Journal of Chromatography, 267 (1983) 409–413
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 15,970

Note

Reversible immobilization of molybdenum cofactor on a gel matrix via sulphydryl groups

RALF R. MENDEL* and ZEREKBAI A. ALIKULOV*
Zentralinstitut für Genetik und Kulturpflanzenforschung, Academy of Sciences, 4325 Gatersleben (G.D.R.)
(Received May 6th, 1983)

The molybdenum cofactor (Mo-co) is a low-molecular-weight, oxygen-sensitive component common to numerous molybdoenzymes (*e.g.*, nitrate reductase, xanthine oxidase, sulphite oxidase, aldehyde oxidase) where it constitutes part of the catalytically active centre[1]. Mo-co bound to cofactor-containing proteins can be released in a functionally active form by heat denaturation of these proteins in the presence of Mo-co-protecting agents (ascorbic acid and/or thiol reagents)[2,3]. Recently, Mo-co from rat liver sulphite oxidase was suggested to contain sulphur[4].

Very recently, Mo-co released from milk xanthine oxidase was shown to contain free sulphydryl groups which are involved in the cofactor-mediated process of subunit dimerization of *Neurospora crassa* nitrate reductase as well as in the correct liganding of molybdenum[5]. The hitherto unknown presence of sulphydryl groups on Mo-co made it possible to investigate conditions for the covalent chromatography of Mo-co in order to immobilize the cofactor via sulphydryl groups on a gel matrix.

Reduced glutathione (GSH) was reported to be a highly efficient stabilizer of Mo-co activity, being superior to other commonly used thiol reagents[5]. Commercially available Thiol-Sepharose 4B (Sepharose–glutathione-2-pyridyldisulphide) contains GSH as an active ligand covalently coupled to the gel matrix. In this work we made use of the appearant high affinity of Mo-co's sulphydryl groups to GSH and a method is described for reversible covalent immobilization of Mo-co on Thiol-Sepharose 4B. Gel-bound Mo-co exhibited a very high stability and could be specifically eluted in a highly active form.

EXPERIMENTAL

Preparation of Mo-co

Mo-co was released from xanthine oxidase by heat treatment as described recently[3]. A 10-μl volume (= 0.1 mg of protein) of milk xanthine oxidase (Serva, Heidelberg, F.R.G.) was diluted in 1 ml of buffer A (50 m*M* potassium/sodium phosphate buffer, pH 7.5, 0.5 m*M* EDTA) containing 1 m*M* ascorbic acid and 25 m*M* sodium molybdate. The solution was thoroughly evacuated, flushed with nitrogen,

* Permanent address: Institute of Botany, Academy of Sciences of Kazakh. SSR, 480100 Alma-Ata 100, U.S.S.R.

0021-9673/83/$03.00

stoppered, incubated for 90 sec at 80°C and chilled on ice. Precipitated protein was removed by centrifugation.

Assay of Mo-co

Mo-co has been assayed by restoration of NADPH-nitrate reductase (E.C. 1.6.6.2) activity in extracts of *Neurospora crassa* mutant *nit*-1. Strain *nit*-1 was grown on NH_4 (as a nitrogen source), induced by nitrate for 5 h and extracted as described earlier[3,6]. The assay of Mo-co was carried out exactly as described previously[3]. A 100-μl volume of *nit*-1 extract and 50 μl of Mo-co were used per assay. The times for complementation and for the nitrate reductase test were 40 and 15–30 min, respectively. One unit of Mo-co activity will reconstitute the *nit*-1 nitrate reductase to 1 nmol/min.

Coupling of Mo-co to the gel matrix

For analytical purposes, about 0.5 g of activated Thiol-Sepharose 4B (Pharmacia, Uppsala, Sweden) was mixed with 10–20 volumes (w/v) of deaerated buffer A containing 0.3 *M* sodium chloride, evacuated, flushed with nitrogen and swollen overnight at 2°C. After extensive washing with the same buffer, about 200 μl of wet settled gel were mixed with 1 ml of Mo-co solution (containing 0.3 *M* sodium chloride), evacuated, flushed with nitrogen and incubated for 20 min at 2°C with slight shaking. Subsequently, the gel particles were sedimented by centrifugation (for 1 min at 1000 *g*) and the supernatant was carefully sucked off. The gel was aerobically washed twice with buffer A (*i.e.*, resuspended in 10 ml of buffer A and sedimented by centrifugation, in each instance). Bound Mo-co could be eluted by resuspending the sedimented gel for 5 min in 1 ml of buffer A containing 5 m*M* GSH and 25 m*M* sodium molybdate. Used gel can be regenerated according to the manufacturer's procedure[7].

