J. Mol. Biol. (1985) 186, 381–391

Novel Thick Filament Protein of Chicken Pectoralis Muscle: the 86 kd Protein

I. Purification and Characterization

Martin Bähler†, Hans M. Eppenberger‡ and Theo Wallimann

Institut für Zellbiologie, Eidgenössische Technische Hochschule ETH-Hönggerberg, CH-8093 Zürich, Switzerland

(Received 14 February 1985, and in revised form 12 July 1985)

A new thick-filament-associated protein, the 86 kd protein, of chicken pectoralis major muscle was isolated from a crude C-protein preparation by a method similar to that used to purify H-protein from rabbit skeletal muscle. However, the protein with an apparent M_r of 86,000 and 370,000 as estimated by gel electrophoresis and gel permeation, respectively, is not related to C-protein and differs from rabbit H-protein by its elution behaviour from hydroxyapatite columns, by its molecular weight, ultraviolet light spectrum, amino acid composition and localization, and by its amount present in myofibrils. The amino acid composition reveals a high content of proline and gel permeation indicates an either highly asymmetric or polymeric structure of the molecule.

Antibodies raised in rabbits against the 86 kd protein were demonstrated by double immunodiffusion and immunoblotting experiments to be specific for this protein. They show no cross-reactivity with any other myofibrillar protein of chicken pectoralis muscle, e.g. myosin, M-band proteins, titin or C-protein, nor did they exhibit a significant cross-reactivity with H-protein from rabbit. The 86 kd protein, which has been purified also by antibody affinity chromatography from a freshly prepared Guba-Straub extract of washed myofibrils, is a specific myofibrillar component located within each half of the A-band.

1. Introduction

Myofibrils, the contractile elements of skeletal muscle, contract and relax through the sliding of thin and thick filaments past each other. Thick filaments are composed mainly of myosin molecules but, in addition, contain several associated minor protein components other than myosin (Starr & Offer, 1971). In electron micrographs of isolated A-bands, 11 prominent transverse stripes are revealed in each half of the A-band (Draper & Hodge, 1949; Huxley, 1967; Hanson *et al.*, 1971; Craig, 1977), also indicating the presence of additional components.

A powerful approach to the identification and isolation of non-myosin components of the thick filament was based on the supposition that these proteins bind to myosin. This idea led Starr & Offer (1971) to examine, by gel electrophoresis, myosin preparations for persistent contaminants. Indeed, these authors recognized a number of protein bands other than myosin, which they labelled alphabetically according to their electrophoretic mobility. Among these, C-protein and H-protein have been isolated (Offer *et al.*, 1973; Offer, 1972; Starr & Offer, 1982, 1983; Yamamoto, 1984) and shown to be components of thick filaments. C-protein has been characterized in detail and was shown to bind to myosin rod, light meromyosin (Moos *et al.*, 1975) and subfragment-2 (Starr & Offer, 1978) as well as to actin (Moos *et al.*, 1978). The binding of C-protein to thin filaments is calcium-dependent and is inhibited by EGTA (Moos, 1981). Furthermore, it has been demonstrated by immunological methods that C-protein accounts for seven or eight of the 11 transverse stripes (stripes 4 or 5 to stripe 11 counting from the middle to the ends of the thick filament) in each half A-band (Offer, 1972; Pepe & Drucker, 1975; Craig & Offer, 1976; Dennis *et al.*, 1984). The other protein isolated, H-protein, has been localized in stripe 3 (Craig & Megerman, 1979; Starr & Offer, 1983).

† Present address: Laboratory of Molecular and Cellular Neuroscience, Rockefeller University, New York, NY 10021-6399, U.S.A.

‡ Author to whom all correspondence should be addressed.

0022-2836/85/220381-11 $03.00/0

In order to resolve the structure and function of the thick filaments, a detailed knowledge about their protein composition is needed. Since there are still some unidentified thick filament components, including M-band proteins (see Strehler *et al.*, 1983), we started to screen crude C-protein preparations of chicken pectoralis major for such additional proteins. Here, we report on the purification, biochemical and immunological characterization of a M_r 86,000 protein from chicken pectoralis muscle. It was purified by techniques virtually identical to those used for H-protein from rabbit skeletal muscle. Nevertheless, it differs by several criteria from H-protein and it is not related to C-protein.

2. Materials and Methods

(a) *Purification of the 86 kd protein*

Crude myosin was prepared from chicken pectoralis major muscle by extraction with Guba–Straub buffer, precipitation with low salt and fractionation with ammonium sulphate, starting with 400 to 500 g of muscle, according to Offer *et al.* (1973). Chromatography on DEAE-Sephadex A-50 yielded crude C-protein in the breakthrough fractions. Myosin, which remains bound to the column, was eluted by a salt gradient, precipitated at low ionic strength and stored at 4°C at a protein concentration of 5 to 10 mg/ml in 40% (w/v) ammonium sulphate, 5 mM-sodium phosphate (pH 7·0), 3 mM-$MgCl_2$, 0·1 mM-EDTA, 0·1 to 0·5 mM-2-mercaptoethanol, 1 to 2·5 mM-ATP (Wallimann & Szent-Györgyi, 1981). Crude C-protein was concentrated by precipitation with ammonium sulphate (55%) and redissolved in 0·3 M-KCl, 10 mM-potassium phosphate (pH 7·0), 1 mM-NaN_3. After removal of residual ammonium sulphate by dialysis, the sample (20 ml at a concentration of approx. 10 mg/ml) was applied to a hydroxyapatite (BRL) column (30 cm × 2·5 cm). Proteins were eluted by a linear phosphate gradient (10 mM to 300 mM-potassium phosphate (pH 7·0), 0·3 M-KCl, 1 mM-NaN_3; with a total volume of 2 l) collecting 3-ml fractions. This scheme essentially followed the outline for the purification of rabbit C, H and X-proteins described by Starr & Offer (1982, 1983). Fractions containing the 86 kd protein were pooled and dialysed against buffer A (50 mM-KCl, 1 mM-$MgCl_2$, 0·5 mM-2-mercaptoethanol, 1 mM-NaN_3, 5 mM-HEPES, pH 7·2) and subsequently concentrated by vacuum dialysis. The concentrated sample (4 ml at a concentration of 2 mg/ml) was applied to a myosin-Sepharose 4B column (10 cm × 1·5 cm). The myosin affinity column was made by coupling, according to the instructions of the manufacturer, 60 mg of DEAE-Sephadex A-50-purified chicken myosin to 6 g of CNBr-activated Sepharose 4B (Pharmacia). After loading the sample on the myosin affinity column, a linear gradient from 50 to 500 mM-KCl in buffer A (total volume of 150 ml) was applied.

