^{1}H NMR study of long and short myosin S2 fragments

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The 270 MHz ^{1}H NMR spectra of rabbit skeletal long and short S2 were indistinguishable at 20°C and 30°C and contained only a small proportion of sharp peaks associated with flexible regions. At 60°C both proteins were denatured and had essentially identical spectra. At 40°C and 50°C the long S2 spectrum contained a marginally greater proportion of sharp peaks, representing not more than 25 residues/chain. Our results are consistent with the presence of a small hinge in long S2 but do not support its containing an extensive region which provides contractile force by a helix–coil transition.

Myosin *Long S2* *Short S2* *NMR* *Hinge* *Fibrous protein*

1. INTRODUCTION

The myosin molecule has two globular heads attached to a long rod-like tail [1]. The heads bind to actin filaments and generate tension while the tails form the backbone of thick filaments and also provide a linkage between these filaments and the heads [2]. During contraction the head moves from the vicinity of the thick filaments to bind to actin and so there must be some flexibility in the part of the tail that forms the connecting linkage. This has given rise to the concept of a 'hinge' [3] or local disruption of the coiled-coil structure. This hinge region may also undergo the active shortening which actually produces contraction [4]. In this instance, it is proposed that a substantial proportion of the myosin rod (~ 150–200 residues) melts and shortens by way of a helix–coil transition.

Limited proteolysis of myosin produces fragments which can be broadly associated with the different functional regions of the molecule [1]. The material thought to correspond to the linkage between the heads and the thick filaments is referred to as S2 (subfragment-2) and by appropriate choice of digestion conditions can be obtained as either a long S2 fragment, of M_r 59 000, or short S2, of M_r 34 000 [5]. Both long and short S2 contain the section of the tail immediately before the heads and long S2 contains in addition the portion of the tail in which the hinge is thought to be located [6,7].

^{1}H NMR spectroscopy affords a powerful method for investigating flexibility and mobility in proteins, since mobile regions give rise to sharp resonances which are easily distinguished from the broader signals deriving from the more rigid bulk of the molecule [8]. We have used this method here to examine long and short S2 over 20–60°C to see if a clearly flexible region associated with the hinge could be identified.

2. MATERIALS AND METHODS

Rabbit skeletal myosin was digested with chymotrypsin to produce myosin rod (the entire tail portion of the molecule) as described [5] and intramolecular disulphide bonds were formed in this material by oxidation with dithio-bis-nitrobenzoic acid [9]. Long and short S2 were produced by tryptic digestion of rod in 0.6 M NaCl, 10 mM Na-phosphate (pH 7) at an enzyme/substrate = 1:50 for 15 min (long S2) or 30 min (short S2) at room temperature. After precipitation of light meromyosin and undigested rod by dialysis to 30 mM NaCl, 5 mM Na-phosphate (pH 6.5), long and short S2 were separated by repeated gel filtration in 0.1 M NaCl, 25 mM Na-phosphate (pH 7) at 4°C using a 96 × 2.6 cm column of Sephacryl

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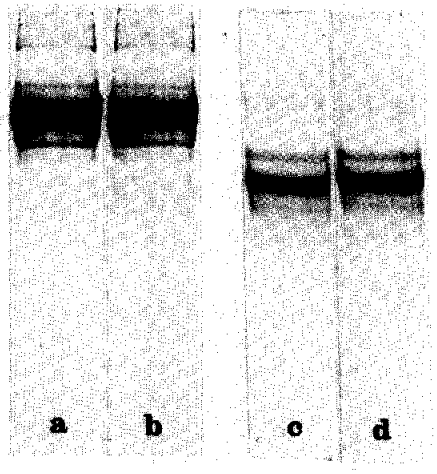

Fig.1. SDS–polyacrylamide gel electrophoresis of the specimens used for NMR before (a,c) and after (b,d) analysis: (a,b) long S2; (c,d) short S2.