RESULTS AND DISCUSSION

The ratio of gel matrix to Mo-co was chosen so that more than 95% of the Mo-co applied was coupled to the gel, *i.e.*, up to 1000 units of Mo-co per millilitre of swollen gel (Table I). Neither a second and third wash of the loaded gel with buffer A nor washing with 1 *M* potassium chloride solution removed detectable amounts of Mo-co, thus demonstrating that non-specific binding did not occur. Addition of 1–5 m*M* GSH and 25 m*M* molybdate to the gel caused immediate and quantitative release of catalytically highly active Mo-co (Table I). Molybdate without GSH is not able to elute bound Mo-co from the gel, but the presence of molybdate is essential for obtaining maximal activity of Mo-co in the subsequent *nit*-1 complementation assay. Therefore, molybdate was added to the elution buffer.

The coupling rection between Mo-co and Thiol-Sepharose was complete within 20 min at 2°C and was not impaired by performing it at room temperature and/or under aerobic conditions (Table II). Coupling was routinely performed in the presence of 0.3 *M* sodium chloride (a range of 0.2–0.5 *M* is advisable), as it was observed that omission of at least 0.2 *M* sodium chloride resulted in an approximately 10% lower efficiency of coupling under standard conditions.

Preparation of Mo-co by heat treatment of xanthine oxidase as the Mo-co source requires the presence of reductants in order to preserve the sulphydryl groups

TABLE I

COVALENT CHROMATOGRAPHY OF Mo-co ON THIOL-SEPHAROSE 4B

Step	*Mo-co activity (units/ml)*
Mo-co before coupling	165
Supernatant after coupling	8
2nd wash with buffer A*	0
Elution with:	
25 m*M* Na_2MoO_4, 1 m*M* Asc**	0
25 m*M* Na_2MoO_4, 1 m*M* Asc, 1 *M* KCl	0
25 m*M* Na_2MoO_4, GSH: 0.5 m*M*	80
1 m*M****	149
5 m*M****	156
10 m*M****	147

* 5 m*M* GSH and 25 m*M* Na_2MoO_4 added after elution.
** Asc = ascorbic acid.
*** Separate elutions with the same gel batch and Mo-co preparation.

of Mo-co[3]. The most powerful reductant for this purpose was found to be 5 m*M* GSH[5] which, however, owing to its interference with the covalent chromatography of Mo-co, had to be replaced with 1 m*M* ascorbic acid, which is also known as a potent Mo-co protector[2,3]. If xanthine oxidase was heat treated in the absence of any reductant, the Mo-co lost its catalytic activity irreversibly. However, if xanthine oxidase was heat treated in the presence of Thiol-Sepharose, *i.e.*, the heat release of Mo-co and the coupling rection were combined into one step, Mo-co was fully stabilized and eluted highly active from the gel (Table III). This protective effect of Thiol-Sepharose is obviously due to the matrix-bound GSH which serves as an active coupling ligand and even in the bound state seems to be strong enough to protect Mo-co. Although attractive, the Mo-co preparation and the coupling reaction were not combined for routine experiments as the gel matrix Sepharose 4B underwent gradual degradation during repeated cycles of heating.

TABLE II

TIME COURSE AND CONDITIONS OF COUPLING OF Mo-co TO THIOL-SEPHAROSE 4B

*Coupling conditions**	*Mo-co activity*** *(units/ml)*
5 min	120
10 min	144
15 min	152
20 min	160
30 min	160
30 min, aerobic	159
30 min at 20°C	160
30 min at 20°C, aerobic	161

* Separate couplings with 200 μl of swollen gel and 1 ml of Mo-co in each instance.
** Determined after elution with GSH and molybdate.

