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Quantitative transfer of the molybdenum cofactor from xanthine oxidase and from sulphite oxidase to the deficient enzyme of the *nit-1* mutant of *Neurospora crassa* to yield active nitrate reductase

Timothy R. HAWKES* and Robert C. BRAY
School of Chemistry and Molecular Sciences, University of Sussex, Falmer, Brighton BN1 9QJ, U.K.

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An assay method is described for measurement of absolute concentrations of the molybdenum cofactor, based on complementation of the defective nitrate reductase ('apo nitrate reductase') in extracts of the *nit-1* mutant of *Neurospora crassa*. A number of alternative methods are described for preparing, anaerobically, molybdenum-cofactor-containing solutions from sulphite oxidase, xanthine oxidase and desulpho xanthine oxidase. For assay, these were mixed with an excess of extract of the *nit-1* mutant, incubated for 24h at 3.5°C then assayed for NADPH:nitrate reductase activity. In all cases, the specific activity of the molybdenum cofactor, expressed as μmol of NO_2^- formed/min per ng-atom of Mo added from the denatured molybdoenzyme, was 25 ± 4, a value that agrees with the known catalytic activity of the nitrate reductase of wild-type *Neurospora crassa*. This indicates that, under our conditions, there was quantitative transfer of the molybdenum cofactor from denatured molybdoenzyme to yield fully active nitrate reductase. Comparable cofactor assay methods of previous workers, apparently indicating transfer efficiencies of at best a few per cent, have never excluded satisfactorily the possibility that cofactor activity arose, not from stoichiometric constituents of the molybdoenzymes, but from contaminants. The following factors were investigated separately in developing the assay: the efficiency of extraction of the cofactor from the original enzyme, the efficiency of the complementation reaction between cofactor and apo nitrate reductase, and the assay of the resultant nitrate reductase, which must be carried out under non-inhibitory conditions. Though the cofactor is unstable in air ($t_{\frac{1}{2}}$ about 15min at 3.5°C), it is stable when kept anaerobic in the presence of sodium dithionite, in aqueous solution or in dimethyl sulphoxide (activity lost at the rate of about 3%/24h at 20–25°C). Studies of stabilities, and investigations of the effect of added molybdate on the assay, permit conclusions to be drawn about the ligation of molybdenum to the cofactor and about steps in incorporation of the cofactor into the apoenzyme. Though the development of nitrate reductase activity is slow at 3.5°C ($t_{\frac{1}{2}}$ 1.5–3h) the complementation reaction may be carried out in high yield, aerobically. This is ascribed to rapid formation of an air-stable but catalytically inactive complex of the cofactor, as a precursor of the active nitrate reductase. Molybdenum does not dissociate from the reduced cofactor in the presence of dithionite, but may do so, reversibly, with loss of activity, in its absence. Under these latter conditions, only if dithiothreitol was present could the cofactor to be reconstituted by addition of molybdate. This is new evidence indicating strongly the ligation of molybdenum to thiolate groups in the active cofactor.

Molybdenum-containing enzymes such as xanthine oxidase, nitrate reductase and sulphite oxidase (but not nitrogenase; Pienkos *et al.*, 1977) share a common factor (Pateman *et al.*, 1964; Nason *et al.*, 1971), which contains molybdenum (Lee *et al.*, 1974). The molybdenum cofactor, as released by denaturation of any of these enzymes, is capable of

* Present address: Agricultural and Food Research Council Unit of Nitrogen Fixation, University of Sussex, Brighton BN1 9RQ, U.K.

activating the inactive form of the assimilatory nitrate reductase present in extracts of the *nit-1* mutant of the fungus *Neurospora crassa* (Nason *et al.*, 1971) (referred to below simply as '*nit-1* extracts'). The cofactor from various sources can also activate a deficient form of sulphite oxidase purified from the livers of rats grown on a tungsten-containing diet (Johnson *et al.*, 1977).

Structural characterization of the molybdenum cofactor has been hampered by its unstable nature. However, partial characterization of two oxidized fluorescent derivatives has indicated that it contains a 2-substituted thieno[3,2-*g*]pterin derivative, related, both structurally and metabolically, to urathione (Johnson *et al.*, 1980*a*,*b*; Johnson & Rajagopalan, 1982; Ishizuka *et al.*, 1983).

Molybdenum cofactor activity exists in two apparently distinct forms. It occurs in molybdoproteins in stable association with protein, and requiring denaturation, for example by treatment with acid, organic solvents or sodium dodecyl sulphate (Nason *et al.*, 1971; L'vov *et al.*, 1975; Pienkos *et al.*, 1977; Johnson *et al.*, 1980*a*), to effect its release. After such release, the cofactor is unstable, and has been shown by many authors, to lose rapidly, in the presence of oxygen, its ability to activate *nit-1* extracts (Johnson, 1980). The second form of the cofactor, apparently stable to oxygen, occurs, for example, in extracts obtained under non-denaturing conditions from rat liver (Johnson *et al.*, 1977), *Escherichia coli* (Amy & Rajagopalan, 1979) or *N. crassa* (Nason *et al.*, 1970). However, this stability is due to the binding of the cofactor to some carrier protein, since separation by gel filtration of the molybdenum cofactor, in extracts of *E. coli*, from a carrier protein of M_r about 40000 (Amy & Rajagopalan, 1979) yielded free cofactor as unstable as, and indistinguishable from, that liberated from purified molybdoenzymes by denaturation.

Complementation of NADPH:nitrate reductase activity in *nit-1* extracts of *N. crassa* has remained the most widely used assay for the molybdenum cofactor (Johnson, 1980). This mutant, defective in the synthesis of molybdenum cofactor, retains (Nason *et al.*, 1971; Coddington, 1976) the structural gene for nitrate reductase. The product of this gene is a protein with a sedimentation coefficient of 4.5S, unable to reduce nitrate but retaining the NADPH:cytochrome *c* reductase activity associated with the holoenzyme (Garrett & Nason, 1969). *In vitro*, molybdenum cofactor is reported (Nason *et al.*, 1970; Lee *et al.*, 1974) to associate with the 4.5S promoters to form 7.9S dimers indistinguishable from the wild-type nitrate reductase with respect to relative molecular mass and substrate affinities. However, the activation of *nit-1* extracts has not, as yet, been placed on a fully quantitative basis for assay of the molybdenum cofactor, and indeed such assays, though widely used, have been described in a review (Johnson, 1980) as being 'rather unquantitative'. (A lack of adequate quantification applies to other features of work on the molybdenum cofactor; e.g. direct measurements of the pterin content of molybdoenzymes have not been reported.)

We describe a quantitative assay for the molybdenum cofactor, based on the activation of *nit-1* extracts. Development of the assay method (described in preliminary form by Hawkes & Bray, 1983) involved re-investigating all factors affecting the activation reaction, including effects of oxygen, of added molybdate (Johnson, 1980) and of NADPH (McKenna *et al.*, 1974; Claassen *et al.*, 1982). The techniques that we describe for handling the cofactor without loss of activity, as well as availability of an assay method yielding absolute rather than relative cofactor concentrations, should assist substantially in further work on its purification and characterization.

