Molybdenum-95 N.M.R. Spectroscopy as a Probe of Biological Systems: the Detection of Tetraoxo- and Tetrathio-molybdate(VI) bound to Bovine Serum Albumin

Stuart Bristow,[a] C. David Garner,*[a] Stuart K. Hagyard,[a] Gareth A. Morris,*[a] John R. Nicholson,[a] and Colin F. Mills[b]

[a] *Department of Chemistry, University of Manchester, Manchester M13 9PL, U.K.*
[b] *Department of Inorganic Biochemistry, Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, U.K.*

The feasibility of using ^{95}Mo n.m.r. spectroscopy to monitor the environment of this nucleus in biochemical systems has been established by the observation of the binding of $[MoO_4]^{2-}$ and $[MoS_4]^{2-}$ to the protein bovine serum albumin.

The use of ^{95}Mo n.m.r. as a routine spectroscopic technique in the characterisation of molybdenum compounds and as a probe of reactivity patterns is now established.[1] However, despite the fact that molybdenum is well-known as a biologically active element,[2] no studies have yet been reported in which the n.m.r. properties of the spin-5/2 nuclei ^{95}Mo and ^{97}Mo have been used to probe the nature of a biological system. In order to establish the feasibility of direct observation of ^{95}Mo n.m.r. signals in biochemical systems, we report here experiments establishing that it is indeed possible to observe signals for ^{95}Mo bound to a protein.

Serum albumins are a well-studied class of proteins known

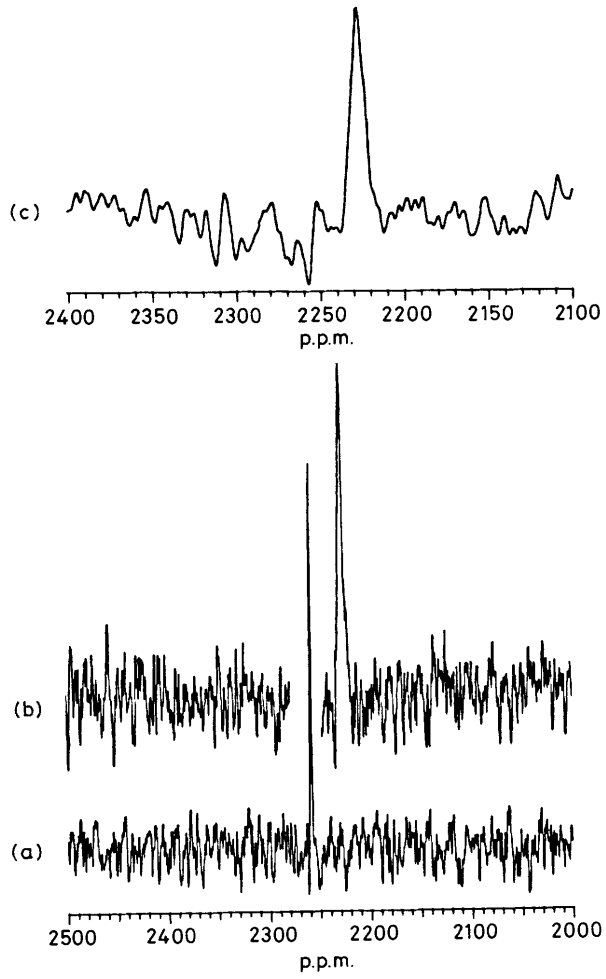

Figure 1. ^{95}Mo N.m.r. spectra of: (a) ^{95}Mo-enriched $[NH_4]_2[MoS_4]$ (3 mM) in D_2O; (b) ^{95}Mo-enriched $[NH_4]_2[MoS_4]$ (3 mM) and BSA (3 mM) in D_2O; (c) natural-abundance $[NH_4]_2[MoS_4]$ (1.5 mM) and BSA (1.5 mM) in D_2O. Acquisition parameters: spectral width = 10 kHz; acquisition time = 0.1 s; pulse width = 25 μs (75°); number of transients = 10^4 for (a) and (b); 10^6 for (c); 100 μs delay between pulse and acquisition. Free induction decay (F.I.D.) weighted by $\exp(-t/0.016)$ (20 Hz exponential line broadening) for (a) and (b), $\exp(-t/0.003)$ (100 Hz exponential line broadening) for (c) and by $1-[\exp(-t/0.001)]$ (convolution difference).

