Biochimica et Biophysica Acta, 358 (1974) 193–207

BBA 67250

ON THE ASSOCIATION OF GLYCOLYTIC COMPONENTS IN SKELETAL MUSCLE EXTRACTS

F. M. CLARKE and C. J. MASTERS

Department of Biochemistry, University of Queensland, St. Lucia, 4067 (Australia)

(Received December 27th, 1973)

SUMMARY

1. In an extension of previous studies which demonstrated the existence of an association of glycolytic components in extracts of rat skeletal muscle, the properties of this complex have been further investigated.

2. It has been shown that this association of glycolytic enzymes may also be observed in extracts of ovine muscle under physiological conditions of pH and ionic strength, and that the association is sensitive to variations of pH and the concentrations of proteins and specific metabolites such as ATP and fructose 1,6-diphosphate. Furthermore, myosin was identified as a necessary component for complex formation under the experimental conditions.

3. The size of the observed complex of glycolytic enzymes increases with increasing protein concentration; at 52 mg protein/ml, $s_{20,w}$ values of over 100 are indicated.

4. These data have been discussed in relation to the possible physiological significance of the multi-enzyme association.

INTRODUCTION

In recent years much consideration has been directed towards the state of association of the individual proteins within the cytoplasm of the cell. Contrary to the previously widely accepted concept that these macromolecular components were freely dissolved within the cellular environment, many authors have suggested that proteins may associate amongst themselves or with the various membranous and particulate components [1–5]. In relation to the enzymes of the glycolytic sequence, for example, the possible existence of a glycolytic complex and the inherent metabolic advantages of such an assembly have been widely canvassed [5–7], and a substantial body of evidence has accumulated from a variety of biological systems indicating that serious consideration of this possibility is warranted [5, 8–11]. In skeletal muscle, for example, many diverse approaches including fractional extraction studies [12, 13], binding studies with purified components (13–15], investigations of press juices [16, 17], moving boundary electrophoretic analyses of protein–protein interactions [9] and histochemical localisations [18] have provided data which suggest that complex formation involving glycolytic enzymes may occur within the muscle fibre.

Until recently, however, definitive proof of enzyme association was lacking. Such data was first obtained in a study of the sedimentation behaviour of a number of glycolytic enzymes in a soluble myogen fraction, direct evidence being provided that complex formation involving glycolytic enzymes was discernible under physiological conditions of pH and ionic strength [19]. Since these observations stood in contrast to the results of other investigations which had failed to detect the presence of any significant degree of multi-enzyme association within rat liver soluble fractions by the use of similar techniques [20, 21], and in view of the potential importance of the observation of complex formation, the nature of the association of glycolytic enzymes within the muscle myogen fraction has been further investigated, and the results of these studies are reported in the present communication.

MATERIALS AND METHODS

Glycolytic enzymes, substrates and cofactors were purchased from Sigma Chemical Co., St. Louis, Missouri. $(NH_4)_2SO_4$ (special enzyme grade) was a product of Schwarz/Mann, Orangeburg, New York; dithiothreitol, was purchased from Calbiochem., San Diego, California; acrylamide and *N,N'*-methylenebisacrylamide were from Eastman Kodak Co., Rochester, New York; while Coomassie BrilliantBlue was purchased from Sigma Chemical Co. Other chemicals were AR Grade.

Preparation of myogen fraction

Myogen preparations were obtained from the freshly excised hind-leg muscles of adult rats or from sheep semitendinosus muscle by the procedure of Beisenherz et al. [22] as described by Arnold and Pette [13]. The only variation of this methodology was the use of 0.1 M potassium phosphate, 0.002 M dithiothreitol, pH 7.5, as the extracting buffer. Freshly excised muscles were minced and immediately extracted with 3 vol. of the above buffer for 20 min with vigorous stirring. This, and all subsequent operations, were performed in the cold at 0.4 °C.

After filtration through two layers of cheesecloth, the preparation was centrifuged at 30 000 rev./min for 60 min using the 30 rotor in a Beckman L2-65 B ultracentrifuge, and the supernatant collected. $(NH_4)_2SO_4$ was then added to this supernatant to give a final concentration of 1.5 M, and the resulting precipitate removed by centrifugation at 10 000 $\times$ *g* for 15 min using an IEC PR-6 refrigerated centrifuge. Solid $(NH_4)_2SO_4$ was then added to achieve a final concentration of 3.2 M while the pH was maintained at 7.0. Following centrifugation the precipitate was dissolved in 0.05 M Tris–Cl, 0.005 M EDTA, 0.005 M dithiothritol, pH 7.5 and then brought to 3.2 M $(NH_4)_2SO_4$ by dialysis against a solution of $(NH_4)_2SO_4$ in the above buffer. The resulting $(NH_4)_2SO_4$ suspension constituted the stock myogen preparation and was stored as such at 4 °C.

Sedimentation analysis

In order to prepare the myogen for sedimentation analysis, a portion of the stock $(NH_4)_2SO_4$ suspension was collected by centrifugation, dissolved in the desired buffer system and then equilibrated with that buffer by extensive dialysis. The buffer system normally used in these studies was 0.005 M imidazole, 0.002 M dithiothreitol, 0.15 M KCl at pH 7.0 or 7.5. Variations in the pH and salt content of this buffer, as

well as other buffer systems, however, were used in the course of the present studies and these are noted in the relevant results sections. Following dialysis, the preparation was clarified by centrifugation at 10 000 × *g* for 15 min in an IEC PR-6 refrigerated centrifuge, and the protein concentration estimated. Examination of this preparation in the electron microscope confirmed the absence of any residual membrane fragments.