Materials and methods

General

Chemicals and biochemicals were generally the purest grades available from Sigma Chemical Co., Poole, Dorset, U.K. and BDH Chemicals, Poole, Dorset, U.K. Dimethyl sulphoxide was distilled as described by McCord & Fridovich (1969) and kept under N_2. Bactoagar was from Difco Laboratories, Detroit, MI, U.S.A., DEAE-cellulose (DE-52) was from Whatman, Maidstone, Kent, U.K., and Sephadex G-25 was from Pharmacia, Hounslow, Middx., U.K. The wild-type strain of *Neurospora crassa* (allele no. STA 4) and the *nit-1* mutant (allele no. 34537) were gifts from the Fungal Genetics Stock Center, Humboldt State University Foundation, Arcata, CA, U.S.A.

Protein was determined by using the biuret method of Gornall *et al.* (1949), with bovine serum albumin as standard.

Maintenance and growth of Neurospora crassa

Strains of *N. crassa* were maintained on 2% (w/v) Bactoagar slopes and grown in liquid culture by following in detail the procedure of Nason *et al.* (1970). Wild-type mycelia were harvested, uninduced for nitrate reductase, from the NH_4Cl-containing growth medium. The *nit-1* mutant cells were transferred to NO_3^--containing induction medium before being harvested. Mycelia were stored at −140°C.

Preparation of extracts from Neurospora crassa

The *nit-1* mutant cells were broken by grinding with a mortar and pestle cooled with liquid N_2.

After the cells had been reduced to a fine powder, approx. 5 vol. of 35 mM-potassium phosphate buffer, pH 7.4 in water, containing 30% (v/v) ethylene glycol, 4% (v/v) propan-2-ol, 1 mM-EDTA, 0.7 mM-dithiothreitol and 0.4 mM-phenylmethanesulphonyl fluoride (as a proteinase inhibitor) was added. (This mixture is referred to below as buffer A.) The mixture was then blended for 2 min, at full speed in an Ato-Mix blender (at about −10°C). The suspension was centrifuged at 25000$g_{av.}$ for 45 min at approx. −4°C, and the supernatant solution was frozen in liquid N_2 and stored in bead form at about −140°C.

Extracts of uninduced wild-type cells were similarly prepared but without centrifugation.

Partial purification of nit-1 extracts

The *nit-1* extracts, from approx. 500 g of cells, were diluted 5-fold in buffer A (to about 8 litres) at −10°C and applied to a DEAE-cellulose column (16 cm × 8 cm diam.), at the same temperature. Washings with approx. 800 ml of the same buffer were discarded, and then the fraction (approx. 500 ml) containing 'apo nitrate reductase' activity (see below for assay) was eluted with buffer A containing 0.3 M-KCl. An equal volume of saturated $(NH_4)_2SO_4$ was added at 0°C, and the mixture was centrifuged (also at 0°C). The clear supernatant solution was discarded, and the pellet was resuspended in 40–50 ml of 25 mM-potassium phosphate buffer, pH 7.3, containing 1 mM-EDTA, 0.7 mM-dithiothreitol, 2% (v/v) propan-1-ol and 0.2 mM-phenylmethanesulphonyl fluoride. Residual ethylene glycol and $(NH_4)_2SO_4$ were removed by gel filtration at 2°C, on Sephadex G-25 equilibrated with the above buffer. The product was then frozen and stored in bead form at −140°C. From a typical purification, crude extract containing 16 g of protein yielded 1.5 g of the partly purified final product. Specific activities (measured after saturation with the molybdenum cofactor; see below) before and after purification were 0.032 and 0.186 μmol of NO_2^- formed/min per mg of protein respectively. The *nit-1* extracts had no measurable NADPH : nitrate reductase activity before the addition of molybdenum cofactor.

FAD-dependent NADPH : cytochrome *c* reductase activity of *nit-1* extracts was measured spectrophotometrically at 550 nm, as described by Garrett & Nason (1969) but with 0.5 mM-EDTA and 350 μM-NADPH. Activity, measured as total activity with FAD added, minus activity without FAD, was found to co-purify with apo nitrate reductase. Our final product from the above purification had an activity of 2.6 μmol of cytochrome *c* reduced/min per mg of protein.

Measurement of NADPH : nitrate reductase activity

Two methods were used. Continuous monitoring at 340 nm of the disappearance of NADPH at 25°C (Garrett & Nason, 1969) was useful to provide a standard by which to check the colorimetric method (below) and to measure initial rates in the determination of the K_m for NO_3^-. Colorimetric determination of NO_2^- in stopped assays was used routinely and was modified from the method of Garrett & Nason (1969). Enzyme was added to a mixture (final vol. 0.5 ml) containing 10 μM-FAD, 0.35 mM-NADPH, 0.1 M-potassium phosphate buffer, pH 7.2, 30 mM-KNO_3 and 5 mM-Na_2SO_3 (an inhibitor of nitrite reductase activity; Amy & Rajagopalan, 1979). The mixture was incubated at 25°C for such a time (10 to 60 min) as to produce between 5 and 60 nmol of NO_2^-, and then the reaction was stopped by the addition of 0.5 ml of 1% (v/v) sulphanilic acid in 20% (w/v) HCl, immediately followed by 0.5 ml of 0.12% napthylethylenediamine dihydrochloride and 0.5 ml of water. After approx. 15 min, the solution was centrifuged to remove turbidity and the absorbance was measured at 540 nm. (Centrifugation did not remove appreciable amounts of the chromophore, since the increment in absorbance due to turbidity was independent of the chromophore concentration.) NO_2^- formation was determined by comparison with standards containing all constituents other than enzyme and between 0 and 60 nmol of NO_2^-. NADPH (but not $NADP^+$) inhibited the colorimetric determination of NO_2^- (Nicholas & Nason, 1957). Inhibition was dependent on the concentration of NADPH (e.g. 0.27 mM-NADPH caused approx. 50% decrease in A_{540}). Since NADPH was depleted during the assay, the amount of NO_2^- formed could not be accurately determined by comparison with standards containing the initial concentration of NADPH. Depletion of NADPH thus resulted in non-linear progress curves with an apparent increase in NADPH : nitrate reductase activity at longer times. This artifact was removed by routine inclusion in the assay mixture of an NADPH-regenerating system [8 mM-DL-isocitrate and 0.43 unit of pig heart isocitrate dehydrogenase (Sigma type IV)]. The colorimetric assay was inhibited by excess of the fungal extract, and therefore no more than 0.25 mg of protein was added.

The continuous and colorimetric assays gave results in good agreement with one another. In agreement with data in the literature for wild-type and for assembled enzyme (Garrett & Nason, 1969; Horner, 1983), K_m for nitrate determined by the continuous assay was about 230 μM, for nitrate reductase formed (under conditions as in the standard cofactor assay described below) from *nit-1*

extracts and molybdenum cofactor from xanthine oxidase.

Molybdoproteins

Sulphite oxidase was partially purified from chicken liver (Lamy *et al.*, 1980) to a specific activity of about 900units/mg. Xanthine oxidase was purified and assayed as described by Hart *et al.* (1970) (see also Bray, 1975, 1982), by using the salicylate denaturation procedure, to give preparations that were 68–81% functional. Concentrations were determined either from colorimetric Mo analyses (Hart *et al.*, 1970) or from measurements of A_{450}. Desulpho xanthine oxidase (less than 1% functional) was prepared by cyanide treatment of the active enzyme, followed by gel filtration (Massey & Edmondson, 1970).