(b) *Purification of C-protein*

C-protein from chicken pectoralis muscle was purified essentially as described by Starr & Offer (1982). Since, after chromatography on hydroxyapatite, chicken C-protein was not as pure as rabbit C-protein, 2 additional purification steps were included. In order to remove residual phosphorylase b, the preparation containing C-protein was passed over 5'-AMP-Sepharose (Pharmacia). Final purification of C-protein was achieved by passing the preparation over a myosin-Sepharose 4B affinity column as described for the 86 kd protein.

(c) *Ultraviolet spectrum of the 86 kd protein*

The ultraviolet spectrum of the 86 kd protein was obtained using a Beckman DB-GT spectrophotometer connected to a W+W recorder 1100.

(d) *Amino acid analysis*

Purified 86 kd protein, after exhaustive dialysis against water and lyophilization, was hydrolyzed in 6 M-HCl in an evacuated tube at 110°C for 24 h. The hydrolysate was analysed with an LC 6000 E Biotronik amino acid analyser.

(e) *Gel filtration*

For gel filtration experiments, a Sephacryl S-300 column (85 cm × 2·2 cm) equilibrated with 0·3 M-KCl, 1 mM-$MgCl_2$, 1 mM-EDTA, 0·5 mM-2-mercaptoethanol, 1 mM-NaN_3, 20 mM-HEPES (pH 7·2) was used.

(f) *Preparation of antibodies*

Preparative 9% (w/v) polyacrylamide slab gels (containing 0·1% (w/v) SDS†), of the pooled fractions (pool I or pool II) eluted from the hydroxyapatite column were run for an additional 2 h after the front marker left the gel. This improved the separation of the 86 kd protein from its stable breakdown product of M_r 79,000 sufficiently well that the 2 protein bands could be cut out separately from the gel. Gel stripes containing approximately 100 μg of each polypeptide were homogenized separately with Freunds adjuvant by passing the gel back and forth between 2 syringes connected by a 12-gauge needle. In addition, purified native 86 kd protein and C-protein (100 to 200 μg) were used for immunizations. Complete and incomplete Freund adjuvant was used for the first immunization and for the subsequent boost of rabbits at intervals of 2 weeks, respectively. Emulsified native proteins were injected intracutaneously and homogenized gel pieces of denatured proteins were injected subcutaneously, both at multiple sites. Collection of antisera was started 2 weeks after the last injection and the sera were stored at −20°C. Antibodies prepared against the denatured 86,000 and 79,000 M_r polypeptide bands cut out separately from SDS/polyacrylamide gels are referred to as α-d86 and α-d79 antibodies, respectively (d standing for denatured). Antibodies against the native 86 kd protein are referred to as α-n86 antibodies (n standing for native).

(g) *Double immunodiffusion*

Ouchterlony tests were carried out in 1·5% agar Noble dissolved in phosphate-buffered saline supplemented with 0·4 M-NaCl. The plates were stained with 0·5% (w/v) Amino black dissolved in 50% methanol, 10% acetic acid for 30 min and destained in 10% methanol, 10% acetic acid.

† Abbreviations used: SDS, sodium dodecyl sulphate; 86 kd protein, etc., a protein of M_r 86,000, etc.; PFKase, phosphofructokinase.

(h) *Electrophoresis*

SDS/polyacrylamide slab gel electrophoresis was performed according to Laemmli (1970). For rapid checking of the protein composition of column eluates, a microslab gel system described by Matsudaira & Burgess (1978) was used.

(i) *Immunoblotting*

Immunoblotting was done according to Towbin *et al.* (1979). Electrophoretic transfer onto nitrocellulose paper was performed overnight at 5 W. The nitrocellulose was air-dried, blocked with 10% (v/v) horse serum in Dulbecco's PBS and then incubated with antibodies. The protein bands recognized by the antibodies were either visualized by fluorescein-conjugated second antibody (Cappel Laboratories) or by [^{125}I]Protein A (2000 cts/min per 10 μl). The latter was obtained from New England Nuclear.

(j) *Affinity purification of antibodies against the 86 kd protein*

Crude C-protein (55 mg) was coupled to CNBr-activated Sepharose 4B according to the instructions of the manufacturer and subsequently packed into a column (14 cm × 1·5 cm). An antiserum against the 86 kd protein (α-n86), which was checked first for its specificity, was passed over the column. Bound antibodies were eluted by 1 M-propionic acid into tubes filled with an equal volume of 1 M-Tris·HCl (pH 10·0) and rapidly dialysed. The affinity purified antibodies (10 mg a.p. α-86) obtained by this method were checked again for their specificity by immunoblotting with washed myofibrils and crude C-protein preparations and were then coupled to CNBr-activated Sepharose 4B.

(k) *Affinity purification of the 86 kd protein*

Chicken pectoralis major myofibrils prepared as described (Wallimann *et al.*, 1977) were extracted for 5 to 15 min with Guba–Straub solution (0·3 M-KCl, 0·1 M-KH_2PO_4, 0·05 M-K_2HPO_4). After centrifugation, the extract was applied to the anti-86 kd antibody-Sepharose column. The column was washed with high-salt buffer (0·6 M-KCl, 0·15 M-potassium phosphate (pH 6·2), 2 mM-$MgCl_2$, 1 mM-2-mercaptoethanol, 5 mM-EDTA) followed by the same buffer at pH 8·0. Elution of bound protein was achieved with 3 M-KI. In an alternative experiment, the Guba-Straub extract was heat-denatured for 10 min at 93°C, left at room temperature for an additional 10 min and then centrifuged at 26,000 *g* at 0 to 4°C. The heat-denatured extract was concentrated by vacuum dialysis and passed over the antibody affinity column. Before elution of bound protein with 3 M-KI, the column was washed with 0·7 M-KCl, 1 mM-$MgCl_2$, 1 mM-2-mercaptoethanol, 1 mM-EDTA, 20 mM-HEPES (pH 7·0).

3. Results

(a) *Purification of the 86 kd protein*

The DEAE Sephadex A-50 column breakthrough from a crude chicken pectoralis myosin preparation, designated crude C-protein, contained, besides a number of minor bands, three major protein bands, C-protein, the 86 kd protein and a M_r 34,000 protein (Fig. 1(b)). Unlike what is found in crude C-protein preparations from rabbit muscle (Offer *et al.*, 1973), the amounts of the other proteins, e.g. the 86 kd and 34 kd proteins were similar to that of C-protein. Protein bands corresponding to C-protein as well as to 86 kd protein were present in washed