After adjustment to the desired protein concentration, normally 1.8 ml of this preparation was centrifuged at 45 000 rev./min for a fixed period of time (normally 60 min) in special 2-ml adaptors in an SW 50L rotor using a Beckman L2-65B ultracentrifuge at 4 °C. Following centrifugation, fractions were collected in sequence from the bottom of the tubes and assayed for enzyme activity. Assays of each fraction were carried out in triplicate for each enzyme. Control centrifugations were carried out under identical conditions using commercially pure samples of each of the enzymes tested.

Myosin preparation

Myosin was prepared from the hind-leg muscles of adult Wistar rats using the methods of Quass and Briskey [23]. The myosin thus prepared was mixed with an equal volume of cold glycerol and held in a freezer at −20 °C. Myosin was recovered from the stock solution by dilution with 10 vol. of cold distilled water and the precipitate collected by centrifugation at 10 000 × *g* for 15 min. The myosin pellet was then dissolved in the desired buffer system which was generally 0.02 M Tris–acetate, 0.5 M KCl, pH 6.8.

F-actin preparation

Actin was prepared from an acetone powder of beef skeletal muscle by the procedure of Spudich and Watt [24]. The preparation of the acetone powder was based on the scheme outlined by Briskey and Fukazawa [24]. Polymerised F-actin in 0.1 M KCl could be stored at 4 °C in the presence of a crystal of thymol for periods of 2–3 weeks without any deterioration in its properties as judged by electron microscopic observation, and its ability to stimulate myosin ATPase activity.

Sodium dodecylsulphate polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate was performed using the system described by Laemmli [26].

The gels were normally fixed and stained by immersion overnight in freshly prepared 50% trichloroacetic acid containing 0.1% Coomassie Brilliant Blue. Destaining was achieved by repeated washing in a gel wash which contained methanol, acetic acid and water in the ratio 5:1:5, by vol.

Assays

(i) Fructose-diphosphate aldolase. Fructose-diphosphate aldolase was assayed by a procedure essentially the same as that described by Blostein and Rutter [27].

(ii) Lactate dehydrogenase. Lactate dehydrogenase was assayed by the procedure of Wroblewski and La Due [28].

(iii) Pyruvate kinase. Pyruvate kinase activity was measured by the procedure of Bücher and Pfleiderer [29].

(iv) Phosphofructokinase. Phosphofructokinase activity was measured by the method of Racker [30].

All these assays were carried out in 0.05 M Tris–HCl pH 7.5 (except phosphofructokinase which was assayed at pH 8.2) in a final volume of 1.0 ml using a Unicam SP800 recording spectrophotometer at 30 °C, and were expressed as v/V values, based on a "constant aliquot withdrawn basis".

(v) Protein. Protein was measured by the method of Lowry et al. [31].

(vi) ATPase. The rate of hydrolysis of ATP was measured by the following procedure. The reaction mixture (final volume 1.0 ml) contained 0.03 M imidazole (pH 6.8), 0.06 M KCl, 1.0 mM $MgCl_2$ or $CaCl_2$ and enzyme sample at the desired level (generally 0.1–0.2 mg/ml). The reaction was initiated by addition of ATP to 1.0 mM. The incubation was performed at 25 °C and usually for 15 min depending on the level of enzyme used, before the reaction was stopped by addition of 0.5 ml of 20% trichloroacetic acid. After centrifugation for 5 min at 2000 × *g*, the amount of inorganic phosphate in the supernatant was estimated by the method of Marsh [32].

(vii) Superprecipitation tests. The superprecipitation of actomyosin suspensions in solutions of low ionic strength was followed using the turbidity assay system essentially as described by Ebashi and Ebashi [33]. Reaction mixtures contained in final concentrations; 0.02 M Tris–maleate (pH 6.8), 0.043 M KCl, 2.0 mM $MgCl_2$ and 0.5–1.0 mg/ml of sample in a final volume of 1.0 ml. The reaction was initiated by the addition of ATP to 0.2 mM and the increase in the absorbance of the solution at 660 nm was followed using a Unicam SP 800 recording spectrophotometer at 30 °C.

RESULTS

As shown in Fig. 1, the sedimentation profile of fructose-diphosphate aldolase, pyruvate kinase and lactate dehydrogenase exhibited an incomplete profile at the top of the tube which corresponded to the sedimentation of the free enzymes, as shown by control centrifugations of the pure enzymes. In addition, each of these enzymes also exhibited a second rapidly sedimenting boundary which occurred in parallel for each of the enzymes tested, but which was not present in the sedimentation profiles of the pure enzymes. Furthermore, when the stabilizing agents D-fructose 1,6-diphosphate and EDTA [34] were incorporated into the buffer, sufficient phosphofructokinase activity was maintained to permit sedimentation analysis of this important glycolytic enzyme. It is evident from the diagram that phosphofructokinase also exhibited a small but significant boundary which sedimented in parallel with the second rapidly sedimenting boundary of the other three enzymes. It may also be noted that approx. 40% of the phosphofructokinase activity sedimented to the bottom of the tube under these conditions. The formation of large aggregates of phosphofructokinase with sedimentation coefficients in the range of 100–200 S and greater has been previously observed under similar buffer conditions [35].

In regard to the relative specificity of this association, it may be worthy of reiteration, that although the glycolytic components form a major portion of the protein in these extracts, the total protein curves depict a significantly lower degree of association than that for the individual enzymes studied [19].

Again, in relation to the data bearing on the influence of individual parameters on the observed complex, it may be noted that the behaviour of all three enzymes (i.e.