Molybdenum-cofactor-containing solutions

Molybdenum cofactor was prepared, kept and manipulated at 20–25°C, under O_2-free conditions (see below), and all solutions of it, and used in preparing it, contained 1–2mM-$Na_2S_2O_4$.

Xanthine oxidase (or sulphite oxidase), in 50mM-Bicine [*NN*-bis-(2-hydroxyethyl)glycine]/NaOH buffer, pH8.2, of known Mo content, was denatured by addition of the enzyme to dimethyl sulphoxide containing small amounts of water and 5mM-potassium phosphate buffer (pH7.8 in water) to give a final water concentration of 3–6% (v/v). In dilute cofactor solutions, as normally prepared, the concentration of Mo was about 1 μM and the denatured protein formed no obvious precipitate. In preparing more-concentrated solutions, large amounts of denatured xanthine oxidase remained undissolved and were removed by low-speed centrifugation to give a clear solution of the cofactor. The maximum concentration of Mo (by colorimetric analysis) in such supernatant solutions was about 15 μM, the balance of any remaining Mo being associated with the pellet.

As alternatives to the above method, where indicated in the text, solutions of cofactor (normally about 1 μM-Mo) were prepared by one of the following procedures. (1) Xanthine oxidase was diluted into aqueous 25mM-potassium phosphate buffer, pH7.4, containing 1mM-EDTA and 6M-guanidinium chloride. Or, (2) xanthine oxidase was diluted into aq. 25mM-potassium phosphate buffer, pH7.4, containing 1mM-EDTA and 1% (w/v) sodium dodecyl sulphate, heated at 80±5°C for 3min and cooled to 0°C: most of the protein and potassium dodecyl sulphate were precipitated and were removed by low-speed centrifugation. Alternatively, (3) the enzyme was diluted into aq. 25mM-potassium phosphate buffer, pH7.4, containing 1mM-EDTA, heated at 80±5°C for 3min and centrifuged to remove denatured protein. Xanthine oxidase was inactivated by all three procedures, and the molybdenum cofactor solutions had no measureable NADPH:nitrate reductase activity before complementation with *nit-1* extract.

O_2-free solutions

Solutions of buffers and solvents were made O_2-free by bubbling with highly purified N_2 gas. Solutions of proteins were made partly anaerobic by a series of gentle evacuations and replacements of the atmosphere above them with N_2 gas. Where appropriate (e.g. in preparing molybdenum cofactor), $Na_2S_2O_4$ was added to the solution. In other cases (e.g. *nit-1* extracts) 0.2M-glucose, 0.2mg of catalase (Boehringer, crystalline suspension)/ml and 0.2mg of glucose oxidase (Boehringer, fungal grade II)/ml were added. Solutions were kept under an N_2 atmosphere in glass vials with rubber stoppers. For storage longer than about 1h, vials were further enclosed in an outer vessel also filled with N_2 gas and stoppered with a rubber seal. This outer vessel contained $Na_2S_2O_4$-containing buffer to scavenge any incoming O_2. Transfers to and from anaerobic solutions were made by using micro-syringes fitted with stainless-steel needles that could penetrate both rubber seals.

Standard procedure for assay of the molybdenum cofactor

Various quantities of molybdenum cofactor were mixed with a fixed quantity of *nit-1* extract, partially purified as described above and assayed as described below. The preferred concentration (see below) of *nit-1* extract gave an activity of 1–4μmol of NO_2^- formed/min per ml of final solution. The *nit-1* extract was diluted with buffer to a fixed final volume (normally 0.3 to 1.0ml) in a series of small vials. The buffer was 25mM-potassium phosphate, pH7.4, containing 1mM-EDTA, 0.7mM-dithiothreitol and 10mM-Na_2MoO_4. (Note that molybdate and dithiothreitol were omitted from some assays, as indicated below.) Molybdenum-cofactor-containing solutions were transferred anaerobically to the vials via a small-volume micro-syringe. This was always the last addition to be made; anaerobiosis was not required subsequently, owing to stabilization of the cofactor by the *nit-1* extract (see the Results section). Complementation was achieved by incubating the samples aerobically for 24h at 3.5°C. The final concentration of dimethyl sulphoxide never exceeded 1% (v/v). After the incubation, samples (normally 5 to 10μl) were removed for colorimetric NADPH:nitrate reductase assays, as described above.

Assay of 'apo nitrate reductase' in nit-1 extracts

This was basically the reverse of the cofactor assay described above. Cofactor-deficient, in-

active, nitrate reductase ('apo nitrate reductase') in *nit-1* extracts was assayed by conversion into the holoenzyme by addition of excess molybdenum cofactor [in this case, supplied by extracts of wild-type *N. crassa*, grown uninduced for nitrate reductase (Nason *et al.*, 1970)], with subsequent assay for NADPH:nitrate reductase activity. A range of volumes of *nit-1* extract (2–50 μl) was added to tubes containing 200 μl of uninduced wild-type extract and 10 mM-Na_2MoO_4. After incubation for 24 h at 3.5°C, samples were removed for colorimetric NADPH:nitrate reductase assays. The specific activity of the *nit-1* extract was calculated from tubes over the range where molybdenum cofactor was in excess, and activity (μmol of NO_2^- formed/min) was proportional to the amount (mg) of *nit-1* extract.

Calculation from data in the literature of the efficiency of transfer of cofactor from xanthine oxidase to yield active nitrate reductase by the complementation of nit-1 extracts

These calculations are based (see the Results section, below) on comparison of the specific activity of the nitrate reductase produced (as μmol of NO_2^- formed/min per ng-atom of Mo in the xanthine oxidase), relative to the specific activity per Mo of wild-type nitrate reductase. Two examples of such calculations follow.

According to Lee *et al.* (1974) (their Table 1), xanthine oxidase (51.5 mg/ml) was diluted 2000-fold, then 2.1-fold with *nit-1* extract, and finally 0.1 ml was used for nitrate reductase assay. This corresponds to 1.23 μg of xanthine oxidase. This was purified by the procedure of Nelson & Handler (1968), and should therefore have been, say, 95% pure, but perhaps containing 30% of demolybdo enzyme. On these assumptions, with M_r 283000 (Bray, 1975), the molybdenum content of the xanthine oxidase would be 4.7 ng-atom of Mo/mg of protein and the quantity of Mo used for the assay would be 5.8 pg-atom. The highest nitrate reductase activity, after complementation with *nit-1* extract, reported by Lee *et al.* (1974) was 68.2 nmol of NO_2^-/10 min. This corresponds to 1.18 μmol of NO_2^-/min per ng-atom of Mo of xanthine oxidase. This is 4.6% of the activity (26 μmol of NO_2^-/min per ng-atom of Mo) of the wild-type enzyme (see below). Thus the efficiency of cofactor transfer was 4.6%.

According to Mendel (1983), 1 mg of purified xanthine oxidase gave a molybdenum-cofactor activity of 393 nmol of NO_2^-/min in normal nitrate reductase assays. Xanthine oxidase was apparently (cf. Alikulov *et al.*, 1980) purified by a modification, said to yield homogeneous material, of the procedure of Hart *et al.* (1970). Making, rather arbitrarily, the assumptions used above about the Mo content, we arrive at an activity of 0.08 μmol of NO_2^-/min per ng-atom of Mo of xanthine oxidase, corresponding to 0.3% of the activity of the wild-type enzyme, and thus to a transfer efficiency of 0.3%.

