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The Identification and Properties of Phosphatases in Skeletal Muscle with Activity towards the Inhibitory Subunit of Troponin, and their Relationship to other Phosphoprotein Phosphatases

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1. Phosphoprotein phosphatases with activity towards the inhibitory subunit of troponin (troponin I), phosphorylase *a* and lysine-rich histone (fraction F1) have been fractionated from rat skeletal muscle by chromatography on Sephadex G-200 and polylysine-Sepharose. Six separate fractions were identified on the basis of substrate specificity and behaviour during chromatography. 2. All fractions showed similar K_m values for any given protein substrate. The K_m for troponin I (5μM) was significantly lower than that previously reported. 3. Phosphatase activities towards troponin I and phosphorylase *a* did not show a requirement for bivalent-metal ions. Two of the fractions with only minor activity towards histone were activated by Mn^{2+}. 4. Discontinuous polyacrylamide-gel-electrophoresis studies indicated that several of the fractions contained more than one phosphatase activity, and additionally showed that several of the activities could exist in different aggregation states. On the basis of these studies at least two phosphatases with activity only towards troponin I were identified. In addition, phosphorylase phosphatase (which has considerable activity towards troponin I) and a general phosphatase with activity towards all three substrates were found. 5. A fraction with mol.wt. of 150000 could be activated by freezing with 2-mercaptoethanol or by heating to 55°C. This activation was accompanied by a decrease in mol.wt. to 25000. 6. The total amount of phosphatase with activity towards troponin I which was extracted would be sufficient to dephosphorylate all the troponin I present in skeletal muscle in approximately 10s.

Troponin, the Ca^{2+}-binding protein of myofibrils, has been shown to be phosphorylated by phosphorylase kinase and cyclic AMP-dependent protein kinase when isolated from skeletal and cardiac muscle (Bailey & Villar-Palasi, 1971; Stull *et al.*, 1972; Pratje & Heilmeyer, 1972; Perry & Cole, 1973; England *et al.*, 1973; Reddy *et al.*, 1973; Cole & Perry, 1975). Both the inhibitory subunit (troponin I) of mol.wt. 23000 (skeletal) or 28000 (cardiac), and the tropomyosin-binding subunit (troponin T) of mol.wt. 37000 (skeletal) or 41000 (cardiac) were reported to be phosphorylated by added kinase. In addition, these subunits were shown to contain endogenous phosphate when isolated (Stull *et al.*, 1972; Perry & Cole, 1973; Cole & Perry, 1975). A hypothesis was proposed that phosphorylation *in vivo* of troponin could modify its properties so as to allow an increase in contractility of muscle (Stull *et al.*, 1972; England *et al.*, 1973). This phosphorylation could be initiated by hormonal stimulation of muscle, particularly by catecholamines. Further evidence for this hypothesis was given by England (1975), who showed that phosphorylation of troponin-I was correlated with changes in contractility in perfused rat heart when stimulated by adrenaline. Also, Rubio *et al.* (1975) have shown a change in the Ca^{2+} sensitivity of cardiac native actomyosin on phosphorylation of troponin. At present, however, the situation is less clear in skeletal muscle (J. T. Stull, personal communication).

The hypothesis described above postulates a phosphorylation/dephosphorylation cycle which requires the presence in muscle of both a kinase and phosphatase active towards troponin, particularly troponin I. In a previous study (England *et al.*, 1972), it was found that a preparation of phosphorylase phosphatase from the 'glycogen particle' of skeletal muscle (Meyer *et al.*, 1970) was also active towards phosphorylated troponin I. This phosphatase preparation was not active towards phosphorylated histone (mixed fraction). However, several studies have suggested the presence in muscle of phosphoprotein phosphatases with broad specificities (Zieve & Glinsmann, 1973; Nakai & Thomas, 1973; Kato & Sato, 1974; Nakai & Thomas, 1974), although other reports have indicated more specific activities (Hurd *et al.*, 1966; Kato & Bishop, 1972; Antoniw & Cohen, 1975). Phosphoprotein phosphatases can also exist in multiple molecular forms (Fischer *et al.*, 1971;

Kato & Sato, 1974; Brandt *et al.*, 1974; Kato *et al.*, 1974; Huang & Glinsmann, 1975), some of which have been reported to be interconvertible.

In the present study we have investigated the nature of phosphoprotein phosphatase activities in skeletal muscle, with particular reference to activity towards troponin I. Additionally, we have attempted to identify various forms of phosphatase activity towards phosphorylase *a* and histone, to discover whether specific phosphatases exist towards the various substrates, or if general phosphatases with activity towards all substrates are present. A fractionation procedure was developed which separated a number of phosphatases of differing molecular size and substrate specificity. At each stage in the fractionation all phosphatase activity was retained, and not just the fractions of highest specific activity, so that minor activities were not lost. This has lead to the identification of phosphatases of both broad and narrow specificities, but extensive purification was not obtained. At least two phosphatases with activity mainly towards troponin I were found which were separate from phosphorylase phosphatase. In addition, a phosphatase with activity towards all three substrates was identified.