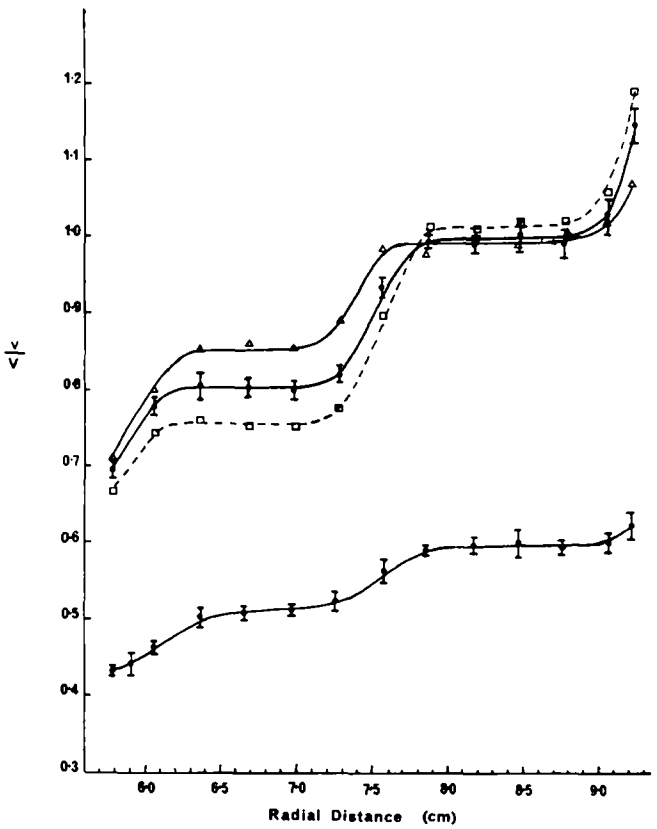

Fig. 1. Enzyme sedimentation in myogen preparation. Myogen (sheep semitendinosus) prepared by dialysis against 0.01 M imidazole, 0.15 M KCl, 0.002 M dithiothreitol (pH 7.5), containing 10^{-5} M fructose 1,6-diphosphate and 10^{-3} M EDTA. Protein concentration 52 mg/ml. Centrifugation at 45 000 rev./min for 30 min at 4 °C. ●, fructose-diphosphate aldolase; □, pyruvate kinase; △, lactate dehydrogenase; ○, phosphofructokinase. Individual values are given as the means of triplicate determinations. For purposes of clarity, the standard deviations of the mean are represented for only fructose-diphosphate aldolase and phosphofructokinase. v/V is the ratio of the activity in each fraction to that in the original myogen sample.

fructose-diphosphate aldolase, lactate dehydrogenase and pyruvate kinase) were studied. The responses of these enzymes were found to be qualitatively similar, however, as indicated in Fig. 3, so in order to facilitate representation, subsequent results have been described in terms of fructose-diphosphate aldolase activity measurements only.

Effect of protein concentration

The size and rate of sedimentation of this second rapidly sedimenting boundary of enzyme activity were demonstrated as dependent on the total protein concentration of the myogen preparation. The profiles in Fig. 2, for example, illustrate that the size and rate of sedimentation of this boundary diminished with decreasing protein concentration. In preparations of low protein concentration (e.g. 4 mg/ml, Fig. 2) no such boundary could be detected at all. Thus the indications are that this boundary does

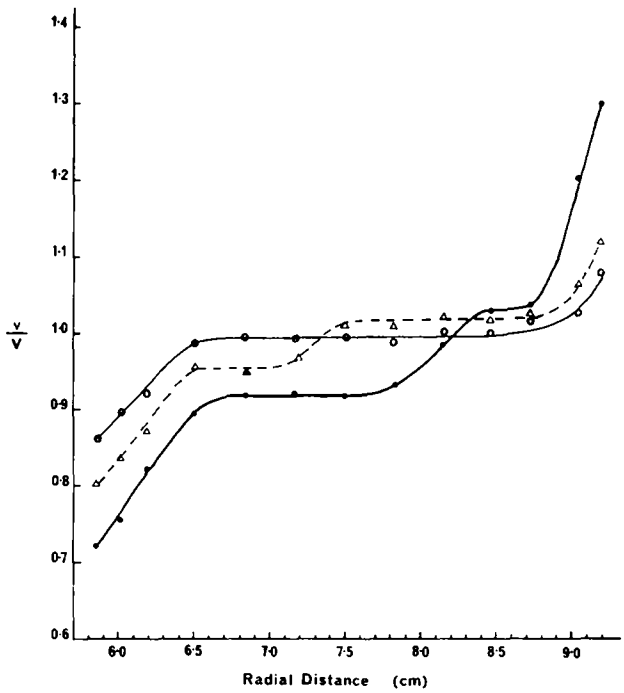

Fig. 2. Effect of protein concentration on enzyme sedimentation in myogen preparations. Myogen (sheep semitendinosus) prepared by dialysis against 0.005 M imidazole, 0.15 M KCl, 0.002 M dithiothreitol (pH 7.5). Centrifugation at 45 000 rev./min for 70 min 4 °C. v/V, fructose-diphosphate aldolase activity ratios. ●—●, 26.5 mg/ml; △—△, 8.0 mg/ml; ○—○, 4.0 mg/ml.

not represent the sedimentation profile of a stable complex, per se, but rather a reaction boundary generated by a rapidly reversible interacting system involving at least the four glycolytic enzymes that were tested.

Effect of metabolites

As previous studies [10, 14] had indicated that certain metabolites may exert specific effects on the interactions between glycolytic enzymes and other macromolecular components of tissues, the influence of some of these metabolites on the present system was investigated. The results presented in Fig. 3, for example, reveal that the addition of D-fructose 1,6-diphosphate (1 mM) altered the sedimentation profiles of each of the three enzymes studied. In particular the size and rate of sedimentation of the second boundary was increased for each of the three enzymes, suggesting that the presence of D-fructose 1,6-diphosphate favoured complex formation under these conditions of high protein concentration. In other experiments, ATP in the absence of Mg^{2+} was found to have the opposite effect to that of D-fructose 1,6-diphosphate, i.e. it decreased the size and rate of sedimentation of the second boundary.