Results

Measurement of 'cofactor activity'

It is first necessary to define 'cofactor activity', as measured by the complementation of *nit-1* extracts to yield NADPH:nitrate reductase activity. The procedure as detailed in the Materials and methods section involved incubation of molybdenum cofactor with a partially purified *nit-1* extract, to permit the complementation reaction to occur, followed by colorimetric assay of the nitrate reductase so formed. Molybdenum cofactor was prepared as a solution of denatured molybdoenzyme. Quantities of cofactor are expressed in ng-atoms of Mo (thus assuming the equivalence of cofactor with Mo in the molybdoenzyme used). For cofactor assays, varying quantities of cofactor were incubated with a fixed quantity of *nit-1* extract. Typical assays are illustrated in Fig. 1 (considered in more detail below). For small additions of cofactor to *nit-1* extract (Fig. 1 inset) the amount of NADPH:nitrate reductase activity (μmol of NO_2^- formed/min) that was formed depended linearly on the amount of molybdenum cofactor added (ng-atoms of Mo). The slope of such graphs yielded our estimate of the 'cofactor activity' expressed in μmol of NO_2^- formed/min per ng-atom of Mo.

To develop the basic procedure into the final quantitative assay, we sought conditions where the cofactor activity was at a maximum. For quantitative transfer, on a one-to-one basis, of the molybdenum cofactor from denatured molybdoenzyme to yield fully active nitrate reductase, cofactor activity would be expected to approach a theoretical maximum of 26 ± 6 μmol of NO_2^- formed/min per ng-atom of Mo, corresponding to the catalytic activity of the nitrate reductase from wild-type *N. crassa* [calculated from the data of Jacob & Orme-Johnson (1980) and Pan & Nason (1978)].

In the standard assay (see the Materials and methods section) excess molybdate was added for incubation of *nit-1* extracts with the molybdenum cofactor. The activity measured is therefore referred to as 'cofactor activity' (rather than 'molybdenum cofactor activity'), since it is limited by cofactor and not by molybdenum.

Some of the experiments we carried out in developing our standard assay are described below. Unless otherwise indicated, all results refer to cofactor prepared from active xanthine oxidase.

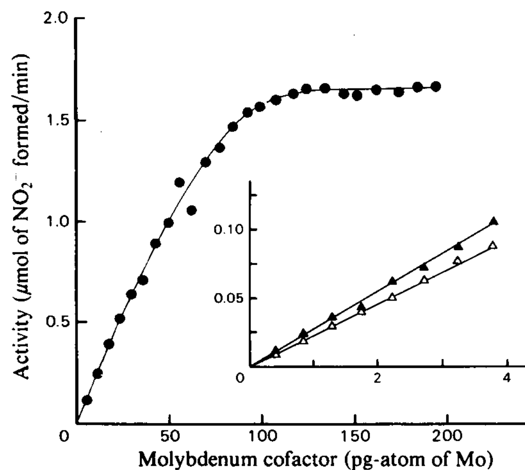


Fig. 1. *Effect of increasing amounts of molybdenum cofactor on the nitrate reductase activity formed from a fixed amount of nit-1 extract*

Nitrate reductase activity was measured after aerobic incubation for 24h at 3.5°C of various quantities of molybdenum cofactor (denatured xanthine oxidase, measured in pg-atom of Mo) with a fixed quantity of partly purified *nit-1* extract. The main graph shows the complete titration curve. For the assay of cofactor activity, only the slope of the initial linear part of the curve is required. This region is shown (for separate experiments) on an expanded scale in the inset graphs. Molybdate and dithiothreitol were present at the following concentrations (mM) respectively: ●, 10, 0.7; ▲, 10, 0.2; △, 0, 0.2; other conditions were as in the standard assay procedure (see the Materials and methods section). In all experiments the concentration of apo nitrate reductase (measured after saturation with excess molybdenum cofactor) corresponded to 1.67 μmol of NO_2^- formed/min per ml. The initial slope in the main graph (corresponding to the cofactor activity in this experiment) was 22 μmol of NO_2^- formed/min per ng-atom of Mo.

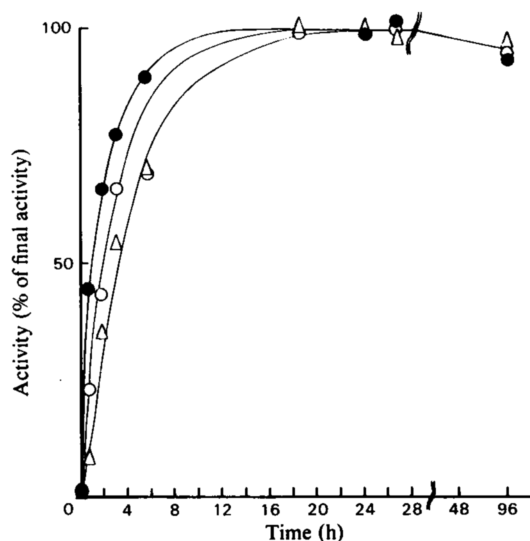


Fig. 2. *Time course of development of nitrate reductase activity*

Various concentrations of molybdenum cofactor (denatured xanthine oxidase) and *nit-1* extract were incubated aerobically at 3.5°C, and samples were removed at intervals via a pre-cooled micro-syringe, for immediate nitrate reductase activity measurements (see the Materials and methods section). Points at time zero correspond to *nit-1* extract and molybdenum cofactor added separately to the NADPH:nitrate reductase assay mixture; 100% on the vertical scale corresponds to the maximum activity in each experiment. Concentrations of molybdenum cofactor (nM-Mo) and of apo nitrate reductase (μmol of NO_2^- formed/min per ml) were respectively: ●, 4.4, 2.40; ○, 4.4, 0.50; △, 1.0, 0.50. [Additional experiments (not illustrated) carried out with the use of the 340nm assay confirmed that the complementation reaction was effectively quenched by the dilution procedure used.]

Optimum conditions for the activation of nit-1 extracts by molybdenum cofactor

Quantitative transfer of the cofactor from xanthine oxidase to nitrate reductase would be expected only under conditions where both the reagents (cofactor and apo nitrate reductase) and the product (holo nitrate reductase) were adequately stable, and under which, furthermore, all the apoenzyme was activatable if excess cofactor was used. Previous workers have carried out the reaction of *nit-1* extracts with molybdenum cofactor at room temperature (Johnson, 1980). However, we found that at 20–25°C *nit-1* extracts were unstable and lost virtually all of their ability to be activated by molybdenum cofactor within 1h. Furthermore, at this temperature, our estimates of cofactor activity were low and irreproducible. We therefore performed complementation experiments at 3.5°C, a temperature at which *nit-1* extracts were relatively stable (less than 25% of the activity lost after 24h). (Stability of the cofactor in the assay is considered later below.)

