Short Communications

Isolation of Microsome-Bound Phosphofructokinase from Frog Skeletal Muscle and its Inhibition by Calcium Ions

By A. MARGRETH, CLAUDIA CATANI and S. SCHIAFFINO

*Institute of General Pathology and 'G. Vernoni' Research Unit for the Study of Physiopathology,
National Research Council, University of Padua, Italy*

(Received 28 December 1966)

Classical views on the location and structural organization of glycolytic enzymes in the intact skeletal-muscle cell (cf. Czok & Bücher, 1960) have been subjected to a certain degree of revision and criticism in recent years. It was shown by electron-microscopic methods (cf. Margreth, Muscatello & Andersson-Cedergren, 1963; Peachey, 1965) that: (i) the inner fluid contained in the membrane-limited spaces of the sarcoplasmic reticulum accounts for a very large portion of the extra-myofibrillar soluble sarcoplasm; (ii) the enzymes of glycolysis are at least in part located in the lumina of the sarcoplasmic reticulum (Fahimi & Karnowsky, 1966). In addition, several lines of evidence indicate that glycolytic enzymes are not freely soluble, as previously thought, and are bound, to various degrees, to muscle ultrastructure (Andersson-Cedergren & Muscatello, 1963; Margreth *et al.* 1963; Amberson, Roisen & Bauer, 1965).

Margreth, Muscatello & Andersson-Cedergren (1963) attached importance to the finding that a certain proportion of phosphofructokinase (EC 2.7.1.11), which is thought to be rate-limiting in glycolysis in muscle and other tissues (cf. Gevers & Krebs, 1966), is tightly bound to fractions derived by fragmentation of the sarcoplasmic reticulum ('microsomes' or 'sarcotubules') in frog skeletal muscle. Peculiarities in the behaviour of rabbit-muscle and -heart phosphofructokinase were later found by Pette & Hofer (1965) by using a method of fractional extraction, and were consistent with the existence of this enzyme in a 'soluble' and in a 'substructure-bound' form. Underwood & Newsholme (1965) reported that 20% of phosphofructokinase activity of liver homogenates is present in the microsomal fraction.

The present communication deals with improvements in the methods of subcellular fractionation of frog skeletal muscle, with a more complete enzymic analysis of microsomal subfractions and with some kinetic properties of microsome-bound phosphofructokinase.

We had previously found that PFK activity was low in frog-muscle homogenates prepared in 0.88-M-sucrose. Buffering of hyperosmotic sucrose with

potassium phosphate, pH 8.0 (20mM) (cf. Ling, Marcus & Lardy, 1965), resulted in the extraction from frog skeletal muscle of larger amounts of protein than sucrose without ions, with about a fivefold greater concentration of phosphofructokinase. However, the potassium phosphate buffer also extracted myofibrillar proteins that interfered with the separation of microsomes by differential centrifugation; this undesirable side effect led us to try other conditions. Disodium EDTA, pH 8.0 (5mM), proved the most potent stabilizer of the frog-muscle enzyme. Extraction of frog skeletal muscle with sucrose-EDTA solution yielded protein in quantities similar to those obtained with sucrose without additions, but with about eightfold higher phosphofructokinase activity.

Under the improved conditions of the present fractionation experiments with 0.88-M-sucrose-5 mM-EDTA medium, 25-30% of phosphofructokinase, as compared with 15-20% of the less active form of the enzyme (Margreth *et al.* 1963), was found to be associated with the 'heavy' microsomes obtained by centrifuging the mitochondria-free supernatant of frog-muscle homogenates at 100 000 *g* for 60 min. Further fractionation of the 100 000 *g* supernatant by differential centrifugation at 150 000 *g* for 60 min. and 120 min. led to the isolation of two additional particulate fractions that contained about 30% and 10% respectively of the total activity in the original extract, leaving about 8% unsedimented in the final soluble supernatant. When examined in the electron microscope, the microsomal fractions that contained the greater proportion of phosphofructokinase activity with about a tenfold factor of concentration appeared composed of tubular and vesicular elements, similar to those described previously (Muscatello, Andersson-Cedergren, Azzone & Decken, 1961; Margreth *et al.* 1963), most of which exhibited a dense amorphous inner content. The 'light' microsomal fraction obtained during centrifuging at 150 000 *g* between 60 min. and 120 min. consisted of very small vesicles embedded in a poorly resolved matrix.

The membrane component of the sarcoplasmic

reticulum to which phosphofructokinase is tightly combined was previously identified with a relaxing factor (Muscatello *et al.* 1961) in frog skeletal muscle. Such peculiar association, the protective effect of the chelating agent EDTA on muscle phosphofructokinase and knowledge of the role played by the sarcoplasmic reticulum in transport and accumulation of Ca^{2+} (cf. Ebashi, 1965) prompted us to investigate whether Ca^{2+} influenced the catalytic properties of phosphofructokinase. Some details of these studies are reported below.