Materials and Methods

Materials

DEAE-cellulose (DE-52) was obtained from W. and R. Balston Ltd., Maidstone, Kent, U.K.; Sephadex G-200, Sepharose 4B and DEAE-Sephadex A50 were from Pharmacia (G.B.) Ltd., London, W.5, U.K. Polylysine (mol.wt. 15000–30000) was purchased from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. Enzymes and other biochemicals were from Boehringer Corp. (London) Ltd., Ealing, London W.5, U.K. Dowex AG-1 X4 was obtained from Bio-Rad Laboratories, St. Albans, Herts., U.K. Other reagents were of the highest purity obtainable.

Preparation of protein substrates and enzymes

Phosphorylase *b* was prepared as described by Fischer & Krebs (1958), and converted into [^{32}P]-phosphorylase *a* by incubation with [γ-^{32}P]ATP and phosphorylase kinase, essentially as described by Cohen (1973). Complete conversion of phosphorylase *b* into *a* was obtained. Excess ATP was removed by dialysis against Dowex AG-1 X4 (Cl^- form) in 20mM Tris/HCl, pH7.0/15mM-2-mercaptoethanol. Phosphorylase kinase was prepared as described by Cohen (1973), with the inclusion of sucrose in the final stages as described by Brostrom *et al.* (1971).

Histone (lysine-rich fraction, F1) was prepared from calf thymus as described by De Nooij & Westenbrink (1962). This contained approximately 0.2mol of protein-bound phosphate per mol of histone F1. A further 0.3–0.4mol of ^{32}P was incorporated by incubation with [γ-^{32}P]ATP and cyclic AMP-dependent protein kinase (Reimann *et al.*, 1971). After incubation the histone was precipitated with trichloroacetic acid and $(NH_4)_2SO_4$ (Kinkade & Cole, 1966), redissolved and dialysed against Dowex AG-1 X4 (Cl^- form). Cyclic AMP-dependent protein kinase (Peak I from the DEAE-cellulose column) was prepared as described by Reimann *et al.* (1971), but omitting the hydroxyapatite step.

Troponin was prepared from rabbit skeletal muscle essentially as described by Greaser & Gergely (1971). Troponin B was obtained from troponin by the method of Hartshorne & Mueller (1968). A typical preparation of troponin B contained 45% of troponin I, 45% of troponin T and 3% of troponin C, with only minor amounts of other contaminating proteins (Fig. 1*a*). Phosphorylation of troponin B was carried out by incubation with phosphorylase kinase

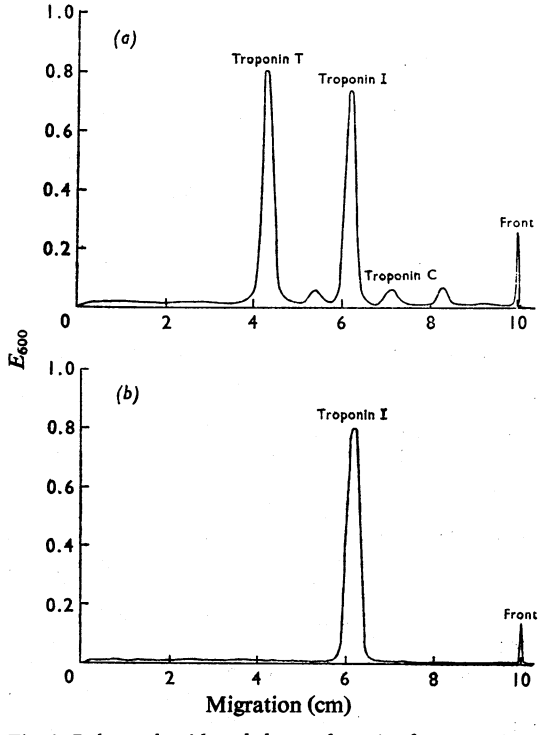


Fig. 1. *Polyacrylamide-gel electrophoresis of troponin B and troponin I from rabbit mixed-skeletal muscle*

Electrophoresis was carried out in 10% (w/v) polyacrylamide gel containing 0.27% (w/v) bisacrylamide, 50mM-sodium phosphate buffer pH7.0, 0.1% sodium dodecyl sulphate (Weber & Osborn, 1969). Gels (*a*) 20μg of troponin B, (*b*) 10μg of troponin I, were stained with Coomassie Brilliant Blue and the E_{600} recorded by using a Gilford linear-transport system attached to a spectrophotometer.