To determine the rate at which holo nitrate reductase was formed at 3.5°C, molybdenum cofactor was mixed with *nit-1* extract and, at intervals, samples were removed for NADPH:nitrate reductase assays. From the data in Fig. 2 and a number of similar experiments, it is clear that, even at the lowest concentrations of apo nitrate reductase (0.2 μmol of NO_2^- formed/min per ml) and of molybdenum cofactor (1.0nM-Mo), the reaction is essentially complete within 24h, and furthermore that the product, holonitrate reduct-

ase, is stable over this time span. Increasing the concentrations of the two reactants accelerated the formation of nitrate reductase (Fig. 2), but far less than would be expected for a second-order reaction.

The optimal pH for the complementation reaction in dilute potassium phosphate buffers was within the range 7.0–7.5. At lower or higher pH values the estimate of the cofactor activity was decreased (by about 65% at pH6.45 and by 25% at pH7.90). Increasing the concentration of phosphate buffer (normally 25mM at pH7.4) also inhibited the cofactor assay (by about 20% at 0.2M). Dimethyl sulphoxide was not inhibitory at concentrations up to 1% (v/v) in the complementation reaction.

Fig. 1 (main graph) describes the activation of a preparation of *nit-1* extract by increasing amounts of molybdenum cofactor. An initial sharp increase of activity (presumably where the molybdenum cofactor is bound stoichiometrically to apo nitrate reductase) is followed by a plateau region (where apo nitrate reductase has been saturated with molybdenum cofactor). The curve has the form of a normal titration curve describing the tight binding of a ligand to a protein. With saturating amounts of molybdenum cofactor (from xanthine oxidase), the maximum specific activity obtained with this particular *nit-1* extract (0.060μmol of NO_2^- formed-/min per mg of protein) was in good agreement with the value obtained (0.064) when extracts of uninduced *N. crassa* (see the Materials and methods section) were used as the source of cofactor. The *nit-1* extract had an FAD-dependent NADPH:cytochrome *c* reductase activity of 0.82μmol reduced/min per mg. Thus this activity was 13 times higher than the NADPH:nitrate reductase activity (after saturation with molybdenum cofactor). This information gives an independent measure of the amount of apo nitrate reductase in the extract. A similar ratio of the two activities (cytochrome *c* reductase activity 12–18 times greater than nitrate reductase activity) has been reported in crude extracts and in purified nitrate reductase from wild-type cells (Garrett & Nason, 1969). The data thus indicate that, under our conditions, with an excess of the molybdenum cofactor, certainly most of the apo nitrate reductase in the *nit-1* extracts is converted into the holoenzyme.

Our primary concern was, however, with the efficiency with which limiting amounts of cofactor were incorporated into apo nitrate reductase, as indicated by the measured cofactor activity. This activity, measured by the slope of the main graph in Fig. 1, was 22μmol of NO_2^- formed/min per ng-atom of Mo. In a series of experiments, with similar conditions of complementation, it was found

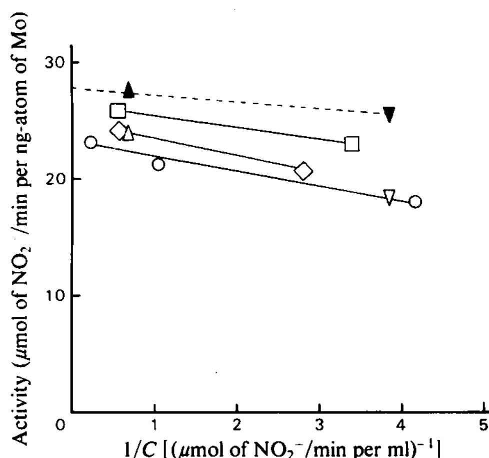


Fig. 3. *Effects on the estimated cofactor activity of O_2 and of the concentration of apo nitrate reductase*

The specific activities of molybdenum-cofactor-containing extracts from xanthine oxidase were measured by complementation of *nit-1* extracts, generally as in the standard assy procedure (Materials and methods section). Cofactor activity is plotted against the reciprocal of the concentration (*C*) of apo nitrate reductase. Different-shaped symbols each refer to results obtained on the same day. The ▲ and ▼ symbols refer to anaerobic complementation (see the Materials and methods section) and the □, ○, ◇, △ and ▽ symbols to aerobic complementation. Concentrations of apo nitrate reductase (μmol of NO_2^- formed/min per ml) were measured as described in the Materials and methods section. The size of the samples used in the NADPH:nitrate reductase assays was adjusted so that comparable amounts of nitrate reductase activity were measured in all experiments.

(Fig. 3) that measured cofactor activity increased somewhat as the concentration of apo nitrate reductase was increased. (It was for this reason that we partly purified and concentrated out *nit-1* extracts.) Thus activities decreased with increasing values of $1/C$, the reciprocal of the apo nitrate reductase concentration, the decrease amounting, e.g. in one case, to 22% for a 16-fold change of concentration. From all the data (for aerobic incubation) shown in Fig. 3, the average cofactor specific activity, extrapolated to an infinite concentration of apo nitrate reductase, was 25$\pm$2.5μmol of NO_2^- formed/min per ng-atom of Mo.

This value seemed surprisingly high since, when incubated on its own under the aerobic conditions used in these assays, the cofactor is unstable (samples losing about 50% of their activity within 15min; see below, Fig. 4 and Table 1, Expts. 23 and 24) on the time scale of the complementation

reaction (see Fig. 2). Conceivably, both our measurement of the cofactor activity and the published catalytic activity of *N. crassa* nitrate reductase were serious underestimates. We therefore tried incubating molybdenum cofactor with *nit-1* extracts under anaerobic conditions where the cofactor would (see below) remain stable. $Na_2S_2O_4$ (1 mM) inhibited the complementation reaction. We therefore maintained *nit-1* extracts anaerobic by using glucose, glucose oxidase and catalase (see the Materials and methods section). This procedure made solutions sufficiently anaerobic to stabilize the cofactor (see below; less than 15% of the activity was lost after 3h at 3.5°C, according to Table 1, Expt. 22). Control experiments indicated that individually these additives did not affect the complementation.

The use of anaerobic conditions of complementation resulted in slightly increased estimates of cofactor activity (broken line in Fig. 3), compared with the values obtained under aerobic conditions, with little, if any, dependence on the concentration of apo nitrate reductase. Thus the activity of $27 \pm 2.0\,\mu$mol of NO_2^- formed/min per ng-atom of Mo from the anaerobic experiments of Fig. 3 was taken to be the maximum ('true') activity of the cofactor from xanthine oxidase. This value is indistinguishable from the catalytic activity of the wild-type nitrate reductase ($26 \pm 6\,\mu$mol of NO_2^- formed/min per ng-atom of Mo), the maximum value that could be expected.

It should be noted that, anaerobically, complementation followed a first-order time course up to about 97% completion (results not shown). Conversely, aerobically, particularly at low concentrations of *nit-1* extract, semi-logarithmic plots for experiments such as those in Fig. 2 indicated reaction rates decreasing with time.

For routine cofactor assays, anaerobic working or extrapolation to infinite apo nitrate reductase concentration were not convenient. Concentrations specified for the standard assay (Materials and methods section) are such, however, that, even without these refinements, within the preferred *nit-1* extract concentration range, no greater than 10–20% diminution of cofactor activity is expected (cf. Fig. 3).

