Isolation, in the intact state, of the pterin molybdenum cofactor from xanthine oxidase

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A procedure is described for isolation of the pterin molybdenum cofactor, in the active molybdenum-containing state, starting from purified milk xanthine oxidase. The method depends on the use of anaerobic-glove-cabinet techniques and on working in aqueous solution, in the presence of 1 mM- $\text{Na}_2\text{S}_2\text{O}_4$. SDS was used to denature the protein, followed by ion-exchange chromatography and gel filtration. The cofactor, obtained at concentrations up to 0.5–1.0 mM, was fully active in the *nit-1* assay [Hawkes & Bray (1984) *Biochem. J.* **214**, 481–493], with a specific activity of 22 nmol of NO_2^- /min per pg-atom of Mo (with 15 % molybdate-dependence). The M_r , determined by gel filtration, was about 610, consistent with the structure proposed by Kramer, Johnson, Ribeiro, Millington & Rajagopalan [(1987) *J. Biol. Chem.* **262**, 16357–16363]. At pH 5.9, under anaerobic conditions, the cofactor was stable for at least 300 h at 20–25 °C.

INTRODUCTION

Molybdenum enzymes (Coughlan, 1980; Spiro, 1985; Bray, 1988) characterized to date fall into two classes: nitrogenase, depending on the iron molybdenum cofactor, and all the other enzymes, which depend on the pterin molybdenum cofactor. The latter is also frequently called simply the 'molybdenum cofactor', or 'molybdopterin' (a term that may be confusing if molybdenum-containing and molybdenum-free forms have to be considered). Existence of the cofactor (Cramer & Stiefel, 1985) was first postulated by Pateman *et al.* (1964), and evidence for its being a di- or tetrahydropterin derivative was first put forward by Johnson *et al.* (1980). Though the holoenzymes of which the molybdenum cofactor forms a part are generally not sensitive to air, the cofactor itself, liberated from them by denaturation, is, in contrast, rapidly destroyed by air. This makes its isolation in an undegraded state extremely difficult. Oxidative degradation involves labilization of the metal, tightly bound (Hawkes & Bray, 1984a) when in the reduced $[\text{Mo(IV)}]$ state but less so in the Mo(VI) state, and oxidation of the reduced pterin. The structure of the cofactor, as deduced from degradation studies by Rajagopalan and co-workers (Kramer *et al.*, 1987), is that of a reduced form of 2-amino-4-hydroxypteridine, with a C_4 side chain in position 6. This chain is reported to bear a phosphorylated primary alcohol in the terminal position, as well as two thiol groups, to which, it is presumed, molybdenum is co-ordinated. Similar structural conclusions have been reached by workers in other laboratories (Ushio *et al.*, 1986; Fish & Massey, 1987).

Notwithstanding this work, it has never been reported that the cofactor has been isolated in the intact fully active state, in concentrated solution, free from denaturing agents. To do this would be a pre-requisite for a variety of physical studies, and some chemical ones, that ought to be carried out before the proposed structure can be fully accepted. Achieving such isolation would involve overcoming problems caused by instability of the

cofactor, as well as those caused by difficulties in assaying it on a fully quantitative basis.

The standard assay for the molybdenum cofactor depends on its incorporation into apo nitrate reductase, present in extracts from the *nit-1* mutant of *Neurospora crassa*, to yield the holoenzyme, which is then assayed colorimetrically for nitrate reductase activity. Hawkes & Bray (1984a) showed that, with appropriate care, the cofactor could be liberated by denaturation and transferred quantitatively from enzymes such as sulphite oxidase and xanthine oxidase to the *nit-1* apo nitrate reductase. Their evidence for this was that the specific activity of the nitrate reductase so produced, namely 25 ± 4 nmol of NO_2^- /min, expressed per pg-atom of molybdenum added from the denatured enzyme, agreed with the known catalytic activity of the wild-type *Neurospora crassa* enzyme.

