The occurrence of α-N-trimethylalanine as the *N*-terminal amino acid of some myosin light chains

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1. INTRODUCTION

The alkali light chains are small subunits of myosin; one is associated with each head region. Rabbit fast-twitch skeletal muscle myosin contains two types, A1 (LC1) and A2 (LC3), which occur in a ratio of ~ 2:1 [1,2]. These differ from one another primarily in that A1 possesses a 41 residue N-terminal 'tail' rich in proline, lysine and alanine residues, although they are otherwise almost identical [3].

Recent NMR studies [4] of rabbit fast muscle myosin S1 isoenzymes revealed an unusual proton resonance of narrow line width at 3.23 ppm. This signal arises from the S1 isoenzyme containing the A1 light chain (S1(A1)), but not from the alternative A2-containing isoenzyme (S1(A2)). Anionic paramagnetic shift reagents induce an upfield shift [5], characteristic of a positively-charged grouping, whereas titration from pH 7–12 did not affect the chemical shift, suggesting that the group did not ionise over this range. Coupled with the chemical shift these data are suggestive of a $-N^+(CH_3)_3$ group, and the resonance was thus tentatively assigned to the *N*-methyl protons of ε-*N*-trimethyllysine [7]. This is a known component of the myosin heavy chain [6], and can be seen similarly in the ^{1}H-NMR spectrum of calmodulin [7]. However, subsequent analyses confirmed that the unknown signal arises not from the heavy chain, but from the associated alkali light chain, A1, which contains no trimethyllysine.

We have now assigned the 3.23 ppm resonance to the *N*-methyl protons of α-*N*-trimethylalanine which is here identified as the N-terminal blocking group of the A1 light chain. ^{1}H-NMR evidence has suggested this tail region to be highly mobile in S1(A1). Stoichiometric addition of actin considerably reduces this mobility [4] as well as causing other large structural changes [4,8]. In particular, the signal at 3.23 ppm disappears, indicating its immobilization in the acto-S1(A1) complex.

2. EXPERIMENTAL

2.1. *Protein and peptide preparations*

Myosin was prepared from fast-twitch rabbit skeletal muscle as in [9]. Light chains were extracted from this essentially as in [10], except that the myosin was dissociated in 9 M urea and the heavy chains precipitated by 63% (v/v) ethanol. The supernatant, containing all 3 light chains, was concentrated and fractionated on DEAE–Sephadex A25, in 4 M urea. The peptide containing residues 1–36 of the A1 light chain was obtained by pepsin digestion [11] and isolated by gel filtration on Sephadex G-25 in 10 mM HCl. It is the only peptide which eluted in the void volume of the column. The N-terminal tripeptide, X–Pro–Lys, was generated by tryptic digestion of fragment 1–36 and was separated from other small peptides by high-voltage paper electro-

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Abbreviations: S1, myosin subfragment 1; S1(A1), S1(A2), myosin subfragment 1 containing either the alkali 1 (A1, LC1) light chain or the alkali 2 (A2, LC3) light chain; ppm, parts per million; NMR, nuclear magnetic resonance; Tml, ε-trimethyllysine

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phoresis [3]. The isolated N-terminal blocking group, X, was obtained from an acid hydrolysate of X–Pro–Lys (6 M HCl, 110°C in vacuo, overnight) by high-voltage paper electrophoresis at pH 2. It was identified by spraying the paper with thymol blue [12] where amino acids appear as yellow spots on red background. Elution from the paper was with 1% formic acid. An authentic sample of α-*N*-trimethylalanine was prepared by refluxing L-alanine in methanol with methyl iodide for 8 h under alkaline conditions [13]. Excess reagents were removed by rotary evaporation in vacuo.

