Hayashi, R. (1977) *Methods Enzymol. 47*, 84–93.
Hayashi, R., Moore, S., & Stein, W. H. (1973) *J. Biol. Chem. 248*, 2296–2302.
Hendon, R. A., & Fraenkel-Conrat, H. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1560–1563.
Horst, J., Hendon, R. A., & Fraenkel-Conrat, H. (1972) *Biochem. Biophys. Res. Commun. 46*, 1042–1047.
Houmard, J., & Drapeau, G. R. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3506–3509.
Hseu, M. J., Guillory, R. J., & Tzeng, M. C. (1990) *J. Bioenerg. Biomembr. 22*, 39–50.
Inglis, A. S. (1980) in *Solid Phase Methods in Protein Sequence Analysis* (Birr, C., Ed.) Proceedings of the 3rd International Conference, Heildelberg, 1979, Elsevier, Amsterdam.
Jeng, T. W., Hendon, R. A., & Fraenkel-Conrat, H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 600–604.
Kouyoumdjian, J. A., Harris, J. B., & Johnson, A. (1986) *Toxicon 24*, 575–583.
Lambeau, G., Barhanin, J., Schweitz, H., Qar, J., & Lazdunski, M. (1989) *J. Biol. Chem. 264*, 11503–11510.
Lee, C. Y., & Ho, C. L. (1980) in *Natural Toxins* (Eaker, D., & Wadström, T., Eds.) pp 539–547, Pergamon Press, Oxford.
Moore, S., & Stein, W. H. (1948) *J. Biol. Chem. 176*, 367–388.
Podell, D. N., & Abraham, G. N. (1978) *Biochem. Biophys. Res. Commun. 81*, 176–185.
Radvanyi, F., & Bon, C. (1982) *J. Biol. Chem. 257*, 12616–12623.
Radvanyi, F., Saliou, B., Lembezat, M. P., & Bon, C. (1989a) *J. Neurochem. 53*, 1252–1260.
Radvanyi, F., Jordan, L., Russo-Marie, F., & Bon, C. (1989b) *Anal. Biochem. 177*, 103–109.
Randolph, A., & Heinrikson, R. L. (1982) *J. Biol. Chem. 257*, 2155–2161.
Renetseder, R., Brunie, S., Dijkstra, B. W., Drenth, J., & Sigler, P. B. (1985) *J. Biol. Chem. 260*, 11627–11634.
Rübsamen, K., Breithaupt, H., & Habermann, E. (1971) *Naunyn-Schmiedeberg's Arch. Pharmacol. 270*, 274–288.
Slotta, K. H., & Fraenkel-Conrat, H. (1938) *Ber. Dtsch. Chem. Ges. 71*, 1076–1081.
Tarr, G. E., Beecher, J. F., Bell, M., & McKean, D. (1978) *Anal. Biochem. 84*, 622–627.
Vital-Brazil, O. (1966) *Mem. Inst. Butantan, Sao Paulo 33*, 981–992.
Vital-Brazil, O., & Excell, B. J. (1971) *J. Physiol. (London) 212*, 34P-35P.

C-Terminal Structure and Mobility of Rabbit Skeletal Muscle Light Meromyosin As Studied by One- and Two-Dimensional ^{1}H NMR Spectroscopy and X-ray Small-Angle Scattering

Hans Robert Kalbitzer,* Kayo Maeda, Andrea Rösch, Yuichiro Maéda,‡ Matthias Geyer, Wolfgang Beneicke, Klaus-Peter Neidig, and Alfred Wittinghofer

Max-Planck-Institute for Medical Research, Department of Biophysics, Jahnstrasse 29, D-6900 Heidelberg, Germany, and European Molecular Biology Laboratory at the German Electron Synchrotron (DESY), Notkestrasse 85, D-2000 Hamburg 52, Germany

Received December 19, 1990; Revised Manuscript Received April 11, 1991

ABSTRACT: Intact rabbit myosin and two different C-terminal fragments of rabbit muscle light meromyosin (LMM) expressed in *Escherichia coli*, LMM-30, and LMM-30C′, were studied by ^{1}H NMR spectroscopy. X-ray small-angle scattering shows that at high ionic strength two polypeptide chains of LMM-30 (which consists of the C-terminal 262 amino acids of myosin heavy chain) or LMM-30C′ (which corresponds to LMM-30 but lacks the last 17 residues) assemble to form an α-helical coiled-coil as it is found also in myosin. The last 12 C-terminal residues of one polypeptide chain of LMM-30 and the last 9 C-terminal residues of the other chain are very mobile. The last 8 residues of the two strands are equivalent from the NMR point of view and unfolded; the valine residues in position 255 in the two strands are not equivalent, suggesting an interaction between the two strands, Ser-252, Arg-253, and Asp-254 are completely immobilized in one of the polypeptide strands and partly mobile in the other. Essentially the same pattern is observed in intact myosin. In spite of the large molecular weights of LMM-30 and LMM-30C′, it is possible to resolve almost all aromatic residues and to determine the pK values of all the 4 tyrosine and of 9 (out of 10) histidine residues. The tyrosine residues in the two strands are equivalent in the two polypeptide chains and both have a pK of 10.5. The pK values of the histidine residues vary between 5.7 and 7.0.

A deeper understanding of muscle contraction can only be obtained if the molecular structure of its contractile elements is known [for reviews, see e.g., McLachlan and Karn (1982), Emerson and Bernstein (1987), and Levine et al. (1990)]. Recently, the structure of G-actin complexed with DNase I has been solved by X-ray crystallography (Kabsch et al., 1990) and a model of F-actin has been derived from X-ray fiber diffraction studies (Holmes et al., 1990). Only a low-resolution X-ray structure of myosin subfragment S1 (Winkelmann et

‡European Molecular Biology Laboratory at DESY.
*To whom correspondence should be addressed.

0006-2960/91/0430-8083$02.50/0

al., 1985) has been reported until now. NMR,[1] which represents an alternative method for obtaining structural information, has also been applied for the study of different structural aspects of myosin (Akasaka et al., 1978; Highsmith et al., 1979; Highsmith & Jardetzky, 1980; Koppitz et al., 1980; Brauer & Sykes, 1981; Shriver & Sykes, 1981; Prince et al., 1981; Highsmith et al., 1982; Eads & Mandelkern, 1984; Roux-Fromy & Cardinaud, 1984; Henry et al., 1985; Bandary et al., 1986; Trayer et al., 1987; Levine et al., 1988; Sommerville et al., 1990; Maeda et al., 1991). Since the spectral resolution of NMR decreases with increasing molecular weight, a useful method for obtaining more detailed structural information on large proteins consists in the expression and study of smaller functional domains. We have expressed two different C-terminal fragments of myosin, LMM-30 and LMM-30C′. LMM-30 consists of the C-terminal 262 amino acids (amino acids 1676–1937 of myosin heavy chain), LMM-30C′ corresponds to LMM-30 but lacks the 17 C-terminal amino acids. In analogy to myosin, two polypeptide chains of LMM-30 or LMM-30C′ probably form a coiled coil (in the following, this basic molecular unit is called "monomer") in solution at high ionic strength. NMR spectroscopy revealed that under these conditions the C-termini of LMM-30 and intact myosin are unfolded and mobile; this unfolded C-terminus appears to be important for the proper assembly of myosin multimers from the coiled-coil precursors (Maeda et al., 1991). In the present paper we will discuss the structural properties of LMM fragments and myosin in more detail.