A complication of transfer and assay of the cofactor is that, as already indicated, molybdenum tends to dissociate from it, except under strongly reducing conditions. Where some dissociation has taken place, this may, under favourable circumstances, be reversed (Hawkes & Bray, 1984a) by addition of high concentrations of molybdate in the complementation reaction between cofactor and apo nitrate reductase. Following loss of molybdenum, the cofactor is readily degraded by oxidative reactions. Though the first stages of such oxidation (perhaps involving -SH groups) may be reversible, the later stages have not been reversed. The extent of reversible dissociation of molybdenum from the cofactor was expressed by Hawkes & Bray (1984a) as the 'percentage dependence on molybdate' of the assay, given as $100[1 - (A_0/A_m)]$, where A_0 and A_m are respectively the activities without and with the addition of molybdate to the complementation medium. They found, for freshly prepared crude cofactor extracts, molybdate dependences of about 15–30 %. Others workers have not always reported comparable data, though rather higher dependences of 20–50 % may be calculated from the work of Wahl *et al.* (1984). Similarly, van Spanning *et al.*

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(1987) and Mendel (1983) both reported high values for molybdate dependence of 90% or more, indicating that the metal had dissociated almost completely from their cofactor preparations.

Aside from the questions of molybdenum loss and cofactor oxidation, the precise nature of the reaction or reactions that take place in the assay when molybdenum cofactor samples are added to *nit-1* preparations, and active nitrate reductase is thereby produced, still remains unclear. Hawkes & Bray (1984a) studied the kinetics of the process and found half-times for completion of the reaction at 3.5 °C of about 1 to 4 h, depending on the conditions. It is not obvious why simple incorporation of a cofactor into an apoenzyme should be so slow. These workers found, however, that the process clearly involves more than one step, since stability of the cofactor increases dramatically as soon as it is mixed with the *nit-1* preparations. On the other hand, as they noted, the work of Horner (1983) on complementation by the cofactor of extensively purified *nit-1* nitrate reductase apoprotein provides some evidence, despite the low specific activities that were observed, that no components other than molybdenum cofactor and apo nitrate reductase are essential for production of holo nitrate reductase. A further complexity of the assay is, however, revealed by the findings by Johnson & Rajagopalan (1987) that crude *nit-1* extracts contain, in addition to apo nitrate reductase, a low-molecular-mass precursor of the cofactor that may be converted enzymically into the cofactor by an enzyme present, for example, in extracts of the *chlA1* mutant of *Escherichia coli*. These workers found that the cofactor precursor was eliminated from *nit-1* extracts by gel filtration on Sephadex G-25. Clearly, therefore, there are advantages of specificity to be gained by including routinely such a gel-filtration step, or more extensive purification of the *nit-1* extracts, into the molybdenum cofactor assay protocol.

Purified, or partly purified, molybdenum enzymes provide a convenient source from which it ought in principle to be a simple matter to isolate the cofactor in the intact state, providing the degree of anaerobiosis that is necessary for its stability can be achieved. Successful purification would be indicated by normal chromatographic criteria of purity of the product, accompanied by high cofactor specific activity and low molybdate dependence in the *nit-1* assay. By these criteria, no satisfactory purification of the intact cofactor has so far been described. Kramer *et al.* (1984) and Hawkes & Bray (1984b) are alone in giving cofactor specific-activity data, but neither group obtained a pure denaturant-free product. As already indicated, the preparations of van Spanning *et al.* (1987) and of Mendel (1983) were highly dependent on molybdate. Although Cramer & Minak (1983) indicated low molybdate dependence of their preparations, their procedure was described in barest outline only.

Using milk xanthine oxidase as starting material, we carried out the purification work described below as a preliminary to physical studies on the intact cofactor. Our molybdenum cofactor assays were carried out as described by Hawkes & Bray (1984a) by using partially purified *nit-1* extracts. Because of the technical problems that are involved, the work is described in some detail. We also present information on the relative molecular mass of the intact cofactor and additional data on its stability.

MATERIALS AND METHODS

Materials

Chemicals and biochemicals were generally the purest grade available from BDH or from Sigma. Sephadex G-10 and G-25 (Superfine grade) and DEAE-Sephacel were from Pharmacia. Xanthine oxidase was prepared by using denaturation with sodium salicylate (Hart *et al.*, 1970; procedure H1,2 of Ventom *et al.*, 1988).