Effect of pH and ionic strength

In previous experiments, pH and ionic strength have been shown to be of

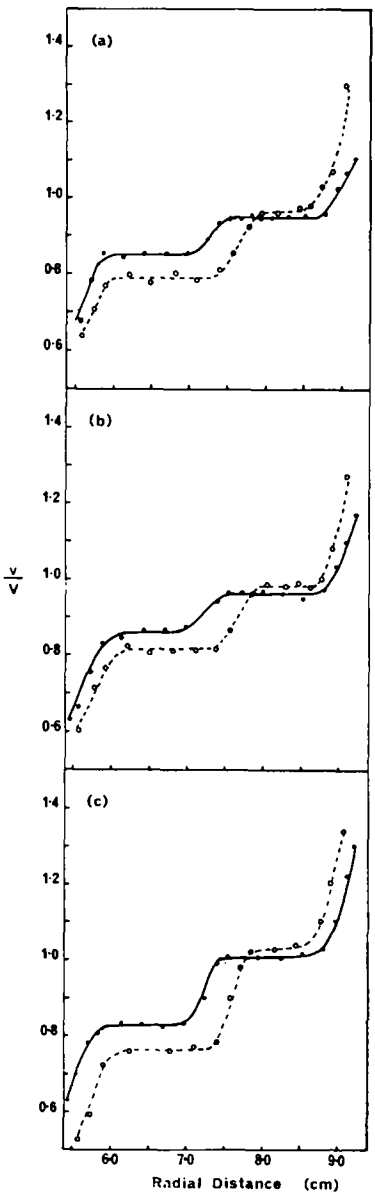

Fig. 3. Effect of fructose 1,6-diphosphate on enzyme sedimentation in myogen preparation. Myogen (rat muscle) prepared by dialysis against 0.005 M imidazole, 0.15 M KCl, 0.002 M dithiothreitol, pH 7.0. Protein concentration 19.0 mg/ml. Centrifugation at 45 000 rev./min for 90 min at 4 °C. (a) Fructose-diphosphate aldolase. (b) Lactate dehydrogenase. (c) Pyruvate kinase. ●, control myogen preparation; ○, myogen preparation containing 1 mM fructose 1,6-diphosphate.

critical importance in determining the degree of interaction between glycolytic enzymes and other cellular components [5, 10, 14]. Consequently, attempts were made to examine the influence of these parameters on 'complex' formation in the present system and the results of these experiments are presented in Figs 4 and 5. To obtain

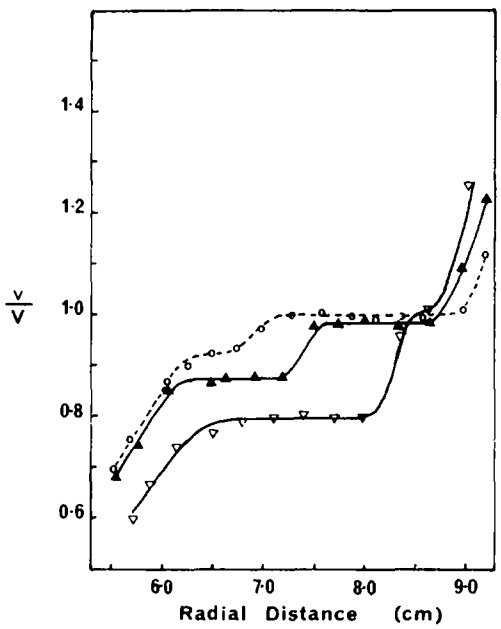

Fig. 4. Enzyme sedimentation in myogen preparations of differing pH. Myogen (rat muscle) prepared by dialysis against 0.005 M imidazole, 0.15 M KCl, 0.002 M dithiothreitol at pH 6.5, 7.0 or 7.5. Protein concentration 20 mg/ml. Centrifugation at 45 000 rev./min for 90 min at 4 °C; v/V, fructose-diphosphate aldolase activity ratios. ○, pH 6.5; ▲, pH 7.0; ▽, pH 7.5.

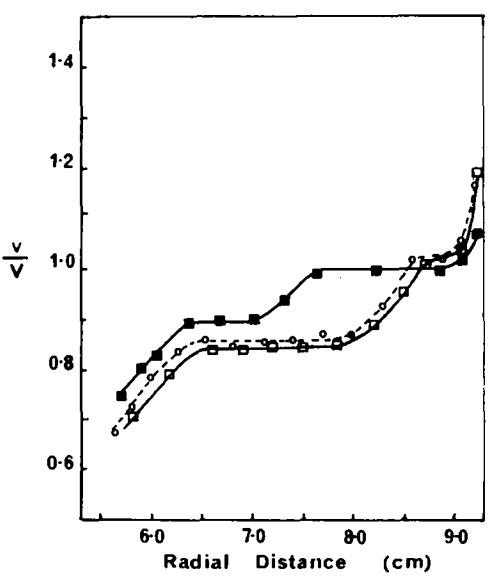

Fig. 5. Enzyme sedimentation in myogen preparations of differing ionic strength. Myogen (rat muscle) prepared by dialysis against 0.005 M imidazole, 0.002 M dithiothreitol (pH 7.5), containing 0.05, 0.15 or 0.25 M KCl. Protein concentration 20 mg/ml. Centrifugation at 45 000 rev./min for 90 min at 4 °C. v/V, fructose-diphosphate aldolase activity ratios. ■, 0.05 M KCl; ○, 0.15 M KCl; □, 0.25 M KCl.

these data, myogen fractions were prepared by dialysis against 0.005 M imidazole, 0.002 M 1,4-dithiothreitol, 0.15 M KCl, at pH values 6.5, 7.0 and 7.5. The dialysed preparations were then clarified and subjected to sedimentation analysis as described in Methods. Similarly, to determine the influence of ionic strength (Fig. 5), myogen preparations were dialysed against 0.005 M imidazole, 0.002 M dithiothreitol (pH 7.5), containing 0.05, 0.15 or 0.25 M KCl, clarified, and then analysed. In each case the myogen preparations were adjusted to a fixed protein concentration prior to analysis. As shown in Fig. 4 a decrease in the pH of the dialysis buffer resulted in a decrease in the size and rate of sedimentation of the second boundary of enzyme activity. Decreasing the ionic strength was also found to produce a similar response (Fig. 5).