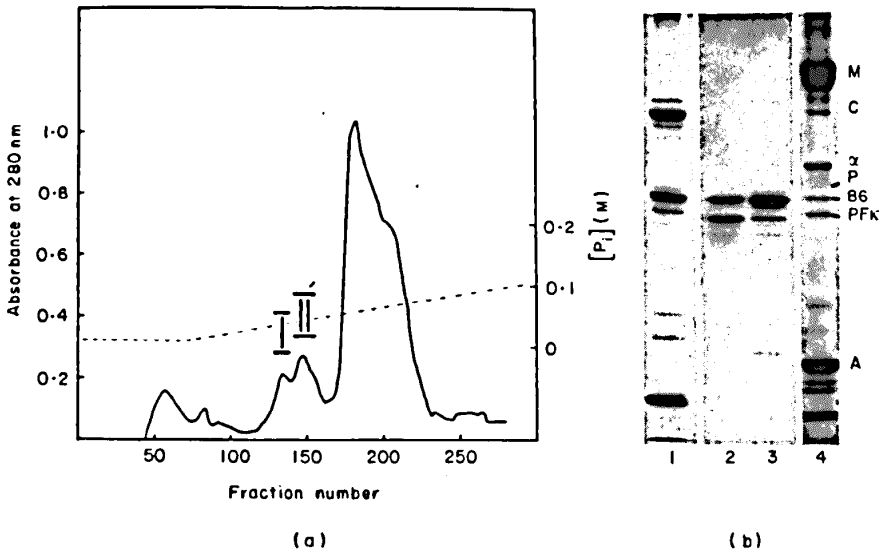

Figure 1. Hydroxyapatite column chromatography. (a) Elution profile from a hydroxyapatite column of crude C-protein that was obtained from the DEAE-Sephadex breakthrough of crude myosin: 200 mg of crude chicken C-protein was applied to the hydroxyapatite column (30 cm × 2·5 cm) and eluted by a linear phosphate gradient (10 to 300 mM-potassium phosphate). (b) SDS/polyacrylamide gel electrophoresis of crude C-protein (lane 1); pooled fractions of peak I (pool I: lane 2); and pooled fractions of peak II (pool II: lane 3). Lane 4, chicken pectoralis major myofibrils. M, Myosin heavy chain; C, C-protein; α, α-actinin; P, phosphorylase b; 86, 86 kd protein; PFk, phosphofructokinase; A, actin.

myofibrils from chicken pectoralis muscle (Fig. 1(b), lane 4), indicating that both are of myofibrillar origin. The elution profile of a crude C-protein preparation from the hydroxyapatite column is shown in Figure 1. The unretarded material eluted first was composed of low molecular weight components. Ahead of the main peak, which was composed mainly of C-protein, a double peak was eluted. The two peaks containing 86 kd protein plus significant amounts of 79 kd protein (pool I) (Fig. 1(b), lane 2) and 86 kd protein plus lesser amounts of the 79 kd protein (pool II; Fig. 1(b), lane 3) were pooled separately. The amount of 79 kd protein relative to that of 86 kd protein present in crude C-protein preparations (Fig. 1(b), lane 1) seemed to increase during chromatography on hydroxyapatite. The 34 kd component was eluted later (now shown). Final purification of 86 kd protein was achieved by passing pool II over a myosin Sepharose-4B affinity column. The elution profile and composition of eluted fractions are shown in Figure 2. First, some minor contaminants were eluted (lane 1), followed by the slightly retarded 79 kd protein (lane 2) and by the 86 kd protein, which eluted in almost pure form in a trailing peak (lanes 4 to 6). Although it was difficult to purify the 86 kd protein without a trace amount of the 79 kd protein, which was later shown by immunological methods to be a stable degradation product, one possibility of separating the 79 kd degradation product from its original 86 kd species was to take advantage of the lower affinity of the 79 kd species for myosin as compared to 86 kd protein. However, even though the 86 kd protein showed myosin binding activity by being significantly retarded on a myosin affinity column, its binding was less than that of C-protein which, under the same conditions, eluted after the 86 kd protein, but also in a long, even more trailing peak (not shown).

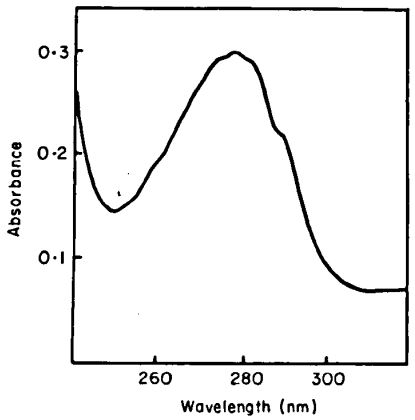

Figure 3. Ultraviolet absorption spectrum of 86 kd protein. The absorption spectrum of the protein was measured at a concentration of 0·5 mg of purified 86 kd protein dissolved in 150 mM-KCl, 2 mM-EDTA, 0·1 mM-2-mercaptoethanol, 1 mM-NaN_3 20 mM-3-(*N*-morpholino) propanesulfonic acid (pH 7·2) with the same buffer as a reference. Protein concentration was measured by the procedure of Lowry *et al.* (1951) using bovine serum albumin as a standard, and the absorption coefficient for 86 kd protein ($E^{1\%}_{280} = 6$) determined accordingly.

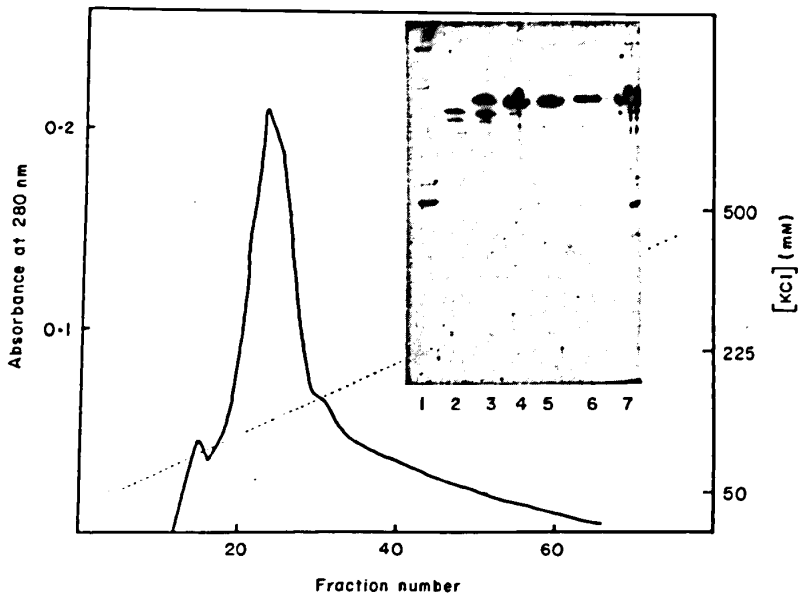

Figure 2. Myosin–Sepharose affinity chromatography. Elution profile from a myosin-Sepharose 4B affinity column of pool II material that was obtained by hydroxyapatite chromatography (see Fig. 1).

Inset: Microslab SDS/polyacrylamide gel electrophoresis of fractions eluted by a linear salt gradient (50 to 500 mM-KCl). Lanes 1 to 6 correspond to fractions 15, 18, 21, 24, 27 and 30. Lane 7 represents pool II material before application to the myosin affinity column.