Anaerobic techniques for cofactor isolation and manipulation

Unless otherwise indicated, all operations were carried out at 20–25 °C, under nitrogen in an anaerobic glove cabinet (Faircrest, Tempo Trading Ltd., Blaina, Gwent NP3 3JW, Wales, U.K.). Residual oxygen, monitored with a 'Coulometer' oxygen sensor (Chandos International, Stockport SK12 4AN, Cheshire, U.K.), was approx. 1.0–1.5 p.p.m. throughout the work. In addition, again unless otherwise indicated, solid $\text{Na}_2\text{S}_2\text{O}_4$ (approx. 1 mM) was added to all solutions. Vessels containing molybdenum cofactor solutions were kept sealed when practicable. Even in the glove box, there was some tendency for $\text{Na}_2\text{S}_2\text{O}_4$ solutions, if exposed for long periods, to lose their reducing ability, so this was checked periodically with Methyl Viologen paper (Eady, 1980). Cofactor samples that did not give a positive response to this test were generally discarded. All plastic materials to come into contact with cofactor solutions were kept in the cabinet for at least 24 h before use. Chromatography columns were packed in the cabinet and washed until the effluent responded to Methyl Viologen paper. Columns were connected to a u.v. monitor and to a fraction collector, both located in the glove cabinet. Incubation for the complementation reaction of the assay (see below) was carried out aerobically, though the samples were made up in the cabinet. Operations carried out anaerobically outside the cabinet included various analyses, centrifugation (in the first stage of cofactor preparation) and freeze-drying. For centrifugation, tubes were filled and tightly sealed in the cabinet before being removed. They were centrifuged without being opened, then returned to the cabinet. Freeze-drying was performed in round-bottomed flasks, filled in the cabinet, that could be closed with 3–4 mm-bore glass stopcocks. Samples were pre-frozen with liquid nitrogen and remained frozen throughout; freeze-drying was not taken to completion.

Assay of molybdenum cofactor activity

Assays were carried out according to the standard assay procedure described by Hawkes & Bray (1984a), by aerobic complementation for 24 h at 3.5 °C of the apo nitrate reductase of partially purified extracts of the *nit-1* mutant of *Neurospora crassa*, to yield active nitrate reductase. Measurements were carried out with and without the addition of Na_2MoO_4 (10 mM) to the complementation medium. The procedure was checked periodically by performing assays on cofactor, freshly liberated (Hawkes & Bray, 1984a) with dimethyl sulphoxide from known small amounts of xanthine oxidase.

Cofactor fluorescence

Fluorescence of the oxidized cofactor, at pH 10.5, following oxidation to fluorescent Form B (Johnson &

Rajagopalan, 1982) was measured at 458 nm, as described by Ventom *et al.* (1988), excitation being at 370 nm.

Analyses

Analysis for molybdenum was carried out colorimetrically after digestion with HClO_4 and H_2SO_4 (Hart *et al.*, 1970). Ninhydrin analysis for total amino acid concentration was performed after alkaline hydrolysis (Hirs, 1967) by the method of Moore & Stein (1955) with norleucine as a standard. When necessary, protein was determined by using the procedure of Lowry *et al.* (1951), with bovine serum albumin as standard. FAD was estimated by measurements of A_{450} [with ϵ (molar absorption coefficient) $11\,300\text{ M}^{-1}\cdot\text{cm}^{-1}$], on samples that had been removed from the cabinet and allowed to become re-oxidized by air. Removal of KCl during gel filtration was checked by conductivity measurements.

Isolation of the molybdenum cofactor from xanthine oxidase

The following procedure was finally adopted. Xanthine oxidase (usually 3–7 μmol in approx. 20 ml; amount calculated from A_{450} by using an ϵ value of $36\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$) that had been dialysed thoroughly against 25 mM-potassium phosphate buffer, pH 7.4, containing 1 mM-EDTA, was made anaerobic in the glove cabinet and 1 mM- $\text{Na}_2\text{S}_2\text{O}_4$ was added. The enzyme was then denatured by the addition of SDS (0.21 g/ μmol of xanthine oxidase) and heating to $80 \pm 5^\circ\text{C}$ for 3 min. The sample was cooled in ice, and the precipitated material was removed by centrifuging at $33\,000 g_{\text{av}}$ for 40 min at 0°C . The supernatant was removed by suction and then clarified by pressure filtration (Amicon PM-10 membrane).