and [γ-^{32}P]ATP as described by Stull *et al.* (1972), but with the addition of 0.15M-KCl. After completion of the incubation, the protein was precipitated by addition of 2vol. of saturated $(NH_4)_2SO_4$ solution, and dialysed against 1mM-HCl. Analysis of the phosphorylated protein by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Weber & Osborn, 1969) showed that approximately 70% of the ^{32}P was present in troponin I, with only minor amounts in other proteins. From 0.5 to 1.0mol of ^{32}P/mol of troponin I could be incorporated.

Owing to the presence of minor phosphorylated components in preparations of troponin B, later experiments were carried out with phosphorylated troponin I. However, each of the experiments reported in the Results section was repeated with several different phosphatase preparations, with either troponin B or troponin I. Essentially no differences in the behaviour of the phosphatase preparations with the two substrates were seen, suggesting that minor phosphorylated proteins in troponin B were not significant substrates for the phosphatases under the assay conditions used. In view of this finding, the term 'troponin I phosphatase' is used in the text, even though some early experiments were performed with troponin B. Troponin I was prepared from troponin as described by Greaser & Gergely (1971) with the modification of Eisenberg & Kielly (1972). Figure 1*b* shows that the purity of troponin I obtained by this method was at least 95%. Phosphorylation of troponin I was carried out as described for troponin B, incorporating between 0.5 and 1.0mol of ^{32}P/mol of troponin I.

Assay of phosphoprotein phosphatase activity

For all incubations the buffer used was 50mM-Tris/HCl, pH7.5, containing 1mM-EDTA, 1mM-theophylline, 15mM-2-mercaptoethanol and 1mg of bovine serum albumin/ml. All incubations were carried out at 30°C. In incubations containing troponin B or troponin I, 0.1M-KCl was included to prevent precipitation of the troponin. All other additions or modifications are described in the Results and Discussion section. A total incubation volume of 60μl was used throughout. At the end of the incubation period the reaction was stopped by the addition of 220μl of silicotungstic acid (9g/litre of 70mM-sulphuric acid), and 20μl of bovine serum albumin (20mg/ml) at 0°C. After precipitation of the protein, ^{32}P in the supernatant was assayed by liquid-scintillation spectrometry. Control experiments showed that these conditions precipitated each of the phosphorylated substrates completely.

Initially, each phosphatase preparation or fraction was assayed to determine the linearity of the time-course of ^{32}P removal. It was found that approximately 20% of the protein-bound phosphate could be removed before the rate of ^{32}P removal became non-linear. Phosphatase fractions were subsequently diluted such that phosphate release was linear over a 3min incubation period. It has been shown (Moir *et al.*, 1974; Huang *et al.*, 1974) that skeletal-muscle troponin I was phosphorylated by phosphorylase kinase at two sites. The major site was a threonine residue, whereas the other site was a serine residue. Experiments with phosphorylase phosphatase (England *et al.*, 1972) showed that the threonine site was dephosphorylated at a much more rapid rate than the serine site. During a 3min incubation period at least 90% of the phosphate removed was from the threonine site. The use of short incubation times therefore suggests that the phosphatase activities measured in these experiments were likely to be specific for the threonine site.

Other methods

[γ-^{32}P]ATP was prepared as described by Glynn & Chappell (1964). Polylysine was coupled to Sepharose 4B as described by Porath *et al.*, (1967). Protein was assayed by the method of Lowry *et al.* (1951), and P_i as described by Itaya & Ui (1966). Discontinuous polyacrylamide-gel electrophoresis was carried out in Tris/HCl/glycine, pH8.9, by the procedure of Gabriel (1967). 30mM-2-mercaptoethanol was included in both pre-electrophoresis and running buffers. After electrophoresis, gels were either stained with Coomassie Brilliant Blue, or transversely sliced into 3mm sections and assayed for phosphatase activity. The slices were ground with 300μl of 0.3M Tris/HCl, pH6.0, and left to extract overnight at 4°C. Phosphorylated substrates were then added, and incubated at 30°C for 30min. The reaction was stopped by the addition of 1ml of silicotungstic acid and albumin as described above. $^{32}P_i$ in the supernatant was assayed by Čerenkov radiation.

