 1b). Absence of change in the electronic spectrum in CH_3CN, on acidification with $0.13 M$ CH_3COOH of the complex, attested that we were dealing with the same species. This shift in cathodic peak potential which responded to an isotope effect with $0.13 M$ CH_3COOD suggested a concerted proton-electron transfer process in the transition state of the electrode reaction (Manchanda *et al* 1991). In CH_3CN containing $0.13 M$ CH_3COOH and $3.5 M$ water, the cathodic peak potential did not change $(-0.77 V)$ but the CV showed that the stabilization of the reduced species as the anodic peak could now be detected (figure 1c). Coulometry and EPR confirmed this irreversible reduction as a two-electron process with the absence of any EPR active Mo(V) species

during controlled potential electrolysis in this medium. The brownish-red solution of electrolysis changed to green and this colour change can be obtained by adding SO_3^{2-} into a solution of $[(C_4H_9)_4P]_2[Mo^{VI}O_2(mnt)_2]$ in CH_3CN–CH_3COOH–H_2O. This reaction has been assessed kinetically which exhibits substrate saturation kinetics at sufficient HSO_3^- concentrations demonstrating parallel enzymatic behaviour. These observations are interpreted in terms of the following reactions:

$$[Mo^{VI}O_2(mnt)_2]^{2-} + HSO_3^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \{MoO(OHSO_3)(mnt)_2\}^{3-}, \qquad (1)$$

$$\{MoO(OH SO_3)(mnt)_2\}^{3-} \overset{k_2}{\to} [Mo^{IV}O(mnt)_2]^{2-} + HSO_4^-. \qquad (2)$$

A double-reciprocal plot (Lineweaver and Burk 1934) gives $V_{max}(=k_2) = 8\cdot134 \times 10^{-2}\,s^{-1}$ and apparent $K_m = 1 \times 10^{-2}$ M in CH_3CN:H_2O (1:1), 0·09 M NaCl at 20°C. Quantification of the products in reaction (2) has been made gravimetrically demonstrating 100% conversion of sulphite to sulphate with the two-electron reduction of $[Mo^{VI}O_2(mnt)_2]^{2-}$ to $[Mo^{IV}O(mnt)_2]^{2-}$. The reduced complex anion (2) was directly prepared (in low yield $\sim$ 20–30%) using a similar method of preparation as described for the oxidized species but at lower pH (2–4) or by reacting MoO_3 and the ligand in water, followed by adding a counter cation. The yield was increased to near quantitative in the presence of excess of SO_3^{2-}. (Diamagnetic, negative ion FAB mass spectrum showing isotopic pattern and the molecular anion centred at $m/Z = 394$; IR (KBr) 930 vs, $v(Mo = 0)$, 2200 vs, $v(CN)$ cm^{-1}; ^{13}C NMR 130·49 (C=C), 118·86 (CN) ppm; λ_{max} (MeCN) 372 ($\varepsilon = 4062$), 491 (187), 602 (110) nm). When pyridine–acetic acid–water was used as solvent, $(pyH)_2[Mo^{IV}O(mnt)_2]$ was isolated.

For the demonstration of the oxidative half-reaction we decided to use $K_3[Fe(CN)_6]$ as this has been successfully used to oxidize the molybenum fraction of sulphite oxidase after tryptic cleavage (Johnson and Rajagopalan 1977). When $(pyH)_2[Mo^{IV}O(mnt)_2]$ dissolved in phosphate–citric acid buffer (pH 8), was treated with two equivalents of $K_3[Fe(CN)_6]$ the green solution immediately changed to red-brown. Addition of $[(C_4H_9)_4P]Br$ in excess into the solution separated a red oil mass which on dissolving in minimum amount of CH_3CN and adding isopropanol–ether precipitated analytically pure $[(C_4H_9)_4P]_2[Mo^{VI}O_2(mnt)_2]$ in 10% yield. This reaction thus confirmed the oxidation of the reduced MoO(IV) species to the oxidized $MoO_2(VI)$ species with the incorporation of the additional oxo-group from water. The ferricyanide oxidation is very fast and much of the compound decomposed with the oxidation of the ligand (Simmons et al 1962). In native sulphite oxidase, ferricyanide causes oxidative modification of the site with the loss of molybdenum (Kessler and Rajagopalan 1974). $[Mo^{IV}O(mnt)_2]^{2-}$ in the buffer (pH 8) reacted with O_2 to generate the oxidized species which is reverted back to the reduced species on adding HSO_3^-. However, this oxidation is a slow process associated with some decomposition of the compound. Furthermore, the oxidation of $[Mo^{IV}O(mnt)_2]^{2-}$ by ferricyanide is completely inhibited by prior adding of one equivalent of KCN which is similar to the inhibition by CN^- to reduced sulphite oxidase (Cohen et al 1971).

Thus, these model compounds demonstrate oxidoreductase activity of sulphite oxidase with SO_3^{2-} as electron donor and $[Fe(CN)_6]^{3-}$ as electron acceptor with the participation of water in these redox reactions. The use of protonated water to facilitate SO_3^{2-} oxidation which is possible simply by using HSO_3^- without acid suggest lengthening of at least one Mo=O bond in close association of the hydrogen in HSO_3^-

by hydrogen bonding. Nucleophilic attack by hydroxide at the sulphur with concerted abstraction of $2e$, H^+ by the $MoO_2(VI)$ would complete the reaction (Durant *et al* 1977), or by direct participation of sulphur lone pair of electrons into the activated $Mo = O$ π^* orbital (Durant *et al* 1977; Williams 1978), the exact interactions are yet to be ascertained. Unlike other model systems (Holm 1990), these oxidized and reduced species do not respond to comproportionation reaction when taken together to yield Mo(V) dimer. Tuning of the potential of molybdenum centre by dithiolene coordination is greatly affected by substituent groups attached to dithiolene. Thus, for $[Mo^{IV}O(S_2C_2(COPh)_2)_2]^{2-}$, one-electron irreversible oxidation at $+0.84$ V vs SCE (Ansari *et al* 1987) is drastically changed to a reversible oxidation at $+0.445$ V vs Ag/AgCl for $[Mo^{IV}O(mnt)_2]^{2-}$ (figure 1d). At this stage we feel that the pterin group attached to the dithiolene moiety in the molybdenum cofactor can effectively tune molybdenum potential as pterins participate in reversible one-electron redox reactions (Scrimgeour 1975). An early electronic spectral data for the $[Mo^{IV}O(mnt)_2]^{2-}$ anion synthesized by different methods (McCleverty *et al* 1969) was apparently contaminated with the *tri*-dithiolene complex (McCleverty *et al* 1968). The two weak $d-d$ transitions at 491 ($\varepsilon = 187$) and 602 (110) nm which shifted in buffer (pH 8) suggest that to identify $d-d$ transitions in reduced sulphite oxidase, the molybdenum fragment should be absolutely haeme-free (Johnson and Rajagopalan 1977). The oxidation of (2) by the biological substrate, $(CH_3)_3NO$, showed quantitative conversion to (1) which is noteworthy in the light of trimethylamine N-oxide reductase (Holm 1990); the details of which will be published later.

Acknowledgements

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