PURIFICATION OF MOLYBDENUM COFACTOR AND ITS FLUORESCENT OXIDATION PRODUCTS

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1. Introduction

Molybdenum is a constituent of various enzymes both in prokaryotic and eukaryotic cells [1–3]. Except for nitrogenase [4] the known molybdo-enzymes are supposed to share a molybdenum-containing cofactor (Mocofactor) [2,3]. This Mocofactor can be extracted from the enzymes by organic solvents [4] or after acidification [2,6] or heat shock [5] by aqueous solutions. After extraction, the Mocofactor is usually present in a low-M_r oxygen-labile form [7,8]. In this free form, Mocofactor can be detected and quantitatively assayed by its ability to complement the defective nitrate-reductase complex of *Neurospora crassa nit1* [5,6]. Because of the high lability of the free cofactor, purification and characterization are difficult. Addition of stabilizing compounds as dithiothreitol and ascorbate does not prevent a loss of activity during purification [7,8], as measured in the complementation assay with *nit1*. Moreover, Mocofactor appears to lose all its molybdenum during purification steps [7,8]. In [9], the loss of Mocofactor activity was accompanied by increasing fluorescence. Excitation and emission spectra of the fluorescent species was recorded and it was suggested to contain a pterin structure [9].

The Mocofactor is also present in the cytoplasm and membranes of *Proteus mirabilis* [6]. Here, we present a fast purification method for Mocofactor in a biologically active state using reversed-phase, high-performance liquid chromatography (HPLC). Furthermore, we demonstrate that as a consequence of oxidation the Mocofactor is converted into at least 2 different fluorescent products. These behave identically for Mocofactor isolated from xanthine oxidase and from membranes of *P. mirabilis*, supporting the idea of a common Mocofactor [3] in different molybdo-enzymes.

2. Materials and methods

Purified xanthine oxidase suspensions (10 mg protein/ml) were obtained from Boehringer (Mannheim).

Proteus mirabilis S503 was grown under anaerobic conditions in the presence of 0.35% nitrate and 1 mM azide as in [6].

Membranes were prepared as usual [6]. Growth conditions of *Neurospora crassa nit1* and preparation of *nit1* extract were as in [6]. Two extraction procedures for Mocofactor were used. Extraction by acidification was done as in [6]. Heat extraction was done as in [5]. The heat-shock took place at 64°C for 1 min in the presence of 1.4 mg SDS/mg protein.

Mocofactor activity was measured by the determination of the formation of nitrate reductase activity in extracts of *N. crassa nit1* [2,5,6]. Reconstitution of the incomplete nitrate reductase complex of *nit1* took place by incubating 100 μl Mocofactor-containing sample, 50 μl *nit1* extract and 100 μl 10 mM potassium-phosphate buffer (pH 7.4) containing 0.5 mM EDTA and 0.5 mM NADPH over 30 min at room temperature. If reconstitution was performed in the presence of molybdate, the buffer contained also 20 mM sodium-molybdate. As measure for cofactor activity, the reconstituted nitrate reductase activity in 100 μl reconstitution mixture was determined by a colorimetrical estimation of the amount of nitrite, produced after 1 h at 20°C.

Excitation and emission spectra were recorded on a

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MC-320M fluorimeter (SLM-Instruments). Spectra were corrected for wavelength-dependent varieties in lamp intensity and for background emission.

Gel permeation chromatography was performed on a Sephadex G-50 fine column (60 cm × 0.9 cm) at 4°C, using anaerobic water as eluent. Separations by means of reversed-phase HPLC were done using an Altex 100 pump and a 25 cm × 4.6 mm (i.d.) stainless-steel column packed with 5 μm Spherisorb ODS. Sample volumes of 160 μl were injected. Elution was performed at room temperature using methanol–water (20:80, v/v) as mobile phase at a flow rate of 0.7 ml/min. Fluorescence was recorded using a Perkin-Elmer fluorescence spectrophotometer 204-A with the excitation and emission wavelength set at 380 nm and 455 nm, respectively.

3. Results

3.1. *Fluorescent properties of Mocofactor after oxidation*

Mocofactor was extracted from a purified xanthine oxidase suspension by the acidification procedure in section 2; Mocofactor extracted in this way has a low M_r [7,8]. The biological activity of the Mocofactor in this extract, as measured in the *N. crassa nit1* complementation assay, appeared to be unstable. After an aerobic incubation of only a few hours at room temperature, all cofactor activity is lost and fluorescence appears. Fig.1 shows an excitation and emission spectrum of the fluorescent species. The same spectrum was found for a Mocofactor-containing extract prepared by heat treatment in the presence of SDS. As long as the Mocofactor is a constituent of the original enzyme, no fluorescence arises at all.

