Isolation and Properties of a Basic Protein from Skeletal-Muscle Sarcoplasm

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1. An isolation procedure for preparing gram quantities of a previously undescribed protein from muscle is presented. 2. The protein amounts to over 2% of the sarcoplasmic proteins of pig longissimus dorsi muscle, but does not correspond to any glycolytic enzyme, nor to any of several other enzymes that have been tested for. 3. The protein is isoelectric between pH 8.5 and 9.0, has mol.wt. 34500 ± 500 and has $E_{281m\mu}^{1\%}$ 20.7. 4. The identities of nearly all the major protein bands obtained in electrophoresis on starch gel of pig longissimus dorsi muscle sarcoplasm have been established.

Much of the water-soluble protein in muscle consists of the 12 glycolytic enzymes, from phosphorylase to lactate dehydrogenase, together with creatine kinase and myoglobin. Czok & Bücher (1960) estimated that at least 70% of rabbit-muscle sarcoplasmic protein was accounted for by the known quantities of 15 enzymes, including the above; moreover, up to 40% as final yield could be obtained in crystalline forms. Nevertheless, some proteins have been isolated from the sarcoplasms of various species that have not been identified with known enzymes. Czok & Bücher (1960) obtained an unidentified nucleotide-containing protein from rabbit muscle, which co-crystallized with two of the glycolytic enzymes. Also, certain fish muscles contain large amounts of an unusual phenylalanine-rich protein (Jebson & Hamoir, 1958; Henrotte, 1960). There must be many uncharacterized proteins in muscle sarcoplasm, and it appears that some of these can amount to a considerable proportion of the total protein; any enzymic functions that these may possess would be expected to be important ones in view of the quantities present.

Electrophoresis in starch gels demonstrates the variety of the proteins present in sarcoplasmic extracts (Giles, 1962; Hartshorne & Perry, 1962; Neelin, 1963; Scopes, 1964a). The main components usually number from 15 to 20, and these can be expected to correspond to the main enzymes mentioned above. A detailed investigation of the starch-gel electrophoretic pattern of pig longissimus dorsi muscle sarcoplasm resulted in identification of nearly all the major components (Scopes, 1964b). Only two proteins, which from their intensity of

staining appeared to be present in a quantity of as much as 2% of the total, were not assigned an enzymic activity. One of these, the most basic of the major components, forms the subject of the present paper; a preliminary report on this basic protein has been presented (Scopes, 1964c).

The nomenclature system previously used for the starch-gel electrophoretic pattern of ox longissimus dorsi muscle sarcoplasm (Scopes, 1964a) divided the pattern into six areas, labelled A to F. Band F3 was indicated as a protein particularly labile in mildly acid conditions; the corresponding protein from pig muscle was rather more basic, and ran the farthest towards the cathode of all the pig proteins. Because of this protein's occurrence in the F-area of the gel in each species shown it has been called 'F-protein'. Details of the purification procedure from pig muscle and of some of the properties of F-protein are presented below; however, no physiological function of the protein has yet been ascertained.

METHODS

Starch-gel electrophoresis. An ice-cooled vertical apparatus, employing the discontinuous buffer system previously described (Scopes, 1964a), was used. The apparatus had a container attached to one side which was filled with ice-water. The water was directly in contact with the $\frac{1}{8}$ in. Perspex plate on which the starch gel had been poured. The apparatus was set up in the cold room, and a potential difference of 400 v could be applied (average 20 v/cm.) without the temperature rising much above 5° in the gel. The current commenced close to 30 mA (4.5 mA/cm.² gel), and fell to 18 mA as the borate front spread through the inner gel. Electrophoresis was continued for 3.5 hr., by which time the discontinuity had traversed 17 cm. of the 18 cm. inner gel. It was necessary to make up the latter with m-urea, which prevented deterioration at the low

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temperatures; also, its handling characteristics were improved.

Identification of protein bands. All glycolytic enzymes from phosphorylase to glyceraldehyde phosphate dehydrogenase, and also lactate dehydrogenase and some other dehydrogenases, were detected by incubation of a starch-gel strip with an NADH- or NADPH-producing assay medium, coupled to reduction of nitroblue tetrazolium in the usual fashion (Fine, Kaplan & Kuftinec, 1963). Other proteins were crystallized or highly purified, and their positions on the starch-gel pattern thus determined (Scopes, 1964b).