to bind a wide variety of inorganic ions. Indications that the association of $[MoO_4]^{2-}$ and $[MoS_4]^{2-}$ with plasma albumin is involved in the metabolic block to copper utilisation induced by molybdenum[3] prompted the choice of molybdate bound to the protein bovine serum albumin (BSA, molecular weight 66 000 dalton) as a suitable system for study. The binding of $[MoO_4]^{2-}$ to BSA has recently been investigated by equilibrium dialysis and polarography.[4]

A solution of ^{95}Mo-enriched $Na_2[MoO_4]$ was prepared by dissolving MoO_3 (96% ^{95}Mo *ex* U.K.A.E.A., Harwell) in NaOH (3 M); ^{95}Mo-enriched $[NH_4]_2[MoS_4]$ was prepared by standard methods.[5] Natural abundance samples of $[MoS_4]^{2-}$ were similarly prepared from $[NH_4]_6[Mo_7O_{24}]\cdot 4H_2O$ (BDH reagent grade) and H_2S (BDH); $Na_2[MoO_4]\cdot 2H_2O$ (BDH reagent grade) and BSA (Sigma, Fraction V, fatty acid free) were used as purchased.

^{95}Mo N.m.r. spectra were recorded by the pulse Fourier transform technique on a Varian XL-300 spectrometer at 19.55 MHz, using a homemade probe with solenoidal coil geometry. Samples in deuteriated water were contained in

Table 1. Chemical shift of ^{95}Mo in $[MoO_4]^{2-}$ and $[MoS_4]^{2-}$ as a function of mole ratio of BSA.

Mole ratio = [BSA]/[Mo]	Chemical shift/p.p.m. $[MoO_4]^{2-}$	$[MoS_4]^{2-}$
0	0.0	2259
0.025	—	2255
0.05	—	2253
0.075	—	2249
0.1	—	2246
0.25	−2.5	2232
0.5	−3.0	2227
0.75	−5.7	—
1.0	−7.0	2226
5.0	−9.8	—

cylindrical tubes of diameter 11.5 mm, length 36 mm, and were not spun; the temperature was 19 ± 1 °C. Chemical shifts were referenced *via* internal D_2O as a secondary reference, to a 2 M solution of $Na_2[MoO_4]$ in D_2O at apparent pH 11. Typical spectra are given in Figure 1. The homemade probe was constructed in an attempt to reduce 'acoustic ringing',[6] which manifests itself as a strongly undulating baseline, and to improve sensitivity. Although the ringing was reduced by this probe, it was necessary to employ convolution difference techniques to obtain a flat baseline.

Two sets of titration experiments were performed similarly by preparing 2.5 mM solutions of $Na_2[MoO_4]$ and 5 mM solutions of $[NH_4]_2[MoS_4]$ and BSA. Various volumes of BSA solution were added to fixed volumes of molybdate solution and made up to the same final volume, giving a range of mole ratios BSA:Mo for both molybdenum species; $[MoO_4]^{2-}$ mixtures were neutralised with HCl. The overall concentrations of molybdenum in the two experiments were 1.25 and 2.5 mM, respectively. The pH of the solutions was in the range 7—8.

The chemical shifts of ^{95}Mo in $[MoO_4]^{2-}$ and $[MoS_4]^{2-}$ as a function of mole ratio of BSA are shown in Table 1 and in Figure 2. The presence of a single signal in each case indicates rapid chemical exchange between the free and bound anion, the observed shift being the weighted average of the free and bound shifts. In both cases clear signals can be seen at millimolar concentrations; transverse relaxation times decrease on binding to about 3 ms, *i.e.* a limiting excess line broadening of 100 Hz. Given the presence of rapid chemical exchange in the systems studied, are the linewidths seen the same as those which would be observed for a kinetically stable $[MoO_4]^{2-}$ (or $[MoS_4]^{2-}$)–BSA conjugate? Providing that chemical exchange is reasonably slow, the limiting linewidth at large BSA:$[MoO_4]^{2-}$ (or $[MoS_4]^{2-}$) mole ratio will be the 'true' linewidth of bound ^{95}Mo plus any contribution from exchange. However, if exchange is extremely rapid it may interfere with the transverse relaxation mechanism. This would require exchange on a timescale of 10—100 ps, which has been shown not to be the case for $^{35}Cl^-$ exchange,[7] and hence is most unlikely to apply to $[MoO_4]^{2-}$ and $[MoS_4]^{2-}$. Thus, the observed linewidths are certainly no less than would be found for $[MoO_4]^{2-}$ (or $[MoS_4]^{2-}$) permanently bound to BSA (*i.e.* not exchanging with the corresponding free anion).