The ultrafiltrate (including rinsings from the membrane) was then applied to an ion-exchange column (DEAE-Sephacel; $2.6\text{ cm} \times 25\text{ cm}$) that had been equilibrated with 25 mM-potassium phosphate buffer, pH 7.4, containing 1 mM-EDTA and 1 mM- $\text{Na}_2\text{S}_2\text{O}_4$. Elution of the cofactor was first with 600 ml of a linear gradient of 0–300 mM-KCl in the same buffer, then with 400 mM-KCl, again in the buffer.

Appropriate fractions (cf. Fig. 1 below) from the ion-exchange column were combined and concentrated by freeze-drying, before the product was applied to a Sephadex G-10 gel-filtration column ($1.6\text{ cm} \times 60\text{ cm}$), equilibrated with 1 mM- $\text{Na}_2\text{S}_2\text{O}_4$ in water adjusted to approx. pH 7.0. Both the load of KCl and the sample volume applied were critical if adequate resolution was to be achieved. The sample was therefore subdivided and gel-filtered in separate portions (not less than five portions when working on the scale indicated). After appropriate fractions had been combined and the cofactor concentrated by freeze-drying, gel filtration on Sephadex G-10 was repeated (with the sample again subdivided into three or more portions), to further reduce contamination with KCl.

The final purification step was gel filtration on a Sephadex G-25 column ($1.6\text{ cm} \times 60\text{ cm}$), again equilibrated with 1 mM- $\text{Na}_2\text{S}_2\text{O}_4$. Appropriate combined fractions from the second purification on Sephadex G-10 were applied to this column, after concentration by freeze-drying. The final product was obtained by appropriate combination of fractions (Fig. 2 below) from

the G-25 column and could be concentrated by freeze-drying and stored frozen in liquid nitrogen.

M_r of the molybdenum cofactor

The relative molecular mass of the cofactor was determined by anaerobic gel filtration on a Sephadex G-25 column ($1.6\text{ cm} \times 60\text{ cm}$) which had been equilibrated with 1 mM- $\text{Na}_2\text{S}_2\text{O}_4$. The column was calibrated by using insulin A chain (M_r 2530); vasopressin (M_r 1084); FAD (M_r 830); biopterin (M_r 237) and phenylalanine (M_r 169). Blue Dextran (Pharmacia) was used to determine the void volume of the column (V_0). Elution volumes (V_e) were recorded with a u.v. monitor. The cofactor was located by activity measurements, as described above, on fractions from the column.

Stability of the cofactor as a function of pH

Experiments were carried out in 25 mM-potassium phosphate buffers of different pH values, containing 1 mM- $\text{Na}_2\text{S}_2\text{O}_4$. Samples of the cofactor (after the second Sephadex G-10 purification step; final concn. about 20 μM) were added to the buffers, in vials that were subsequently sealed with rubber closures. At intervals, samples were removed through the closures for cofactor assay, the vials remaining in the glove cabinet throughout. Each time a sample was removed, it was checked for reducing ability with Methyl Viologen paper. An apparently positive response was obtained up to 300 h in all experiments, except at the two extreme pH values. Even here, however, the $\text{Na}_2\text{S}_2\text{O}_4$ survived for somewhat longer than did significant amounts of cofactor activity, the last positive responses to Methyl Viologen, at pH 11.1 and 2.8, being recorded at 5 and 20 h respectively. At the end of the experiment, the molybdenum content of the solutions was determined colorimetrically, so that cofactor specific activities could be calculated.

RESULTS AND DISCUSSION

Isolation of the molybdenum cofactor

The aim of the work was to prepare relatively large quantities (e.g. 1 μmol) of ungraded molybdenum cofactor in concentrated solution (e.g. 1 mM), as would be required for a variety of physical studies. It is obviously necessary that the preparations should be highly active and that the purity should approach 100 %.

We selected xanthine oxidase as the starting material because of its ready availability (Hart *et al.*, 1970). Hawkes & Bray (1984a) investigated different means of liberating the cofactor from this and other enzymes but prepared only dilute solutions (e.g. 15 μM). Consideration of their work indicated that denaturation with SDS might be appropriate for our purposes. In preliminary experiments we established, furthermore, that concentrated cofactor solutions could be prepared in the presence of SDS.

