Study of the phosphorylatable light chains of skeletal and gizzard myosins by nuclear magnetic resonance spectroscopy

B. A. LEVINE,* H. S. GRIFFITHS,† V. B. PATCHELL‡ and S. V. PERRY‡

*Inorganic Chemistry Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QR, U.K., and Departments of †Biochemistry and ‡Physiology, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

³¹P and ¹H n.m.r. studies of the phosphorylatable light chains from rabbit fast skeletal and chicken gizzard muscles in the isolated state and in the intact myosin molecule indicate that the *N*-terminal region of the light chain containing the sites of phosphorylation has independent segmental flexibility. The ionization behaviour of serine phosphate in both rabbit skeletal and chicken gizzard P light chains exhibits co-operativity and is compatible with the phosphate group being influenced by neighbouring positively charged side-chains. No marked difference in phosphate ionization behaviour was apparent between the monophosphorylated P light chains of rabbit skeletal and chicken gizzard myosins. From ¹H and ³¹P n.m.r. studies of the overall conformation, side-chain ionization properties and the spectral effects of titration with an anionic paramagnetic reagent bound at the basic *N*-terminal region, it is concluded that Thr-18 and Ser-19 are phosphorylated in the bisphosphorylated P light chain of gizzard myosin, the latter residue being the site of monophosphorylation. In the presence of F-actin the mobility of the serine phosphate of the P light chain of intact gizzard myosin was reduced. No interaction between the isolated P light chain and F-actin was however detected. These results are discussed with reference to the observed conformational features of the P light chain.

INTRODUCTION

The precise manner in which the phosphorylation of the P (LC2 or DTNB) light chain of myosin regulates the Mg²⁺-activated ATPase of actomyosin is far from clear. Despite the similarity of structure and function of myosin and the kinase systems in striated and smooth muscles, the role of phosphorylation appears to be different in the two muscle types. Whereas phosphorylation of the P light chain is essential for the activation of the actomyosin ATPase in smooth muscle, in striated muscle it is not (see Hartshorne & Siemankowski, 1981; Perry *et al.*, 1984; Stull *et al.*, 1985 for reviews).

One difference between the skeletal and smooth muscle systems is that, whereas in fast skeletal muscle there is no evidence of a second site of phosphorylation, a bisphosphorylated form of gizzard P light chain has been reported (Cole *et al.*, 1985) and confirmed (Ikebe & Hartshorne, 1985).

The amino acid sequences close to the sites of phosphorylation of the P light chains of myosin from striated and smooth muscle are very similar. It was originally reported that the phosphorylation site of rabbit fast skeletal muscle myosin was one of an adjacent pair of serine residues in a duodecapeptide obtained by chymotryptic digestion of the P light chain (Perrie *et al.*, 1973). Subsequently, the determination of the complete amino acid sequence of the P light chain identified this pair of serines as residues 14 and 15 (Collins, 1976; Maita *et al.*, 1981). The corresponding phosphorylation sites on the P light chain of chicken gizzard myosin as shown in this work are Thr-18 and Ser-19, the latter

being the site of phosphorylation in monophosphorylated gizzard P light chain. It has been presumed that the homologous site on rabbit fast skeletal P light chain, Ser-15, is the site of phosphorylation of the light chain by skeletal myosin light-chain kinase (Kendrick-Jones & Jakes, 1977).

Activation of the actomyosin ATPase of smooth muscle does not depend on the phosphorylation of the second site, but Ikebe & Hartshorne (1985) consider that phosphorylation of this site increases the ATPase activity, whereas Cole *et al.* (1985) found no clear correlation between high ATPase activity of gizzard actomyosin and second site phosphorylation.

A number of reports (see Kendrick-Jones & Scholey, 1981, for review) suggest that the differences in the regulation of contraction in the various muscle types depend upon the nature of the myosin light chains. To explore further this possibility as it relates to the P light chain we have undertaken a comparative study of these light chains from skeletal and smooth muscle myosins by high resolution ³¹P and ¹H n.m.r. Using intrinsic side-chain ionization properties and extrinsic paramagnetic species as structural probes it has been possible to describe the environment of the phosphorylated side-chains. Further, the ¹H n.m.r. spectral features of the molecules reflect subtle conformational differences between the light chains of the two muscle types. These differences are discussed in the light of the known light-chain and actin binding sites on the myosin head. Some aspects of this work have been briefly reported (Perry *et al.*, 1985; Levine *et al.*, 1987).

MATERIALS AND METHODS

Preparation of muscle proteins

Myosin from rabbit fast skeletal muscle was prepared by the method of Perry (1955), modified by initial extraction in 0.6 M-KCl (Pires *et al.*, 1974), and used after three precipitations. The chicken gizzard myosin was the SL preparation of Cole *et al.* (1982). Actin was prepared from rabbit fast skeletal and chicken gizzard muscles by the method of Spudich & Watts (1971). It was stored as the freeze-dried protein and polymerized for use by dissolving in 50 mM-KCl/2 mM-MgCl₂.

P light chains of myosin

The P light chains were isolated from rabbit fast skeletal muscle and chicken gizzard myosins by the method of Grand & Perry (1983), dialysed against 50 mM-ammonium bicarbonate, freeze dried and stored at -20 °C. The purity of preparations was monitored by electrophoresis in 8 M-urea, pH 8.3. Preparations used for ³¹P n.m.r. studies were at least 80–90 % pure and for ¹H n.m.r. spectroscopy only preparations better than 90 % pure were used.