Experiments with other affinity matrixes showed some noteworthy behaviour of C-protein and 86 kd protein in that they both were retained on 5'-AMP-Sepharose 4B columns in the presence of 50 mM-NaCl but were both readily eluted under the same conditions by increasing the salt concentration to 0·5 M-NaCl. However, neither of the two proteins was eluted from this matrix by AMP or NADH added to the low salt buffer. In addition, both proteins bound under low salt conditions to reactive red-120-agarose (Sigma), which was reported to be useful for the isolation of nucleotide-requiring enzymes and were both eluted with 0·5 M-salt (not shown here).

(b) *Ultraviolet spectrum*

The spectrum of 86 kd protein has a peak at 278 nm with two symmetrical shoulders relative to the peak wavelength at 275 nm and 281 nm (Fig. 3). The spectrum exhibits also a pronounced shoulder at 290 nm, suggesting a relatively high content of tryptophan. The protein concentration was measured by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard, and an extinction coefficient of $E^{1\%}_{280} = 6$ was determined for 86 kd protein.

(c) *Molecular weight*

An apparent M_r of 86,000 was estimated for the protein by comparing its electrophoretic mobility on 7·5% polyacrylamide gels in the presence of 0·1% SDS to that of standards with known M_r values (Fig. 4). Gel permeation experiments (Fig. 5), however, yielded a much higher apparent M_r of approximately 370,000; that is, the 86 kd protein

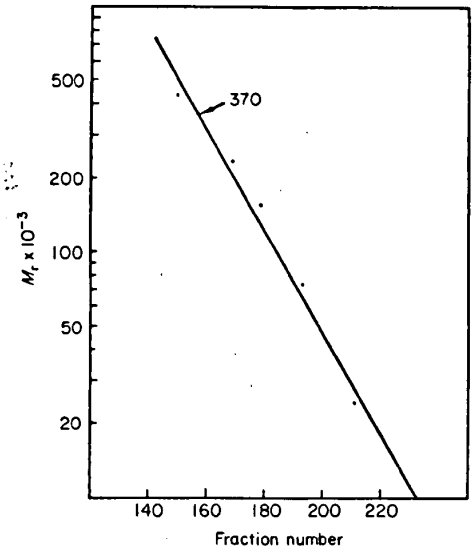

Figure 5. Determination of the molecular weight by gel filtration. Purified 86 kd protein was passed over a Sephacryl S-300 column (85 cm × 2·2 cm) pre-equilibrated in 0·3 M-KCl, 1 mM-$MgCl_2$, 1 mM-EDTA, 0·5 mM-2-mercaptoethanol, 1 mM-NaN_3, 20 mM-HEPES (pH 7·2). The column was calibrated with the following molecular weight standards: Ferritin (440,000), pyruvate kinase (237,000), aldolase (158,000), transferrin (74,000) and α-chymotrypsinogen (24,500). The arrow points to the peak fraction of the 86 kd protein, which corresponds to a molecular weight of 370,000.

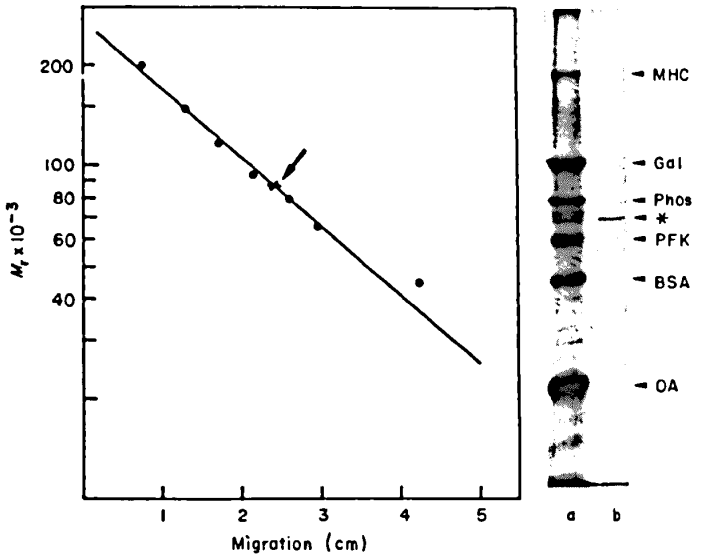

Figure 4. Determination of the apparent molecular weight by SDS/polyacrylamide gel electrophoresis. The 86 kd protein (*) was run (b) alone or (a) was premixed with molecular weight standards on an SDS/7·5% polyacrylamide slab gel. Myosin heavy chain (MHC, 210,000), C-protein (C, 140,000), β-galactosidase (Gal, 116,000), phosphorylase b (Phos, 94,000), phosphofructokinase (PFK, 80,000), bovine serum albumin (BSA, 66,000) and ovalbumin (OA, 45,000) were taken as M_r standards. The graph shows a semi-logarithmic plot of the molecular weights of these proteins *versus* electrophoretic mobility and indicates an apparent molecular weight of the purified thick filament protein of 86,000 (arrow).

was eluted slightly behind C-protein, which runs under these conditions with an apparent M_r of 400,000. These data suggest either a highly asymmetric or a polymeric structure for 86 kd protein.

(d) *Amino acid composition*

In Table 1 the amino acid composition of chicken 86 kd protein is compared with that of myosin and myosin-associated proteins isolated from rabbit muscle. Myosin-associated proteins, especially 86 kd protein and rabbit H-protein, are rich in proline. 86 kd protein has a somewhat lower serine and leucine and higher lysine content than rabbit H-protein. Compared to rabbit C-protein, 86 kd protein has more alanine and proline and less valine. The amount of several amino acids present in rabbit X-protein, rabbit red C-protein and rabbit myosin differs considerably when compared with chicken 86 kd protein.

(e) *Immunological characterization*

Ouchterlony double immunodiffusion plates with anti-86 kd serum (α-n86) placed in the central well and serially diluted crude C-protein, still containing 86 kd protein, in the outer wells exhibited a single precipitin line (Fig. 6(a)). However, when purified C-protein instead of crude C-protein was placed in the outer wells, no precipitin line was formed (Fig. 6(b)). If antiserum to 86 kd protein (α-n86) was placed in the middle well and purified C-protein alternating with 86 kd protein was placed in the outer wells, only the homologous arrangement formed a precipitin line (Fig. 6(c)), indicating that anti-86 kd protein antibody does not cross-react with C-protein. Alternatively, if crude C-protein was placed in the central well and antisera against C-protein and against 86 kd protein were alternated in the outer wells, single crossing precipitin lines were formed (Fig. 6(d)). A complete crossing-over with no fusion of precipitin lines was formed also by a square arrangement of C-protein opposite to anti-C-protein antiserum and 86 kd protein opposite to anti-86 kd protein antiserum (α-n86), both tests indicating immunological non-identity of the two proteins.