Effect of NADPH on the complementation reaction

Previous workers (Johnson, 1980; Alikulov *et al.*, 1980; Claassen *et al.*, 1982) have often included NADPH when incubating *nit-1* extracts with the molybdenum cofactor, since this was said to stimulate the cofactor activity (McKenna *et al.*, 1974). In contrast, under our conditions (standard assays as described in the Materials and methods section, under aerobic conditions and including molybdate), including 0.5mM-NADPH in the complementation reaction mixture led to a 35% decrease in the cofactor activity.

Comparison of different molybdoenzymes as sources of molybdenum cofactor and of different methods of extracting the cofactor

Samples of molybdenum cofactor were prepared from the different enzymes as described in the Materials and methods section and assayed by the standard procedure. Specific activities of 23.5 ± 3.0, 20.5 and $28.3\,\mu$mol of NO_2^- formed/min per ng-atom of Mo were obtained for cofactor from xanthine oxidase, desulpho xanthine oxidase and sulphite oxidase respectively. The last two values refer to single preparations only, and the data probably do not indicate any significant differences between the three sources of cofactor.

In a further experiment, molybdenum-cofactor-containing solutions, prepared by diluting xanthine oxidase to a final concentration of about $1\,\mu$M-Mo in dimethyl sulphoxide, guanidinium chloride or sodium dodecyl sulphate (see the Materials and methods section) had indistinguishable cofactor activities of $25.0 \pm 1.0\,\mu$mol of NO_2^- formed/min per ng-atom of Mo. [For the extract in guanidinium chloride, correction had to be made for inhibition (30%) caused by the small amount (approx. 30mM) of this reagent carried over into the complementation reaction.] However, heat treatment (see the Materials and methods section) of a solution of xanthine oxidase (also about $1\,\mu$M-Mo) released into solution less than one-third of the cofactor activity released by the other three treatments.

Stability of the active cofactor and its association with molybdenum

It has been widely reported (Johnson, 1980) that the cofactor activity is stimulated by molybdate, and all the assays we have described thus far included 10mM-molybdate during complementation with *nit-1* extracts. When this was omitted the cofactor activity was diminished (cf. Fig. 1 inset). Stimulation required relatively high concentrations of molybdate (10mM rather than 0.1 mM; see also Lee *et al.*, 1974), and this was effective only at the start of the 24h complementation period. Stimulation is presumably due to the reconstitution by molybdate of demolybdo, but otherwise intact, cofactor molecules. Thus the degree of stimulation by molybdate should provide an index of the proportion of such molecules that are present. In the experiments described in Table 1 and Fig. 4 we investigated effects of variations in the conditions under which molybdenum cofactor was stored ('preincubation') and under which it was subsequently assayed by complementation with *nit-1* extract. We measured both the total cofactor

Table 1. *Effects of various conditions, during storage and during the complementation reaction with nit-1 extract, on the measured activity of the molybdenum cofactor*

Molybdenum cofactor (prepared from xanthine oxidase by treatment with $Na_2S_2O_4$-containing dimethyl sulphoxide as described in the Materials and methods section) was stored or preincubated under various conditions as indicated. It was then assayed, by complementation with *nit-1* extract, either under the conditions of the standard assay (in air and with 0.7 mM-dithiothreitol and 10 mM-molybdate) or under other conditions, as indicated. Activities are expressed as percentages of that of freshly prepared molybdenum cofactor, assayed under standard conditions. The percentage dependence on molybdate is expressed as $100[1-(A_0/A_m)]$, where A_0 and A_m are respectively activities without and with molybdate. Errors are the range of values where several experiments were carried out, or for single experiments, the range of possible slopes for assay curves such as those in Fig. 1 (inset). A minus sign in the 'Air' column indicates anaerobic conditions (see the Materials and methods section). 'DTT' is dithiothreitol; one plus sign indicates that it was present, initially, at 0.05–0.15 mM; two plus signs indicate 0.7–1.2 mM. The concentration of molybdenum cofactor in dimethyl sulphoxide (Me_2SO) was 250–800 nM; in the aqueous preincubations it was 6–20 nM.

Expt. no.	Preincubation conditions: Solvent	Air	$S_2O_4^{2-}$	DTT	Temp. (°C)	Time	Conditions during complementation with *nit-1* extract: Air	DTT	Activity (% of control)	Dependence on MoO_4^{2-} (%)
1-6*	Me_2SO	−	+	−	20-25	2h†	+	++	100*	30±12
7-13	Me_2SO	−	+	−	20-25	2h†	+	+	91±9	17±7
14	Me_2SO	−	+	−	20-25	2h†	+	−	35±5¶	0±5
15, 16	Me_2SO	−	+	−	20-25	2h†	−‡	++	116±7‖	44±4
17, 18	Me_2SO	−	+	−	20-25	7 days	+	+	74±6	14±3
19	Me_2SO	+	−	−	20-25	1 day	+	++	0	−
20, 21	Aq§	−	+	−	3.5	1 day	+	+	87±8	15±4
22	Aq§	−‡	−	++	3.5	3h	+	+	82±9	50±10
23, 24	Aq§	+	−	++	3.5	15min	+	++	55±10	53±10
25, 26	Aq§	+	−	−	3.5	15min	+	+	20±5	17±5

* Control for other experiments: freshly prepared molybdenum cofactor assayed under standard conditions.
† Freshly prepared molybdenum cofactor.
‡ Maintained anaerobic with glucose oxidase (see the Materials and methods section).
§ The solvent was 25 mM-potassium phosphate buffer, pH 7.4, 1 mM-EDTA and less than 1% dimethyl sulphoxide.
‖ From the data in Fig. 3.
¶ The *nit-1* extract was gel-filtered to remove dithiothreitol.

activity and its dependence on molybdate (on a scale such that zero corresponds to equal activities with and without molybdate and '100% dependence' corresponds to no activity without added molybdate).

Molybdenum cofactor kept, as prepared, in the presence of $Na_2S_2O_4$ was substantially stable with respect to both cofactor activity and dissociation of molybdenum. (In Table 1, comparing Expts. 7–13 with Expts. 17, 18, 20 and 21 shows less than 4% loss of total cofactor activity/day, with no progressive increase in its dependence on molybdate.) As noted above, the complementation reaction used in these assays could not, unfortunately, also be carried out with $Na_2S_2O_4$ present. Thus we take the backbround molybdate-dependencies observed (17% in Expts. 7–13 and 30% in Expts. 1–6) to arise from dissociation of molybdenum occurring during the complementation process (see below). The molybdenum cofactor, when kept under aerobic conditions (i.e. as for complementation in the standard assay but without *nit-1* extract), lost activity rapidly (see Fig. 4 and Table 1, Expts. 19 and 23–26). With progressive degradation residual activity became almost completely molybdate-dependent (up to 80%; compare Fig. 4 ▲ and △). This was not the case, however, when dithiothreitol was absent (compare Fig. 4 ● and ○). Thus, although aerobic degradation led to loss of molybdenum, dithiothreitol was required then, to preserve ability of the cofactor to be re-activated by the subsequent addition of molybdate. This phenomenon is also apparent in the slight increase (from 17 to 30%) in the molybdate-dependence of the activity of similar samples of molybdenum cofactor upon increasing the concentration of dithiothreitol during complementation (compare in Table 1 Expts. 1–6 with Expts. 7–13). In the extreme case, no molybdate-dependence was observed when dithiothreitol was omitted (Table 1, Expt. 14). (However, the total cofactor activity was low in this experiment, possibly owing to instability of apo nitrate reductase in the absence of dithiothreitol.)