Estimations. Tyrosine and tryptophan in the purified protein were estimated spectrophotometrically by the method of Goodwin & Morton (1946). Tryptophan was chemically estimated by the method of Spies & Chambers (1948). Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951); routine measurements were made by reading the extinction at 280 m μ , by using $E_{280\text{m}\mu}^{1\%}$ 10.3 for the (dialysed) whole sarcoplasm and $E_{280\text{m}\mu}^{1\%}$ 20.5 for the purified F-protein.

RESULTS

Isolation of F-protein

Muscle extract. Pig longissimus dorsi muscle, preferably from a 2-3-year-old animal, was excised shortly after death and cooled on ice. The pH of the muscle before homogenization was usually in the range 6.3-6.9. In one case the pH was 5.5 1hr. after death; this muscle showed the characteristics of 'watery pork' (Ludvigsen, 1954; Bendall & Lawrie, 1964), and the yield of F-protein was low, having been mostly denatured during the rapid post-mortem pH fall.

A 2 kg. portion of muscle was chopped into small pieces and homogenized in 2 vol. of cold iso-osmotic (3%, v/v) glycerol, containing EDTA (2mM), for 1min. in a Waring Blender. The homogenate was adjusted to pH 6.5 with M-tris or N-acetic acid and then centrifuged at 15000g for 10min. at 0°. The residue was discarded and the supernatant passed through glass wool to remove fat particles. The resultant fraction I had volume 4200ml. and contained 20-23mg. of protein/ml.

First acetone fractionation. Fraction I, pH 6.5, was cooled to below 5° and 1800ml. of cold (-20°) acetone added quickly. The temperature of the

mixture rose to about 12°; it was placed in a room at -10° and left to cool overnight. In the cold room (1°) a further 1650ml. of cold acetone was added over a period of 20min., while stirring, to make the total acetone concentration 45% (v/v). Stirring was continued for a further 20min. and then the mixture centrifuged in batches (5000g for 5min. at -7°). The residue contained about half the F-protein and over 90% of the other protein of the sarcoplasm. The supernatant was made 65% (v/v) with respect to acetone by quickly adding the requisite amount of cold acetone (approx. 1700ml.) and left to stand at -10°. After 1hr. the precipitated protein had sedimented and the bulk of the slightly cloudy supernatant could be decanted. The remaining slurry was centrifuged (10000g for 5min. at -10°) and all the supernatant discarded. This precipitate was dissolved in cold cacodylate buffer, pH 6.8 (40mM-cacodylic acid-32mM-sodium hydroxide), and made to 500ml. with the buffer.

The 0-45% acetone precipitate was resuspended in 2000ml. of cold (-10°) 40% (v/v) acetone, containing cacodylic acid (20mM), and stirred vigorously for 30min. The mixture was centrifuged (5000g for 5min. at -7°) and the residue discarded. The supernatant was made 65% with respect to acetone, and the protein recovered as before and combined with the first 45-65% acetone fraction. The solution was centrifuged briefly at 0° to remove a small insoluble residue. The resultant fraction II had volume 1000ml. and contained 14mg. of protein/ml.

CM-Sephadex adsorption. Fraction II was passed through a CM-Sephadex C-50 column (2.5cm. $\times$ 10cm.), equilibrated with the cacodylate buffer, at 1°. The unadsorbed protein (fraction IIA) contained little or no F-protein and was discarded. The absorbed protein was desorbed with a single change to a borate buffer, pH 8.5 (0.2M-boric acid-30mM-sodium hydroxide), and the eluant collected until dilution lowered the total extinction at 280m μ to 10. The resultant fraction III had volume 320ml. and contained 5mg. of protein/ml.

Second acetone fractionation. The pH of fraction III was lowered to 7.0 with M-cacodylic acid, and cold acetone added to give 30% (v/v) concentra-

Table 1. *Purification of F-protein from 2 kg. of pig muscle*

Experimental details are given in the text.

Fraction	Volume (ml.)	Protein (mg.)	F-protein* (mg.)	Recovery* (%)	Purifica- tion*
I: pH 6.5 iso-osmotic glycerol extract	4200	90000	2000	100	$\times$ 1.0
II: combined 65% acetone ppts.	1000	14400	1400	70	$\times$ 4.4
III: desorbed from CM-Sephadex	320	1600	1300	65	$\times$ 36
IV: 50-62% acetone, passed through DEAE-cellulose	50	1050	1000	50	$\times$ 43

* Estimated (see the text).

tion. The solution was left to stand at -10° overnight. Further cold acetone was added slowly over a period of 20 min. to make the total acetone concentration 50% (v/v). The mixture was centrifuged (5000g for 5 min. at -7°), and the supernatant was made 62% (v/v) with respect to acetone and centrifuged at -10° . The latter precipitate was dissolved in the minimum amount of cold dilute tris buffer, pH 8.0 (2 mM-acetic acid-3 mM-tris), and this solu-

tion then passed through a small (1 cm. $\times$ 2 cm.) DEAE-cellulose column equilibrated with the same buffer. The unadsorbed protein was collected as the final preparation. The resultant fraction IV had volume 50 ml. and contained 21 mg. of protein/ml.

