THE P-LIGHT CHAIN OF RABBIT SKELETAL MUSCLE MYOSIN: A ^{31}P NMR STUDY

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1. Introduction

^{31}P NMR studies have revealed that in glycogen phosphorylase *a* the phosphate group of phosphoserine-14 forms a salt bridge to probably an arginine residue and thus fixes the flexible N-terminal peptide to the enzyme (detailed in [1,2]). In troponin T the phosphate group of phosphoserine-1 may have the opposite function namely to prevent an interaction of the N-terminal domain with the rest of the molecule [3]. This latter phosphate group shows one main ^{31}P NMR signal of a narrow band width [4]. Furthermore, it changes its chemical shift with pH as free phosphoserine indicating that this phosphate is exposed to the solvent and can freely rotate even though it is attached to a protein of M_r 90 200. Other phosphate groups of phosphoproteins may also be classified in one of the two types above. Here, we show that the phosphate group of phosphoserine-14 or -15 which is present in the 'P-light chain' (LC_2) of rabbit skeletal muscle myosin [5–7] behaves similarly to phosphoserine-1 in troponin T.

2. Materials and methods

Myosin from rabbit skeletal muscle was prepared as in [8]. Protein was determined according to [9]. The Ca^{2+} content of the solutions employed was determined by atomic absorption spectrometry and the desired concentrations of Ca^{2+} were adjusted by EGTA according to [4].

SDS–Polyacrylamide gel electrophoresis was done according to [10] and in presence of 6 M urea as in [5]. Total phosphate content was determined as in [11].

^{31}P NMR spectra were recorded with a Bruker WH-180 widebore superconducting spectrometer in the Fourier transform mode at 72.86 MHz with broadband proton decoupling (0.4 W). Cooling to 0°C was achieved by blowing cold dry air through the probe head and around the shim coils. A concentric 5 mm NMR tube containing ^2H_2O was employed as field/frequency lock. For all spectra the exponential line broadening used prior to Fourier transformation was 10 Hz. All linewidth data are corrected for this line broadening effect. Chemical shifts are expressed with respect to 85% H_3PO_4 as external reference. For further experimental details see [4].

Standard buffers were 50 mM tris-(hydroxymethyl)-aminomethane (Merck), pH 7.5 and 8.5, and 10 mM 3-(*N*-morpholino)-propane-sulfonic acid (Serva) pH 6.0. EGTA was purchased from Sigma, urea (analytical grade) was obtained from Serva.

3. Results and discussion

Native freshly isolated rabbit skeletal muscle myosin was used as material and was not further fragmented in its proteolytic subfragments. Partial proteolysis may cleave peptide linkages of the light chains which potentially can alter the interaction of the light chain phosphate group with other amino acid side chains of myosin. The isolated material contained av. 0.5 mol phosphate/mol protein as determined following repeated precipitation with trichloroacetic acid and oxidative digestion. The observation of several phosphate signals which are seen in the ^{31}P NMR spectrum as described below shows the difficulty in interpreting the phosphate content of phosphopro-

Abbreviations: EGTA, ethylene glycol bis(β-aminomethyl-ether) *N,N,N',N'*-tetraacetic acid; SDS, sodium dodecylsulphate

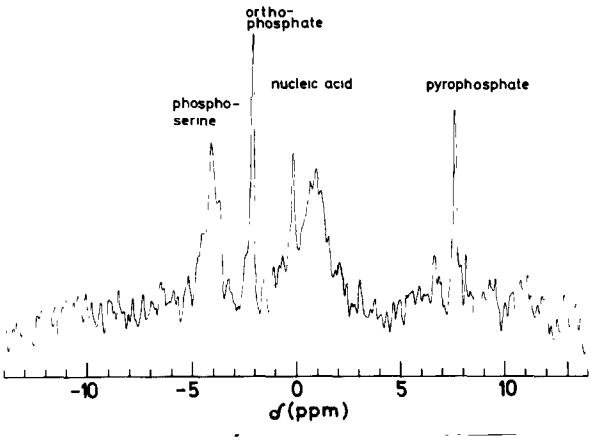

Fig.1. ^{31}P NMR spectrum of freshly prepared rabbit skeletal muscle myosin. The enzyme (35 mg/ml) was dissolved in 0.5 M KCl, 50 mM tris-(hydroxymethyl)-aminomethane, 15 mM 2-mercaptoethanol, 2 mM EGTA (pH 7.5). The spectrum taken at 0°C represents 26 000 scans with a repetition time of 1.7 s. The signals show the following chemical shifts: pyrophosphate, 6.8 ppm; nucleic acid, ~0.0 ppm; orthophosphate, −2.7 ppm; phosphoserine, −4.6 ppm.