TABLE III

PROTECTING EFFECT OF REDUCTANTS ON Mo-co ACTIVITY DURING HEAT RELEASE

Additive to buffer A	*Mo-co activity (units/ml)*
25 m*M* Na_2MoO_4 + 1 m*M* ascorbic acid*	166
25 m*M* Na_2MoO_4*	0
25 m*M* Na_2MoO_4 + Thiol-Sepharose**	159

* Mo-co was heat-released in the presence of the additives indicated (conditions exactly as described under Experimental) and immediately tested for activity.

** 200 μl of swollen gel per 1 ml of heat-treatment mixture (NaCl omitted). After heat treatment, coupling was continued for 30 min as described under Experimental. Mo-co activity was determined after elution with GSH and molybdate.

The coupling of Mo-co to matrix-bound GSH increased the stability of the Mo-co several-fold. At 2°C, gel-bound Mo-co exhibited at least a ten-fold higher stability compared with that of the free form (half-life more than 5 days *versus* 12 h) (Table IV). At 20°C, a similar ratio between the stabilities of free and gel-gound Mo-co was observed, although the half-lives were generally lower. The data in Table 4 show that tempereature has a remarkable effect on the oxygen sensitivity of bound Mo-co. The same observation has been reported for free Mo-co in non-aqueous solvents[8]. Under aerobic and anaerobic conditions, molybdate exerted a stabilizing effect on Mo-co. At 20°C, anaerobic storage of gel-bound Mo-co in the presence of 25 m*M* molybdate turned out to be the most efficient variant for preserving the high activity of Mo-co.

TABLE IV

HALF-LIVES OF FREE AND GEL-BOUND Mo-co UNDER DIFFERENT CONDITIONS OF STORAGE

Storage conditions	*Half-life (h)*
2°C:	
Free*, $-O_2$, Asc + Mo**	12
GSH + Mo	26
Bound***, $+O_2$, + Mo	$\gg 120$
20°C:	
Free*, $-O_2$, Asc + Mo	4
GSH + Mo	20
Bound***, $-O_2$	81
$-O_2$ + Mo	>120
$+O_2$	54
$+O_2$ + Mo	68

* Free = heat-released Mo-co not coupled to gel.

** Concentrations used throughout: Asc = 1 m*M* ascorbic acid; Mo = 25 m*M* Na_2MoO_4; GSH = 5 m*M*.

*** Bound = heat-released Mo-co coupled to Thiol-Sepharose 4B as described under Experimental and stored under the conditions indicated. Mo-co activity was determined after elution with GSH and molybdate.

In conclusion, the highly stable gel-bound Mo-co could serve as a means for studying the interactions between immobilized Mo-co and Mo-co-binding proteins or protein domains, as well as for elucidating the physical and chemical properties of immobilized Mo-co.

ACKNOWLEDGEMENTS

We are indebted to Dr. A. J. Müller for critically reading the manuscript. This research was supported by a research award of the Academy of Sciences of the G.D.R. to Z.A.A.

REFERENCES

1 J. L. Johnson, in M. Coughlan (Editor), *Molybdenum and Molybdenum-containing Enzymes*, Pergamon Press, Oxford, 1980, pp. 345–383.
2 Z. A. Alikulov, N. P. Lvov, S. H. Burikhanov and V. L. Kretovich, *Izv. Akad. Nauk SSSR, Ser. Biol.*, No. 5 (1980) 712–718.
3 R. R. Mendel, *Phytochemistry*, 22 (1983) 817–819.
4 J. L. Johnson and K. V. Rajagopalan, *Proc. Nat. Acad. Sci. U.S.*, 79 (1982) 6856–6860.
5 Z. A. Alikulov and R. R. Mendel, *Biochim. Biophys. Acta*, submitted for publication.
6 R. R. Mendel, Z. A. Alikulov, N. P. Lvov and A. J. Müller, *Mol. Gen. Genet.*, 181 (1981) 395–399.
7 *Affinity Chromatography*, Pharmacia, Uppsala, 1979.
8 P. T. Pienkos, V. K. Shah and W. J. Brill, *Proc. Nat. Acad. Sci. U.S.*, 74 (1977) 5468–5471.