The Copper–Molybdenum Antagonism in Ruminants. IV. Reaction of Thiomolybdate Ions with Proteins and Metalloproteins

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(Received June 22, 1987)

Administration of thiomolybdate salts, $M_2[MoO_xS_{4-x}]$ ($M = NH_4^+$ or Na^+, $x = 0–2$) to both ruminants and non-ruminant animals causes marked changes in the distribution of copper within blood plasma [1–3]. The evidence points to the thiomolybdate ion being principally associated with the protein albumin [4]. Inhibition of the activity of Cu enzymes has also been implicated [5, 6]. The use of $(NH_4)_2MoS_4$ in treating the Cu overload of Wilson's disease patients also shows that a non-absorbable Cu form appears in blood plasma [7].

We have reported results on the nature of the interactions between the thiomolybdate ions and Cu(II) compounds in aqueous media [8, 9]. We now wish to report our observations on the interactions involving Cu–albumin and Cu–thionein and the corresponding Cu-free proteins. These observations complement the more biochemical studies mentioned above.

Experimental

$(NH_4)_2MoO_2S_2$, K_2MoOS_3 and $(NH_4)_2MoS_4$ were prepared and characterised as previously described [8]. Bovine serum albumin (Fluka Ltd.) was used without further purification, analysis showed it to contain a small amount (*ca.* 0.1 ppm) of copper contaminant. Cysteine-protected bovine albumin monomer was purchased from Miles Laboratories Ltd., and used without further purification. A solution of Cu(I)–thionein was prepared as follows: Zn–thionein (0.037 g)† was dissolved in water (4 cm^3) and the pH adjusted to *ca.* 1.5 with conc. HCl. To this was added a freshly prepared aqueous solution of $Cu^I(CH_3CN)_4ClO_4$ (0.096 g in 25 cm^3 aqueous tris-acetate buffer, pH 7.4, containing 1 mol dm^{-3} CH_3CN). 3 cm^3 of the mixture was passed through a Sephadex G-25 column and eluted with water under anaerobic conditions. Elution of the Cu(I)–thionein was monitored by UV absorption. The fractions containing product were pooled, made up to 50 cm^3 distilled water and stored at 0 °C under an N_2 atmosphere. The Cu(I) concentration of the final solution was calculated as being 3.9×10^{-4} mol dm^{-3}, giving *ca.* 9–10 mol Cu per mol protein. All reactions involving the Cu(I)–thionein were carried out under anaerobic conditions. All other reagents were of 'AnalaR' or equivalent grade.

The extent of association of the thiomolybdate ions with the proteins was determined by ultrafiltration using either an immersible molecular separator (Millipore) with a nominal molecular weight limit of 10 000 or a Diaflo UM2 ultrafilter with a nominal molecular weight limit of 1000. Aqueous solutions of the 1:1 Cu(II)–albumin complex were freshly prepared by the mixing of solutions of $CuCl_2 \cdot 2H_2O$ and albumin (Cu(II) 1.0×10^{-4} mol dm^{-3}, albumin 5.8×10^{-4} mol dm^{-3}). All solutions were monitored by UV/Vis spectroscopy. Reactions were initiated by the rapid mixing of freshly prepared solutions at 25 °C. The reactant mixtures were buffered to pH 7.4 and contained 0.15 mol dm^{-3} NaCl.

UV/Vis spectra were recorded with a Perkin Elmer 555 spectrophotometer and ESR spectra with a Bruker ER-200D instrument using liquid-N_2 frozen samples.

Results

Thiomolybdate Anion Binding to Albumin Protein

Membrane filtration experiments showed that the thiomolybdate ions bind to albumin (no added metal ions). The binding is relatively weak with association constants of the order of unity (conditions: pH 7.4, 0.15 mol dm^{-3} NaCl, 25 °C). This value is only approximate because of complications from hydrolysis of the thiomolybdate ions, the hydrolytic reaction rate following the order $MoO_2S_2^{2-} > MoOS_3^{2-} > MoS_4^{2-}$. No changes were observed in the UV/Vis spectra of the thiomolybdate ions on initial binding to albumin. Further experiments with MoS_4^{2-} showed the protein binding to be dependent on the NaCl concentration, the extent of binding increasing with decreasing salt concentration. The same results were obtained with the cysteine-blocked albumin protein.