Fraction IV was dialysed at 0° against a pH 7.8 buffer (0.4 M-boric acid-0.15 M-tris-0.1 mM-EDTA), in which it retained its properties for several weeks.

Fig. 1. Composition of protein fractions during preparation of F-protein: starch-gel electrophoretic patterns (only major bands shown). Experimental details are given in the text. On the left are given the identities of the bands. F, 'F-protein'; LDH, lactate dehydrogenase; PPH, phosphopyruvate hydratase (enolase); PGI, phosphoglucose isomerase; PK, pyruvate kinase; PGAK, phosphoglycerate kinase; ALD, aldolase; GAPDH, glyceraldehyde phosphate dehydrogenase; PH, phosphorylase; PFK, phosphofructokinase; D, 'D-protein'; PGM, phosphoglucomutase; TPI, triose phosphate isomerase; CK, creatine kinase; PGAM, phosphoglycerate mutase; MB, myoglobin; ALB, (serum) albumin. Fraction I, iso-osmotic glycerol-EDTA extract; fraction II, combined 65% acetone precipitates; fraction IIA, fraction II after passing through CM-Sephadex column; fraction III, desorbed from CM-Sephadex column; fraction IV, final preparation.

Since electrophoretic analysis was the only means of detecting F-protein, each fraction was submitted to starch-gel electrophoresis and the relative proportions and concentrations of the protein in each fraction were estimated in a semi-quantitative fashion. Recovery could be estimated by diluting the fractions to the equivalent volume of fraction I, and comparing the intensities of the F-protein bands after electrophoresis. The summary of the purification procedure given in Table 1 rests on the assumptions that in this particular preparation the recovery was 50% and that the final fraction was 96% pure. These values indicate that the F-protein constituted 2.2% of the sarcoplasmic protein; a lower recovery assumption would raise this percentage, and vice versa.

Fig. 1 shows the starch-gel electrophoretic patterns of the individual fractions. All major bands are shown, together with their identification. 'D-protein', except for its electrophoretic mobility, behaves similarly to Czok & Bücher's (1960) unidentified nucleotide-containing protein from rabbit, since it can be isolated by the same procedure.

No satisfactory conditions for crystallization of F-protein have been found. A few thin plate-like crystals were formed in an ammoniacal ammonium sulphate suspension, but the protein tended to coagulate rather than crystallize.

Properties of F-protein

Behaviour in starch-gel electrophoresis, and purity of preparation. The protein exhibited two principal bands on the starch gel, and when freshly prepared the main one (F in Fig. 1) constituted about 90% of the total. The minor component (E) could not be separated from the major one by a variety of ion-exchange columns and other techniques. Moreover, on storage the F band became converted into component E, and after more prolonged storage (at 0° or -20°) the pattern split into several components in the E-F area. This deterioration was slowed in the presence of EDTA, but was accelerated by pH values above 8.5. Thus it appears that component E is a slightly modified form of F, perhaps a result of firm anion-binding during preparation and storage. To check that the multiplicity of bands was not due to polymerization, a well-aged preparation, which showed about five components on the starch gel, was subjected to an ultracentrifuge run. The sedimentation pattern (Fig. 2) was sufficiently symmetrical at all stages to rule out the possibility of polymerization. The sedimentation coefficient, S_{20} , calculated from this single run was 3.8s.

The position that the F-protein ran to on the gel at pH 8.5 and 5° was slightly ahead of the position that a neutral substance was carried to by electro-

Fig. 2. Sedimentation diagram of F-protein. The conditions used were: 1% protein in 0.1 M-potassium phosphate buffer, pH 7.0; sedimentation for approx. 30 min. at 52 640 rev./min.

osmosis. Thus it must have been negatively charged at this pH. But at pH 9.0 it ran behind the neutral point; also, it could be adsorbed on DEAE-cellulose at pH 9.0 but not at pH 8.5. From these observations it appears likely that the isoelectric point of F-protein is in the range 8.5-9.0.