2.2. *NMR Spectroscopy*

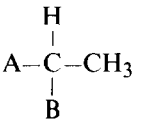
^{1}H-NMR experiments were performed on a 300 MHz Bruker spectrometer operated in the Fourier-transform mode as in [4]. Samples of either freeze-dried or vacuum desiccated material were dissolved in ^2H_2O buffers containing 10 mM phosphate (pH 7) (uncorrected). Chemical shifts are given in ppm from sodium 3-trimethylsilyl-(2,2,3,3-$^2H_4$) propionate as internal standard and measurements were made at 297 K.

3. RESULTS

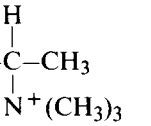
As judged by its narrow linewidth, the unassigned resonance at 3.23 ppm must arise from a residue of exceptional mobility. ^{1}H-NMR studies on isolated light chains showed it to be a component of A1 but not A2. It thus seemed likely that the unknown signal arises from the 41 residue 'difference peptide' and this was confirmed in the spectrum of an A1 fragment containing residues 1–36 (fig.1A). In order to accommodate the postulated $-N^+(CH_3)_3$ group, it was considered that A1 might possess a trimethylated amino acid at its N-terminus. Such a residue would be difficult to detect by conventional methods, since it lacks a primary amino group. Furthermore, the N-terminal blocking group of A1 has never been identified, although A2 was shown to be *N*-acetylated [3]. With this in mind, the strategy for identification of the unknown resonance centered on the isolation of a small N-terminal peptide, X–Pro–Lys. This peptide was separated by high-voltage electrophoresis and was identified by its equimolar content of proline and lysine and by lack of a free α-amino group.

X was shown to be a modified amino acid by high-voltage electrophoresis at pH 2.0 of an acid hydrolysate of X–Pro–Lys. The paper was sprayed with the pH indicator dye, thymol blue, which detects amino acids by the buffering action of free carboxyl groups [12]. The (X,Pro,Lys) acid hydrolysate yielded 3 spots, 2 corresponding to proline and lysine; whereas the third migrated with a mobility slightly less than aspartic acid, consistent with a single positive charge at pH 2.0. Ninhydrin stained only proline and lysine.

Fig.1B shows the ^{1}H-NMR spectrum of X–Pro–Lys. Two signals which do not originate from the proline and lysine residues are immediately apparent; the singlet resonance of 9 proton intensity at δ = 3.23 ppm and the doublet methyl group resonance of 3 proton intensity at δ = 1.60 ppm. The nature of the coupling pattern for these two signals was confirmed using two-pulse spin-echo techniques. The double nature of the resonance at δ = 1.60 ppm can only arise from coupling to a single proton attached to an adjacent carbon. This was confirmed by spin decoupling experiments, irradiation at δ = 1.60 ppm leading to the collapse of a multiplet signal at δ = 4.50 ppm typical of C_α–H protons. Reciprocal effects were observed upon irradiation at δ = 4.50 ppm which lead to the collapse of the doublet signal to a singlet. Thus the observed coupling pattern and chemical shifts point to a



moiety. Nuclear Overhauser experiments [14] were undertaken to investigate the proximity of the $-N^+(CH_3)_3$ group to the –CH and $-CH_3$ group protons. Irradiation of the $-N^+(CH_3)_3$ signal led to positive Overhauser enhancements on both –CH and $-CH_3$ groups signals (fig.1C), thereby confirming the



connectivity of the X moiety, α-*N*-trimethylalanine.

Identification of the X moiety as trimethylalanine is supported by comparison of the ^{1}H-NMR spectrum of X as isolated from the X–Pro–Lys tripep-

tide with the spectrum of synthetic trimethylalanine (fig.1D,1E). Spin decoupling effects and Overhauser enhancements observed for the isolated moiety were in agreement with the data obtained for the tripeptide. Anionic probe effects (not shown) were also consistent with the structure of X as α-*N*-tri-