Phosphorylation of myosin

Myosin light-chain kinase was prepared from rabbit fast skeletal muscle by the method of Nairn & Perry (1979) and from chicken gizzard muscle by the method of Adelstein & Klee (1981).

Rabbit skeletal myosin (20 mg/ml) dissolved in 50 mM-Tris/HCl (pH 7.6), 10 mM-MgCl₂, 1 mM-dithiothreitol (DTT), 1 mM-CaCl₂, 5 mM-ATP, calmodulin (10 µg/ml) was incubated with skeletal light-chain kinase (5 µg/ml) for 10–30 min at 30 °C. The solution was added to an equal volume of 2 M-KCl and applied to a column of Sepharose 4B (1.5 cm × 80 cm for 40 mg of myosin) equilibrated with 0.8 M-KCl, 20 mM-imidazole/HCl (pH 7.0), 1 mM-EGTA, 1 mM-DTT, 0.01 % (w/v) NaN₃. Fractions of the main peak of myosin eluted with the same buffer from several columns run concurrently were combined to obtain the amount of protein required for ³¹P n.m.r. study.

Chicken gizzard myosin was phosphorylated by incubation in 50 mM-imidazole/HCl (pH 7.0), 10 mM-MgCl₂, 5 mM-ATP, 1 mM-CaCl₂, 1 mM-DTT, calmodulin (100 µg/ml) either by endogenous kinase or by the addition of gizzard light-chain kinase (5 µg/ml). After incubation until the required level of phosphorylation was reached, usually 5–10 min at 30 °C, an equal volume of 2 M-KCl, 2 mM-EDTA, 2 mM-EGTA, 2 mM-ATP was added. Kinase was removed by gel filtration on Sepharose 4B as described by Cole *et al.*, (1983) and the fractions of the main peak of myosin combined.

The phosphorylated samples of skeletal and gizzard myosins were dialysed after gel filtration against 50 mM-KCl, 20 mM-imidazole/HCl (pH 7.0), 1 mM-EDTA, 1 mM-DTT and precipitated by dilution with 5 vol. of water at 2 °C. Immediately before n.m.r. study, the myosin precipitate was dissolved in an equal volume of 2 mM-EGTA, 40 mM-imidazole/HCl (pH 8.2). 10 mM-ATP, 2 mM-DTT in ²H₂O.

Phosphorylation of isolated P light chains

Rabbit skeletal muscle P light chain (10 mg/ml) was incubated in 50 mM-Tris/HCl (pH 7.6), 10 mM-MgCl₂, 1 mM-CaCl₂, 1 mM-DTT, 5 mM-ATP, calmodulin

(10 µg/ml) with skeletal light-chain kinase (5 µg/ml) for 10 min at 30 °C. Phosphorylation was stopped by the addition of an equal volume of 10 % (w/v) trichloroacetic acid, the precipitated protein separated by centrifugation and dissolved in 9 M-urea (10 mg/ml). The solution of phosphorylated light chain was applied to a column of Sephadex G-25 (2 cm × 45 cm, 20 mg of protein) equilibrated against 50 mM-ammonium bicarbonate. The protein peak eluted by the same buffer was freeze-dried.

Chicken gizzard P light chain (10 mg/ml) was incubated in 50 mM-imidazole/HCl (pH 7.0), 10 mM-MgCl₂, 1 mM-CaCl₂, 1 mM-DTT, 5 mM-ATP, calmodulin (100 mg/ml) with gizzard light-chain kinase (50 mg/ml). Mono-phosphorylated P light chains were usually obtained after 5 min incubation and bisphosphorylated after 40 min at 30 °C under these conditions. The phosphorylated light chain was isolated and freeze-dried as described for the skeletal muscle P light chain.

Electrophoresis

Protein preparations were monitored by electrophoresis on 8 % (w/v) polyacrylamide, 1 % (w/v) SDS, 0.1 M-Tris/Bicine, pH 8.3 (Weeds *et al.*, 1975) or in 12.5 % (w/v) Acrylogel, 8 M-urea, 25 mM-Tris/80 mM-glycine, pH 8.3 (Perrie & Perry, 1970) to determine the extent of phosphorylation. Gels were scanned using a Zeineh soft-laser densitometer.

N.m.r. methods

³¹P and ¹H n.m.r. spectra were recorded at 149 MHz and 300 MHz respectively using Bruker Spectrospin instruments operating in the Fourier transform mode. Pulse and collect observations were carried out using pulse widths of 20 µs (³¹P) and 4 µs (¹H) corresponding to a 70° pulse angle. An interpulse relaxation delay of 2.5 s was introduced when collecting ³¹P n.m.r. data. Accumulations were made using 8K data points and spectral widths of 3 kHz. ¹H n.m.r. two-pulse Carr Purcell spin-echo spectra were obtained using a (90 τ–180 τ) pulse sequence with a delay time (τ) of 60 ms and a relaxation delay of 2 s. In this way signals with relaxation times (*T*₂) notably longer than 60 ms could be effectively resolved. The signal multiplicity (*J*, spin–spin coupling constant) of the latter signals introduces phase modulation in the resulting spectra (Campbell *et al.*, 1975) which allows the multiplicity of these resonances to be identified.