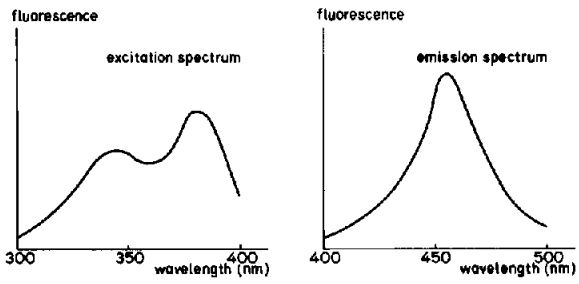

Fig.1. Excitation and emission spectrum of an aerobically incubated Mocofactor-containing extract prepared by acidification of xanthine-oxidase: excitation wavelength, 380 nm.

The development of fluorescence appeared to be dependent on the presence of oxygen during the incubation. If a free Mocofactor-containing extract was incubated under anaerobic conditions during several days, cofactor activity was still present and no fluorescence was observed. The appearance of fluorescence in a Mocofactor-containing extract could be accelerated by bubbling air through the solution, but it was always relatively slow in comparison with the expiration of the cofactor activity (fig.2). This indicates that upon oxidation the Mocofactor is first converted into a biologically inactive form, which does not fluoresce and subsequently into fluorescent products.

3.2. *Purification of Mocofactor in a biologically active state by means of HPLC*

Because of the time-consuming procedures required

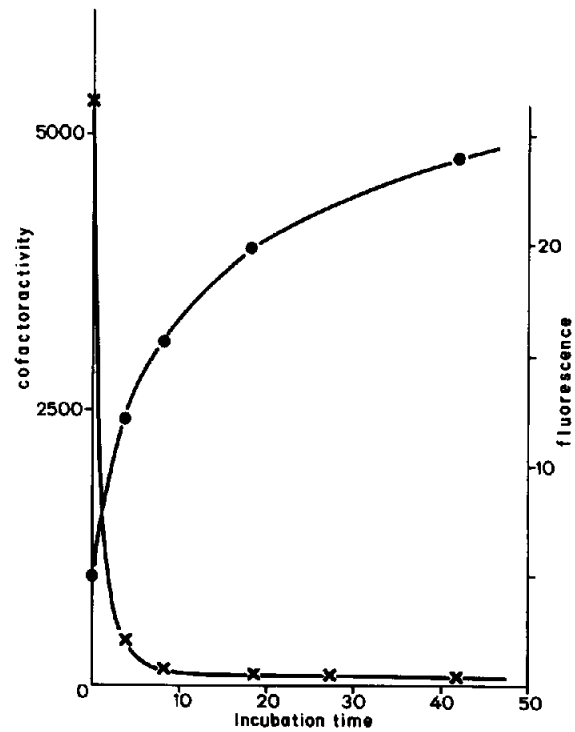

Fig.2. Decay of cofactor activity and development of fluorescence during aerobic incubation at 4°C in a Mocofactor-containing extract prepared by acidification of xanthine oxidase. Cofactor activity of the extract is expressed in NADPH-dependent nitrate reductase activity/mg xanthine oxidase protein after incubation of the extract with *nit1* extract in the presence of 8 mM molybdate as in section 2. Fluorescence was measured as the emission intensity (in arbitrary units) at 455 nm, using 380 nm as wavelength of excitation.

for the purification of Mocofactor by means of conventional gel permeation chromatography [4,7,8], the use of anaerobic eluents [4] or the addition of compounds to stabilize the Mocofactor in a biologically active state [7] is necessary to prevent a poor recovery. By means of HPLC, separation of extracts containing Mocofactor can be performed within 10 min, using a mixture of methanol and water as eluent. As a consequence, detection of cofactor activity is possible even after elution under aerobic conditions in the absence of stabilizing compounds.

Purification of Mocofactor in a biologically active form was done on a Spherisorb ODS column. A Mocofactor-containing suspension, prepared by acidification of xanthine oxidase was quickly separated in the HPLC system. As eluent a mixture of methanol and water (20:80, v/v) was used. Fig.3 shows that the biologically active Mocofactor eluted in one peak.