Table 1
pH Dependence of ^{31}P chemical shift of signal I in rabbit skeletal muscle myosin and of phosphoserine

pH	Chemical shift (ppm)	
	Signal I of myosin	Phosphoserine
8.5	−4.6	−4.6
7.5	−4.6	−4.6
6.0	−2.9	−3.0

teins as determined by direct phosphate analysis. A typical ^{31}P NMR spectrum of native myosin shows 4 main absorption bands (fig.1). Two of these signals with chemical shifts of −2.7 and 6.8 ppm can be identified as orthophosphate and pyrophosphate, respectively, by adding the corresponding salts. They are present as contaminants from the phosphate–pyrophosphate buffer used for extraction of the myosin from the fibrils. Other contaminants are phosphodiesters which absorb near 0.0 ppm. Characteristi-

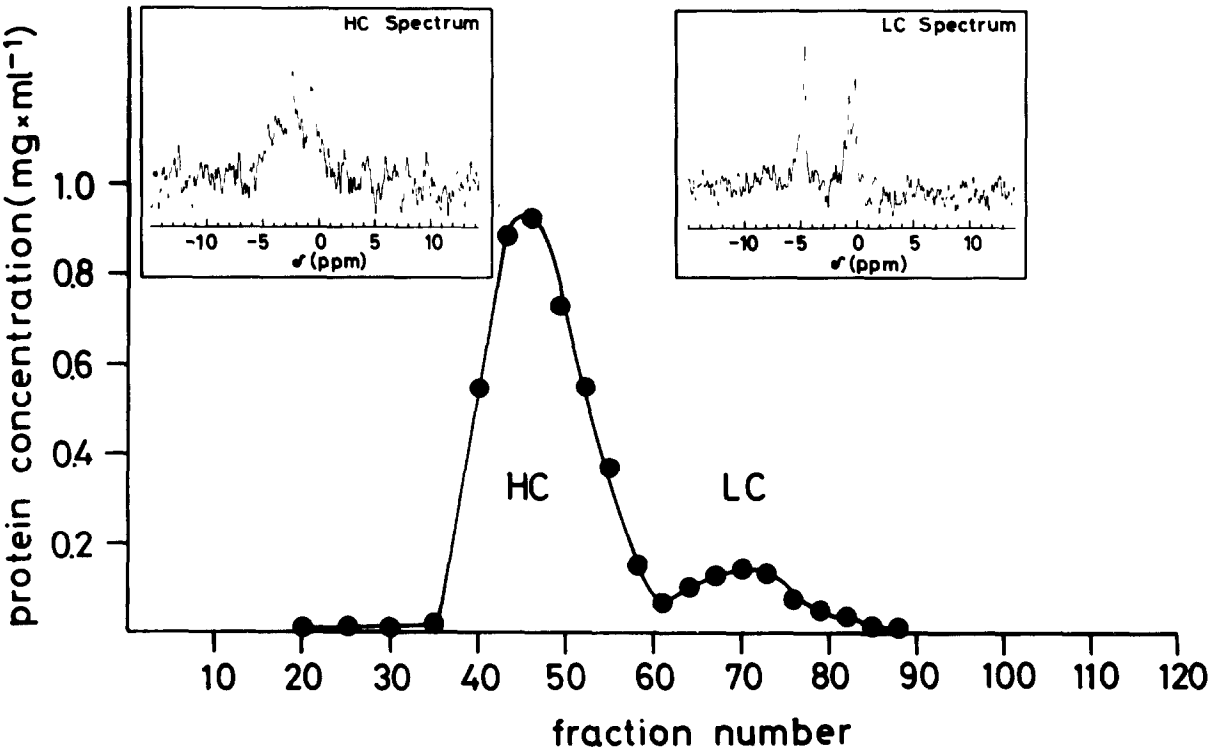

Fig.2. Separation of SDS denatured myosin on Sephadex G-200 into heavy-chains (HC) and light-chains (LC). 15 ml myosin (13.8 mg/ml) were denatured in 1% SDS, 50 nM tris-(hydroxymethyl)-aminomethane, 1.5 mM mercaptoethanol (pH 7.5) and were applied to a column (50 × 750 mm) which was equilibrated in the same buffer. Fractions of 13 ml were collected and their content identified by gel electrophoresis (see fig.3). The ^{31}P NMR spectra of the heavy chain fraction (97 000 scans) and of the light chain fraction (198 000 scans) are shown as inserts.