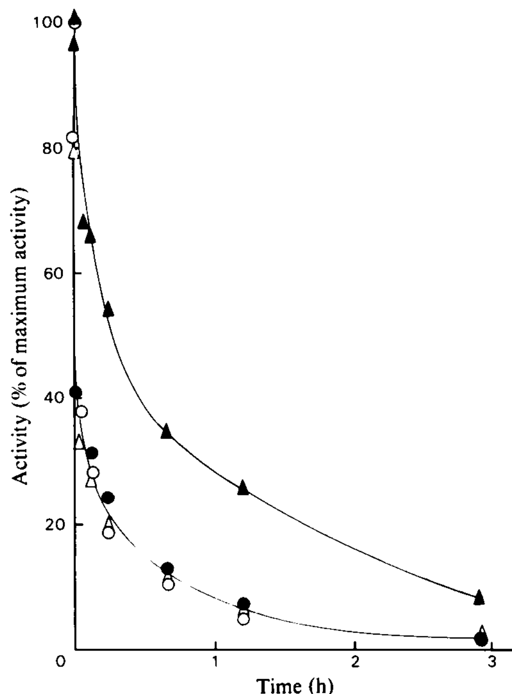


Fig. 4. *Inactivation of the molybdenum cofactor under aerobic conditions in aqueous solution: effects of dithiothreitol and of molybdate*

Samples of molybdenum cofactor, as normally prepared (see the Materials and methods section), were diluted to final concentrations of 7nM-Mo and 0.75% (v/v) dimethyl sulphoxide, with aerobic aqueous buffer (25mM-potassium phosphate, pH7.4, containing 1mM-EDTA), with or without the addition of dithiothreitol. After various times of preincubation at 3.5°C, cofactor assays were carried out on the reaction mixtures by complementation with *nit-1* extract, with or without the addition of molybdate and dithiothreitol. The ▲ symbols correspond to 1mM-dithiothreitol in the preincubation, with 1mM-dithiothreitol and 10mM-molybdate present during complementation. For the other experiments, concentrations (mM) of dithiothreitol in the preincubation and of dithiothreitol and molybdate during complementation were respectively: △, 1, 1, 0; ●, 0, 0.2, 10; ○, 0, 0.2, 0. All activities are expressed as percentages of those at zero time, in the experiment having molybdate present during complementation and dithiothreitol present throughout. Zero time corresponds to samples in which the cofactor was diluted directly into complete complementation mixture already containing *nit-1* extract.

Molybdenum cofactor solutions that were kept anaerobic by using glucose oxidase were only slightly less stable with respect to cofactor activity than when dithionite was used (Table 1, Expt. 22). However, the increase in the molybdate-dependence observed after either preincubation (Table 1, Expt. 22) or complementation (Table 1, Expts. 15 and 16) under these conditions indicates that anaerobiosis, in the absence of dithionite, is insufficient to prevent dissociation of molybdenum from the cofactor.

It should be noted that molybdate may not be the molybdenum species responsible for the restoration of activity to demolybdo cofactor, since, under our conditions, molybdate was found to react slowly with dithiothreitol, causing the solution to turn yellow, presumably owing to the formation of a binuclear Mo(V) complex (Steifel, 1977). However, in partly preserving ability of the cofactor to be reconstituted by a subsequent addition of molybdate (Fig. 4 and Table 1, Expts. 23 and 24), dithiothreitol was clearly acting on the cofactor and not on molybdate.

In additional experiments we found that the extent of molybdate-dependence was not dependent on the molybdoenzyme used (xanthine oxidase, desulpho xanthine oxidase or sulphite oxidase), or on the method used to extract the cofactor (i.e. treatment with dimethyl sulphoxide, guanidinium ions or sodium dodecyl sulphate). We failed to confirm our preliminary finding (Hawkes & Bray, 1983) that cofactor from xanthine oxidase, prepared without the usual salicylate denaturation step (Hart *et al.*, 1970), differed with regard to molybdate-dependence from other samples.

Discussion

As detailed in the Results section, we achieved essentially quantitative transfer of the molybdenum cofactor from xanthine oxidase to the apo nitrate reductase of *N. crassa nit-1* extracts. This depended on (1) extracting the cofactor completely from the xanthine oxidase or sulphite oxidase, (2) carrying out the complementation under conditions where both the reactants and the product were adequately stable, and (3) assaying the resulting nitrate reductase under non-inhibitory conditions. In developing the procedure we eliminated various problems relating to all three of these stages. The quantitative nature of the transfer is shown by the specific activity of the nitrate reductase formed, being the same (on a per-molybdenum basis) as that of the holoenzyme from wild-type *N. crassa*.

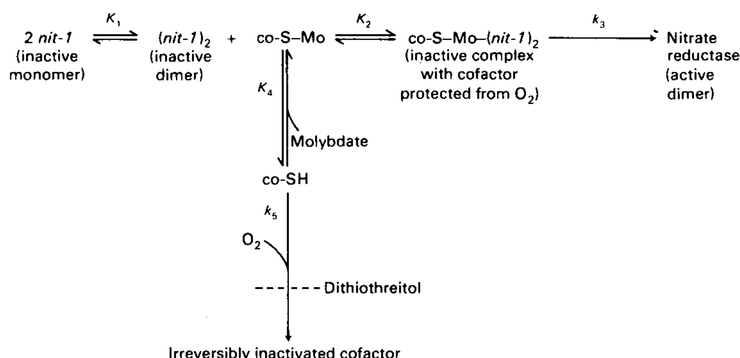
It is not easy, from the literature, to compare our efficiency of cofactor transfer with that achieved by other workers (see, e.g., Lee *et al.*, 1974; Pienkos *et al.*, 1977; Amy & Rajagopalan, 1979; Johnson *et al.*, 1980*c*; Claassen *et al.*, 1982; Mendel, 1983), but it seems that only very low effi-

ciencies were achieved by others. Extracting numerical data from the literature is difficult, since the low specific activities of the nitrate reductase formed have not been emphasized, but wherever possible we made the calculations (see the Materials and methods section). We conclude that Lee *et al.* (1974) achieved a transfer efficiency of about 5%, that no workers have presented evidence showing definitely higher transfer efficiencies, and that elsewhere efficiency has been, at best, about the same, or sometimes substantially lower. Thus, on the assumption of one cofactor molecule per atom of molybdenum, our assay method permits, for the first time, direct and absolute quantification of concentrations of the active cofactor, and establishes rigorously the important point, that cofactor activity arises from a stoichiometric constituent of xanthine oxidase rather than from some contaminant.