Mocofactor purified in this way was able to complement defective *nit1* nitrate reductase even in the absence of molybdate. Complementation was stimulated by addition of 8 mM molybdate to the same degree as usually reported for Mocofactor present in crude extracts. Apparently, Mocofactor does not loose all its molybdenum during this purification. If this freshly prepared xanthine oxidase extract was chromatographed in the same system with fluorescence detection using 380 nm as excitation wavelength and 455 nm as emission wavelength, hardly any fluorescence was observed.

3.3. *Purification of fluorescent compounds from aerobically incubated Mocofactor*

When a Mocofactor-containing extract prepared by heat shock of xanthine oxidase in the presence of SDS was incubated aerobically during a few hours at room temperature, all cofactor activity as measured in the *nit1* assay had disappeared. This was accompanied by the development of a fluorescent signal having excitation and emission maxima at 380 and 455 nm, respectively. To obtain more information on the homogeneity of the fluorescent species, we separated the fluorescent compounds present in the extract by means of HPLC again using methanol-water (20:80, v/v) as eluent (fig.4). Two main peaks were found. These peaks did not alter if the aerobic incubation was continued for several weeks; that is, at least two stable fluorescent oxidation products with different hydrophobic properties are formed during aerobic incubation.

To prove that these products were formed from Mocofactor the HPLC procedure was repeated with purified Mocofactor, obtained as follows. A Mocofactor-containing extract was prepared by heat-

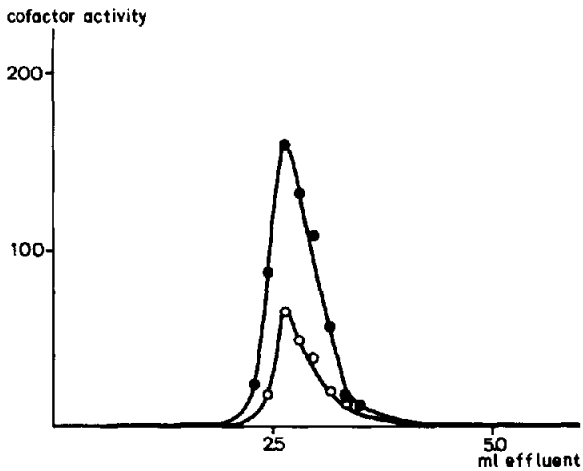

Fig.3. Elution pattern of biologically active Mocofactor extracted by acidification from xanthine oxidase. Fractions of 0.175 ml were pooled and cofactor activity was assayed, as in section 2. (x——x) Cofactor activity, expressed in NADPH-dependent nitrate reductase activity/ml fraction after reconstitution in the presence of 8 mM molybdate. (o——o) As in (x——x) but after reconstitution in the absence of molybdate.

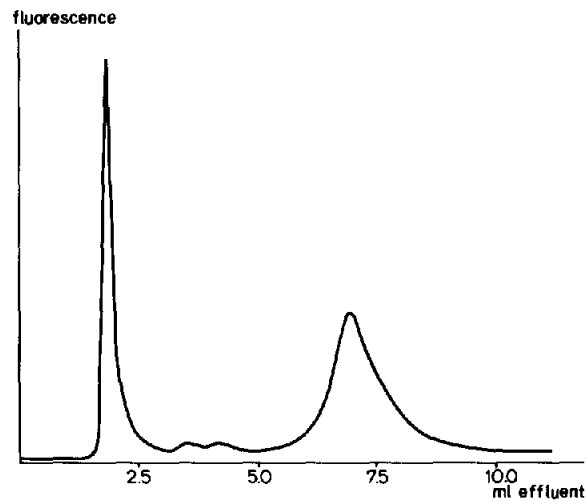

Fig.4. Elution pattern of fluorescent compounds present in a Mocofactor-containing extract after aerobic incubation. The extract was prepared by heat shock of xanthine oxidase in the presence of SDS. HPLC with fluorescence detection was done as in section 2.

shock of xanthine oxidase in the presence of SDS. Mocofactor from this extract was purified by fractionation on a Sephadex G-50 column using anaerobic water as eluent. Mocofactor eluted in the biologically active form as a low-M_r compound. Cofactor activity could only be detected if reconstitution with *nit1* was done in the presence of molybdate, that is, Mocofactor lost its molybdenum during this purification step. Afterwards all fractions were aerobically incubated at room temperature during at least 2 days and next analysed in the HPLC system using methanol–water (20:80, v/v) as eluent. Only fractions initially containing cofactor activity showed a chromatogram with two fluorescent peaks at the same retention as found for the crude oxidized extract (cf. fig.4). Fluorescence spectra of both peaks showed excitation and emission maxima at 380 and 455 nm, respectively as was the case for the spectra of the crude xanthine oxidase extract (cf. fig.1).