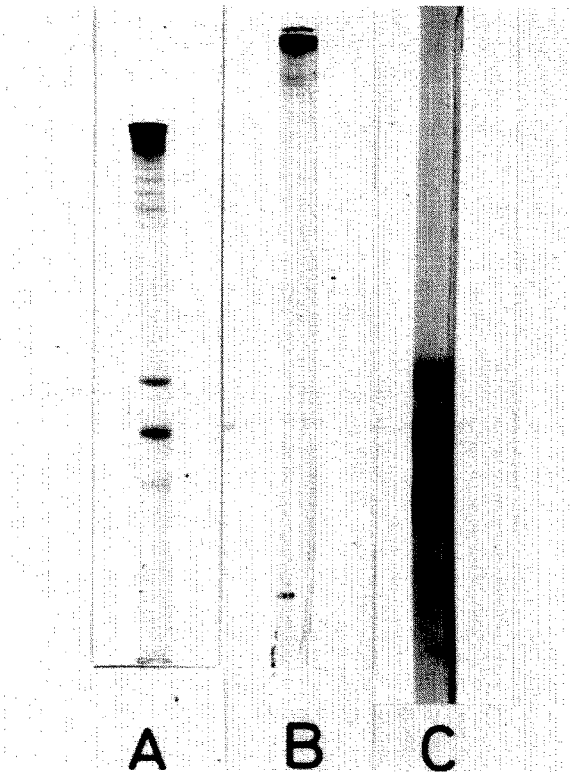

Fig.3. Polyacrylamide gel (10%) electrophoresis of unfragmented and fragmented myosin. Electrophoresis was carried out as in section 2: (A) Unfragmented myosin in the presence of SDS: (B) heavy chain fraction; (C) light chain fraction after separation on Sephadex G-200.

cally their chemical shift does not change over pH 6.5–8.5 (not shown). Their broad linewidth and their high molecular weight (see fig.2) characterize the material as nucleic acid. The signal at –4.6 ppm is characteristic for phosphoserine [4]. Its chemical shift changes with pH in a manner identical to free phosphoserine (see table 1). It indicates that this phosphate group is exposed to the solvent. The phosphate group of phosphoserine-1 in rabbit skeletal muscle troponin T behaves analogously [4]. The linewidth of the myosin phosphoserine signal is ~40 Hz which is ~10-fold broader than that of troponin T. The Stokes-Einstein relationship predicts that τ_c and, hence, the linewidth are proportional to molecular weight when the phosphate group is bound tightly to the protein. For this case we expect a ^{31}P linewidth of ~140 Hz for the phosphoserine in myosin (M_r ~500 000) using the data in [12]. Thus, the experimental linewidth of ~40 Hz indicates a significant internal mobility of phosphate group about the C–O bond, since the ^{31}P linewidth is significantly reduced from the predicted value due to the additional modulation of ^{1}H–^{31}P internuclear vectors and chemical shift tensor orientation. The signal at –4.6 ppm can be identified as phosphoserine of the P-light chain. Gel filtration over Sephadex G-200 of sodium dodecylsulphate denatured myosin separates the heavy chain from the light chain-fraction (fig.2). The break through material contains the heavy chains (see SDS gel pattern fig.3). Correspondingly, the ^{31}P NMR spectrum shows only nucleic acids which absorb near 0.0 ppm with the above absorption characteristics. No phosphoserine signal was detected. The second peak contains the known 3 light chains (see SDS gel pattern, fig.3). The ^{31}P NMR spectrum of this material contains as a characteristic signal at –4.6 ppm that of phosphoserine. In the denatured form lacking specific interactions it shows a linewidth which is considerably narrowed to ~10 Hz in comparison to native myosin. In addition some contaminating nucleic acid of lower molecular weight is present. The phosphoserine signal of native myosin disappears nearly completely after incubation either for 30 h at 0°C or for a few hours at 20°C. Concomitantly in the urea gel electrophoresis system [5] the faster migrating P-light chain disappears and combines with the more slowly migrating band (not shown).

Therefore, this disappearance of the ^{31}P NMR signal directly reflects the hydrolysis of phosphoserine by the highly specific myosin light chain phosphatase which is present in the native myosin preparation [13]. The phosphoserine signal at –4.6 ppm of native myosin does not change if the spectrum is recorded either in buffer containing 10^{-8} M or 10^{-5} M free Ca^{2+}. Again this behaviour is analogous to that of phosphoserine in troponin T [4]. It excludes that the phosphoserine is involved directly in binding of Ca^{2+} to the P-light chain.

In accordance with this interpretation it has been shown [14] that the phosphorylated and dephosphorylated P-light chains exhibit an identical affinity for Ca^{2+} if they are present in native myosin. In contrast isolated P-light chains lose affinity for Ca^{2+} considerably upon phosphorylation [15]. However, isolated P-light chains change their conformation upon complexation in holomyosin [16].

The presence of this phosphate in the N-terminal region, i.e., on serine-12 or -15 of the P-light chain [6,7] might prevent an interaction of the N-terminal

domain with other parts of the myosin molecule as can be concluded in analogy to the effect of phosphoserine-1 present in troponin T [4,5]. Another possibility could be that the phosphoserine of the P-light chain interacts with Ca^{2+} bound by actin as proposed [17] which may be revealed by further ^{31}P NMR studies of a reconstitued actomyosin.

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