Materials and Methods

Protein Expression and Purification. LMM-30 was expressed in *Escherichia coli* and purified as described by Maeda et al. (1991). LMM-30 consists of the 262 C-terminal amino acids of rabbit skeletal myosin heavy chain, which is numbered from the N-terminus of myosin heavy chain from residue 1676 to residue 1937 (Maeda et al., 1987). LMM-30C′ is identical with LMM-30 but lacks the 17 C-terminal amino acids. Sequencing of the N-termini of these fragments revealed that the N-terminal methionine is removed in *E. coli*. However, for a better comparison with the data already published (Maeda et al., 1991), the original numbering of the amino acids according to the DNA (including the methionine residue) will be used here.

Intact rabbit fast skeletal muscle myosin containing two heavy chains, two alkali light chains, and two regulatory light chains was isolated from rabbit back and leg muscles according to the method of Margossian and Lowey (1982). After extraction for 10 to 12 min, the extract was diluted to collect myosin. For purification, myosin was resuspended to a high salt concentration and precipitated by dilution.

X-ray Small-Angle Scattering. The small-angle X-ray scattering profiles were obtained at the beam-line X-33 of EMBL at DESY, Hamburg, with the synchrotron X-radiation from the electron beam of DORIS operated at 3.656 GeV, 40–80 mA. The specimen-to-detector distance was 2.8 m. Exposure time was between 15 and 30 min on the one-dimensional position-sensitive detector of wire-per-wire type (Hendrix et al., 1982). The protein, at concentrations between 3 and 15 mg/mL, was dissolved in 0.6 M KCl, 1 mM NaN_3, 1 mM DTE, and 25 mM HEPES, pH 7.4. Before the measurements, the solution was centrifuged at 8000–10000*g* for 10 min. The experiments were performed at 288 K. The samples were contained in cuvettes that have two mica windows to let the X-ray beam pass. In order to subtract the instrumental background and to monitor the shift of the beam position, before and after each exposure of the protein solution intensity profiles were recorded from the same cuvette filled with the protein-free buffer solution. When the intensity profiles of the controls before and after the measurement normalized for the incident beam were not identical, the data were discarded.

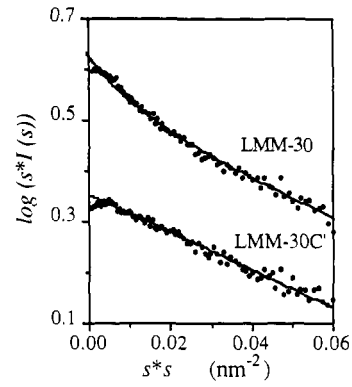

FIGURE 1: Guinier plots of X-ray small-angle scattering from LMM-30 and LMM-30C′. The intensity $I(s)$ is a function of the normalized scattering angle s, which is defined by $s = (2 \sin \theta)/\lambda$ with θ the scattering angle and λ the wavelength. The intensity I is given in arbitrary units, that is, $\log sI$ is defined only ± an arbitrary constant. The solid lines show a line fit to the data with $R_g = 0.66$ nm assuming a single population of monomers (LMM-30) or a mixture of monomers, dimers, tetramers, and hexamers occurring in a ratio of 0.94:0.02:0.02:0.02 (LMM-30C′).

NMR Measurements. ^{1}H NMR spectra were recorded with a Bruker AM-500 NMR spectrometer operating at 500 MHz. The water signal was suppressed by selective presaturation. DQF-COSY, *z*-filtered ROESY, NOESY, and *z*-filtered TOCSY spectra were recorded according to Rance et al. (1983), Rance (1987), Jeener et al. (1979), and Braunschweiler and Ernst (1983), respectively. Semisoft TOCSY and NOESY experiments were performed by replacing the first 90° pulse by a half-Gaussian pulse of approximately 1 ms duration followed by a hard 90° purging pulse (Kessler et al., 1989). In all experiments, phase-sensitive detection in the t_1 direction was obtained by using time-proportional phase increments (TPPI) (Marion & Wüthrich, 1983).

The NMR samples contained 0.025 mM myosin or 1 mM LMM fragment in a buffer solution of 10 mM potassium phosphate and 0.6 M KCl. The chemical shifts are refered to the internal reference (trimethylsilyl)perdeuteropropionic acid (TSP). The pH value of the solution was varied by adding appropriate amounts of DCl or KOD to the solution. The pH values were measured with a combination glass electrode and were not corrected for isotope effects. The pK values and chemical shifts in fully protonated and deprotonated form were obtained by fitting the data to the modified Henderson–Hasselbalch equation [see, e.g., Schlemmer et al. (1988)]. The two-dimensional NMR spectra were processed with the program AURELIA.

Results

Global Structure of LMM-30 and LMM-30C′ from Small-Angle X-ray Scattering. Assuming a rod structure for LMM-30 and LMM-30C′, the diameter of the rod can be

[1] Abbreviations: DQF-COSY, double-quantum filtered correlated spectroscopy; DTE, dithioerythritol; LMM, light meromyosin; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; ROESY, rotating frame Overhauser spectroscopy; TOCSY, total correlation spectroscopy; Tris, tris(hydroxymethyl)aminomethane; TSP, (trimethylsilyl)perdeuteropropionic acid.

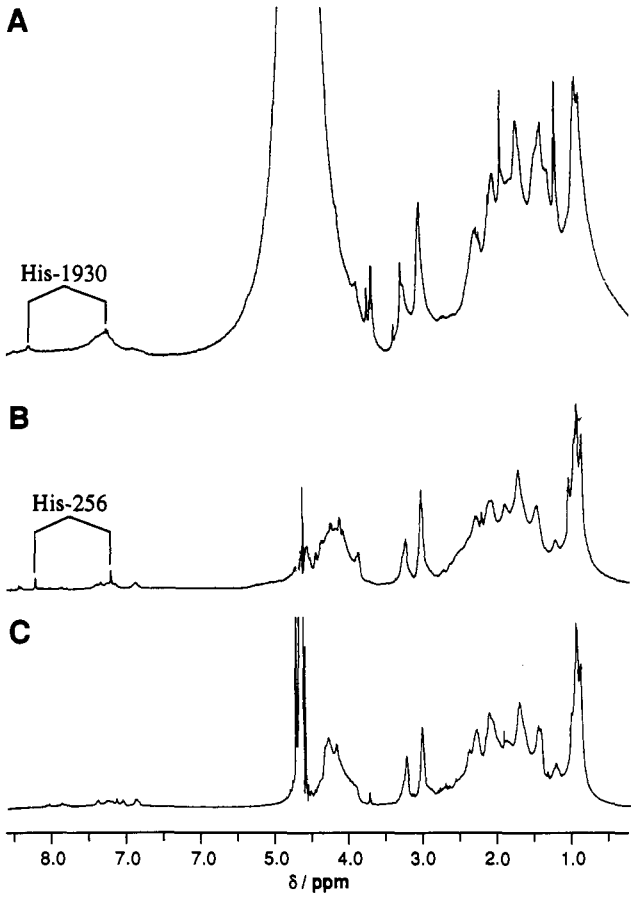

FIGURE 2: 500-MHz ^{1}H NMR spectra of LMM fragments and intact myosin. (A) 0.025 mM intact rabbit skeletal muscle myosin, (B) 1 mM LMM-30, (C) 1 mM LMM-30C′ in 0.6 M KCl and 10 mM potassium phosphate in 99.75% D_2O; the temperature was 308 K, at pH 6.6 (A, B) or 7.3 (C). The signal-to-noise ratio was improved by exponential multiplication with a line broadening of 0.5 Hz. Acquisition time for (A) was approximately 14 h, for (B) and (C), 30 min.