The freeze-dried light chains (2–3 mg/ml) were dissolved in 25 mM-ammonium bicarbonate/0.3 mM-DTT in ²H₂O for n.m.r. study. Sample volumes were 2.5 ml for ³¹P and 0.5 ml for ¹H n.m.r. The number of accumulations (obtained at ambient probe temperature, 26–27 °C) per spectral run was in the range of 10⁴ for ³¹P and 10³ for ¹H in order to obtain the signal-to-noise levels appropriate for the specific aim of the particular experiment (chemical shift data or paramagnetic relaxation effects). ³¹P n.m.r. spectra of solutions of intact myosin (10–20 mg/ml, see above) were accumulated for (1–4) × 10⁴ scans using an interpulse relaxation delay of 1 s and a 60° pulse width. Chemical shift values are quoted with respect to trimethyl phosphate as external reference (³¹P n.m.r.) or 3-trimethylsilylpropanesulphonate as internal reference (¹H n.m.r.).

Titration curves were carried out over a pH range of 6–9. The chemical shift position of the fully ionized protein phosphate in each case was readily determined by

extrapolation of the data obtained in the alkali pH range. These values were consistent with ^{31}P n.m.r. data for serine and threonine phosphates previously reported in proteins (e.g. Vogel & Bridges, 1983; Brauer & Sykes, 1984). Precipitation of the proteins below pH 6 precluded direct or similar determination of the chemical shift position associated with the fully protonated state of each protein phosphate group. To obtain a numerical evaluation of apparent $\text{p}K_a$ values rather than a qualitative comparison with the parameters found for the free monoester phosphates, chemical shift values for the fully protonated species were inferred from the data for serine phosphates (overall ionization induced shift of 4 ± 0.5 p.p.m.; Vogel & Bridges, 1983; Brauer & Sykes, 1984). The least-square fit of the titration data using these end-point values is reported. All pH values quoted were direct meter readings obtained using a micro-electrode placed in the n.m.r. tube. The pH was adjusted by addition of small aliquots of stock solutions of 1 M NaOH or ^2HCl .

RESULTS

Isolated skeletal muscle P light chain

A single resonance deriving from a monoester phosphate species was observed by ^{31}P n.m.r. As would be expected from the previous assignment of Ser-15 as the site of phosphorylation (Perrie *et al.*, 1973; Kendrick-Jones & Jakes, 1977), the pH titration behaviour of the signal was typical of serine phosphate (Fig. 1). The signal from Ser-15 shows a titration midpoint closely similar to that observed for free serine phosphate (Fig. 1) with an estimated apparent $\text{p}K_a = 5.8 \pm 0.2$. Its pH titration profile however, shows more co-operative characteristics while the different shielding environment compared with free serine phosphate, as reflected in the chemical shift of the ^{31}P resonance of Ser-15 (Fig. 1), suggests that the phosphate group experiences an electronic environment different to that expected from purely solvent surroundings. The observed linewidth (~ 10 Hz), however, indicates that segmental mobility is available to the phosphate moiety of Ser-15.

Segmental flexibility of other side-chains of the *N*-terminal tail of the P light chain of skeletal muscle was also indicated by spectral data obtained using ^1H n.m.r. Here two-pulse spin-echo methods (see n.m.r. methods) were used to filter signal intensity on the basis of spin-spin relaxation times reflecting the intrinsic dynamics of the corresponding groups. The signals observed by this pulse technique derive from groups with greatest segmental freedom. Further, the spectra obtained using this pulse procedure show phase modulation. It is thereby possible to correlate the observed signals with amino acid type on the basis of resonance position and signal multiplicity (Levine *et al.*, 1979). Thus the signals originating from the most mobile groups derived largely from the *N*-terminal blocking group, α -*N*-trimethylalanine (Henry *et al.*, 1985), lysine ϵCH_2 and alanine βCH_3 groups, all of which residues are located in the *N*-terminal region (Fig. 2).

Notably absent from this spectrum of the most segmentally mobile side-chains is a signal derived from threonine γCH_3 . This implies that the region of primary structure possessing marked segmental flexibility does not extend to Thr-24, the threonine residue closest to the *N*-terminus. Further, the lack of spectral contribution

Fig. 1. Effect of pH on the chemical shift of the ^{31}P monoester phosphate resonances of the P light chains of myosin

Ser-15 of rabbit fast skeletal muscle myosin ($\square$), Ser-19 of chicken gizzard myosin ($\circ$) and free serine phosphate ($\bullet$). Isolated light-chain concns. 2–3 mg/ml, $T = 300$ K and creatine phosphate as internal chemical shift standard. The titration end-points were determined by extrapolation to high pH and by reference to the overall shift upon ionization (4 ± 0.5 p.p.m.) found in previous studies of monoester phosphates (Brauer & Sykes, 1984; Vogel, 1983; Vogel & Bridges, 1983).

from threonine γCH_3 groups also indicates that segments of the structure encompassing the remaining threonine residues of the molecule (Thr-32, 50, 78, 82, 98, 111, 122, 123 and 158) possess differentially restricted mobility compared to the *N*-terminal region. The conclusion that these other regions of the structure of the skeletal muscle light chain are relatively more structured is supported by the observed ^1H n.m.r. spectrum (Fig. 3). Specific spectral features, illustrated in Fig. 3, point to local structural elements, e.g. the spread in resonance energy for the phenylalanine side-chains indicative of proximal disposition of some of these hydrophobic groups from different parts of the primary structure. Taken overall these data indicate that the skeletal muscle P light chain possesses a characteristic but motile tertiary fold from which extends the *N*-terminal tail that possesses segmental mobility independent of the rest of the molecule.