Despite non-quantitative cofactor transfer in earlier work, the assay has been widely employed to make semi-quantitative comparisons of different procedures used in the extraction, and reagents used in the stabilization, of the cofactor (Lee *et al.*, 1974; Pienkos *et al.*, 1977; Alikulov *et al.*, 1980; Mendel *et al.*, 1982; Mendel, 1983). Not surprisingly, although there is broad agreement on qualitative phenomena (e.g. the stabilization of the cofactor under anaerobic conditions and in the presence of reducing agents such as thiols or ascorbate), the quantitative data are equivocal. At variance with previous work, we find that heat treatment releases only 30% of molybdenum cofactor from xanthine oxidase (rather than 95%; Alikulov *et al.*, 1980; Mendel *et al.*, 1982) and that NADPH inhibits (rather than activates; McKenna *et al.*, 1974; Claassen *et al.*, 1982) the complementation of *nit-1* extracts by the cofactor. Our results place on a firmer footing earlier evidence (Johnson, 1980; Johnson *et al.*, 1980*c*) that active xanthine oxidase, desulpho xanthine oxidase and sulphite oxidase all yield equivalent amounts of a single cofactor. The dependence of the cofactor activity on the addition of molybdate, which yields information on the proportion of active cofactor molecules in the demolybdo form, provided no evidence for the presence of substantial amounts of demolybdo cofactor in any of the enzyme preparations that we used.

Since the cofactor is unstable in air, we attempted to carry out both its extraction and subsequent complementation with *nit-1* extracts under anaerobic conditions. We found that extraction of the cofactor could be carried out without loss of activity, in a number of different ways, by working in the presence of $Na_2S_2O_4$. This presumably not only acts as an oxygen scavenger, but also ensures that the cofactor remains in the fully reduced state. Though the use of dithionite could not be extended to the complementation reaction, we found, nevertheless, that optimum results for this were also achieved under anaerobic conditions, obtained by the use of glucose and glucose oxidase.

Further discussion relating to the stability of the cofactor is conveniently carried out in relation to Scheme 1, which shows our suggested mechanism (based to some extent on earlier proposals; e.g. Lee *et al.*, 1974; Johnson & Rajagopalan, 1982) for incorporation under various conditions of the cofactor into the apo nitrate reductase of *nit-1*



Scheme 1. *Suggested mechanism for the incorporation of the molybdenum cofactor into nitrate reductase during complementation of nit-1 extracts*

It is assumed that apo nitrate reductase ('*nit-1*') and molybdenum cofactor ('co-S–Mo') are the only species involved. Molybdenum-free cofactor is 'co-SH'; oxidation states of molybdenum and cofactor have not been defined. The rates at which equilibria, governed by K_1, K_2 and K_4, are established are not necessarily rapid; k_3 and k_5 represent slower reactions. Indirect involvement, only, of the cofactor in dimerization (Nason *et al.*, 1970) of the (4.5S) apo nitrate reductase is indicated. The possible existence of additional pathways is not excluded; these might include, e.g. (Amy & Rajagopalan, 1979), reaction of co-SH with $(nit\text{-}1)_2$.

extracts. In qualitative terms, at least, the scheme is consistent with all available data.

A dramatic stabilization of the cofactor must occur when it is added aerobically to the *nit-1* extract at 3.5°C, since, though the complementation reaction takes many hours to go to completion (Fig. 2), nevertheless the cofactor has a lifetime measured in minutes (Table 1) on its own under these conditions. We assume (Scheme 1) that the cofactor is first protected from oxygen damage by the rapid formation of an inactive complex in which the cofactor is stabilized. For simplicity this is here taken to be with apo nitrate reductase. After formation of the complex (via the equilibrium governed by K_2), the complex slowly isomerizes (via the rate-limiting reaction governed by k_3) into active nitrate reductase (a step that is taken to be irreversible since there is no evidence that active nitrate reductase dissociates at low concentrations to yield free molybdenum cofactor). The near-quantitative assay of the cofactor, under aerobic conditions, despite its inherent instability, can be understood in terms of a high value of the association constant, K_2, in comparison with the constants K_4 and k_5, which govern the degradative pathway. Under the conditions of our standard assay, with excess molybdate, the equilibrium governed by K_4 lies in favour of the cofactor having molybdenum bound to it. Under these circumstances, in the presence of oxygen and dithiothreitol, the rate for the oxidative inactivation of the cofactor is controlled by the reaction governed by k_5, inhibited only partly by dithiothreitol.

The dependence of cofactor activity, under aerobic conditions, on the concentration of apo nitrate reductase (Fig. 3) can be understood in terms of low concentrations of *nit-1* extract resulting in incomplete formation, via K_2, of the stabilized complex, and hence favouring the degradative pathway, via K_4 and k_5. Conversely, under anaerobic conditions, with excess molybdate, the only route for degradation of the cofactor, via the reaction governed by k_5, would be eliminated. Therefore all the cofactor added would be effectively incorporated into active nitrate reductase.

Horner (1983) has purified the *nit-1* apoprotein extensively. Though he reported a low specific activity, his preparations were, nevertheless able to undergo complementation with the molybdenum cofactor. This provides some evidence for our unsupported assumption that no components other than molybdenum cofactor and apo nitrate reductase are essential for complementation.

The effects of dithiothreitol, with regard to its stimulation of molybdenum-dependence of the assay, are most simply understood (Scheme 1), in terms of its stabilizing one or more thiolate groups in the molybdenum-free cofactor, with these groups functioning, in the intact coenzyme, as ligands of the metal. This is consistent with conclusions from e.p.r. by Meriwether *et al.* (1966) and from X-ray-absorption extended fine structure by Bordas *et al.* (1980) and by Cramer *et al.* (1981) that most of the ligands of molybdenum in xanthine oxidase are sulphur, as well as with the finding by Johnson & Rajagopalan (1982) that sulphur is present in inactive cofactor. According to Scheme 1, it is the concentration of the demolybdo cofactor, co-SH, present during the complementation reaction, that will determine the magnitude of the molybdenum-dependence of the assay, with oxygen destroying co-SH and with dithiothreitol stabilizing it. Our data showing thiol-dependence of the stimulation of the assay by molybdate provide the most direct evidence (cf. Johnson & Rajagopalan, 1982) so far available for thiolate ligands of molybdenum in the cofactor.

However, in order to reconcile all of our data with Scheme 1, a further point has to be considered. Oxidation states of molybdenum [presumably Mo(IV), Mo(V) or Mo(VI)] and of the cofactor (tetrahydro-, dihydro- or normal pteridine) are for simplicity omitted from Scheme 1, but are clearly important in relation to cofactor degradation and to molybdenum loss from it. The kinetics of aerobic cofactor decomposition (Fig. 4) indicate, not surprisingly, a multi-step process. It seems that dissociation of molybdenum does not take place until some oxidation has taken place. When maintained in the reduced state, with dithionite present, the molybdenum cofactor could be diluted to about 20nM (Table 1, Expts. 20 and 21), and kept in aqueous solution for 24h without affecting either the activity or its molybdenum-dependence. This confirms that molybdenum does not dissociate significantly from the dithionite-reduced cofactor, indicating the dissociation constant, K_4, to be vanishingly small. However, in the absence of dithionite, anaerobic conditions, though preserving cofactor activity, did not prevent considerable dissociation of molybdenum (Table 2, Expt. 22). Presumably a reversible oxidation takes place, leading to diminished affinity for molybdenum (K_4 perhaps about 1 mM), so accounting for the requirement for molybdate at high concentrations.

We conclude that the observed effects of O_2 and of dithiothreitol on the dependence of cofactor activity on molybdate are all readily understandable in terms of Scheme 1, when this is modified by the further assumption that K_4 is dependent on the oxidation state of either the organic part of the cofactor, or of its molybdenum, or of both of these.

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