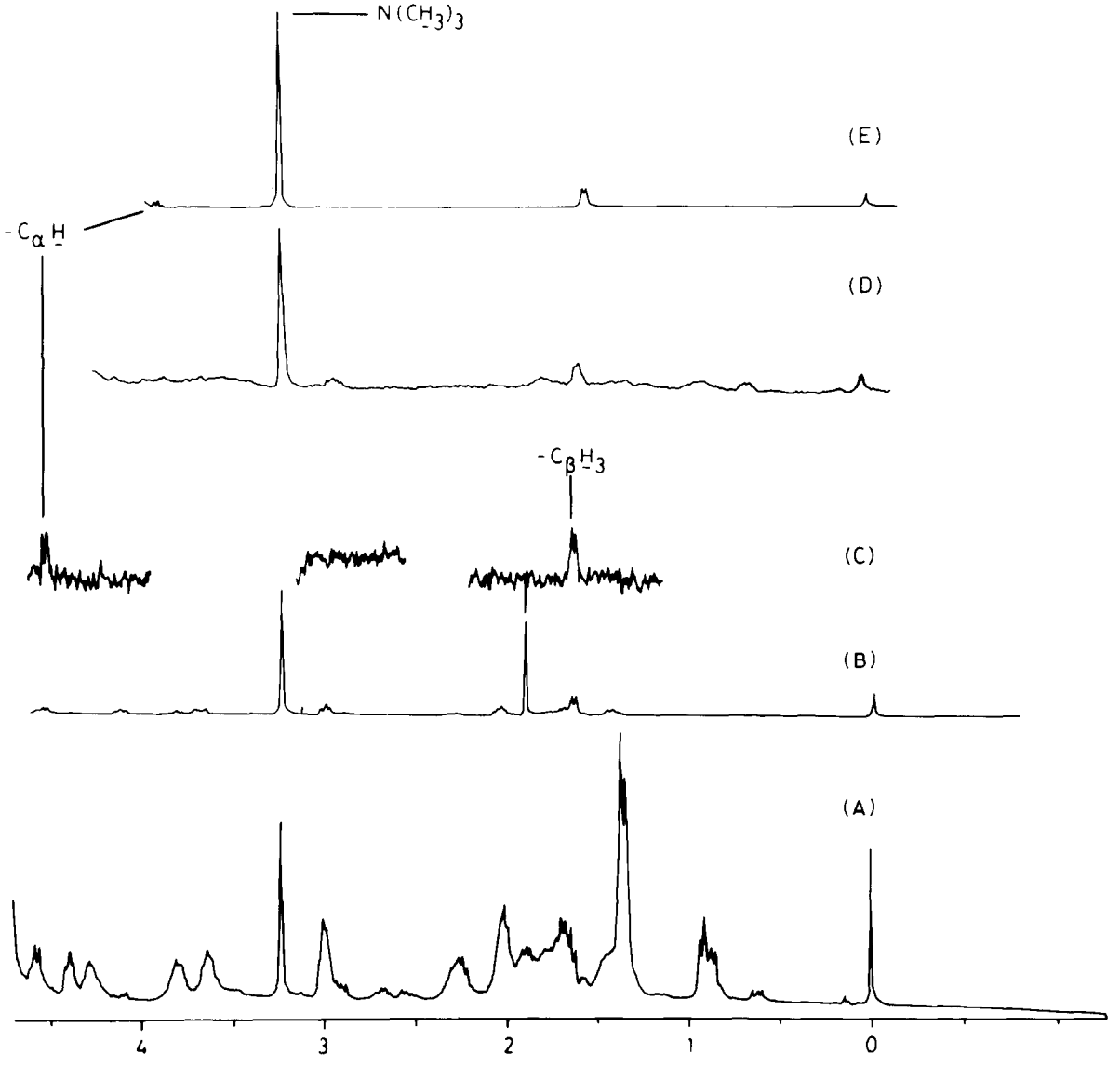


Fig.1. 300 MHz ^{1}H-NMR spectra used to identify the nature of the N-terminal blocking group (X) of the A1 light chain as α-*N*-trimethylalanine: (A) peptide containing residues 1–36 from the rabbit fast muscle myosin A1 light chain; (B) N-terminal peptide, X–Pro–Lys, generated by trypsin digestion of fragment 1–36. Elution from the paper was with 1% acetic acid (residual signal at δ = 1.89 ppm); (C) Nuclear Overhauser difference spectrum [14] showing positive enhancements for both $C_\alpha H$ and $C_\beta H_3$ signals upon irradiation of the $-N^+(CH_3)_3$ signal of X–Pro–Lys; (D) isolated N-terminal blocking group (X) separated from an acid hydrolysate of X–Pro–Lys; (E) synthetic trimethylalanine. The $C_\alpha H$ signals in (D) and (E) are shifted upfield from those in (B) and (C) due to the proximity of the negatively-charged free carboxyl-group in the isolated amino acid.

methylalanine. Furthermore, separate chromatographic and electrophoretic analyses on X showed that it always co-migrated with authentic α-*N*-trimethylalanine.

4. DISCUSSION

N-Terminal methylations are apparently a rare event. There are two other examples reported in the literature of quaternized α-amino groups in proteins: cytochrome *c*–557 of the protozoan *Crithidia oncopelti* is blocked by *N*-dimethyl proline [15],16], whereas *N*-trimethylalanine occurs at the *N*-terminus of the *E. coli* ribosomal protein L11 [17]. The occurrence of *N*-dimethyl proline at the *N*-terminus of the protozoan cytochrome presents some interesting parallels with A1. Both molecules possess N-terminal extensions; the 41 residue tail of A1 is not present in the A2 [3], whereas cytochrome *c*-557 contains a 10-residue N-terminal segment not found in vertebrate cytochromes *c* [15]. Furthermore, the α-*N*-methylated amino acid (X) occurs in the sequence X–Pro–Lys–Lys in A1, X–Pro–Tml in the cytochrome and X–Lys–Tml in L11 [18], i.e., at the terminus of a highly basic region.