The antibodies against 86 kd protein (α-n86; α-d86; a.p. α-86) were shown by immunoblotting to recognize only one band of M_r 86,000 in the particulate fraction of chicken breast muscle and in washed chicken pectoralis major myofibrils (Fig. 7). Hence, 86 kd protein is not a soluble sarcoplasmic protein but is of myofibrillar origin. Furthermore, the 86 kd protein is not a degradation product of a higher molecular weight protein such as C-protein, nebulin, titin, M-band proteins or myosin. No antibody cross-reaction with any other antigen, e.g. AMP deaminase, phosphofructokinase, C-protein or rabbit H-protein was detected. Antibodies prepared against the 86 kd and against the 79 kd polypeptide (α-d79) bands, which were cut out separately from gels, were shown by immunoblotting both to recognize the 86 kd as well as the 79 kd protein, the latter being the most abundant contaminant during purification of 86 kd protein (Fig. 7, lanes 3′ and 4′). Due to its lower binding affinity for myosin, most of the 79 kd polypeptide could be removed by a myosin-affinity column (Fig. 2). However, with freshly prepared myofibrils, both antibodies recognized on immunoblots only one band with an

Table 1

Amino acid composition of chicken 86 kd protein compared with myosin and myosin-associated proteins from rabbit

Amino acid	86 kd protein	H-protein†	C-protein†	X-protein†	Red C-protein‡	Myosin§
Asp	72	60	83	86	96	85
Thr	65	56	51	50	62	44
Ser	58	77	49	44	52	39
Glu	111	108	101	95	107	157
Gly	54	63	61	55	63	40
Ala	107	101	57	65	72	78
Val	61	60	90	63	61	43
Met	12	8	15	18	16	23
Ile	37	40	44	57	55	42
Leu	51	68	60	57	58	81
Tyr	22	18	25	33	32	20
Phe	23	25	32	27	28	29
Lys	75	52	74	96	83	92
His	23	16	12	15	14	16
Arg	43	45	38	40	43	43
Pro	110	91	61	60	63	22
Cys	–	9	13	11	–	–
Trp	–	19	19	16	–	–

All values are given as no. residues per 10^5 g.
† Starr & Offer (1983).
‡ Callaway & Bechtel (1981).
§ Lowey & Cohen (1962).

apparent M_r of 86,000. We conclude, therefore, that the 79 kd protein copurified with the 86 kd protein is a stable degradation product of the latter. This is supported by the fact that more 79 kd protein is formed during purification, especially on hydroxyapatite, as well as on ageing or prolonged storage of 86 kd preparations.

In conclusion, immunodiffusion tests as well as immunoblots prove the specificity of the antiserum against 86 kd protein and demonstrate clearly that the 86 kd protein and C-protein represent two immunologically distinct proteins. In addition, the 86 kd protein seems to be a specific marker for striated muscle, since no cross-reacting antigen was detected by immunoblotting in chicken gizzard, brain, liver or kidney (not shown).

(f) *Immunoblots with rabbit psoas myofibrils*

Since the purification procedure used here for the 86 kd protein from chicken is virtually identical to that for H-protein from rabbit muscle, immunoblotting experiments were performed with rabbit psoas myofibrils, as well as with a Guba–Straub extract of these myofibrils. However, no significant cross-reaction of all the antibodies directed against chicken 86 kd protein with H-protein of M_r 69,000 or with any other protein of rabbit psoas myofibrils was detected (Fig. 8). In contrast, by the same technique, antibodies against chicken C-protein cross-reacted to a low extent with rabbit C-protein, which was found to migrate slightly faster than chicken C-protein on gel electrophoresis (Fig. 8).

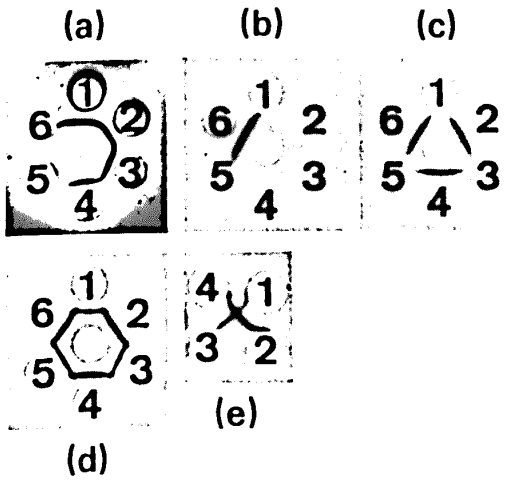

Figure 6. Double-immunodiffusion. Centre wells in Ouchterlony plates (a), (b) and (c) all contained antiserum against 86 kd protein. Peripheral wells in plate (a): crude C-protein at 1·5 mg/ml, which also contained 86 kd protein (well 1), followed by 1 : 2 serial dilutions in wells 2 to 6. Peripheral wells in plate (b) contained purified C-protein without 86 kd protein at 0·3 mg/ml (well 1) and 1 : 2 serial dilution thereof in wells 2 to 5, followed by well 6 containing crude C-protein at 0·5 mg/ml. Peripheral wells in plate (c) contained purified C-protein at 0·3 mg/ml in wells 1, 3 and 5 alternating with purified 86 kd protein at 0·25 mg/ml in wells 2, 4 and 6). Note the absence of cross-reactivity of anti-86 kd protein antibody with purified C-protein. The centre well in plate (d) contained crude C-protein. In the peripheral wells were placed anti-C-protein antiserum (wells 1, 3 and 5) alternating with anti-86 kd protein antiserum in wells 2, 4 and 6. In plate (e), 86 kd protein at 0·25 mg/ml (well 3) was placed opposite to anti-86 kd protein antiserum (well 1) and purified C-protein at 0·3 mg/ml (well 2) opposite to anti-C-protein antiserum (well 4). Note the cross-over of precipitin lines in plates (d) and (e), indicating immunological non-identity of 86 kd protein with C-protein.

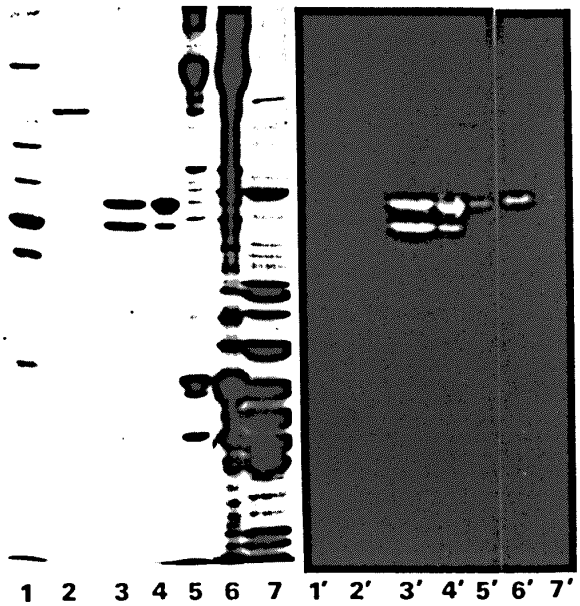

Figure 7. Immunoblot of proteins electrophoretically transferred from gels to nitrocellulose paper stained with anti-86 kd protein antibody. Lane 1, molecular weight markers: myosin heavy chain (210,000), β-galactosidase (116,000), phosphorylase b (94,000), rabbit phosphofructokinase (80,000), bovine serum albumin (66,000), and ovalbumin (45,000); lane 2, purified chicken C-protein; lane 3, pool I from the hydroxyapatite fractionation; lane 4, 86 kd protein; lane 5, chicken pectoralis major myofibrils; lane 6, particulate fraction of homogenized muscle; and lane 7, soluble fraction of homogenized muscle were run parallel on identical SDS/8% polyacrylamide gels. The gel on the left was stained for protein with Coomassie blue (lanes 1 to 7). The gel on the right (lanes 1' to 7') was transferred to nitrocellulose paper and stained by indirect immunofluorescence with anti-86 kd protein antibodies. Note the presence of only a single band at the 86 kd position in the myofibrillar (lane 5'); and the particulate fraction (lane 6').