Isolated gizzard muscle P light chain

The monoester phosphate resonance of Ser-19 of chicken gizzard P light chains was monitored as a function of pH using both the monophosphorylated and bisphosphorylated forms of the protein. Its ionization behaviour in the monophosphorylated molecule showed

Fig. 2. ^1H n.m.r. two-pulse spin-echo spectra of myosin P light chains

(a) Rabbit fast skeletal muscle myosin P light chain; (b) chicken gizzard myosin P light chain. Since signals from the *N*-terminal residues [*N*-acetyl, (a), and $-\text{N}(\text{CH}_3)_3$, (b)] are readily observed together with signals of groups deriving from the *N*-terminal portion of the primary structure (e.g. lysine, alanine), the *N*-terminal segment of each light chain (sequence shown) can be seen to possess significant mobility with respect to the rest of the molecular configuration whose corresponding signals are absent. $T = 300 \text{ K}$, $\text{pH} = 7.9$, $\tau = 60 \text{ ms}$.

notable co-operativity [co-operativity parameter (n) < 1] with a calculated apparent $\text{p}K_a = 5.6 \pm 0.2$ (Figs. 1 and 4) and was more noticeably dependent upon ionic strength than was found for Ser-15 of the skeletal muscle P light chain. Concomitant with a salt-induced shift in $\text{p}K_a$ to 6.1 ± 0.2 (0.15 M-NaCl) there occurred a loss in the apparent co-operativity of the ionization of the serine phosphate moiety (Fig. 4). These observations indicate that the ionization characteristics of the phosphate group of Ser-19 are influenced by proximal positively charged side-chains rather than by proximal groups titrating in the same pH range, the effect of added salt being to shield the phosphate moiety from the positive charge. This conclusion, which is relevant also to the observed behaviour of Ser-15 of the skeletal muscle P

light chain, may be correlated with the highly basic composition of the *N*-terminus of both skeletal and gizzard muscle P light chains, and is in keeping with the observed shift in resonance position of the serine phosphate signal to low field when compared with free serine phosphate. The implication therefore, that the *N*-terminal segment of the light chain, though possessing a large degree of segmental mobility, adopts a more restricted set of conformations than available to a random coil, was verified by the observation that correspondence of both resonance position and $\text{p}K_a$ for the phosphate group with that of free serine phosphate was observed when titrations were carried out in the presence of 8 M-urea.

When preparations of bisphosphorylated gizzard myosin P light chain containing some monophosphorylated protein ($\sim 1.7 \text{ mol of P/mol}$) were examined, three distinct ^{31}P resonances were resolved (Fig. 5). The possibility of contamination by adenosine monophosphate (^{31}P n.m.r. studies on myosin, see below) was eliminated since incubation with 5'-nucleotidase did not alter the spectrum. Direct comparison with the spectral data for the monophosphorylated light chain showed that the signal furthest downfield (Fig. 5) derived from Ser-19 of the monophosphorylated form. Correlation with the chemical shift position obtained with a known sample of threonine phosphate indicated that the resonance furthest upfield (signal 3) was derived from a phosphorylated threonine side chain. The remaining monoester phosphate signal was therefore assigned to Ser-19 of the bisphosphorylated species.

It is apparent, in view of the different resonance energy position of Ser-19 of the bisphosphorylated species under the same pH conditions compared with the monophosphorylated form, that phosphorylation at the second site (threonine) on the molecule alters the electronic environment in the vicinity of Ser-19. This observation could result either from an allosteric effect (i.e. long-range conformational influence of further phosphorylation) or it could be the consequence of direct interaction between the two phosphorylated side-chains by virtue of their proximity in the primary structure. To distinguish between these two possibilities, the ionization characteristics of the bisphosphorylated gizzard muscle P light-chain were studied. Variation of solution pH showed that the two phosphorylated groups of the protein displayed differently co-operative proton dissociation (Fig. 6) with apparent $\text{p}K_a = 6.6 \pm 0.2$ for the threonine phosphate group, a value higher than was observed for free threonine phosphate. Also in the bisphosphorylated form Ser-19 showed different co-operativity parameters and an apparent $\text{p}K_a = 6.1 \pm 0.2$ compared with a value of 5.6 ± 0.2 found for this residue in the monophosphorylated form, Figs. 4 and 6.