Our final purification procedure, which was carried out under rigorously controlled anaerobic conditions in a glove cabinet, and working in the presence of $\text{Na}_2\text{S}_2\text{O}_4$ throughout, is described in the Materials and methods section, and results are summarized in Table 1 and in Figs. 1 and 2. It is based on conventional chromatographic procedures. Isolating the cofactor involves separating it from SDS, from denatured protein, from liberated FAD and iron, from salts added during ion-exchange and finally (a problem that was not anticipated

Table 1. Isolation of the molybdenum cofactor from xanthine oxidase

The purification was carried out as described in the text. At each stage, the molybdenum content and the cofactor activity of the sample were measured. Cofactor specific activities, with molybdate added to the complementation medium (see the Materials and methods section), are expressed as nmol of NO_2^- /min per pg-atom of Mo. Activities were also measured without added molybdate; 'percentage dependence on molybdate' is expressed as $100(1 - A_0/A_m)$, here A_m and A_0 are respectively the activities with and without molybdate. The procedure was checked periodically by performing assays on cofactor freshly liberated with dimethyl sulphoxide from known small quantities of xanthine oxidase. The specific activity so obtained was 23.1 ± 2.0 (range), with an average molybdate dependence of 9%. Amino acids were estimated with ninhydrin after alkaline hydrolysis (see the Materials and methods section).

Purification step	Volume (ml)	Specific activity (nmol of NO_2^- /min/pg-atom of Mo)	Dependence on MoO_4^{2-} (%)	Mo (μmol)	Amino acids (nmol/ng-atom of Mo)	Recovery of Mo (%)
(Xanthine oxidase)	30	23.1*	7*	3.03	—	(100)
Heating with SDS	28	22.5	9	2.91	—	96
Centrifugation	27	22.1	9	2.69	—	88
Ultrafiltration	30	22.0	10	2.40	—	79
DEAE-Sephacel	180	22.0	11	0.85	—	28
1st Sephadex G-10	70	22.1	12	0.62	—	20
2nd Sephadex G-10	40	22.0	13	0.51	12–14†	17
Sephadex G-25	20	22.0	15	0.22	< 0.5‡	7

* Measurements on an aliquot denatured with dimethyl sulphoxide.

† Range of values from a number of experiments; amino acid analyses indicated a non-stoichiometric and variable composition.

‡ Any contamination with amino acids could be further reduced if a lower recovery was acceptable (cf. Fig. 2).

Fig. 1. Purification of the molybdenum cofactor by ion-exchange chromatography on DEAE-Sephacel

SDS-treated xanthine oxidase was applied to the column (2.6 cm $\times$ 25 cm) as described in the Materials and methods section. The column was eluted, first with a KCl gradient, then with 400 mM-KCl. The elution pattern following the application of the 400 mM-KCl, is shown. Symbols: ■, molybdenum concentration (μM); O, molybdenum cofactor activity (nmol of NO_2^- produced/min per 5 μl aliquot, assayed without added MoO_4^{2-}); ●, cofactor specific activity (nmol of NO_2^- /min per pg-atom of Mo); ▲, [FAD] (μM). The double-headed arrow indicates fractions that were combined.

at the outset) from peptide impurities, presumably arising from the use of proteolytic enzymes in the xanthine oxidase preparation.

For the denaturation step, to avoid having to remove

a large excess of SDS, we related the amount of the denaturant used to that (approx. 1.4 g of SDS/g of protein) which is expected (Creighton, 1985) to bind to the protein. Centrifugation, followed by pressure

Fig. 2. Gel filtration of the molybdenum cofactor on Sephadex G-25

Cofactor-containing fractions from gel filtration on Sephadex G-10 were combined, concentrated and applied to a Sephadex G-25 column (1.6 cm $\times$ 60 cm). Symbols: ■, molybdenum concentration (μM); ○, molybdenum cofactor activity (nmol of NO_2^- produced/min per 5 μl aliquot, assayed without added MoO_4^{2-}); ▽, fluorescence at 435 nm (after oxidation) (arbitrary units); ▼, amino acid concentration (μM ; from ninhydrin analysis); □, A_{280} (from a u.v. monitor). The double-headed arrow indicates fractions that were combined.

filtration, as described in the Materials and methods section, was effective in removing most of the SDS micelle and the denatured protein. If the pressure filtration was omitted, the cofactor failed to bind to the ion-exchange column in the subsequent purification step.