Fig. 1. Inhibition by Ca^{2+} of frog-muscle phosphofructokinase and its reversal by EDTA. A 10% homogenate of frog leg muscles in 0.88 M-sucrose-5 mM-disodium EDTA, pH 8.0, was centrifuged at 30000 *g* for 30 min. to remove myofibrils and the bulk of mitochondria. The supernatant fluid was further centrifuged at 80000 *g* for 30 min. and the sediment, which contained microsomes and mitochondrial fragments, was discarded. The microsomal fraction obtained by centrifuging the 80000 *g* supernatant at 100000 *g* for 60 min. was used as the enzyme source. Phosphofructokinase activity was assayed essentially as described by Ling *et al.* (1965). The reaction medium in a 1 cm. light-path cell (final volume 3 ml.) contained: tris-HCl buffer, pH 8.0, 33.3 mM; sodium fructose 6-phosphate, 1 mM; sodium ATP, 0.5 mM; MgCl_2 , 1 mM; NADH, 0.1 mM; 50 μg . of crystalline aldolase and 10 μg . of mixed crystals of α -glycerophosphate dehydrogenase and triose phosphate isomerase (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany). At zero time, 50 μl . (6 μg . of protein) of microsomal suspension in 0.88 M-sucrose-5 mM-EDTA, pH 8.0, was added and E_{340} was recorded on a model L SR Sargent recorder fitted to a Beckman DU spectrophotometer. At the times indicated, CaCl_2 (3 μmoles in 20 μl .) and then EDTA (each addition 1.5 μmoles in 10 μl .) were added in succession. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

At pH 8.0, and with optimum concentrations of substrates and of Mg^{2+} (1 mM-fructose 6-phosphate, 2 mM-ATP and 6 mM- MgCl_2), as employed in the conventional assay medium, Ca^{2+} had a marked inhibitory effect on microsome-bound phosphofructokinase from frog muscle. The adding of Ca^{2+} in molar ratio 1:12, 1:6, 1:3 and 1:1 with respect to Mg^{2+} inhibited phosphofructokinase activity by 25, 40, 55 and 75–80% respectively. On the other hand, with 0.5 mM-ATP and with initial equimolar concentrations of Mg^{2+} and Ca^{2+} (both 1 mM), the degree of inhibition exerted by Ca^{2+} on phosphofructokinase was progressively lowered by raising the concentration of Mg^{2+} in the medium, and was almost completely overcome when Mg^{2+} exceeded Ca^{2+} by 15-fold. With the same initial concentrations of ATP, Mg^{2+} and Ca^{2+} as above, the inhibited enzyme could be reactivated by the addition, subsequent to Ca^{2+} , of equimolar amounts of EDTA (Fig. 1).

Inhibition of frog-muscle phosphofructokinase by Ca^{2+} was independent of the concentration of fructose 6-phosphate. Agents reported to exert allosteric effects on mammalian muscle phosphofructokinase (cf. Mansour, Wakid & Sprouse, 1966) did not counteract, to any appreciable extent, the inhibition by Ca^{2+} of the frog enzyme. These included AMP, ADP, 3',5'-(cyclic)-AMP (all at 1 mM) and inorganic phosphate (10 mM). Our present results suggest competition between Ca^{2+} (or calcium ATP) and Mg^{2+} (or magnesium ATP) at the ATP substrate site of phosphofructokinase. Lowry & Passonneau (1966) have reported that brain phosphofructokinase is also inhibited by Ca^{2+} .

It has been calculated that Ca^{2+} may accumulate in the internal lumina of the sarcoplasmic reticulum up to a concentration of 20 mM (Ebashi, 1965) during muscle relaxation. Assuming an average intracellular concentration of free Mg^{2+} in frog skeletal muscle of about 3 mM (Nanninga, 1961), the above concentrations of Ca^{2+} would inhibit a large proportion of the phosphofructokinase activity. The displacement of Ca^{2+} from the membrane-limited spaces of the sarcoplasmic reticulum into the myofibrillar milieu, after muscle excitation, might release the enzymic activity from the inhibitory action of Ca^{2+} .

According to recent speculations (Helmreich, Danforth, Karpatkin & Cori, 1965), the turning on and off of the activity of muscle phosphorylase, during the contraction-relaxation cycle, would be mediated by the movements across the reticulum membranes of Ca^{2+} , which activates phosphorylase b kinase. 'Compartmentation' of phosphofructokinase in the sarcoplasmic reticulum away from phosphorylase, which is supposedly located in the ground substance external to the reticulum, and the effects exerted by Ca^{2+} in opposite directions

on these two enzymes, would provide an additional mechanism for their tightly co-ordinated changes in activity in stimulated frog muscle.