The higher $\text{p}K_a$ of the threonine phosphate group is likely to result from the presence of neighbouring negative charge and in view of the ionization behaviour of Ser-19 in the bisphosphorylated light chain compared with the monophosphorylated form, these data suggest that the two phosphate groups are in spatial proximity thereby altering the local electronic environment of Ser-19 and leading to the observed electrostatic communication between these two phosphorylation sites. Further, the segmental mobility available to the threonine phosphate side-chain as reflected in its ^{31}P n.m.r. signal linewidth suggests that it too is derived from the *N*-terminal tail of

Fig. 3. Conventional ^1H n.m.r. spectra of myosin P light chains

(a) Rabbit fast skeletal myosin P light chain; (b) chicken gizzard myosin P light chain. Although the spectra generally reflect the absence of a unique overall fold, the greater spread in resonance energy of signals from specific groups, e.g. the aromatic side-chains of the gizzard P-light chain (c), indicates that the proteins adopt distinguishably motile configurations in solution (see text). This is confirmed by the observation that denaturation with 8 M-urea leads to a marked increase in signal intensity from groups not derived from the *N*-terminus. Isolated unphosphorylated light chains (2–3 mg/ml), $T = 300\text{ K}$, $\text{pH} = 7.9$.

the molecule that extends away from the rest of the protein structure (i.e. the phosphate group is on Thr-9, 10 or 18).

As may be expected from the high proportion of positively-charged residues at the *N*-terminal of the P light chain, this region of the molecule adopts a relatively flexible, extended configuration. To localize more precisely the site of threonine phosphorylation, the protein was titrated with the anionic paramagnetic reagent, $\text{Cr}(\text{CN})_6^{3-}$. The spectral relaxation effects induced by the probe vary inversely with distance (r) away from the bound species. The signal linewidth broadening of groups in the vicinity of the probe is proportional to r^{-6} . The choice of this probe reagent was based on the isotropic nature of its spectral perturbation and on its ability to bind at the highly basic *N*-terminal segment of the P light chain molecule while being unable to interact directly with phosphate groups. The primary sequence of this extended segment (Fig. 2) shows that both Thr-9 and Thr-10 are flanked by lysine residues while Thr-18 does not occur in such a densely basic sequence, the closest positively-charged centre being that of Arg-16. Titration of the bisphosphorylated gizzard muscle P light chain with $\text{Cr}(\text{CN})_6^{3-}$ showed that the signals primarily perturbed by the binding of this anionic relaxation probe were derived from groups located at the more mobile *N*-terminal segment of the molecule. This is illustrated in

Fig. 7 where ^1H n.m.r. resonances of lysine, alanine, threonine and the *N*-acetyl group are seen to be notably relaxed, consistent with probe binding at the *N*-terminal region which is expected to possess net attraction for the anionic reagent. These observations provided a basis for distinguishing between Thr-9 or 10 and Thr-18 as the phosphorylated residues. The former occur closer than Thr-18 and Ser-19 to the positively charged centres that act as binding sites for the paramagnetic anionic probe on the essentially extended structure characteristic of the *N*-terminal region. Titrations were therefore similarly carried out using ^{31}P n.m.r. to monitor the relative proximity of the serine and threonine phosphate to the bound probe. These experiments showed that the relaxation of the threonine phosphate resonance as indicated by the progressive perturbation during titration with $\text{Cr}(\text{CN})_6^{3-}$ closely matched that of the signal deriving from Ser-19. In view of the flexible, extended nature of the *N*-terminal segment these data cannot have come about if the threonine phosphate was at positions 9 or 10 which are flanked by lysine side-chains. Rather, both phosphate groups on the P light chain must be located at similar radial distances from the bound probe. It is therefore concluded that Thr-18 is the second site of phosphorylation on the gizzard light chain, consistent with the observed ionization characteristics of these two phosphorylated side-chains.

Fig. 4. Effect of salt on the ionization behaviour of the ^{31}P n.m.r. signals of the phosphate group of Ser-19 of the chicken gizzard P light-chain

P light chain (●); P light-chain in the presence of 0.15 M-NaCl (○); protein at 3–4 mg/ml. H^+ and H^- represent chemical shifts in the protonated and fully ionized forms. Treatment of the data in this way enables the derivation of both pK_a (intercept on pH axis) and co-operativity parameter, n (slope). The addition of salt is seen to result in typical Henderson–Hasselbach titration character ($n = 1$), as well as a return of the observed pK_a towards normal. These linear graphs are least-square fits of the experimental data using a range of values (4 ± 0.5 p.p.m.) for the overall ionization induced shift ($\delta_{\text{H}^-} - \delta_{\text{H}^+}$) to determine δ_{H^+} which could not be directly observed due to precipitation of the proteins at pH values < 6 . Values of δ_{H^-} were obtained as described in the legend to Fig. 1.

The marked segmental flexibility of the *N*-terminal segment of the P light chain of gizzard muscle myosin does not extend beyond Met-24 in the primary structure (Figs. 2 and 3). The rest of the molecule shows ^1H n.m.r. spectral features characteristic of a relatively stable overall fold, e.g. resolved in the spectrum of the P light chain of gizzard muscle myosin (Fig. 4) are differentially shifted signals of $-\text{CH}_3$ groups ($\delta \sim 0.4$ p.p.m.) as well as the resonances of the aromatic groups of tyrosine and phenylalanine. These differential shifts result from different electronic shielding environments experienced by the corresponding groups. The spread in resonance energy reflects the relative disposition of these $-\text{CH}_3$ groups and aromatic side-chains in the fold possessed by the main portion (residues 24–171) of the gizzard myosin P light chain. Similarly, a relatively stable hydrophobic core has been observed in ^1H n.m.r. studies of the alkali light chains (LC1 and LC3) of skeletal muscle myosin (Chaussepied *et al.*, 1986). The ^1H n.m.r. spectrum of the P light chain of skeletal myosin by contrast shows a much less pronounced dispersion of aromatic signals than its gizzard myosin counterpart (Fig. 3). This spectral difference was consistently observed in several light-chain preparations. The broad signal envelope observed

Fig. 5. ^{31}P n.m.r. spectrum of the chicken gizzard myosin P light chain showing the signals of Ser-19 in the mono-phosphorylated and bisphosphorylated forms

Myosin P light chain $\sim 70\%$ bisphosphorylated, 3 mg/ml, pH = 7.25, $T = 298\text{K}$. Creatine phosphate (CP) used as internal shift standard.