Thiomolybdate Anion Binding to Copper–Albumin

Mixing of MoS_4^{2-} and 1:1 Cu:albumin solutions resulted in decreased absorbance of the MoS_4^{2-} peaks at 466 and 316 nm with new peaks appearing at 500 and 350 nm. These changes were relatively slow (see Fig. 1). The final absorbance at 500 nm

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†Kindly supplied by Dr. I. Bremner, Rowett Research Institute, Aberdeen.

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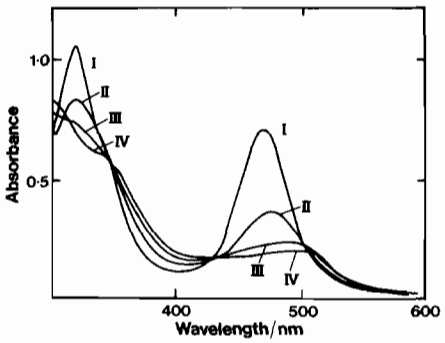

Fig. 1. Effect of addition of MoS_4^{2-} to Cu–albumin solutions at 25 °C. Reactant concentrations both 1.0×10^{-4} mol dm^{-3}. Spectra recorded after (I), 1 min, (II) 11 min, (III) 1 h, and (IV) 24 h.

was linearly related to the initial MoS_4^{2-} concentration. ESR spectra showed the disappearance of the Cu^{II} signal (see ref. 9) as MoS_4^{2-} was added. The rate and extent of the disappearance of the ESR signal exactly paralleled the changes in the UV/Vis spectra. From these changes, the stoichiometry of the reaction appeared to be 1:1 MoS_4^{2-}:Cu–albumin. No insoluble products were obtained at any stage.

The di- and trithiomolybdates exhibited similar ESR and UV/Vis spectroscopic changes to that of MoS_4^{2-} on their mixing with Cu–albumin. These reactions were not followed in detail, however, because of competing hydrolytic reactions.

Thiomolybdate Anion Binding to Metallothioneins

The ultrafiltration experiments, after making allowance for the binding of the anions to the UM2 membranes used, showed there was no reaction or binding of the thiomolybdates with zinc–thionein.

With Cu(I)–thionein, however, reaction occurred which, judged by the UV/Vis spectra, mirrored those obtained with Cu(II)–albumin. Thus, with MoS_4^{2-} the absorbances at 416 and 315 nm decreased and new bands appeared at 510 and 350 nm (compare Fig. 1). The stoichiometry of this reaction, as judged from the disappearance of the 466 nm peak, appeared to be 2:1 Cu–thionein:MoS_4^{2-}. For $MoO_2S_2^{2-}$ and $MoOS_3^{2-}$, the stoichiometry appeared to be 1:1. No precipitates were observed.

Conclusions

The results clearly show that the thiomolybdate ions bind via ionic interactions to albumin protein and not via covalent disulphide bond formation. With Cu–albumin specific reaction occurs with the Cu(II) ion leading to ternary $MoO_xS_{4-x}^{2-}$–Cu(I)–albumin complexes. The changes observed in the ESR and UV/Vis spectra closely parallel those reported earlier using low-molecular Cu(II) complexes [9]. However, no insoluble products were obtained with the proteins and there was no finding of a Mo(V) ESR signal, the latter could be due to the greater dilution of the protein solutions.

The observations parallel those made by Mason and co-workers [2] who showed that after introducing thiomolybdate ions into the rumen of ruminants, these ions appeared in the plasma bound to albumin. More recently, Woods and Mason [10] have shown that $MoOS_3^{2-}$ can reversibly form an $MoOS_3^{2-}$–Cu–albumin complex but conclude that this may not involve the known Cu binding site of albumin. The reduction of Cu(II) to Cu(I) was not reported in these studies [2, 10].

The facile reaction between thiomolybdates and Cu remarkably occurs with both Cu oxidation states. This is reflected in the reactions with Cu–thionein. These latter reactions warrant further studies to assess their physiological importance, particularly in view of recent reports of increased biliary excretion of Cu following intravenous administration of MoS_4^{2-} to sheep [11].

It is likely then that these ternary complexes play an important role in the systemic interactions of thiomolybdates and copper which are of importance in the Mo–Cu antagonism of ruminants and in the use of $(NH_4)_2MoS_4$ in the treatment of Wilson's disease patients.

Acknowledgements

D.E.P. was supported by a Leicestershire Education Authority research assistantship. Part of the work was done at the Rowlett Research Institute, Aberdeen, under the auspices of a Frank Horne award (to N.J.C.). We also thank Dr. J. Mason for his preprint of ref. 10.

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