Ion-exchange chromatography on DEAE-Sephacel (Fig. 1) removed residual protein (detected by Folin analysis), which emerged during preliminary washing of the column (not illustrated in Fig. 1). The cofactor was then eluted as a rather broad peak, which was followed by, and overlapped with, another molybdenum-containing peak, most likely inorganic molybdenum, followed finally by FAD as a sharp peak (Fig. 1).

Removal of the very substantial quantities of KCl introduced by the ion-exchange procedure proved quite troublesome. Concentration of the combined cofactor-containing fractions (Fig. 1) was necessary to avoid working with excessively dilute solutions, but the high density of the KCl solutions then led to poor resolution on gel-filtration columns. We eventually found a three-step gel filtration procedure to be the most effective. In this, low-molecular-mass contaminants, including most of the KCl and some amino acid or peptide impurities, were removed by two purification steps on Sephadex G-10 (not illustrated). This was followed by gel filtration on Sephadex G-25, to remove high-molecular-mass impurities, including (Fig. 2) peptides (M_r approx 1600) that were invariably present (Table 1) in our samples. Whereas the peptides (distinguished by ninhydrin analysis after alkaline hydrolysis) absorbed significantly at 280 nm, the cofactor, by contrast, had little absorption at this wavelength (Fig. 2). Cofactor activity, molybdenum and fluorescence after oxidation all coincided in the final purification step and, in agreement with Kramer *et al.* (1984), the final cofactor fractions were free from amino acids. However, since resolution from the peptide peak was incomplete under the conditions used (Fig. 2), recovery of the cofactor was rather poor at this stage (Table 1).

The most noteworthy feature of the purification (Table 1) is the high specific activity of the cofactor, expressed per pg-atom of molybdenum, that was maintained without significant diminution from the starting material

(xanthine oxidase), through to the final product. The value for the final product, namely 22.0 nmol of NO_2^- /min per pg-atom of Mo, is within the limiting range found by Hawkes & Bray (1984a). Furthermore, even for our final sample (Table 1), the dependence of the cofactor activity on molybdate, of 15%, was less than that generally observed by these workers for freshly prepared unpurified cofactor extracts. No doubt this was due to our using a glove box that was not available in the earlier work. What is more, even this value of 15% may exaggerate the extent of degradation of our samples as prepared, since some of the dependence could well arise from oxidation and dissociation of the molybdenum in the very dilute solutions used in the assay. Even so, it is interesting that the molybdate dependence (Table 1) increased steadily as the purification progressed, from a starting value of 7% to the final 15%, indicating a real and progressive, though very small, deterioration. Clearly, however, all the data indicate that our final product is indeed close to being pure, fully functional, molybdenum cofactor.

The overall yield of the cofactor, expressed as recovery of molybdenum from the starting xanthine oxidase, was 7%, a value that could undoubtedly be increased by relatively minor changes in the chromatographic procedures. For many purposes, of course, complete elimination of KCl and of peptides would not be necessary.

Solubility of the molybdenum cofactor

Pteridine derivatives tend to have low solubilities in all solvents. In keeping with this, Hawkes & Bray (1984a,b) did not succeed in preparing solutions of the molybdenum cofactor in dimethyl sulphoxide more concentrated than about 15–20 μM . So far as we are aware, there are no reports in the literature of cofactor solutions more concentrated than this having been prepared in any solvent. We found, however, that the cofactor has high solubility in aqueous solutions. In a number of experiments, when working at 20–25 $^{\circ}\text{C}$ we concentrated samples to 0.5–1.0 mM with no indications that the solubility limit had been reached. In further preliminary experiments, however, we found that, at least in the

presence of KCl, cofactor activity could be precipitated from aqueous solution by addition of methanol at 0 °C, and re-dissolved without loss of activity; this could be important in further work.