Fig. 6. Effect of pH on the chemical shift position of the ^{31}P n.m.r. signals of serine (○) and threonine (●) phosphate in the bisphosphorylated chicken gizzard P light chain

Data presented as in Fig. 4. Conditions as for Fig. 5. Note the different co-operativity parameters for each signal and the difference between the ionization behaviour of Ser-19 in the mono- and bis-phosphorylated molecules (cf. Fig. 4).

for signals of tyrosine and phenylalanine side-chains is unlikely to be a consequence of polymerization. The spectrum was unaffected by the presence of a large excess of DTT while relatively sharp signals are observed elsewhere in the spectrum (Fig. 3a and Fig. 2). Rather, the observed lineshape suggests the presence of a relatively ill-defined/unstable fold of the main portion of

Fig. 7. ^1H n.m.r. two-pulse spin-echo spectra of the chicken gizzard myosin P light chain during titration with the anionic relaxation reagent, $\text{Cr}(\text{CN})_6^{3-}$.

Isolated P light chain approx. 70% bisphosphorylated (3 mg/ml), $T = 298\text{ K}$, $\text{pH} = 7.3$. Perturbation of the signals deriving from groups close to the probe binding site is seen as a decrease in intensity (enhanced relaxation) in spectra (a)–(c). Note the relative effects on lysine ϵCH_2 and δCH_2 , alanine βCH_3 and Thr- γCH_3 as compared with the signal of Arg- δCH_2 groups. The signals here shown derive from the segmentally mobile N-terminal tail (cf. Fig. 2).

the isolated P light chain of skeletal myosin. This structural distinction in tertiary folding character may be a consequence of specific residue substitutions in the two largely homologous primary sequences (e.g. the replacement of threonine in the skeletal P light chain by phenylalanine at position 116 in the smooth muscle protein). Such substitutions may well be of functional relevance since the distinction exists in that region of the molecule that anchors the P light chain on to the myosin head.

^{31}P n.m.r. studies on intact myosin

All the studies described above were carried out on P light chains isolated from myosin. ^{31}P n.m.r. studies on intact myosin present considerable technical problems due to the difficulty in obtaining concentrations that are high enough to give adequate signal to noise with moderately short accumulation times since the P light chain represents only 7% of the myosin molecule. It has been concluded in previous ^{31}P n.m.r. studies of rabbit skeletal myosin that serine was the phosphorylation site (Koppitz *et al.*, 1980). Contamination of myosin preparations by inorganic phosphate and nucleotide monophosphates, particularly AMP, which resonate in the

same spectral range as serine or threonine phosphates also presents difficulties in the interpretation of ^{31}P n.m.r. observations. The nucleotides are present in myosin preparations that have been incubated with ATP to phosphorylate the light chain and are produced by adenylate kinase contamination, small traces of which are difficult to eliminate from preparations of myosin.

Direct identification of the phosphate monoester of serine in monophosphorylated smooth and skeletal muscle myosins was made by observing the effects on the ^{31}P n.m.r. spectrum of the addition of phosphatase and, in a separate experiment, 5'-nucleotidase which would remove a presumptive AMP signal (Fig. 8). The observed triplet character and spectral linewidth of the serine phosphate ($\sim 35\text{ Hz}$ at half-height of the signal) indicated that the phosphate group on the P light chain in intact myosin possesses notable segmental mobility whilst its resonance position (downfield of free serine phosphate) reflects the deshielding effect of proximal charge (as found for the isolated light chains, see above). Confirmation that Ser-19 is the primary phosphorylation site in smooth myosin was obtained by observing the ^{31}P n.m.r. spectral properties of the P light chain isolated from a similar smooth myosin preparation. This showed serine phosphate to be the only monoester phosphate present ($> 90\%$) and the signal displayed ionization behaviour identical to that of the monophosphorylated form obtained by phosphorylation of the isolated gizzard P light chain.

Addition of actin at low salt in the absence of ATP and Ca^{2+} to gizzard myosin did not perturb the serine phosphate signal. Upon titration with F-actin the signal progressively broadened indicating reduced mobility of the phosphate group of Ser-19 (Fig. 8). Comparable ^{31}P n.m.r. studies with isolated gizzard muscle P light chain gave no evidence of an alteration in the segmental mobility of the serine phosphate group in the presence of F-actin. Likewise, ^1H n.m.r. data also showed that no binding occurred between F-actin and the isolated light chain and that the segmental flexibility of the N-terminal region of the isolated monophosphorylated gizzard muscle P light chain was unaffected by the addition of F-actin.