The $[MoS_4]^{2-}$ curve in Figure 2 fits well to a simple model of 6 equivalent binding sites per BSA, with binding constants about 2 $dm^3\ mmol^{-1}$. For $[MoO_4]^{2-}$ such a crude model is clearly inadequate; Arora *et al.*[4] reported the existence of two distinct types of site, with slightly stronger binding than that observed here.

The crucial parameter governing the observability or otherwise of a quadrupolar nucleus such as ^{95}Mo in a given

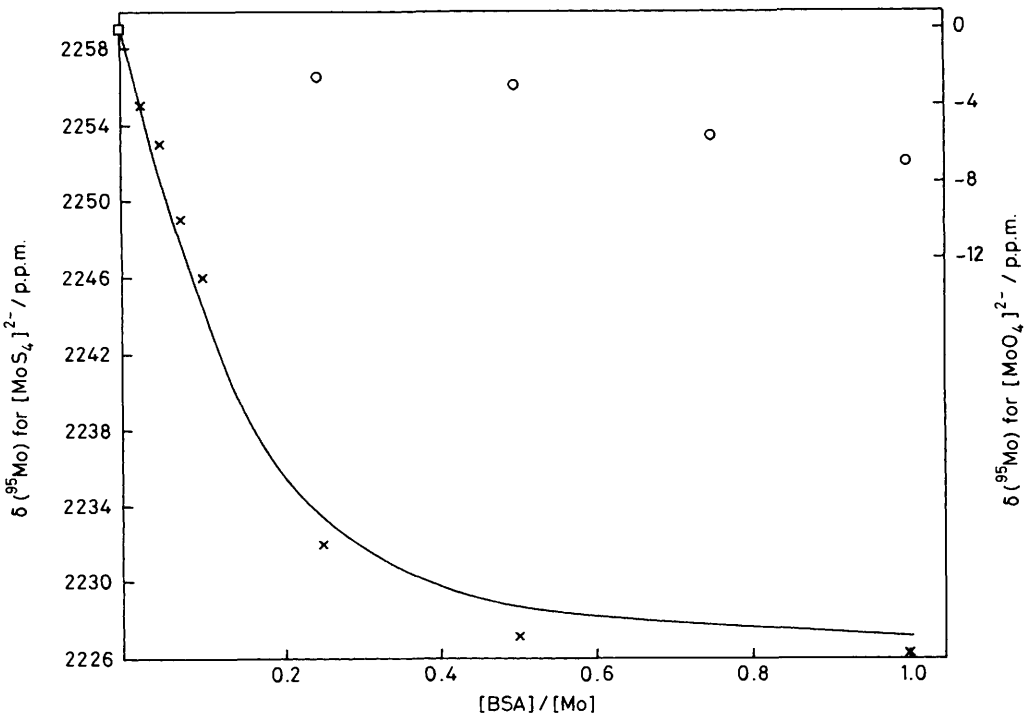

Figure 2. ^{95}Mo N.m.r. chemical shift of $[MoS_4]^{2-}$ (×) and $[MoO_4]^{2-}$ (○) as a function of mole ratio of BSA. The solid line is a curve calculated for $K = 2$ dm^3 mmol^{-1} and $n = 6$.

sample is linewidth, which in turn depends on the magnitude of the electric field gradient at the nucleus and on the rotational correlation time. Not only does an increase in the linewidth decrease peak height and hence degrade signal-to-noise ratio, but also it makes the discrimination between signals and 'acoustic ringing' more difficult: this is often the limiting factor for ^{95}Mo. The spectra reported here establish that, if molybdenum sites in metalloproteins have a reasonably symmetrical ligand environment (*i.e.* a low electric field gradient at the nucleus), it should be possible to observe natural abundance ^{95}Mo signals at submillimolar concentrations. Where enrichment is possible, the detection limit should fall to 100 μM, *i.e.* about 10 p.p.m. Since the chemical shift of molybdenum is known to be highly sensitive to its chemical environment,[1] the spectra reported here confirm the considerable potential of ^{95}Mo n.m.r. spectroscopy in the study of molybdenum-containing proteins and coenzymes.

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