Using the same procedures we tried to determine if Mocofactor present in membrane-bound enzymes in *P. mirabilis* showed properties identical to Mocofactor extracted from xanthine oxidase. As shown in [6], membranes purified from *P. mirabilis* grown under anaerobic conditions in the presence of nitrate and azide contain enzyme-bound Mocofactor in high amounts as a consequence of the extremely strong derepression of nitrate reductase synthesis [10] observed under these conditions. The Mocofactor extracted from these membranes by heat treatment in the presence of SDS was also purified on a Sephadex G-50 column. The retention volume for Mocofactor was exactly the same as found for Mocofactor from xanthine oxidase. Fractions which contained cofactor activity were aerobically incubated during at least 2 days. Next the fluorescent products, formed during this incubation were analysed in the HPLC system with methanol–water (20:80, v/v) as eluent. Two peaks were found at virtually the same retention volumes as observed for peaks from Mocofactor extracted from xanthine oxidase (fig.5). Fluorescence spectra of both peaks showed an excitation and emission maximum at 380 and 455 nm, respectively.

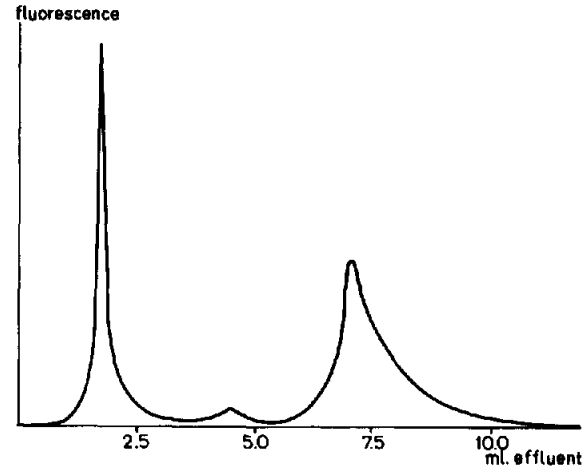

Fig.5. Elution pattern of fluorescent compounds present in a purified Mocofactor-containing extract after aerobic incubation. Mocofactor was extracted by heat shock in the presence of SDS from a membrane suspension, prepared from *P. mirabilis*, grown under anaerobic conditions in the presence of nitrate and azide. Afterwards, Mocofactor, present in this extract, was purified on Sephadex G-50. HPLC with fluorescence detection was carried out as in section 2.

4. Discussion

Thus, Mocofactor can be purified in a biologically active state using reversed-phase HPLC. The cofactor can be purified partly in the molybdo-form although elution takes place in the absence of molybdate. In conventional gel permeation chromatography, this cofactor is obtained in demolybdo-form under these circumstances [7].

This study confirms the observation [9] that fluorescence occurs by oxidation of Mocofactor present in a non-enzyme-bound form. The corrected fluorescence spectra recorded after oxidation show excitation and emission maxima at 380 and 455 nm, respectively, which are also reported for pterins. Other cofactors which contain pterin structures generally only play their biological role if they are present in the fully reduced tetrahydro-form, which is oxygen-labile [11]. Upon oxidation, one or more dihydro-forms are produced before the fully oxidized and fluorescent form will be present, as known for instance for tetrahydrobiopterin [12]. We have observed that the development of fluorescence does not occur at the same moment as the loss of cofactor activity. Probably, one or more non-fluorescent and biologically inactive products were formed as a first step in the oxidation of Mocofactor. On the basis of data known for biopterin [12], one may expect the non-fluorescent product to be a dihydro-form.

After continued aerobic incubation of Mocofactor HPLC analysis showed the presence of 2 fluorescent

peaks with Mocofactor from xanthine oxidase and from bacterial membranes. The fluorescence spectra of both peaks display excitation and emission maxima at 380 and 455 nm, respectively. Obviously, at least 2 pterin structures with different hydrophobic properties are formed by oxidation of Mocofactor. These results confirm the idea of the presence of an identical Mocofactor in various molybdo-enzymes [2,3].

Acknowledgements

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