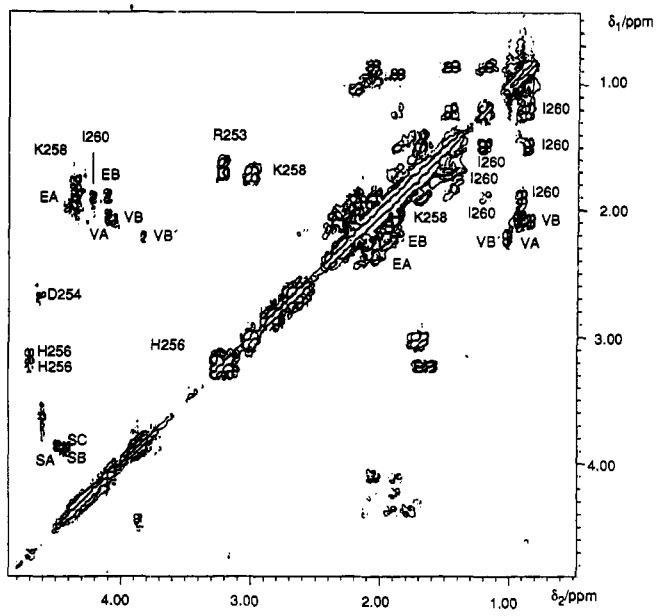

FIGURE 3: Part of a DQF-COSY spectrum of the C-terminal light meromyosin fragment LMM-30. The sample contained 1 mM LMM-30 in 0.6 M KCl and 10 mM sodium phosphate buffer in D_2O. The temperature was 308 K, at pH 6.6. The resolution enhancement by Lorentzian-to-Gaussian transformation (Ferrige & Lindon, 1978) was optimized for line widths of 4.5 Hz. Improvement of the signal-to-noise and signal-to-artifact ratio was achieved by additive symmetry enhancement (Neidig & Kalbitzer, 1991). Spin systems that occur only once in the C-terminal sequence are labeled according to their position in the sequence; the other spin systems are labeled arbitrarily with A, B, etc. The total acquisition time was 26 h.

determined from the small-angle X-ray scattering. The scattering intensity of LMM-30C′ results in a linear Guinier plot (Figure 1), the slope of which gives a radius of gyration (R_g) of the cross sections of the rods (Guinier, 1963). As a result of $n = 4$ independent experiments one obtains an R_g of 0.658 ± 0.016 nm. This value corresponds to a solid rod with a diameter of 1.5–2.0 nm and is consistent with two polypeptide chains in a coiled-coil structure. The linearity of the Guinier plot indicates that only LMM-30C′ monomers exist in solution at high ionic strength. The Guinier plot of LMM-30 is slightly curved upward in the lower angle region ($s^2 < 0.02$ mm^{-2}), converging to a straight line with a slope corresponding to $R_g = 0.70 \pm 0.01$ nm ($n = 4$) in the higher angle region ($s^2 > 0.02$ mm^{-2}). The fact that the profile deviates from the linearity only in the region with $s^2 < 0.02$ mm^{-2} indicates that LMM-30 predominantly consists of monomers with a diameter between 1.5 and 2.0 nm, a diameter that is identical with that of the monomers of the LMM-30C′ preparation. The curve fitting of the data indicates that more than 94% of the particles must have this dimension. Although it is not yet known in detail what causes the upward curvature in the region of $s^2 < 0.02$mm^{-2}, the curved profile is easily accounted for by a minimal amount of higher aggregates (dimers, tetramers, ...) of LMM-30 that coexist with the monomers (Figure 1).

Resonance Assignments of the C-Terminal Residues of LMM-30. The ^{1}H NMR spectrum of LMM-30 contains a group of surprisingly sharp resonances with a line width of the order of 4–5 Hz that are not contained in the spectrum of LMM-30C′, which lacks the C-terminal 17 amino acids (the sequence of the last 20 amino acids in LMM-30 is Gln-Val-Asn-Lys-Leu-Arg-Val-Lys-Ser-Arg-Asp-Val-His-Ser-Lys-Val-Ile-Ser-Glu-Glu). Without resolution enhancement, the absence of these lines can most easily be noticed in the aromatic region of the spectrum where a pair of sharp histidine ring resonances are missing in the spectrum of LMM-30C′ (Figure 2). Likewise, the sharp Hα resonances of LMM-30 between 3.5 and 5 ppm visible in Figure 2 cannot be observed in the spectrum of LMM-30C′. However, this region of the spectrum of LMM-30C′ exhibits a few new resonances with relatively small line widths. Careful analysis of the resolution-enhanced spectra shows that all sharp resonances that can be identified unequivocally in the one-dimensional spectra of LMM-30 are missing in LMM-30C′. This suggests that these resonances come from amino acids located in the C-terminus of LMM-30. The corresponding spin systems were identified by two-dimensional spectroscopy and are indicated in the DQF-COSY spectrum shown in Figure 3. The amino acids belonging to these spin systems match well the composition of the C-terminus of LMM-30 (Table I). All evidence taken together, it is very likely that these sharp resonances are due to the 12 C-terminal amino acids of LMM-30. A method for independently proving our proposal would be the measurement of sequential NOEs and the comparison of the sequence obtained from NMR with the sequence in question. However, no sequential amide–amide or amide–Hα NOEs in the laboratory or rotating frame could be detected (with a mixing time of 0.15 s and 36 h of data aquisition per NOESY or ROESY spectrum). However, with the above assumption, the sequential assignments of the amino acids that occur only once in the C-terminal sequence (Arg-253, Asp-254, His-256, Lys-258, Ile-260) is trivial. (In Figure 3, the spin systems of