A further observation deriving from these experiments was that decreasing pH or ionic strength was accompanied by an increase in precipitate formation in the myogen preparations. Concomitant with this increased precipitation there was a greater removal of protein and enzyme activities from these preparations on clarification (cf. Methods). For example, only 3.0–3.5% of the total protein and 1.0–1.4% of the total fructose-diphosphate aldolase activity were removed on clarification of the normal preparation dialysed against buffer containing 0.15 M KCl, whereas clarification of preparations dialysed against buffer containing 0.05 M KCl resulted in the removal of 6–7% of the total protein along with 4–5% of the total fructose-diphosphate aldolase activity. Thus it appeared possible that the observed effects of decreasing pH and ionic strength may not have been a direct reflection of the influence of these parameters on complex formation, but rather indicative of the removal of some components which were essential for complex formation, and which displayed a decreased solubility under the varied experimental conditions.

This possibility was investigated and the results of the experiments presented in Fig. 6 served to reinforce the likelihood of the latter interpretation. Two myogen samples were prepared, one by dialysis against 0.005 M imidazole, 0.002 M dithiothreitol (pH 7.5) buffer containing 0.15 M KCl, the other by dialysis against the same buffer without added salt. Following the dialysis and clarification procedures (cf. Methods), KCl was then added to the sample prepared in the absence of salt to give a final concentration of 0.15 M. Both samples were then subjected to the normal sedimentation analysis. The sample prepared in the presence of 0.15 M KCl yielded a normal sedimentation profile containing a second rapidly sedimenting boundary of enzyme activity (Profile A in Fig. 6), whereas no such boundary could be detected in the sedimentation profile of the sample prepared in the absence of salt (Profile B in Fig. 6). As both samples were analysed at the same ionic strength, it was concluded that the failure to observe this boundary was due to the removal of some component or components, which were critical to enzyme association during the low ionic dialysis procedure.

Reconsideration of the procedure used to extract the myogen fraction from the muscle mince, in the light of the available literature on the extractability of muscle proteins, suggested that the preparation might contain trace quantities of structural proteins such as myosin.

Comparison of the electrophoretic patterns of myosin and myogen on sodium dodecylsulphate polyacrylamide gels was consequently carried out and revealed the presence in the myogen preparation of a high-molecular-weight polypeptide compo-

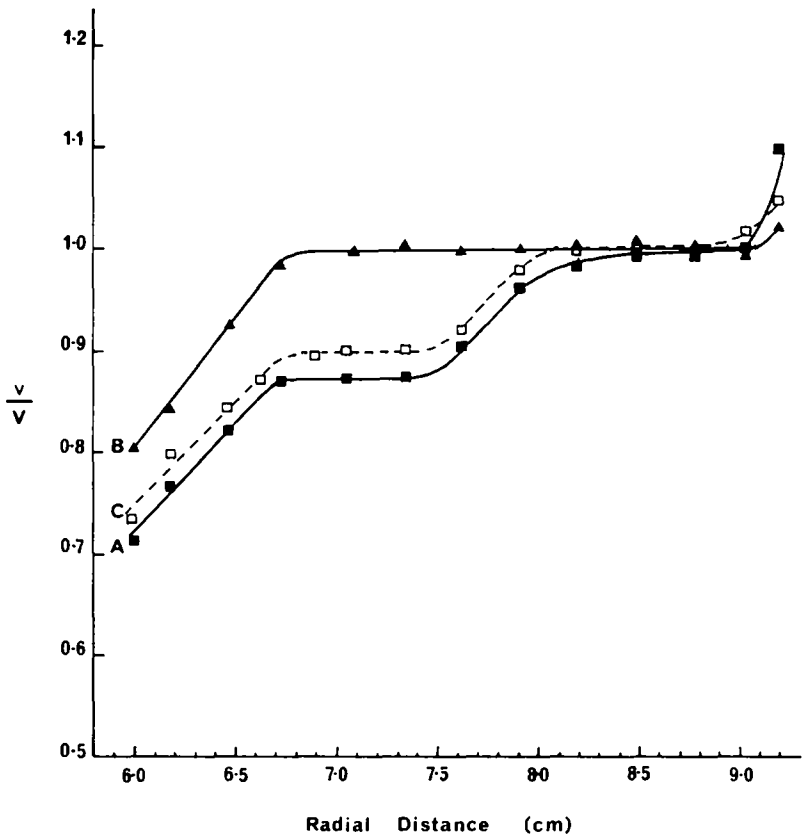

Fig. 6. Enzyme sedimentation in modified myogen preparations. (A) ■, normal myogen prepared by dialysis against 0.005 M imidazole, 0.15 M KCl, 0.002 M dithiothreitol, pH 7.5. Protein concentration 20 mg/ml. (B) ▲, myogen prepared by dialysis against 0.005 M imidazole, 0.002 M dithiothreitol, pH 7.5. The preparation was then clarified as described in Methods and KCl added to a final concentration of 0.15 M. Protein concentration 20 mg/ml. (C) □, Myogen preparation B as described above, mixed with approx. 5 mg of purified myosin and then equilibrated with 0.005 M imidazole, 0.15 M KCl, 0.002 M dithioreitol (pH 7.5) by dialysis and clarified. Protein concentration 20 mg/ml. Centrifugation at 45 000 rev./min for 60 min at 4 °C. Myogen from sheep semitendinosus muscle. v/V, fructose-diphosphate aldolase activity ratios.

nent whose electrophoretic mobility was identical with that of the heavy chains of myosin (Fig. 7), and which constituted approx. 3% of the total protein present. In addition, these electrophoretic analyses revealed that after dialysis of the myogen against low-ionic-strength buffer, this band was no longer evident in the electrophoretic pattern of the clarified myogen preparation, but was notably prominent in the electrophoretic pattern of the precipitate which formed during such dialysis.