(g) *Extract of myofibrils according to Guba–Straub from chicken pectoralis major*

The main proteins extracted by Guba-Straub solution from chicken myofibrils (Fig. 9(a), lane 7) were myosin (M), the two M-band proteins of

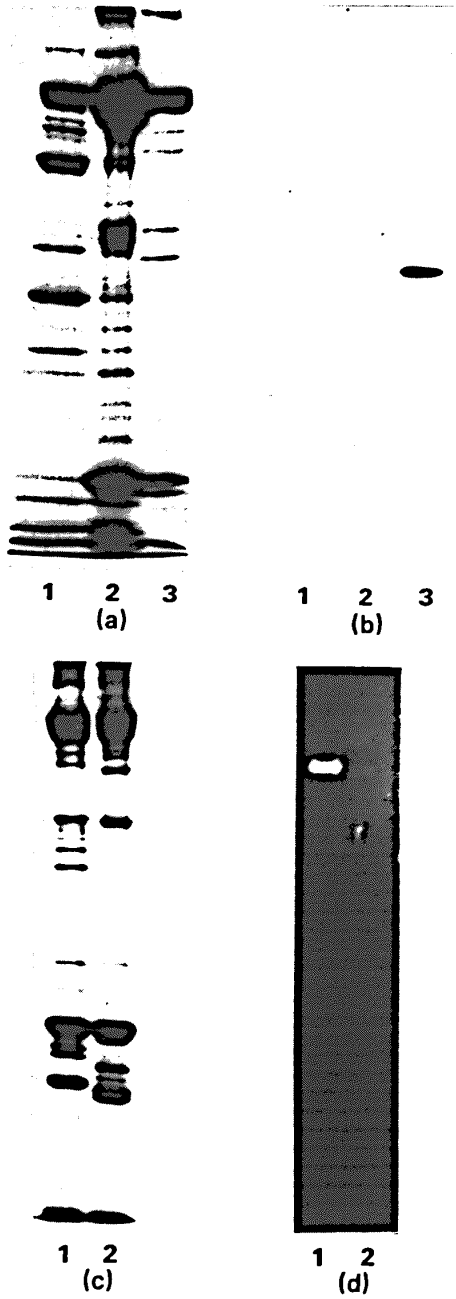

Figure 8. Cross-reactivity of antibodies directed against chicken pectoralis 86 kd protein and against chicken C-protein with rabbit myofibrillar proteins on immunoblots. (a) and (b) Parallel SDS/7·5% polyacrylamide gels of a Guba-Straub extract of the rabbit myofibrils (50 μg; lane 1), rabbit myofibrils (60 μg; lane 2) and chicken pectoralis major myofibrils (20 μg; lane 3) were run. (a) Proteins were stained with Coomassie blue or (b) transferred electrophoretically to nitrocellulose paper for reaction with anti-chicken 86 kd protein antibodies followed by [^{125}I]Protein A. (c) and (d) Parallel SDS/8% polyacrylamide gels of chicken pectoralis major (lane 1) and rabbit psoas myofibrils (lane 2) were run. They were either (c) stained with Coomassie blue or (d) transferred to nitrocellulose paper and reacted with anti-chicken C-protein antibodies followed by fluorescein-conjugated second antibodies.

M_r 165,000 and 185,000 (seen here as one band below the myosin heavy chain (M)), C-protein (C), phosphorylase b (heavy band above the asterisk) and, only barely separated from phosphorylase b, the 86 kd protein (indicated by an asterisk), which can be seen more clearly on washed myofibrils (Fig. 9(a), lane 6). In addition, besides some actin (A), M-line bound MM-creatine kinase (just below actin) and tropomyosin (T), considerable amounts of phosphofructokinase (P) were extracted. Phosphorylase b (apparent M_r 94,000) and phosphofructokinase (PFK, apparent M_r 80,000) were identified by immunoblotting with the respective antibodies (Heizmann & Eppenberger, 1978; M. Bähler, unpublished results). Phosphorylase differs from 86 kd protein by its apparent M_r value (Fig. 9(a), lanes 6 and 7) and by a number of biochemical criteria, e.g. the protein band corresponding to phosphorylase, but not the 86 kd protein, could be removed specifically by 5′-AMP-Sepharose. These data, together with the difference in the localization of the two proteins, since phosphorylase was localized in the I-band (Heizmann & Eppenberger, 1978) whereas 86 kd protein was localized in the A-band (Bähler *et al.*, following paper), indicate that 86 kd protein is not related to phosphorylase.

(h) *Affinity purification of 86 kd protein*

A Guba-Straub extract of washed chicken pectoralis myofibrils that was obtained within two to four hours of killing the chicken and that contained essentially all of the known thick-filament-associated proteins including 86 kd protein (Fig. 9(a), lane 7; 9(b), lane 2) was applied to an anti-86 kd protein antibody (a.p. α-86) affinity column. After washing with high-salt buffer, the first fractions of the 3 M-KI eluate contained, besides the 86 kd protein, significant amounts of precipitated phosphofructokinase (Fig. 9(a), lane 2). The latter was identified by immunoblots with anti-chicken PFKase and by its solubility characteristics. It aggregated at physiological or lower than physiological ionic strength and could be redissolved only by the addition of ATP or fructose-6-P (Parmeggiani *et al.*, 1966; Massey & Deal, 1973). In contrast to PFKase, 86 kd protein, which was eluted later, was perfectly soluble under low-salt conditions or even in pure distilled water. Since PFKase has been localized in the I-band (Sigel & Pette, 1969) and anti-86 kd antibodies are shown to label, within the A-band, a specific region of thick filaments (see Bähler *et al.*, 1985. the accompanying paper), the binding of PFKase to the antibody column was considered to be unspecific, especially as PFKase is known to be a "sticky" protein (Bronstein & Knull, 1981). This notion is supported by the fact that in the subsequent fractions rather pure 86 kd protein was eluted. Most importantly, no C-protein or any peptide comparable to rabbit H-protein was retained by the anti-86 kd protein antibody (Fig. 9(a), lanes 2 to 5). If the