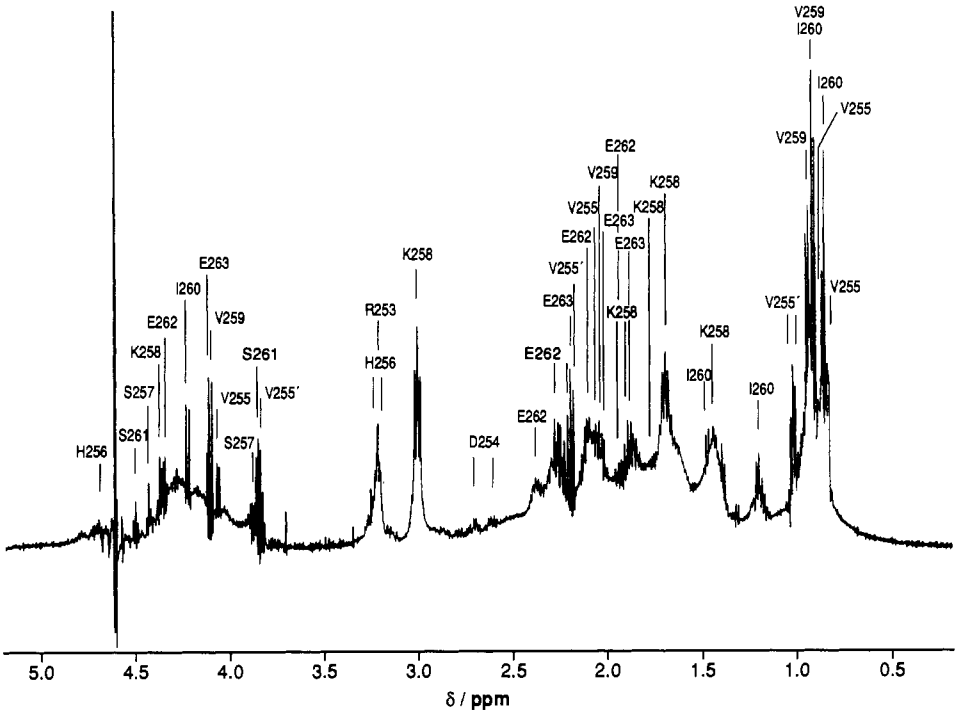

FIGURE 4: Assignments of the C-terminal resonances in the light meromyosin fragment LMM-30. The sample contained 1 mM LMM-30 in 0.6 M KCl and 10 mM sodium phosphate buffer in D_2O. The temperature was 308 K, at pH 6.6. The resolution enhancement by Lorentzian-to-Gaussian transformation (Ferrige & Lindon, 1978) was optimized for line widths of 4.5 Hz. The resonances are labeled according to their assignments described under Results.

Table I: Assignments of the C-Terminal Amino Acids in LMM-30[a]

	chemical shifts δ (ppm)					
residue	H	Hα	Hβ	Hγ	Hδ	Hε
Ser-252	–[b]	4.40	3.86			
			3.86			
Arg-253	–[b]	–[b]	–[b]	1.67	3.23	
				1.67	3.23	
Asp-254	–[b]	4.62	2.69			
			2.61			
Val-255	8.05	4.06	2.09	0.85		
				0.84		
Val-225′	–[b]	3.82	2.21	1.04		
				1.03		
His-256	–[b]	4.71	3.17		6.98	7.68
			3.28			
Ser-257	8.43	4.42	3.94			
			3.88			
Lys-258	8.37	4.36	1.85	1.46	1.71	3.02
			1.78	1.46	1.71	3.02
Val-259	8.20	4.09	2.05	0.95		
				0.92		
Ile-260	8.29	4.21	1.90	1.47	0.86	
				1.21		
				0.92		
Ser-261	8.36	4.49	3.86			
			3.86			
Glu-262	8.48	4.35	2.11	2.37		
			1.94	2.25		
Glu-263	8.02	4.10	2.04	2.20		
			1.89	2.20		

[a] 1 mM LMM-30 in 0.6 M KCl and 10 mM phosphate buffer in D_2O, pH 6.6, at 308 K. Amide protons were detected in 90% H_2O/10% D_2O, at 298 K. The assignments given and experimental conditions correspond to those of Figure 4. [b] Resonances too broad to be identified.

Table II: *J* Couplings of the C-Terminal Amino Acids in LMM-30[a]

	J coupling (Hz)	
residue	H–Hα	Hα–Hβ
Val-255	5.8	7.0
Val-255′		7.4
His-256		5.0, 8.9
Ser-257		5.4
Lys-258	6.6	
Val-259	7.4	8.3
Ile-260	8.3	8.3
Ser-261	7.4	5.4
Glu-262	8.3	
Glu-263	7.4	

[a] 1 mM LMM-30 in 0.6 M KCl and 10 mM phosphate buffer in D_2O, pH 6.6, at 308 K. Amide protons were detected in 90% H_2O/10% D_2O, at 298 K.

these amino acids are already labeled with these numbers; the other spin systems are labeled with A, B, ...) Nevertheless, these resonances can be tentatively assigned to their position in sequence from additional evidences. The Hα and the methyl resonances of Val-B and Val-B′, but not of Val-A, show small but significant pH-dependent shifts that parallel the deprotonation of the ring system of His-256. A probable explanation of this cotitration is a direct interaction with a neighboring histidine ring. This points to Val-255 being Val-B and Val-B′. With similar arguments Ser-C can be assigned to position 257, because it also cotitrates with His-256. Ser-A and Ser-B can be tentatively assigned to positions 261 and 252, respectively, since Ser-B shows only a relatively weak cross peak in the COSY spectrum and rather broad lines in the 1D spectra indicating that it is located near the more rigid (less mobile) coiled-coil part of LMM-30. Whereas Glu-A shows almost exactly the chemical shifts of an internal residue in a random coil peptide, the resonances of Glu-B are shifted to higher field, an effect that is expected for a C-terminal residue in such a peptide (Bundi & Wüthrich, 1979). This allows the tentative assignment of Glu-A to position 262 and Glu-B to position 263. (The chemical shift values of the C-terminal residues are given in Table I; Figure 4 shows the assignments in the one-dimensional spectrum.) The amide–Hα and Hα–Hβ coupling constants of some of the residues could be determined from the 1D-spectra and are summarized in Table II. Interestingly,

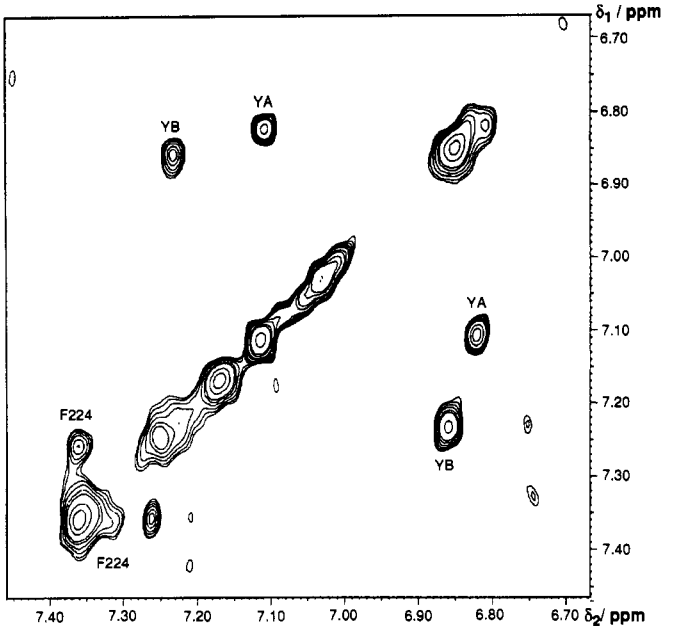

FIGURE 5: Semisoft TOCSY spectrum of the aromatic residues of LMM-30C′. Semisoft TOCSY of 1 mM LMM-30C′ in 0.6 M KCl and 10 mM potassium phosphate in D_2O, pH 7.3. The temperature was 308 K, and the isotropic mixing time was 20 ms. The acquisition time was 44 h. Improvement of signal-to-noise and signal-to-artifact ratio was achieved by additive symmetry enhancement (Neidig & Kalbitzer, 1991). Resonances are labeled as in Figures 6 and 7 and Table III.

sharp resonances from the N-terminal amino acids cannot be observed.