The purity of the preparation, judged on the starch gel, was estimated to be about 95% (bands E and F combined). The protein was unstable in mildly acid conditions. This resulted in a low yield from 'watery pork' muscle (see the isolation procedure). At pH 4.50 and 30° it denatured rapidly, with first-order denaturation rate kinetics (k_1 0.14 min.⁻¹, by using the method described for the denaturation of creatine kinase; Scopes, 1965), until only 4% of the initial protein remained. This undenaturable protein corresponded to the small impurities detected on the starch gel, and so it was concluded that the preparation was 96% pure F-protein.

Ultraviolet spectrum. The protein contains an unusually high proportion of tryptophan, resulting in high absorption at about 280 m μ . The $\lambda_{\max}$ is 281 m μ , and $E_{281}^{1\%}$ is 20.7, based on the freeze-dried dry weight. This value is considerably higher than for any other sarcoplasmic protein that has been reported. The u.v. spectrum in the range 200-320 m μ is shown in Fig. 3. Tryptophan 'shoulders' at 292 and 220 m μ are evident, and clearly no nucleotide is associated with the protein; the E_{281}/E_{260} ratio is 2.0.

Tyrosine and tryptophan contents were estimated as: tyrosine, 35.0 ± 0.5 residues/mol. of mol.wt. 10^5 (spectrophotometric); tryptophan 26.3 ± 0.3 residues/mol. of mol.wt. 10^5 (spectrophotometric) and 26.1 ± 0.2 residues/mol. of mol.wt. 10^5 (chemical).

Since there is good agreement between the chemical and spectrophotometric estimates of tryptophan, errors in the latter method due to buried or interacting residues exhibiting abnormal spectra are presumed insignificant.

Fig. 3. Ultraviolet spectrum of F-protein in the ranges 200–240 m μ and 240–320 m μ . At $\lambda_{\max}$, 281 m μ , $E_{281\text{m}\mu}^{1\%}$ is 20.7; at $\lambda_{\min}$, 250 m μ , $E_{250\text{m}\mu}^{1\%}$ is 6.9.

Molecular weight. Molecular-weight determinations by osmotic pressure were carried out on solutions containing 4–16 mg. of protein/ml. by using a simple osmometer (Adair, 1961). The extrapolated mol.wt. was $36\,000 \pm 2000$. By using this value, a more accurate estimate could be made from the tyrosine/tryptophan ratio of the protein (above). The ratio of these two amino acids is exactly 4:3, with 3 tryptophan residues/mol. representing a minimum mol.wt. of $11\,500 \pm 150$. Since the osmotic-pressure measurements gave a value of 36000 the molecule must contain 9 tryptophan residues and 12 tyrosine residues, and a more accurate value for the mol.wt. is $34\,500 \pm 500$.

Additional observations. (a) Amino acid analysis. A preliminary amino acid analysis revealed no marked abnormalities. The basic residues (excluding histidine) exceeded the acidic ones by 2–3/mol.; the histidine content was rather high (6% of hydrated amino acids).

(b) Partial specific volume. The density of a 2.35% solution was measured by using a pycnometer, and the partial specific volume calculated to be 0.732.

(c) Enzymic character. The protein's non-identity with any of the glycolytic enzymes is demonstrated in Fig. 1. In addition, the protein was assayed for the following activities: malate dehydrogenase, isocitrate dehydrogenase, β -hydroxybutyrate dehydrogenase, creatine kinase, adenosine triphosphatase, trypsin and several of the glycolytic enzymes. No enzymic activity was found.

DISCUSSION

No enzymic assay was available for F-protein, and analysis was only possible by using the electrophoretic technique. Nevertheless, it was soon clear in what ways it behaved differently to the other

proteins in the sarcoplasm; its basic nature pointed the way to the main step in the purification procedure, with CM-Sephadex.

The protein amounts to over 2% of the sarcoplasmic protein in pig longissimus dorsi muscle. Similar proteins exist in ox and rabbit muscle (Scopes, 1964a), but they are present in slightly lower proportions in these species. Application of the isolation procedure described above to rabbit sarcoplasm was only partially successful, and the method would need some modification.

The possibility exists that F-protein could be an inactivated form, a sub-unit, or an isoenzyme of one of the major sarcoplasmic proteins for which no specific staining technique was available. This seems unlikely in view of its properties (in particular, the high tryptophan content), which significantly differ in detail from the main proteins that have been isolated.

Since several of the most important proteins in muscle, e.g. phosphoglucose isomerase and phosphoglycerate mutase, each amount to only about 1% of the total sarcoplasmic protein (Czok & Bücher, 1960), i.e. less than F-protein, then if the latter has any enzymic nature it may well be of fundamental importance to the normal functioning of the muscle.

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