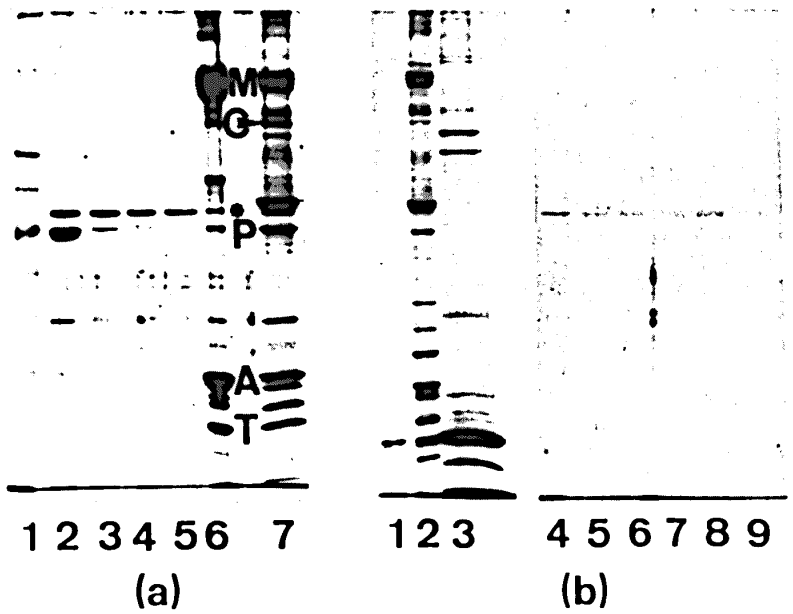

Figure 9. Affinity purification of 86 kd protein by antibody. (a) SDS/polyacrylamide gel electrophoresis of fractions eluted by 3 M-KI from an anti-86 kd protein antibody-affinity column (lanes 2 to 5 corresponding to fractions 5 to 8) after passing a Guba-Straub extract (lane 7) obtained from washed chicken pectoralis myofibrils (lane 6) over the column. Molecular weight markers in lane 1 are: myosin heavy chain (210,000), β-galactosidase (116,000), phosphorylase b (94,000), rabbit phosphofructokinase (80,000), bovine serum albumin (66,000) and ovalbumin (45,000). M, myosin heavy chain; C, C-protein; *, 86 kd protein; P, phosphofructokinase; A, actin; T, tropomyosin. (b) SDS/polyacrylamide gel electrophoresis of fractions eluted from the anti 86 kd protein affinity column by 3 M-KI (lanes 4 to 9) after passing the concentrated supernatant of a heat-denatured Guba-Straub extract (lane 3) over the antibody column. Original extract and supernatant after heat-denaturation are shown in lanes 2 and 1, respectively.

concentrated supernatant of a heat-denatured Guba-Straub extract (Fig. 9(b), lane 3) was applied to the anti-86 kd affinity column, essentially pure 86 kd polypeptide was eluted by 3 M-KI (Fig. 9(b), lanes 5 to 9). The fraction eluted towards the end of the peak showed a striking doublet on polyacrylamide gels (Fig. 9(b), lanes 7 to 9), which was observed sometimes during conventional purification, indicating a possible modification of the protein. Except for tropomyosin, which is known to be very heat-stable (Bailey, 1948), most of the other proteins present in the original Guba–Straub extract (Fig. 9(b), lane 2) were denatured and subsequently removed by centrifugation. However, some of the 86 kd protein resisted the treatment, indicating a certain degree of heat-stability of the 86 kd protein.

(i) *Quantitation of 86 kd protein in myofibrils*

Proteins of well-washed myofibrils were separated on SDS/polyacrylamide gels (Fig. 1(b), lane 4) and, after staining with Fast Green, quantitated by densitometry. The amount of 86 kd protein calculated relative to the amount of actin was estimated to be $0{\cdot}0435 \pm 0{\cdot}01$ ($n = 3$) by weight. Hence, assuming that actin comprises 22% of the myofibrillar protein mass (Yates & Greaser, 1983), the content of 86 kd protein in the myofibrils is approximately 1%. Compared to C-protein, which accounts for 2% of the myofibrillar protein (Offer *et al.*, 1973), 86 kd protein is present at a roughly equimolar ratio to C-protein.

4. Discussion

Crude C-protein preparations from rabbit muscle have proven to be a very valuable starting material for the isolation of thick-filament-associated minor proteins, like C-protein, H-protein and X-protein (Starr & Offer, 1983). Titin (M_r several million), supposedly a component within the myofibrillar sarcomere of the elastic cytoskeletal lattice, has been purified from crude C-protein preparations (Wang *et al.*, 1984). In this study, we have used crude C-protein preparations from chicken muscle as a source material. Compared to rabbit, crude C-protein preparations from chicken contains a larger number of proteins and, in addition, some of the chicken proteins seem to be different from those of rabbit.

We have purified an 86 kd protein, one of the most prominent proteins, besides C-protein, present in crude C-protein preparations from chicken pectoralis muscle. Like rabbit H-protein, this protein was eluted from the hydroxyapatite column well ahead of C-protein. Compared to H-protein, however, it elutes at a somewhat higher concentration of phosphate. Furthermore, in relation

to rabbit H-protein, which exhibits an apparent M_r of 69,000 on polyacrylamide gels (Starr & Offer, 1983) or 74,000 (Yamamoto, 1984), the molecular weight of the isolated chicken protein was estimated by the same method to be 86,000±2000. On the other hand, the amino acid composition of purified 86 kd protein showed a high proline content, as is typical for the other thick-filament-associated proteins known to date, therefore also suggesting a low α-helical content for the 86 kd protein. However, compared to H-protein, the amino acid composition of 86 kd protein differs considerably in the amount of lysine, leucine and serine. In addition, significant differences between 86 kd protein and H-protein are revealed by their different ultraviolet light spectra. The spectrum of 86 kd protein has a peak value at 278 nm and two symmetrical shoulders at 275 nm and 281 nm relative to the peak value, whereas the spectrum of H-protein has a peak value at 281 nm and only one shoulder at about 277 nm (Starr & Offer, 1983). Due to the fact that 86 kd protein has a higher proline content, it cannot be decided at the moment whether the M_r value of 370,000 obtained by gel permeation for the native protein is indicative of a highly asymmetric polypeptide chain or whether the 86 kd polypeptide exists in polymeric form.