DISCUSSION

A feature of the N-terminal region of the P light chains of both rabbit skeletal and chicken gizzard myosins is segmental mobility that is independent of the rest of the molecule. In this respect the P light chain resembles the N-terminal tail of the LC1 (A1) light chain of myosin that is also found to extend away from the main body of the light chain (Henry *et al.*, 1985). The flexible segment on both P light chains includes the phosphorylation sites. Extrapolation from the proton n.m.r. results (Fig. 2) indicates that the flexible segment does not extend to Met-20 of the rabbit fast skeletal light chain or Met-24 of the gizzard light chain since no signals corresponding to these residues were observed in the spin-echo spectra. Thus the flexible N-terminal regions are similar in the striated and smooth muscle P light chains although the latter are slightly longer due to the extra four residues at the N-terminus.

Taken overall these spectral studies also identify a functionally relevant structural difference between the P light chains of rabbit skeletal and chicken gizzard myosin.

Fig. 8. ^{31}P n.m.r. spectrum of monophosphorylated chicken gizzard myosin

Myosin (20 mg/ml) was monophosphorylated with kinase under conditions described in the Materials and methods section. (a) Insert (i) shows, at $8\times$ the gain, the signal of Ser-19 and AMP (*). The latter signal is lost upon addition of 5'-nucleotidase. Insert (ii), at $4\times$ the gain of (i), after incubation with phosphatase resulting in the loss of the signal of Ser-19, shown using resolution enhancement methods to possess triplet character. (b) Addition of F-actin in the presence of 5'-nucleotidase leads to progressive broadening of the signal of Ser-19.

Fig. 9. Comparative structural features of the P light chain of smooth (chicken gizzard) and skeletal (rabbit) myosin

The sites of phosphorylation are located towards the C-terminal end of the flexible N-terminal of each molecule. The rest of the protein structure is less flexible with a relatively compact hydrophobic core in the case of the gizzard light chain.

Much greater conformational flexibility is shown by the core of the skeletal P light chain. Here the hydrophobic groups adopt a less well-organized configuration as shown by the greater signal overlap (Fig. 3). By contrast,

the well-resolved spectral spread for the ^1H n.m.r. signals of aromatic groups of the chicken gizzard P light chain is indicative of distinct environments and reflects a well-defined fold of the body of the molecule from which

extends the *N*-terminal. This structural feature may well facilitate 'action-at-a distance' in the gizzard P light chain. The n.m.r. studies confirm our preliminary conclusion that the two sites of phosphorylation of the gizzard muscle P light chain are adjacent (Perry *et al.*, 1985) as have sequence studies on isolated peptides (Ikebe *et al.*, 1986; A. J. G. Moir, V. B. Patchell & S. V. Perry, unpublished results), i.e. Thr-18 and Ser-19 are the phosphorylation sites in bisphosphorylated chicken gizzard P light-chain (Fig. 9).

The fact that the activation of gizzard muscle actomyosin ATPase occurs as a consequence of phosphorylation in this flexible region of the light-chain structure, and that the segmental freedom of the phosphate group of Ser-19 *in situ* is much restricted in the presence of F-actin implies an important role for this region of the P light chain. It is interesting therefore, in view of the marked difference in effect of phosphorylation on the actin-activated ATPase in the two myosins that the ¹H n.m.r. studies reveal subtle differences in overall conformation of the two P light chains (Fig. 3). The ¹H and ³¹P n.m.r. studies, however, have not shown any gross difference in conformation of either of the two isolated P light chains upon monophosphorylation. These observations imply that the difference in behaviour of smooth and striated myosin may be associated with subtle conformational effects transmitted through the P light chain as a result of phosphorylation at its flexible *N*-terminal tail, to elicit differences in response of some regions of the myosin heavy chain.

The effect of actin on the mobility of the serine phosphorylation site of the P light chain *in situ* could be explained by direct interaction of actin with the *N*-terminal region. The failure to observe this effect with the isolated light chain implies that such interaction may only occur when the light chain is in its normal association with the heavy chain of myosin. The decrease in mobility observed with myosin could therefore be the consequence of direct interaction with actin of the P light chain, or the collapse of the tail onto the rest of the P light chain, or by possible indirect effects transmitted over a distance by interaction with the myosin heavy chain (or alkali light chain) at another region of the P light chain. It is noteworthy in this respect that the *N*-terminal of the P light chain has been located at the head-rod junction of the myosin molecule (Hardwicke *et al.*, 1983; Winkelmann *et al.*, 1983; Sutoh *et al.*, 1986), away from both ATP-binding site and the sites of interaction between actin and the myosin heads, while the *C*-terminal of the P light chain appears to bind to a 10–12 kDa segment of the myosin head that also interacts with actin (Mitchell *et al.*, 1986). This segment of the myosin head extends towards the head-rod junction.

These relative positions of the phosphorylatable *N*-terminal of the P light chain and its *C*-terminal interaction site with the myosin head close to an actin-binding site suggest a transduction mechanism for the phosphorylation event akin to the structural mode of triggering described for calcium binding to calmodulin (Levine & Dalgarno, 1983; Dalgarno *et al.*, 1984). Definition of the structural consequences of phosphorylation of the P light chain of smooth muscle myosin remains the subject of further investigation.