This work was supported by a grant from the Muscular Dystrophy Associations of America Inc.

- Amberson, W. R., Roisen, F. J. & Bauer, A. C. (1965). *J. cell. comp. Physiol.* **66**, 71.
- Andersson-Cedergren, E. & Muscatello, U. (1963). *J. Ultrastruct. Res.* **8**, 391.
- Czok, P. & Bücher, Th. (1960). *Advanc. Protein Chem.* **15**, 315.
- Ebashi, S. (1965). In *Molecular Biology of Muscular Contraction*, p. 197. Ed. by Ebashi, S., Oosawa, F., Sekine, T. & Tonomura, Y. Amsterdam: Elsevier Publishing Co.
- Fahimi, H. D. & Karnowsky, M. J. (1966). *J. Cell Biol.* **29**, 113.
- Gevers, W. & Krebs, H. A. (1966). *Biochem. J.* **98**, 720.
- Helmreich, E., Danforth, W. H., Karparkin, S. & Cori, C. F. (1965). In *Control of Energy Metabolism*, p. 299. Ed. by Chance, B., Estabrook, R. W. & Williamson, J. R. New York: Academic Press Inc.
- Ling, K. H., Marcus, F. & Lardy, H. A. (1965). *J. biol. Chem.* **240**, 1893.
- Lowry, O. H. & Passonneau, J. V. (1966). *J. biol. Chem.* **241**, 2268.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Mansour, T. E., Wakid, N. & Sprouse, H. M. (1966). *J. biol. Chem.* **241**, 1512.
- Margreth, A., Muscatello, U. & Andersson-Cedergren, E. (1963). *Exp. Cell Res.* **32**, 484.
- Muscatello, U., Andersson-Cedergren, E., Azzone, G. F. & Decken, A. von der (1961). *J. biophys. biochem. Cytol.* **10**, Suppl. 4, 201.
- Nanninga, L. B. (1961). *Biochim. biophys. Acta*, **54**, 338.
- Peachey, L. D. (1965). *J. Cell Biol.* **25**, 209.
- Pette, D. & Hofer, H. W. (1965). In *Control of Energy Metabolism*, p. 71. Ed. by Chance, B., Estabrook, R. W. & Williamson, J. R. New York: Academic Press Inc.
- Underwood, A. H. & Newsholme, E. A. (1965). *Biochem. J.* **95**, 868.

Biochem. J. (1967) **102**, 37c

Use of Iodide Salts in the Isolation of Antibodies and the Dissolution of Specific Immune Precipitates

By STRATIS AVRAMEAS and THÉRÈSE TERNYNCK

Laboratoire de Chimie des Protéines and Laboratoire d'Immunochimie, Institut de Recherches Scientifiques sur le Cancer, Villejuif (Val de Marne), France

(Received 22 December 1966)

The observation that concentrated solutions of sodium chloride inhibited the formation of specific pneumococcal polysaccharide-antibody precipitate (Heidelberger, Kendall & Teorell, 1936) led to methods employing concentrated sodium chloride solutions to dissociate specific pneumococcal precipitates or agglutinates (Heidelberger & Kendall, 1936; Heidelberger & Kabat, 1938). This method has been used also to dissociate specific protein immune precipitates (Oudin & Grabar, 1944). No attempts have been made to dissociate protein antigen-antibody complexes with other halogen salts after it was reported that thiocyanate, iodide and bromide ions inhibited the formation of egg albumin-rabbit antibody complexes (Kleinschmidt & Boyer, 1952a,b).

In the present report, iodide salt solutions were found effective in dissolving immune precipitates formed by protein antigens. By employing iodide salt solutions it was also possible to separate anti-

bodies from immunosorbents. These observations are the topic of this communication, in which the effect of iodide salts on isoagglutinins from human serum is also reported.

Methods. The salts used were of analytical grade when possible or of the highest purity available. Immuno-electrophoresis (Grabar & Williams, 1953), electrophoresis in polyacrylamide-agarose gel (Uriel, 1966), quantitative precipitation tests (Heidelberger & Kendall, 1935) and preparation of rabbit antisera (Avrameas & Ternynck, 1967) were carried out according to previously published methods. Insoluble human immunoglobulin G, used as immunosorbent for the isolation of antibodies to immunoglobulin G, was prepared by copolymerization at pH 4.7 of human immunoglobulin G and rabbit serum albumin, by using ethyl chloroformate as a polymerizing agent (Avrameas & Ternynck, 1966, 1967).

Dissolution of specific precipitates by halogen