The 86 kd protein was shown to be entirely of myofibrillar origin and was extracted from washed myofibrils together with myosin and C-protein. In addition, the purified protein displayed myosin-binding activity which, however, was weaker than that of C-protein. It is unlikely that 86 kd protein is a proteolytic fragment of other proteins such as myosin, C-protein, M-band proteins, titin or nebulin, since immunoblotting and affinity purification experiments revealed no higher molecular weight bands. In addition, the antibodies that were shown by several criteria to be specific for the 86 kd protein did not cross-react with any of the proteins mentioned above, nor did anti-C-protein cross-react with the 86 kd protein. As judged by electrophoresis of myofibrils stained for protein, the amount of 86 kd protein was similar to that of C-protein, whereas the content of H-protein in rabbit myofibrils has been reported to be five to nine times less than C-protein (Starr & Offer, 1983; Yamamoto, 1984). Even though anti-86 kd protein antibodies stained, by indirect immunofluorescence, the middle parts of each half-A-band of myofibrils, similar to anti-C and anti-H-protein antibodies, the localization of 86 kd protein is clearly different from the latter two, as is shown in the accompanying paper (Bähler *et al.*, 1985).

In conclusion, 86 kd protein represents a novel thick-filament-associated protein different from C and H-protein. Since our antibodies do not cross-react with any protein of rabbit psoas myofibrils, the question of whether there exists a corresponding protein in rabbit muscle remains to be determined. Conversely, we do not know whether a protein analogous to H-protein is present in chicken muscle. However, there is a band with an apparent M_r of 70,000, similar to H-protein, present on SDS/polyacrylamide gels of chicken myofibrils. But the identity of this band needs to be determined. We cannot rule out the possibility that the chicken analogue of the rabbit H-protein could be the 86 kd protein.

Except for myosin-binding activity (to myosin-Sepharose), which is reflected by the fact that the 86 kd protein is a persistent contaminant in myosin preparations from chicken, we have not been able to attribute a specific function to the 86 kd protein. It might be involved in thick filament assembly or act in a mechanical way, or it may have a regulatory or enzymatic function, e.g. by controlling the movement of cross-bridges. All of these functions were considered as possibilities for C, H and X-protein (Starr & Offer, 1983). Future work on this protein will include studies with regard to these hypothetical functions. However, we can confidently say that 86 kd protein differs from H-protein and is not related to C-protein, but is a genuine, new thick-filament associated protein located within the A-band of the sarcomere at very specific sites. This is described in detail in the accompanying paper (Bähler *et al.*, 1985).

We thank Mrs Hanni Moser for technical assistance and Mrs Monika Wirth for the amino acid analysis. We are especially obliged to Dr Gerald Offer for careful reading and helpful suggestions on the manuscript. We also wish to thank Mrs E. Abächerli, R. Amatore and M. Leuzinger for typing the manuscript. This work was supported by ETH training grant no. 0.330.081.30/8 to M.B. and by a Swiss National Science Foundation grant no. 3.707-8.80 given to H.M.E.

References

Bähler, M., Eppenberger, H. M. & Wallimann, T. (1985). *J. Mol. Biol.* **186**, 393–401.

Bailey, K. (1948). *Biochem. J.* **43**, 271–279.

Bronstein, W. W. & Knull, H. R. (1981). *Canad. J. Biochem.* **59**, 494–499.

Callaway, J. E. & Bechtel, P. J. (1981). *Biochem. J.* **195**, 463–469.

Craig, R. (1977). *J. Mol. Biol.* **109**, 69–81.

Craig, R. & Megerman, J. (1979). In *Motility in Cell Function, Proc. First John M. Marshall Symp. Cell Biol.* (Pepe, F. A., Sanger, J. W. & Nachmias, V. T., eds), pp. 91–102, Academic Press, New York.

Craig, R. & Offer, G. (1976). *Proc. Roy. Soc. ser. B*, **192**, 451–461.

Dennis, J. E., Shimizu, T., Reinach, F. C. & Fischman, D. A. (1984). *J. Cell Biol.* **98**, 1514–1522.

Draper, M. H. & Hodge, A. J. (1949). *Aust. J. Exp. Biol. Med. Science.* **27**, 465–503.

Hanson, J., O'Brien, E. J. & Bennet, P. M. (1971). *J. Mol. Biol.* **58**, 865–871.

Heizmann, C. W. & Eppenberger, H. M. (1978). *J. Biol. Chem.* **253**, 270–277.

Huxley, H. E. (1967). *J. Gen. Physiol.* **50** (suppl.), 71–83.

Laemmli, U. K. (1970). *Nature (London)*, **277**, 680–685.

Lowey, S. & Cohen, C. (1962). *J. Mol. Biol.* **4**, 293–308.

Lowry, O. H., Rosebrough, N. G., Farr, A. L. & Randall, R. J. (1951). *J. Biol. Chem.* **193**, 265–275.

Massey, T. H. & Deal, W. C. Jr (1973). *J. Biol. Chem.* **248**, 56–62.

Matsudaira, P. T. & Burgess, D. R. (1978). *Anal. Biochem.* **87**, 286–296.
Moos, C. (1981). *J. Cell Biol.* **90**, 25–31.
Moos, C., Offer, G., Starr, R. & Bennett, P. (1975). *J. Mol. Biol.* **97**, 1–9.
Moos, C., Mason, C., Bestermann, J., Feng, I. N. & Dubin, J. (1978). *J. Mol. Biol.* **124**, 571–586.
Offer, G. (1972). *Cold Spring Harbor Symp. Quant. Biol.* **37**, 87–96.
Offer, G., Moos, C. & Starr, R. (1973). *J. Mol. Biol.* **74**, 653–676.
Parmeggiani, A., Luft, J. H., Love, D. S. & Krebs, E. G. (1966). *J. Biol. Chem.* **241**, 4625–4637.
Pepe, F. A. & Drucker, B. (1975). *J. Mol. Biol.* **99**, 609–617.
Sigel, P. & Pette, D. (1969). *J. Histochem. Cytochem.* **17**, 225–237.
Starr, R. & Offer, G. (1971). *FEBS Letters*, **15**, 40–44.
Starr, R. & Offer, G. (1978). *Biochem. J.* **171**, 813–816.
Starr, R. & Offer, G. (1982). *Methods Enzymol.* **85**, 130–138.
Starr, R. & Offer, G. (1983). *J. Mol. Biol.* **170**, 675–698.
Strehler, E. E., Carlsson, E., Eppenberger, H. M. & Thornell, L. E. (1983). *J. Mol. Biol.* **166**, 141–158.
Towbin, H., Staehelin, T. & Gordon, J. (1979). *Proc. Nat. Acad. Sci., U.S.A.* **76**, 4350–4354.
Wallimann, T. & Szent-Györgyi, A. G. (1981). *Biochemistry*, **20**, 1176–1187.
Wallimann, T., Turner, D. C. & Eppenberger, H. M. (1977). *J. Cell Biol.* **75**, 297–317.
Wang, K., Ramirez-Mitchell, R. & Palter, D. (1984). *Proc. Nat. Acad. Sci., U.S.A.* **81**, 3685–3689.
Yamamoto, K. (1984). *J. Biol. Chem.* **259**, 7163–7168.
Yates, L. D. & Greaser, M. L. (1983). *J. Mol. Biol.* **168**, 123–141.

Edited by H. E. Huxley