Integration of the Narrow Resonance Lines. The relative concentrations of the visible C-terminal residues (causing the sharp resonances described above) were determined by comparing the integrals of their matched filtered resonances (Ferrige & Lindon, 1978). Using the filtered spectra for the determination of relative integrals is an allowed procedure, since the Fourier transform of a signal by an arbitrary real function does not influence the integrals (except for a normalization constant). Since the complete spin systems are known from the two-dimensional experiment, only the integral of one (of course, the best resolved and superposition-free) resonance per spin system has to be determined. The relative concentrations of (NMR-visible) Ser-252, Arg-253, Asp-254, Val-255, and Val-255′ are only one half of those of the remaining eight C-terminal residues. The absolute concentrations of the residues responsible for the sharp peaks in the NMR spectrum were estimated by comparison of their integrals with the integral of the methyl group of the internal reference TSP. In the limits of error (±10%) the relative concentrations of Ser-252, Arg-253, Asp-254, Val-255, and Val-255′ are 1 per monomer; the relative concentrations of the other C-terminal amino acids are 2 per monomer. The same results were obtained when comparing the integrals of the sharp resonances of the C-terminal residues with the broad but clearly visible resonances of the tyrosine and phenylalanine ring systems of LMM-30.

Aromatic Residues in LMM-30 and LMM-30C′. The downfield parts of the spectra of LMM-30 and of LMM-30C′ appear indistinguishable if one neglects the two sharp resonances of His-256 that are only present in the spectrum of LMM-30. Although the line widths of these resonances are considerably broader (of the order of 20 Hz), they can even be observed in suitably adapted two-dimensional NMR spectra. Figure 5 shows a semisoft TOCSY spectrum of

Table III: Chemical Shifts and pK Values of Aromatic Residues in LMM-30 and LMM-30C′[a]

residue	atom	pK	δ_{AH} (ppm)	δ_{A^-} (ppm)
His-256[b]	δ2	6.70	7.35	6.98
	ε1		8.65	7.68
His-A	δ2	7.07	7.38	6.97
	ε1		8.62	7.68
His-B	δ2	7.02	7.38	6.97
	ε1		8.62	7.68
His-C	ε1	6.70	8.63	7.77
His-D	δ2	6.50	7.34	7.00
	ε1		8.65	7.72
His-E	δ2	6.45	7.38	6.97
	ε1		8.67	7.70
His-F	ε1	5.7	8.30	7.79
His-G	δ1	6.2	8.70	7.70
Phe-224	δ		7.25	
	ε		7.38	
	ζ		7.32	
Tyr-A	δ	10.5	7.12	(6.94)[c]
	ε		6.82	6.52
Tyr-B	δ	10.5	7.22	7.07
	ε		6.85	6.54

[a] Absolute errors: pK, ± 0.1; δ_{AH} for histidine residues, ± 0.02 ppm and tyrosine residues (for His-C) ± 0.07 ppm; δ_{A^-} for histidine and tyrosine residues, ± 0.01 ppm (Hε of Tyr-A ± 0.1). The last digits of the pK values given are only significant concerning the relative errors. The sample contained 1 mM LMM-30C′ in 0.6 M KCl and 10 mM potassium phosphate in D_2O. The temperature of the measurement was 308 K. Resonances (residues) are labeled as in Figures 5–7. [b] Only contained in LMM-30. [c] Not enough data available for a precise fit (see Figure 6).

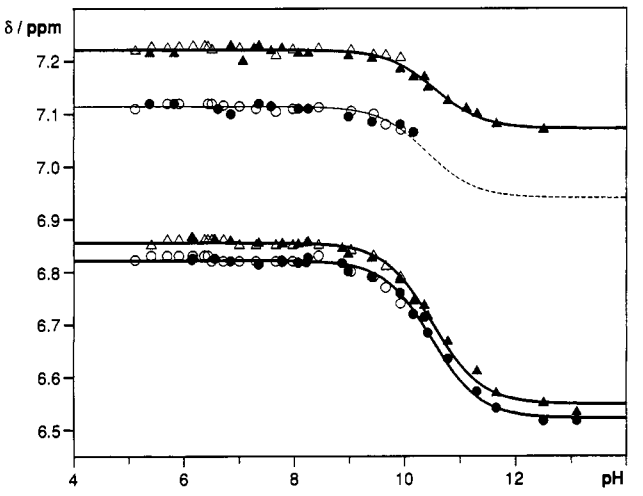

FIGURE 6: pH dependence of the tyrosyl chemical shifts in LMM-30 and LMM-30C′. The chemical shifts δ of the Hδ and Hε resonances of tyrosine residues in LMM-30 and LMM-30C′ are plotted as function of the pH. The curves depicted were calculated with the parameters given in Table III. (○) Tyr-A in LMM-30, (●) Tyr-A in LMM-30C′, (△) Tyr-B in LMM-30, (▲) Tyr-B in LMM-30C′. Conditions: 1 mM protein in 0.6 M KCl, and 10 mM potassium phosphate in D_2O, at 308 K. At pH > 10, only the data from LMM-30C′ were of sufficient quality for the unequivocal identification of the tyrosine resonances.

LMM-30C′ recorded with a rather short isotropic mixing time of 20 ms. The spin systems of the phenylalanine (Phe-244) and the two tyrosine (Tyr-180 and Tyr-206) residues contained in one LMM-30C′ polypeptide chain can be identified (Table III). These assignments were supported by the observation of the corresponding NOE cross peaks in a semisoft NOESY experiment (data not shown) and by the characteristic pH dependence of the chemical shifts of the tyrosine resonances (Figure 6). The chemical shifts at low and high pH and the pK values of Tyr-A and Tyr-B are identical in the two LMM fragments (Table III). The (principally existing) inequivalence

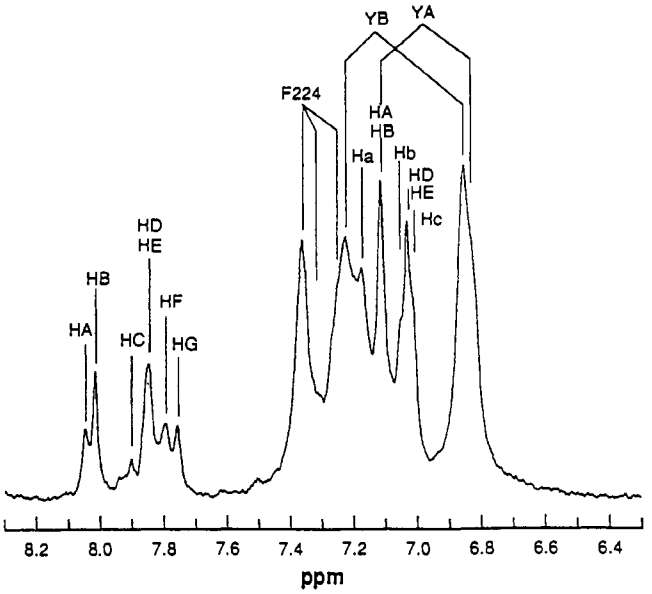

FIGURE 7: Downfield part of the ^1H NMR spectrum of LMM-30C′. The sample contained 1 mM LMM-30C′ in 0.6 M KCl and 10 mM potassium phosphate in D_2O, pH 7.3, at 308 K.