The presence of myosin in the myogen preparation was further substantiated by the observation of other typical myosin-like characteristics on analyses of the precipitate obtained by dialysis against low-ionic-strength buffer. These properties included (i) superprecipitation phenomena on mixing with F-actin and ATP under suitable solvent conditions, (ii) the presence of Mg^{2+}- and Ca^{2+}-activated ATPase

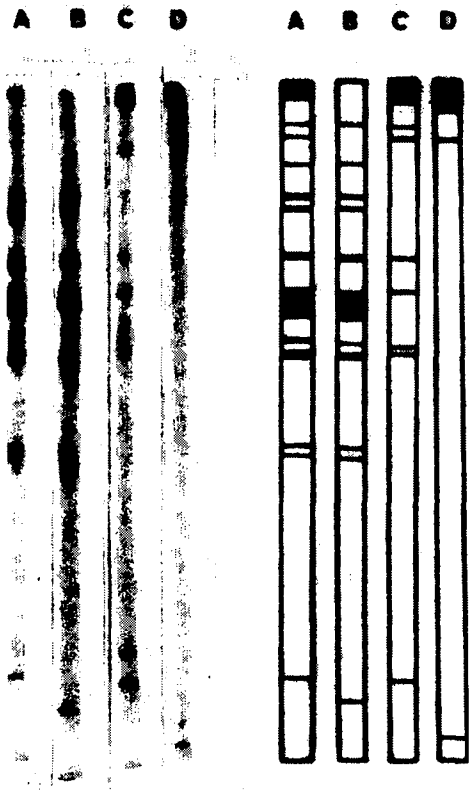

Fig. 7. Sodium dodecylsulphate gel electrophoretic patterns of myogen preparations. A, normal myogen fraction prepared in 0.15 M KCl. (B), myogen fraction prepared at low ionic strength. C, precipitate obtained on clarification of preparation B. D, purified rat muscle myosin. Left, photograph of gels. Right, diagramatic representation of gels.

activities and the influence of actin on these activities, and (iii) the observation on electron microscope examination of typical myosin-like filaments.

In order to measure superprecipitation, the turbidity assay procedure of Ebashi and Ebashi [33] was employed. In Fig. 8b a copy of a recorder tracing is provided showing the response obtained on addition of ATP to a mixture of F-actin and the low-ionic-strength myogen precipitate. For comparison, Fig. 8a shows the response recorded on addition of ATP to a suspension of synthetic actomyosin under the same assay conditions. Both preparations exhibited comparable behaviour showing a sharp rise in turbidity on addition of ATP. The F-actin–myogen precipitate system yielded a more rapid response which may have been due to the presence of other proteins in this system. Seraydarian et al. [36] have observed that the turbidity response of actomyosin suspensions in this assay system is extremely sensitive to the presence of such additional components.

The presence of myosin in the low-ionic-strength precipitate obtained from the myogen preparation was also indicated by the measurement of the ATPase activity of this fraction. The low-ionic-strength precipitate fraction was found to possess significant Mg^{2+}- and Ca^{2+}-stimulated ATPase activities which were similar to those

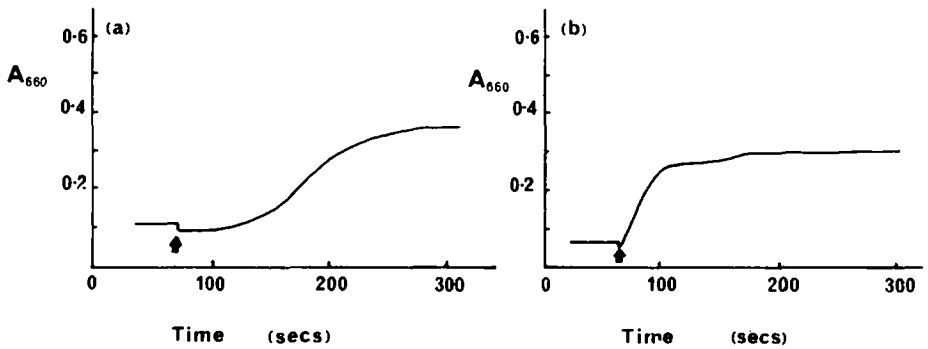

Fig. 8. Superprecipitation assays: Comparison of synthetic actomyosin and an actin–myogen low-ionic-strength precipitate mixture. a, synthetic actomyosin. 0.4 mg myosin; 0.133 mg F-actin; total protein concentration 0.53 mg/ml. b, F-actin–myogen precipitate mixture. 0.78 mg myogen precipitate; 0.133 mg F-actin; total protein concentration 0.918 mg/ml. Assay conditions as described in Methods. Arrows indicate addition of 0.2 mM ATP.

of myosin in that the Ca^{2+}-stimulated activity was 5–10 times greater than the Mg^{2+}-stimulated activity. Further similarity to myosin was noted on addition of actin. The presence of actin resulted in a 4–5-fold increase in the Mg^{2+}-stimulated ATPase activities of both the myosin and the low-ionic-strength precipitate fraction, while little or no effect was noted in regard to the Ca^{2+}-stimulated activities of either protein preparation. The results obtained with myosin samples and low-ionic-strength precipitates from myogen are presented in Table I.