We thank the Medical Research Council, the American Muscular Dystrophy Association and the Muscular Dystrophy

Group of Great Britain for financial support of various aspects of this work. We are grateful to Professor R. J. P. Williams for helpful discussions.

REFERENCES

- Adelstein, R. E. & Klee, C. B. (1981) *J. Biol. Chem.* **256**, 7501–7509
- Brauer, M. & Sykes, B. D. (1984) *Methods Enzymol.* **107**, 36–81
- Campbell, I. D., Dobson, C. M., Williams, R. J. P. & Wright, P. E. (1975) *FEBS Lett.* **57**, 96–99
- Chaussepied, P., Mornet, D., Audemard, E., Kassab, R., Goodearl, A. J., Levine, B. A. & Trayer, I. P. (1986) *Biochemistry* **25**, 4540–4547
- Cole, H. A., Grand, R. J. A. & Perry, S. V. (1982) *Biochem. J.* **206**, 319–328
- Cole, H. A., Patchell, V. B. & Perry, S. V. (1983) *FEBS Lett.* **158**, 17–20
- Cole, H. A., Griffiths, H. S., Patchell, V. B. & Perry, S. V. (1985) *FEBS Lett.* **180**, 165–169
- Collins, J. H. (1976) *Nature (London)* **259**, 699–700
- Dalgarno, D. C., Klevit, R., Levine, B. A. & Williams, R. J. P. (1984) *Trends Pharmacol. Sci.* **84**, 266–271
- Grand, R. J. A. & Perry, S. V. (1983) *Biochem. J.* **211**, 267–272
- Hardwicke, P. M. D., Walliman, T. & Szent-Gyorgi, A. G. (1983) *Nature (London)* **301**, 478–482
- Hartshorne, D. J. & Siemankowski, R. E. (1981) *Annu. Rev. Physiol.* **43**, 519–530
- Henry, G. D., Trayer, I. P., Brewer, S. & Levine, B. A. (1985) *Eur. J. Biochem.* **148**, 75–82
- Ikebe, M. & Hartshorne, D. J. (1985) *J. Biol. Chem.* **260**, 10027–10031
- Ikebe, M., Hartshorne, D. J. & Elzinga, M. (1986) *J. Biol. Chem.* **261**, 36–39
- Kendrick-Jones, J. & Jakes, R. (1977) in *Myocardial Failure* (Riechker, G., Weber, A. & Goodwin, J., eds.), pp. 28–40 Springer Verlag, Berlin and Heidelberg
- Kendrick-Jones, J. & Scholey, J. M. (1981) *J. Muscle Res. Cell Motil.* **2**, 347–372
- Koppitz, B., Feldmann, K. & Heilmeyer, L. M. G. (1980) *FEBS Lett.* **117**, 199–202
- Levine, B. A., Moore, G. R. M., Ratcliffe, R. G. & Williams, R. J. P. (1979) in *Chemistry of Macromolecules IIA* (Offord, R. E., ed.), vol. 24, pp. 77–141, University Park Press, Baltimore
- Levine, B. A. & Dalgarno, D. C. (1983) *Biochim. Biophys. Acta* **726**, 187–204
- Levine, B. A., Griffiths, H. S., Patchell, V. B. & Perry, S. V. (1987) *J. Muscle Res. Cell Motil.* **8**, 75
- Maita, T., Chenn, J.-I. & Matsuda, G. (1981) *Eur. J. Biochem.* **117**, 417–424
- Mitchell, E. J., Jakes, R. & Kendrick-Jones, J. (1986) *Eur. J. Biochem.* **161**, 25–35
- Nairn, A. C. & Perry, S. V. (1979) *Biochem. J.* **179**, 89–97
- Perrie, W. T. & Perry, S. V. (1970) *Biochem. J.* **119**, 31–38
- Perrie, W. T., Smillie, L. B. & Perry, S. V. (1973) *Biochem. J.* **135**, 151–164
- Perry, S. V. (1955) *Methods Enzymol.* **2**, 582–588
- Perry, S. V., Cole, H. A., Hudlicka, O., Patchell, V. B. & Westwood, S. A. (1984) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **43**, 3015–3020
- Perry, S. V., Griffiths, H. S., Levine, B. A. & Patchell, V. B. (1985) *Adv. Protein Phosphatases* **2**, 3–18
- Pires, E., Perry, S. V. & Thomas, M. A. W. (1974) *FEBS Lett.* **41**, 292–296
- Spudich, J. A. & Watts, S. (1971) *J. Biol. Chem.* **246**, 4866–4871

- Stull, J. T., Nunnally, M. H., Moore, R. L. & Blumonthal, D. K. (1985) *Adv. Enzyme Regul.* **23**, 123–140
- Sutoh, K., Yamamoto, K. & Wakabayashi, T. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 212–216
- Vogel, H. J. (1983) *Biochemistry* **22**, 668–674
- Vogel, H. J. & Bridges, W. A. (1983) *Can. J. Biochem. Cell Biol.* **61**, 363–369
- Weeds, A. G., Hall, R. & Spurway, N. C. (1975) *FEBS Lett.* **49**, 320–324
- Winkelman, D. A., Lowey, S. & Press, J. P. (1983) *Cell* **34**, 295–306

Received 17 September 1987/9 March 1988; accepted 6 May 1988