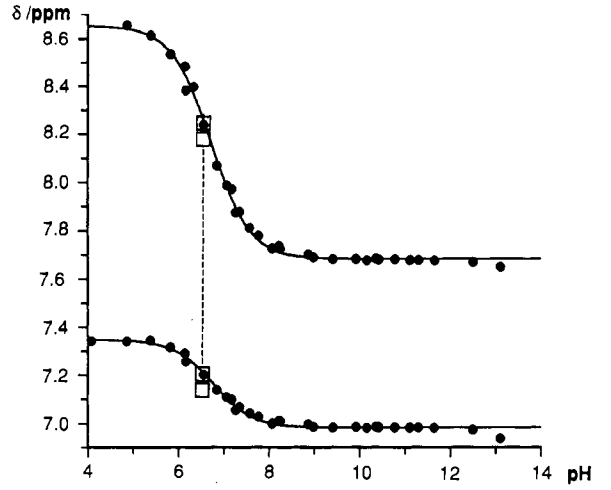

FIGURE 8: pH dependence of chemical shifts of His-256 in LMM-30. The chemical shifts δ of the Hδ2 and Hϵ1 resonances of His-256 of LMM-30 are plotted as function of the pH (●). The curves depicted were calculated with the parameters given in Table III. Conditions: 1 mM protein in 0.6 M KCl and 10 mM potassium phosphate in D_2O, at 308 K. The open squares represent the chemical shifts of presumed His-1930 of native myosin. The two pairs of data points probably correspond to this histidine residue in the two major isoforms of myosin (designated as His-A and His-B in Figure 10) and coincide at this pH (vertical broken line) with the chemical shifts of His-256 of LMM-30.

of tyrosine residues in the two protomers is not reflected in an observable heterogeneity of their chemical shifts; they are not distinguishable (Figure 7) from the NMR point of view.

The histidine ring resonances can be identified from their singlet structure and their typical pH-dependence of chemical shifts. The titration curve of His-256 in LMM-30 is shown in Figure 8. Four additional histidine residues (His-15, His-106, His-171, His-219) are present in the polypeptide chain of LMM-30 and LMM-30C′, that is, up to 10 pairs of Hδ2 and Hϵ1 resonance lines can be expected in the NMR spectra, if the environments of the histidine rings in the two polypeptide strands are different. Figure 7 shows that at least some of the histidine residues are not completely equivalent in the two polypeptide chains. From the integrals and the pH dependence, seven of the eight Hϵ1 resonances can be identified and only one of the resonance lines expected could not be located unequivocally. It is probably buried by the resonances of His-D, His-E, or His-F. The Hδ1 resonances belonging to these histidine residues can be assigned from their corresponding pK values. For the remaining Hδ1 resonances (labeled in Figure 7 with a, b, c), the pH dependence could not be determined with sufficient accuracy, therefore they could not be assigned to their corresponding Hϵ2 resonances. The resonances of the ring protons of His-A and His-B have very similar NMR characteristics (Figure 7, Table II), they are probably due to histidine residues in the same sequence position in the two polypeptide chains. The same is true for the pair His-D and His-E.

C-Terminal Resonances of Intact Myosin. As shown above, the sharp resonances of a histidine ring system with the same characteristics as His-256 in LMM-30 can be observed even in intact myosin. This suggests that these resonances are due to the C-terminal histidine in myosin, His-1930. They have approximately the same line width of 4.5 Hz in whole myosin and LMM-30. This is also true for a couple of resonances in the aliphatic part of the spectrum of myosin. By enhancement of those resonances with a line width of approximately 4.5 Hz in the spectrum of myosin by appropriate time domain filtering (Figure 9), it is possible to identify resonances that can be assigned most probably to the C-terminal residues of myosin. However, the resonances of the Ser-1926, Asp-1928, and Val-1929′ (Val-B′ in LMM-30) could not be identified positively in the spectrum of myosin, especially the methyl signals of Val-B′ (in LMM-30) are not visible. These are precisely the resonances that also show signs of restricted mobility in LMM-30. They are probably too broad to be detected in intact myosin because of an increased effective correlation time in this very large molecule (molecular weight 460 000). Interestingly, the Hϵ resonances of Lys-1932 show signs of a spectral heterogeneity that is probably due to the sequence heterogeneity (Thr ↔ Ser) at position 1931 of the main isoforms of myosin that are contained in our preparation (Maeda et al., unpublished results). A closer look at the histidine resonances His-1930 (Figure 10) shows the same heterogeneity in the NMR spectrum for the other neighbor of Thr-1931 and Ser-1931, respectively. In addition to the very sharp lines of low molecular weight impurities (labeled with a star in Figure 9) some other relatively narrow lines can be observed, some of which may arise from residues of the myosin light chains (see Discussion).

Discussion

For the interpretion of the NMR data, it is essential to know the aggregation state of the LMM fragments in solution. This information could be obtained from the X-ray small-angle scattering data. The same radius of gyration of approximately 0.66 nm was obtained for the two fragments LMM-30 and LMM-30C′, indicating that the main aggregation state is identical in the two preparations. The diameter of the cross section of 1.5–2 nm obtained is consistent with a particle of α-helical coiled-coil structure consisting of two polypeptide chains but not with the occurrence of a single α-helical chain. Thus the main population found in solution under the conditions of the NMR experiment consists of monomers, although the LMM-30 preparation contains a small population of higher aggregates. At low ionic strength, LMM-30 as well as LMM-30C′ form tactoids that show striations with a spacing of approximately 15 or 29 nm (data not shown). These spacings of striation, which originate from the molecular packing within the tactoid, are also found on tactoids formed from intact myosin molecules. Such a striation pattern is

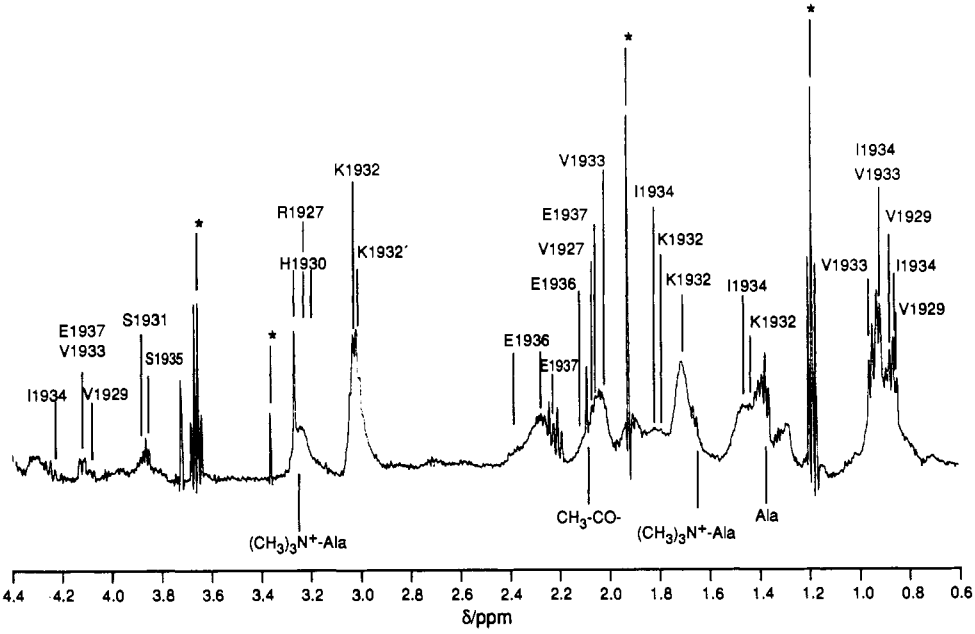

FIGURE 9: C-terminal residues of intact myosin. 500-MHz ^{1}H NMR spectrum of 0.025 mM intact rabbit skeletal myosin in 0.6 M KCl, 10 mM potassium phosphate, pH 6.6, at 308 K (same data as in Figure 2A, but processed in a different way). Resonance lines with line widths of about 4.5 Hz were enhanced by subtracting part of the broad background [multiplying the free induction decay by $1-0.9e^{-t/(\pi LB)}$, LB = 100 s], followed by a Lorentzian-to-Gaussian transformation (multiplication of the free induction decay by $e^{t/(\pi LB)-bt^2}$, with LB = 4.5 s and b = 5.4 s^{-2} (Ferrige & Lindon, 1978). A star (★) indicates low molecular weight impurities with line widths of the order of 1 Hz.