Stability of the molybdenum cofactor

Our successful purification of the molybdenum cofactor confirms the conclusion of Hawkes & Bray (1984a) that it is relatively stable under anaerobic conditions in the presence of sodium dithionite. To obtain more quantitative information, and to study the effect of pH on the stability, we carried out the experiment described in the Materials and methods section and illustrated in Fig. 3. Results reveal both substantial stability of the cofactor at 20–25 °C in dilute phosphate buffers at the optimal pH, and sharply diminishing stability at high, and to a slightly lesser extent, at low, pH values. In all cases where partial loss of activity occurred, there was also an increase in molybdate dependence (shown by a greater decrease in activity when molybdate was absent from the assay than when it was present).

Fig. 3. Stability of the molybdenum cofactor as a function of pH

Samples of the cofactor (approx. 20 μM) were incubated anaerobically in the presence of 1 mM-dithionite in 25 mM-potassium phosphate buffer at the pH values indicated, at 20–25 °C. Samples were removed at the times shown and cofactor assays were performed in the presence (O) or absence (●) of molybdate; results are expressed as cofactor specific activities in nmol of NO_2^-/min per pg-atom of Mo.

Loss of activity (assayed with molybdate present) in 300 h was undetectable (i.e. less than about 5%) at pH 5.9. There was, however, a small but significant increase in molybdate dependence (to about 18%) at this pH value. Stability was comparable, at pH 7.4, or no more than marginally less good. On the other hand, at pH 8.8, stability was markedly poorer, the cofactor showing a half-life, in the assay with molybdate, of around 50 h, and with molybdate dependence increasing to about 60% in the later stages of the experiment. At the highest pH value studied, 11.1, 80% decomposition occurred in 5 min, with almost complete (90–95%) molybdate dependence, even of this low activity. At the other extreme pH value, 2.8, stability was a little higher, corresponding to a half-life for the cofactor in the assay with molybdate of perhaps 1 h.

The above data apply to molybdenum cofactor concentrations of about 20 μM . Though we did no systematic studies, several further observations point to diminished stability of the cofactor in more dilute solutions, indicating furthermore that the instability may be due, despite our precautions, to oxidative damage. (In this context, it may readily be calculated that the total quantity of oxygen circulating at any time in the glove cabinet, when operating at 1 p.p.m. of O_2 , approaches 0.1 mmol, a quantity that exceeds by more than an order of magnitude the total quantity of molybdenum cofactor in the box, even at the start of one of our larger preparations.) Lessened stability at low concentration is suggested by our sometimes finding diminished cofactor specific activities in the wings of chromatographic peaks. Another pointer in this direction was an unsuccessful experiment in which we removed from the cabinet cofactor fractions that had been filled into spectro-

Fig. 4. Determination of M_r for the molybdenum cofactor by gel filtration on Sephadex G-25

$\log M_r$ values for the standards indicated (see the Materials and methods section) were plotted against elution volume (V_e/V_0). A Sephadex G-25 column (1.6 cm $\times$ 60 cm) was used. The position of the bar corresponds to the elution volume for molybdenum cofactor activity, its upper and lower ends corresponding to M_r values of 700 and 520 respectively.

photometer cells and sealed, following gel filtration into 0.1 mM- $\text{Na}_2\text{S}_2\text{O}_4$, with a view to recording the u.v. spectrum of the intact cofactor. The more dilute samples so examined all showed absorption in the region of 310 nm, characteristic of oxidized pteridines. This absorption was not present in the stronger cofactor samples, in keeping with greater stability in more concentrated solution.

M_r of the molybdenum cofactor

We used gel filtration on Sephadex G-25 to determine the M_r of the active molybdenum cofactor. Results are presented in Fig. 4 and indicate the M_r to be approx. 610 ± 90 . (Limits of error are not readily calculated, since our data are towards the lower end of the working range of Sephadex G-25, where a linear relationship is not necessarily expected.) M_r for the structure proposed for the cofactor by Kramer *et al.* (1987) is 523 (with hydroxyl ligands on molybdenum in place of oxo groups, for the metal in the quadrivalent state). Our data are therefore consistent with the structure proposed by these workers.

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