The discovery of trimethylalanine in rabbit myosin represents the first account of α-*N*-methylated amino acid in a higher organism. It is quite possible, however, that N-terminal methylations are considerably more widespread than current data predict. The very nature of the substitution yields derivatives which will be undetectable by routine techniques. In this context, it is interesting to note that the ^{1}H-NMR spectra of the phosphorylatable (DTNB, LC2) light chain of rabbit fast muscle myosin and LC1 (an A1-type light chain) from bovine cardiac ventricular muscle both contain the characteristic resonance at 3.23 ppm, suggesting that this unusual amino acid may be found in many of the myosin light chains.

It should be noted that trimethylalanine is contained within a highly mobile region of S1(A1) which forms part of the actin binding site [4]. Analogies exist between this region of the A1 light chain and certain DNA binding proteins. In the cases cited below, the isolated proteins contain a structured central core with 1 (or 2) highly basic terminal regions containing large amounts of proline and alanine. These terminal regions show little tertiary structure in the isolated proteins, as judged by NMR spectroscopy (e.g., see [19]), but are immobilized to some extent in the presence of DNA [20]. Examples include H1 and H1-type histones [19–21] and certain viral capsid proteins [22]. The association of unstructured, mobile charged regions with negatively charged (at physiological pH) fibrous structures such as F-actin and DNA presents some interesting parallels. It is tempting to speculate that these represent repeated independent exploitation of a successful evolutionary binding strategy.

Myosin is already a classic example of a methylated protein, containing 2 mol 3-methylhistidine [23], 2 mol ε-monomethyllysine and 4 mol ε-trimethyllysine/mol [6]. All these modifications occur on the heavy chain. The addition of trimethylalanine to the list may represent the most significant modification to date in that its participation in the actomyosin interaction represents by far the best example, as far as we are aware, of a precise structural role for any methylated amino acid in eukaryotic proteins. Its characteristic NMR signal, which is clearly visible in ^{1}H-NMR spectra of both S1(A1) and the parent myosin molecule should serve as a useful probe for actomyosin interaction in the future.

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