S-300 operated at 10 ml/h and taking 5 ml fractions. Fig.1 shows the purity of these samples as assessed by SDS–polyacrylamide gel electrophoresis using 10% gels as described [9]. The long S2 was composed primarily of M_r 59 000 material whereas the short S2 was composed primarily of M_r 34 000 material. Both samples had a small quantity of M_r 45 000 material which is generally present [7] and probably represented an intermediate digestion product. In addition, the short S2 contained a small quantity of material of M_r ~30 000. Neither sample contained appreciable quantities of low M_r material and neither was significantly degraded by the somewhat prolonged treatment at 20–60°C required to record the NMR spectra.

Samples for NMR were made up to ~0.1 mM in 1 M NaCl, 50 mM Na-phosphate (pH 7) and ^1H_2O exchanged for ^2H_2O by repeated freeze-drying. ^1H NMR spectra were obtained at 270 MHz using a Bruker WH270 spectrometer. Quadrature phase detection was used with a 4.2 kHz spectral width and 0.4 s pulse intervals. The free-induction decay was recorded in 4096 data points and 1000 decays were averaged for each spectrum. Before Fourier transformation, the free-induction decay was multiplied by an exponential corresponding to a line broadening of 5 Hz and the data table extended to 8192 points with zeros. Chemical shifts were measured from an internal dioxane standard and expressed relative to 2,2-dimethylsilapentane-5-sulphonate, taking the dioxane resonance as 3.71 ppm from dimethyl-silapentane sulphonate.

3. RESULTS

Fig.2 shows the aliphatic regions of the 270 MHz ^1H NMR spectra of oxidised long and short S2 over 20–60°C. Essentially the same result was obtained when the intramolecular disulphide bonds of these samples were reduced with 1 mM dithiothreitol.

At 20°C and 30°C the spectra for long and short S2 were remarkably similar. In both, relatively narrow signals having linewidths of ~20–30 Hz can be seen, particularly at 0.9 and 3.0 ppm, superimposed on a broad envelope of overlapping resonances. Calculations indicate that these sharp signals are extremely unlikely to derive from the rigid bulk of the molecule. Long S2 is ~75 nm long whereas short S2 is ~40 nm long. Both molecules are ~2 nm diam. and at room temperature have an α-helical coiled-coil conformation [7,10]. Rigid rods of these dimensions would have rotational correlation times of the order of 10^{-7} s for rotation about their long axis [11] and $>2 \times 10^{-6}$ s for end-over-end tumbling [12] and would be expected to give rise to featureless NMR spectra with linewidths >1 kHz (see [8]).

The signals at 0.9 and 3.0 ppm most probably arise from the methyl protons of valine, leucine and isoleucine and from the ε-CH_2 protons of lysine, respectively. The sharpness of these lines will arise in part from side-chain motion, but it is unlikely that this alone would be sufficient to account for the observed linewidths (see [8,13]) and so some degree of backbone motion is likely. However, the number of residues giving rise to these signals at 20°C and 30°C was small, since the area of these sharp signals was only ~3–5% of the total area of the spectrum upfield of 3.7 ppm. Both the overall appearance of these spectra and the intensity of the sharp component are very similar to those of intact myosin rod ([16]; M.S., G.C.K.R. unpublished).

At 60°C the spectra of long and short S2 were again virtually identical since at this temperature both molecules had been completely denatured [7,14,15] and their amino acid compositions are very similar [6]. However at 40°C and 50°C the

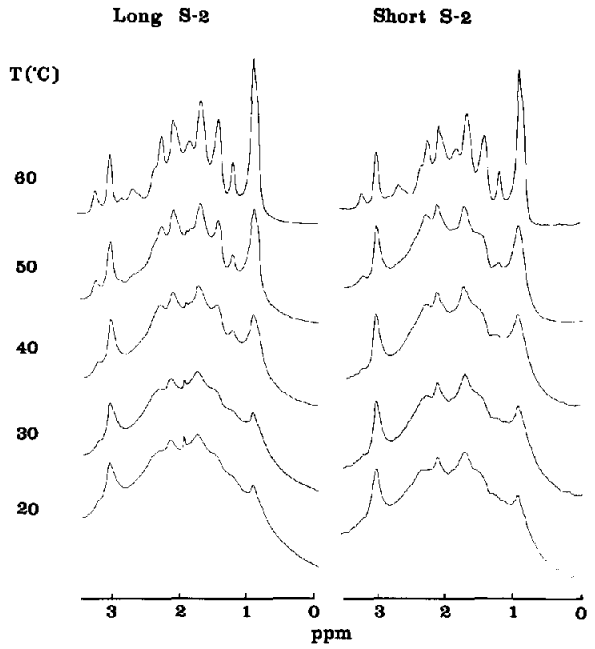

Fig.2. The aliphatic region of the 270 MHz ^{1}H NMR spectra of long S2 (left) and short S2 (right) recorded over 20–60°C. Spectra obtained at 20°C after cooling from 60°C were indistinguishable from those shown.