Finally, in order to confirm that addition of purified myosin to a myogen preparation which had been depleted of that protein by the low-ionic-strength dialysis procedure, would interact with the glycolytic enzymes so as to result in the observation of a second rapidly sedimenting boundary, a pellet of approx. 5 mg of myosin was redissolved in a myogen preparation which had been prepared by dialysis against low-ionic-strength buffer and clarified prior to addition of KCl to 0.5 M. This recon-

TABLE I

ATPase ACTIVITIES OF MYOSIN AND LOW-IONIC STRENGTH PRECIPITATE FRACTIONS FROM MYOGEN

Incubations were performed at 25 °C for 15 min under the assay conditions described in Methods section. Ratio of myosin to actin and myogen precipitate to actin was 3:1. Myosin and myogen precipitate fraction were prepared from rat muscle. Each value represents the mean of triplicate determinations obtained with four different preparations.

μMoles P_i/min per mg protein

Myosin		Myosin + actin		Myogen precipitate		Myogen precipitate + actin	
Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}
0.017		0.092		0.019		0.072	
0.025	0.193	0.096	0.218	0.013	0.08	0.056	0.082
0.019	0.190	0.085	0.227	0.016	0.081	0.058	0.078
0.020	0.192	0.100	0.230	0.012	0.096	0.060	0.085

stituted preparation was then extensively dialysed against the normal dialysis buffer containing 0.15 M KCl, clarified, and then subjected to sedimentation analysis.

The results of this experiment are incorporated in Fig. 7c in order to allow direct comparison with the sedimentation profiles of normal and myosin-depleted preparations. It is evident from these data that addition of myosin to the myosin-depleted preparation did result in the reappearance of the second rapidly sedimenting boundary of enzyme activity characteristic of which was the normal preparation. Electrophoretic analysis on sodium dodecylsulphate polyacrylamide gels confirmed the presence of myosin in the 'reconstituted' preparation of myogen which produced this profile.

DISCUSSION

The degree of intramolecular organization within the glycolytic enzyme sequence is a topic of wide biological import, but one which has remained controversial and unamenable to conclusive solution over many years [5, 6]. In a classic attempt at resolving this question, De Duve [21] applied exacting sedimentation criteria to the behaviour of enzymes from rat liver, but failed to uncover any evidence of the presence of a glycolytic particle and was forced to the conclusion that the enzymes of this sequence were molecularly dispersed in the cytosol.

It was of some interest in this connection, then, when the presence of an association of glycolytic enzymes was subsequently demonstrated in a soluble myogen preparation from rat skeletal muscle [19]. The present studies serve to confirm these latter observations, to extend our understanding of the nature of this complex and the factors affecting enzyme association, and to allow comment on the reasons why the existence of such aggregates has not been substantiated previously.

It is evident from the present data, for example, that both protein and salt concentrations are of prime importance in preserving the definition of this enzyme assemblage. Many of the previously observed associations of glycolytic enzymes with structural components have been subject to the criticism that they have only been observed under low-ionic-strength conditions which would tend to diminish any in vivo significance of the phenomenon [21]. The present data, however, demonstrate that the enzyme association in myogen is preserved at isotonic ionic strength, provided that the protein concentration is also high; both of which conditions are typical of the cellular environment.

A further feature of interest in the present results is the marked influence of certain metabolites on the stability of the enzyme aggregate. The choice of D-fructose 1,6-diphosphate and ATP for these studies was governed by the known high degree of specificity of these metabolites both in regard to their influence on the aggregation of the key control enzyme phosphofructokinase [35, 37] and in relation to their effect on the association of other glycolytic enzymes with the structural components of mammalian tissues [10, 14]. It has been established previously, for example, that fructose-diphosphate aldolase was released from its association with the particulate fraction of rat skeletal muscle in a highly specific manner by D-fructose 1,6-diphosphate [38]; in the present system, however, this same metabolite appeared to favour the involvement of fructose-diphosphate aldolase and other glycolytic enzymes in complex formation. Antithetically, ATP displayed the opposite direction of effect to fructose 1,6-diphosphate in both of the above situations. The influence of fructose

1,6-diphosphate and ATP on the association of glycolytic components in the present experiments, then, would appear to be more reminiscent of the influence which these two metabolites exert on the aggregation properties of phosphofructokinase [35, 37] rather than the characteristics of enzyme adsorption.

One of the significant features of the present investigation has been the identification of myosin as a necessary component for complex formation under the experimental conditions. The inclusion of small quantities of this solubilized structural component in the myogen extracts has enabled the definitive sedimentation criteria of De Duve [21] to be applied to the myogen system, and established the presence of an association of these glycolytic enzymes in skeletal muscle extracts. There is some previous evidence in the literature of a slight solubility of myosin over the range of ionic strength from 0.0 to 0.25 [16, 39]. Furthermore, it may be noted that the interaction of glycolytic enzymes with myosin is not an entirely novel observation. Arnold and Pette [13] have previously observed a reversible binding of fructose-diphosphate aldolase to myosin in a low-ionic-strength system although, the affinity of aldolase for myosin was found to be much less than the affinity of fructose-diphosphate aldolase for F-actin, the other major structural protein of muscle. Additionally, Amberson and Bauer [9] have provided some indication, by means of moving boundary electrophoresis, that components of the myogen may interact with myosin; as their electrophoretic analyses were also performed under high-ionic-strength conditions, the results obtained would appear to correlate well with the present sedimentation data.

From the present studies, too, a comment may be derived in relation to the size of the observed complex. De Duve [21] has calculated that a glycolytic particle made up of one molecule of each of the glycolytic enzymes would have molecular weight of the order of $1.6 \cdot 10^6$, and an approximate sedimentation coefficient ($s_{20,w}$) of 50. It is of interest, then, to note that the present results indicate that the size of the enzyme aggregate increases with increasing protein concentration, and that at the highest protein concentration in these experiments (52 mg/ml) which is still less than cellular concentrations, the complex has an indicated $s_{20,w}$ value well in excess of 100.