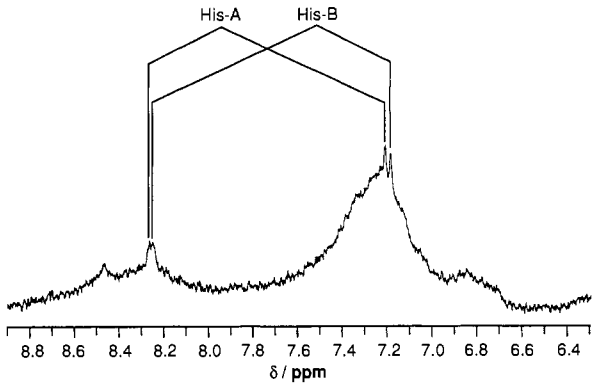

FIGURE 10: Sequence heterogeneity in the C-terminus of myosin. In an expanded view of Figure 2A, the aromatic residues in native myosin are shown. Signal-to-noise improvement was achieved by exponential multiplication with a line broadening of 0.5 Hz. The ring resonances of presumed His-1930 in the two isoforms are labeled arbitrarily with A and B.

typically obtained from the assembly of α-helical coiled-coil molecules but not from the assembly of single straight α-helical polypeptide chains.

The chemical shifts as well as the calculated pK values of the aromatic residues located in the putative α-helical coiled-coil part of the protein are identical in the two LMM-fragments LMM-30 and LMM-30C′. Since chemical shifts are very sensitive for structural changes, most probably the coiled-coil structure and the mutual arrangement of the polypeptide chains are identical in the two fragments and are not perturbed by the presence or absence of the C-terminal amino acids.

The conclusion that the sharp resonances of LMM-30 have to arise from amino acids located in its C-terminal stretch of 17 amino acids that is missing in LMM-30C′ is straightforward since these resonances are missing in the spectra of LMM-30C′ (although it has the same structure as LMM-30, see above). The agreement of the spin systems identified with the amino acid composition of the stretch of the last 12 and 9 residues, respectively, makes it very likely that the assignment to just these amino acids is correct. The agreement of chemical shifts, line widths, and coupling constants of these resonances in LMM-30 and intact myosin is so excellent that it is very likely that they have to be assigned to the same entity, namely the C-terminus of LMM-30 and intact myosin. Sharp resonances have been described before in intact myosin as well as fragments or components of myosin and were assigned to the myosin head region or the hinge region [see, e.g., Akasaka et al. (1978), Highsmith et al. (1979), Koppitz et al. (1980), Highsmith et al. (1982), Brauer and Sykes (1981), Shriver and Sykes (1981), Prince et al. (1981), Eads and Mandelkern (1984), Henry et al. (1985), and Trayer et al. (1987)]. The singlet resonance at 3.26 ppm and the doublet resonance at 1.65 ppm could correspond to the trimethylamino and the β-methyl groups of the N-terminal N-trimethylalanine residues found in the myosin light chains LC-1 and LC-2 (Roux-Fromy & Cardinaud, 1984; Henry et al., 1985; Trayer et al., 1987). The singlet at 2.09 ppm could come from the N-acetyl group of the N-terminal amino acids in light chains LC-3 (Henry et al., 1985). The doublet resonance at 1.37 ppm could be caused by the methyl groups of the mobile alanine residues of subfragment S1 (Prince et al., 1981; Henry et al., 1985; Bhandary et al., 1986; Trayer et al., 1987). The small differences of a few hundredths of ppm of the chemical shifts reported for these compounds (3.23, 1.63, 2.05, and 1.37 ppm) can most probably be attributed to the different experimental conditions. However, most of these lines designated as "sharp" have line widths of the order of 50 Hz, that is, one order of magnitude broader than the lines we refer to as "sharp". The question arises why these resonances have not been observed earlier. For LMM the answer is very easy: In conventionally prepared proteolytic fragments of myosin, it is extremely likely that the mobile C-terminus is cleaved off during the digestion [see, e.g., Nyitray et al. (1983)]. For myosin itself the answer is probably that nobody did look for such sharp lines, that one needs a very good signal-to-noise ratio with rather low concentrations of myosin (low viscosity of the solution!), and that an exponential filtering matched to a line width of 50 Hz will quench these sharp lines.

When all the data are taken together the following picture emerges: In intact myosin as well as in the complete C-terminal fragment LMM-30, the last 9 C-terminal residues of one strand and the 12 C-terminal residues of the other strand are mobile, whereas in the shortened C-terminal fragment LMM-30C′ only the last 2 residues show signs of increased mobility. Beginning with His-256 (His-1930 in myosin), the time-averaged structures of the two C-termini are identical from the NMR point of view. The chemical shift values and coupling constants are rather close to the random-coil values (Bundi & Wüthrich, 1979) but nevertheless show small differences. Especially the somewhat higher amide–Hα coupling constants would suggest a higher population of more extended conformations. The complete absence of sequential ROESY and NOESY peaks in this part of the sequence excludes the existence of a unique three-dimensional structure and suggest that the C-termini are most probably unfolded (not uniquely folded). (The missing of detectable NOEs in the laboratory frame is expected even for a well-structured peptide when the motional correlation times responsible for the dipolar relaxation are in the range where the sign of the NOE in the laboratory frame changes. Indeed, the T_2 relaxation times derived from the line widths of the sharp resonances indicate that correlation times in this part of the structure are near this point. However, in the rotating frame, sequential NOEs should be observed even with a high mobility of C-terminus, if the C-terminus is well-structured.) The valine residues in position 255 (position 1929 in intact myosin) in the two strands are inequivalent in the NMR spectrum but mobile, suggesting an interaction between the two strands. Ser-252, Arg-253, and Asp-254 are completely immobilized in one of the strands (all resonances are too broad to be detectable) and partly mobile in the other strand (only part of the resonances are sharp enough to be detectable, see Table I). This is consistent with a parallel arrangement of the two α-helices of LMM [as confirmed also for the fragments LMM-30 and LMM-30C′ by electron microscopy (data not shown)]. The periodicity of 7 found in the distribution of hydrophobic and charged or polar amino acid residues suggests that the myosin monomer could consist of a whole family of α-helical coiled-coil structures with the two polypeptide chains shifted by multiples of 7 relative to each other (Crick, 1953; McLachlan & Karn, 1982, 1983; Cohen & Parry, 1990). Such a heterogeneity can be excluded from the NMR data that show only one dominant structure.

Interestingly, the mobile, unfolded part of one of the C-termini is three residues longer than the other one. In his model for packing two α-helices within a coiled-coil molecule, Crick (1953) proposed that the apolar residues at positions a and d of the repeating unit $(abcdefg)_n$ of one chain are meshed with the counterparts of the other chain in an interdigitating manner in the interface area ("knobs into holes" model). With two identical polypeptide chains that are packed parallel and in register, as in our case the LMM part of intact myosin or the LMM fragments LMM-30 and LMM-30C′, the model implies that the general molecular packing is symmetrical (although from the NMR point of view only an antiparallel arrangement of the two chains would grant their complete equivalence) and the two tails are in register. Our data suggest an asymmetrical arrangement, since 9 residues of one chain and 12 residues of the other chain are mobile. This is hardly accounted for by Crick's model, no plausible explanation is readily available until we know details of the molecular packing from new experiments.