spectrum from long S2 contained a larger proportion of sharp signals than that from short S2. This was clearest for the peaks at about 1.3 and 1.5 ppm, which were quite distinct peaks in the long S2 spectrum but which were little more than shoulders in the short S2 spectrum. Close inspection also indicated that the resonances at 2.2, 2.35 and 3.3 ppm were sharper in the long S2 spectrum at these temperatures. At these temperatures the structure of the molecules will be partially melted [7,14,15]. Protein unfolding is commonly observed to be a slow process on the NMR time scale [8] and the absence of any progressive shifts of the resonances with temperature is consistent with this. It follows that, at temperatures at which a protein is partially denatured, the spectrum will consist of sharp resonances, with chemical shifts characteristic of a random coil, superimposed on the much broader resonances (with chemical shifts reflecting the structure of the protein) from the native regions of the protein [8]. This is clearly reflected in the progressively increasing proportion of sharp signals in the spectra (fig.2) as the temperature was increased.

The larger proportion of sharp signals in the long S2 spectrum compared with the short S2 spectrum seen at 40°C and 50°C, but not at 20°C and 30°C, thus indicates the existence of a region or regions in long S2 which have a lower thermal stability than short S2 and which melt at a lower temperature, which is in qualitative agreement with other melting studies [7,14,15]. If one assumes that the part of the molecule common to both fragments shows the same melting behaviour in both, then this region of lower thermal stability must be in the ~225 residues present in long S2 but not in short S2. However, from the spectra in fig.2 it is clear that this region of lower thermal stability can only represent a small fraction of these extra residues. The additional sharp lines at 1.3, 1.5 and 3.3 ppm in the long S2 spectrum (which probably derive from alanine, threonine and arginine residues respectively) together constituted only 1.5% (at 40°C) to 2.5% (at 50°C) of the total area of the long S2 spectrum upfield of 3.7 ppm. Although these estimates are very approximate and, because of errors in establishing an accurate baseline, could be in error by as much as a factor of 2, they clearly set an upper limit of ~5% of the long S2 molecule or about 25 residues for the size of this area of lower thermal stability.

4. DISCUSSION

Our study has detected small regions of flexibility in both long and short S2 at 20°C and 30°C, but there is no obvious difference between the 2 molecules under these conditions which could be attributed to the presence of a region of random coil or similarly flexible structure in the hinge region of long S2. While both spectra contain some sharp peaks, these make up only a very small proportion of the signal and we cannot, for example, entirely exclude the possibility that these peaks could derive from a contaminant in both samples which could not be detected by gel electrophoresis. At 40°C and 50°C there was a small difference between the spectra from the 2 molecules which indicated that there is a region of long S2 which has a lower thermal stability than short S2. This region must be in the extra portion of the long S2 molecule in which a hinge is thought to be located, but

our results suggest that it only comprises a small component of this difference peptide, amounting to not more than ~25 residues. If this region does represent the hinge in the myosin rod, it is therefore clearly not very extensive and it is not easy to reconcile this result with proposals for a region of ~150–200 residues in long S2 which could, by melting to a random coil conformation, provide the shortening of the cross-bridges required for muscle contraction [4]. Of course, our results do not exclude the possibility that the coiled-coil could melt to a contracted but non-flexible conformation and so would be compatible with a modified model which incorporated this feature. The existence of a small region of flexibility such as that indicated by our results here is consistent with the original proposal for a hinge to enable movement of the cross-bridges between thick and thin filaments [3] and would also be consistent with the general regularity of the myosin sequence in this region [17].

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