Also, it may be noted that while the present data do not rule out the possibility that the enzymes studied may associate amongst themselves under different experimental conditions, they do lead to the conclusion that the association of glycolytic enzymes is favoured by the presence of myosin. An important point which needs to be made in regard to the present data, though, is that myosin may more aptly be regarded as one of the more soluble structural components of muscle rather than the most plausible physiological adsorbent in this tissue. As has been discussed previously, Arnold and Pette [13] have demonstrated that fructose-diphosphate aldolase and other glycolytic enzymes exhibit a marked preferential binding to other major structural protein, F-actin, under in vitro conditions. In addition, Sigel and Pette [8] using histochemical techniques have localised the majority of the glycolytic enzymes within the I bands of the myofibril where the major structural protein is F-actin. Finally, in recent experiments in this laboratory (Clarke, F. M. and Masters, C. J., unpublished observations), significant adsorption of the majority of glycolytic enzymes to F-actin has been observed under physiological conditions of ionic strength and high protein concentration.

ACKNOWLEDGEMENTS

These investigations were supported in part by funds from the Australian Research Grant Committee.

REFERENCES

1 Atkinson, D. E. (1969) Current Topics in Cellular Regulation (Horecker, B. L. and Stadtman, E. R., eds), Vol. 1, Academic Press, New York pp. 29–43
2 Ling, G. N. (1969) Int. Rev. Cytol. 26, 1–61
3 Kempner, E. S. and Miller, J. H. (1968) Exp. Cell. Res. 51, 141–156
4 Sols, A. and Marco, R. (1970) Current Topics in Cellular Regulation (Horecker, B. L. and Stadtman, E. R., eds), Vol. 2, pp. 227–273, Academic Press, New York
5 Green, D. E., Murer, E., Hultin, H. O., Richardson, S. H., Salmon, B., Brierley, G. P. and Baum, H. (1965) Arch. Biochem. Biophys. 112, 635–647
6 Hubscher, G., Mayer, R. J. and Hansen, H. J. (1971) Bioenergetics 2, 115–118
7 Reed, L. J. and Cox, D. J. (1966) Ann. Rev. Biochem. 35, 57–84
8 Sigel, P. and Pette, D. (1969) Histochem. Cytochem. 17, 225–237
9 Amberson, W. R. and Bauer, A. C. (1971) J. Cell. Physiol. 77, 281–299
10 Clarke, F. M. and Masters, C. J. (1972) Arch. Biochem. Biophys. 156, 673–683
11 Mowbray, J. and Ottaway, J. H. (1973) Eur. J. Biochem. 36, 369–379
12 Bauer, A. C., Pette, D., Roisen, F. and Amberson, W. R. (1964) Fed. Proc. 23, 310
13 Arnold, H. and Pette, D. (1968) Eur. J. Biochem. 6, 163–171
14 Arnold, H. and Pette, D. (1970) Eur. J. Biochem. 15, 360–366
15 Arnold, H., Henning, R. and Pette, D. (1971) Eur. J. Biochem. 22, 121–126
16 Amberson, W. R., Bauer, A. C., Philpott, D. E. and Roisen, F. (1964) J. Cell. Comp. Physiol. 63, 7–21
17 Amberson, W. R., Roisen, F. and Bauer, A. C. (1965) J. Cell. Comp. Physiol. 66, 71–90
18 Arnold, H., Nolte, J. and Pette, D. (1969) J. Histochem. Cytochem. 17, 314–320
19 Clarke, F. M. and Masters, C. J. (1973) Biochim. Biophys. Acta 327, 223–226
20 Anderson, N. G. and Green, J. G. (1967) Enzyme Cytology (Roodyn, D. B., ed.), pp. 475–509, Academic Press, New York
21 De Duve, C. (1972) Structure and Function of Oxidation Reduction Enzymes (Akeson, A. and Ehrenberg, A., eds), pp. 715–728, Pergamon Press, Oxford and New York
22 Beisenherz, G., Boltze, H. J., Bücher, T., Czok, R., Garbade, K. H., Meyer-Arend, E. and Pfleiderer, G. (1953) Z. Naturforsch. 8b, 555–563
23 Quass, D. W. and Briskey, E. J. (1968) J. Food Sci. 33, 180–189
24 Spudich, J. A. and Watt, S. (1971) J. Biol. Chem. 246, 4866–4871
25 Briskey, E. J. and Fukazawa, T. (1971) Adv. Food Res. 19, 279–360
26 Laemmli, U. K. (1970) Nature 227, 680–685
27 Blostein, R. and Rutter, W. R. (1963) J. Biol. Chem. 238, 3280–3285
28 Wroblewski, F. and La Due, J. S. (1955) Proc. Soc. Exp. Biol. Med. 90, 210–213
29 Bücher, T. and Pfleiderer, G. (1955) Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds), Vol. 1, pp. 435–440, Academic Press, New York
30 Racker, E. (1947) J. Biol. Chem. 167, 843–854
31 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. S. (1951) J. Biol. Chem. 193, 265–275
32 Marsh, B. B. (1959) Biochim. Biophys. Acta 32, 357–361
33 Ebashi, S. and Ebashi, F. (1964) J. Biochem. Tokyo 55, 604–613
34 Wakid, N. and Mansour, T. E. (1965) Mol. Pharmacol. 1, 53–61
35 Parmeggiani, A., Luft, J. H., Love, D. S. and Krebs, E. G. (1966) J. Biol. Chem. 241, 4625–4637
36 Seraydarian, K., Briskey, E. J. and Mommaerts, W. F. (1967) Biochim. Biophys. Acta 133, 399–411
37 Mansour, T. E. and Ahlfors, C. F. (1968) J. Biol. Chem. 243, 2523–2533
38 Clarke, F. M., Masters, C. J. and Winzor, D. J. (1970) Proc. Aust. Biochem. Soc. 3, 66
39 Edsall, J. T. (1930) J. Biol. Chem. 89, 289–313