The aromatic residues in the putative α-helical part of LMM-30 show rather small line widths for a protein with a molecular weight of 61 283. This is mainly the consequence of highly anisotropic rotational reorientation of the molecule but may be partly due to the fact that the coiled-coil is not completely rigid. Some of the aromatic residues are equivalent from the NMR point of view, which could mean that they do not take part in the interchain interaction.

As shown earlier (Maeda et al., 1991), the mobile C-terminal amino acids appear to play a role in the assembly of myosin monomers to thick filaments: The removal of the C-terminus of LMM-30 severely disturbs the aggregation to higher polymers, a feature that is most prominent at higher pH values. A possible role of the unfolded C-terminus in myosin could be that it might function as a kind of cement that fills open spaces in the polymeric structure that exist after the aggregation of the coil-coiled monomers. However, the existence of unfolded and highly mobile structures may not be a rarity found only in rabbit myosin. Although secondary structure predictions from sequence data alone are rather dubious, they give at least a hint at which secondary structure elements are favored. An analysis of the known myosin sequences shows that the C-termini are at least not simply α-helical (McLachlan & Karn, 1982, 1983; Cohen & Parry, 1990).

As a generalization, one could conclude that unfolded and mobile termini are a general property of fibrous proteins and are important as adaptive elements for the proper assembly. An analysis of the sequences of α-helical coiled-coil proteins by Cohen and Parry (1990) shows that the C-termini (and sometimes also the N-termini) are most probably not α-helical. The question of whether or not these structures are really unfolded and mobile can only be answered by future experiments.

Acknowledgments

We thank K.C. Holmes for continuous support of this work and helpful discussions. We are grateful to M. H. C. Koch for providing the sample cuvette for our X-ray experiments. We also thank R. Hofmann for sequencing the C-termini of the LMM fragments. One of us (A.W.) was supported by the Deutsche Forschungsgemeinschaft (Wi 371/3-2).

References

Akasaka, K., Konrad, M., & Goody, R. S. (1978) *FEBS Lett. 96*, 287–290.

Bhandari, D. G., Levine, B. A., Trayer, I. P., & Yeadon, M. E. (1986) *Eur. J. Biochem. 160*, 349–356.

Brauer, M., & Sykes, B. D. (1981) *Biochemistry 20*, 2060–2064.

Braunschweiler, L., & Ernst, R. R. (1983) *J. Magn. Reson. 53*, 521–528.

Bundi, A., & Wüthrich, K. (1979) *Biopolymers 18*, 285–297.

Cohen, C., & Parry, D. A. D. (1990) *Proteins 7*, 1–15.

Crick, F. H. C. (1953) *Acta Crystallogr. 6*, 689–697.

Eads, T. M., & Mandelkern, L. (1984) *J. Biol. Chem. 259*, 10689–10694.

Emerson, C. P., & Bernstein, S. I. (1987) *Annu. Rev. Biochem. 56*, 695–726.

Ferrige, A. G., & Lindon, J. C. (1978) *J. Magn. Reson. 31*, 337–340.

Guinier, A. (1963) in *X-Ray Diffraction*, W. H. Freeman, San Francisco, 319–350.

Hendrix, J., Fürst, H., Hartfield, B., & Dainton, D. (1982) *Nucl. Instrum. Methods 201*, 139–144.

Henry, G. D., Trayer, I. P., Brewer, S., & Levine, B. A. (1985) *Eur. J. Biochem. 148*, 75–82.
Highsmith, S., & Jardetzky, O. (1980) *FEBS Lett. 121*, 55–60.
Highsmith, S., Akasaka, K., Konrad, M., Goody, R. S., Holmes, K., Wade-Jardetzky, N., & Jardetzky, O. (1979) *Biochemistry 18*, 4238–4244.
Highsmith, S., Wang., C.-C., Zero, K., Pecora, R., & Jardetzky, O. (1982) *Biochemistry 21*, 1192–1197.
Holmes, K. C., Popp, D., Gebhard, W., & Kabsch, W. (1990) *Nature 347*, 44–49.
Jeener, J., Meier, B. H., Bachmann, P., & Ernst, R. R. (1979) *J. Chem. Phys. 71*, 4546–4553.
Kabsch, W., Mannherz, H. G., Suck, D., Pai, E. F., & Holmes, K. C. (1990) *Nature 347*, 37–44.
Kessler, H., Anders, U., Gemmecker, G., & Steuernagel, S. (1989) *J. Magn. Reson. 85*, 1–14.
Koppitz, B., Feldmann, K., & Heilmeyer, L. M. G. (1980) *FEBS Lett. 117*, 199–202.
Levine, B. A., Griffiths, H. S., Patchell, V. B., & Perry, S. V. (1988) *Biochem. J. 254*, 277–286.
Levine, B. A., Moir, A. J. G., Trayer, I. P., & Williams, R. J. P. (1990) *Top. Mol. Struct. Biol. 15*, 171–209.
Maeda, K., Sczakiel, G., Hofmann, W., Menetret, J.-F., & Wittinghofer, A. (1989) *J. Mol. Biol. 205*, 269–273.
Maeda, K., Rösch, A., Maëda, Y., Kalbitzer, H. R., & Wittinghofer, A. (1991) *FEBS Lett. 281*, 23–26.
Margossian, S. S., & Lowey, S. (1982) *Methods Enzymol. 85*, 55–71.
Marion, D., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 113*, 967–974.
McLachlan, A. D., & Karn, J. (1982) *Nature 299*, 226–229.
McLachlan, A. D., & Karn, J. (1983) *J. Mol. Biol. 164*, 605–626.
Neidig, K.-P., & Kalbitzer, H. R. (1991) *J. Magn. Reson. 91*, 155–164.
Nyitray, L., Mocz, G., Szilagyi, L., Balint, M., Lu, R. C., Wong, A., & Gergely, J. (1983) *J. Biol. Chem. 258*, 13213–13220.
Prince, P., Trayer, H. R., Henry, G. D., Trayer, I. P., Dalgarno, D. C., Levine, B. A., Cary, P. D., & Turner, C. (1981) *Eur. J. Biochem. 121*, 213–219.
Rance, M. (1987) *J. Magn. Reson. 74*, 557–564.
Rance, M., Sorensen, O. W., Bodenhausen, G., Wagner, G., Ernst, R. R., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 117*, 479–485.
Roux-Fromy, M., & Cardinaud, R. (1984) *FEBS Lett. 172*, 198–204.
Schlemmer, H., Sontheimer, G. M., & Kalbitzer, H. R. (1988) *Magn. Reson. Chem. 26*, 260–263.
Shriver, J. W., & Sykes, B. D. (1981) *Biochemistry 20*, 2004–2012.
Sommerville, L. E., Henry, G. D., Sykes, B. D., & Hartshorne, D. J. (1990) *Biochemistry 29*, 10855–10864.
Trayer, I. P., Trayer, H. R., & Levine, B. A. (1987) *Eur. J. Biochem. 164*, 259–266.
Winkelmann, D. A., Mekeel, H., & Rayment, I. (1985) *J. Mol. Biol. 181*, 487–501.