Results and Discussion

Fractionation of phosphatase activity

Preparation of extract. Five male Wistar rats, weighing 200–300g, were decapitated and the hind-limb muscles rapidly removed and placed on ice. All subsequent steps were carried out at 0–4°C. The muscle was homogenized in 2.5vol. of 25mM-Tris/HCl, pH7.5, 4mM-EDTA, 15mM-2-mercaptoethanol, and centrifuged at 10000*g* for 30min. The pH of the supernatant was re-adjusted to 7.5, and $MgCl_2$ added to a final concentration of 5mM. This was centrifuged at 100000g for 90min, and the supernatant dialysed for a short period against 25mM-Tris/HCl, pH7.5, 1mM-EDTA, 5mM-$MgCl_2$, 30mM-2-mercaptoethanol.

DEAE-cellulose chromatography. The dialysed extract (approx. 400ml) was applied to a column (4cm×9cm) of DEAE-cellulose equilibrated with the dialysis buffer described above. The column was washed with 10 bed-vol. of the same buffer, and phosphatase activity eluted with 300ml of the same buffer containing 0.5M-NaCl. The protein was precipitated by addition of 2vol. of saturated $(NH_4)_2SO_4$ solution, collected by centrifugation, dissolved in 10ml of 50mM-Tris/HCl, pH7.5, 0.5M-NaCl, 60mM-2-mercaptoethanol, and dialysed briefly against the same buffer. This procedure was used simply as a method of removing contaminating protein, and not for fractionating phosphatase activities. Previous studies (Kato & Bishop, 1972; Abe & Tsuiki, 1974) have used DEAE-cellulose chromatography to partially separate phosphatase activities, but in the present study only a slight, variable separation could be obtained.

Sephadex G-200 chromatography. The dialysed solution from step 2 was applied to a 2cm×80cm column of Sephadex G-200 equilibrated with the dialysis buffer. Fig. 2 shows the profiles of phosphatase activities towards phosphorylase *a*, histone and troponin I. Three fractions of activity could be identified. Fraction I, which had a mol.wt. of greater than 250000 (calcd. from its elution volume), had activity predominantly towards troponin I, with little activity towards the other substrates. Fraction II was a broad region with mol.wt. from 50000 to 250000, which contained a distinct peak of phosphatase activity with mol.wt. of approx. 120000. This fraction showed activity towards all three substrates. Fraction III (mol.wt. 20000–50000) showed activity towards troponin I and phosphorylase *a*, but little activity towards histone. The contents from each fraction were pooled, dialysed against 50mM-Tris/HCl (pH7.5)/30mM-2-mercaptoethanol/0.3M-sucrose, and stored frozen until further fractionation.

Table 1 shows the total and specific activities of the three fractions from this step after dialysis, and also for the previous steps in the fractionation. Fraction I was a relatively minor fraction that contained less than 10% of the phosphatase activity towards troponin I. Fraction II contained most of the activity towards troponin I, all of the activity towards histone, and approximately 50% of the activity towards phosphorylase *a*. Fraction III contained the remainder of the activities towards troponin I and phosphorylase *a*. Previous reports have suggested a large range of molecular weights for phosphatase activities. Kato & Sato (1974), Kato *et al.* (1974) and Brandt *et al.* (1974) have reported phosphatase activities towards phosphorylase *a* and histone with mol.wts. of 150000 to 300000. Kato & Bishop (1972) found a mol.wt. of approx. 60000 for histone phosphatase from muscle, and phosphorylase phosphatase could be disaggregated to a form with a mol.wt. of 30000 (Brandt *et al.*, 1974). The present study appears to have separated phosphatase

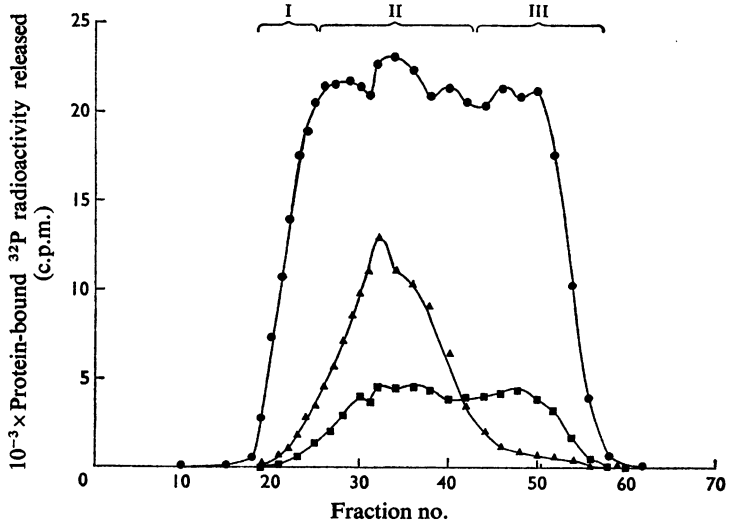


Fig. 2. *Separation of phosphoprotein phosphatase activities by chromatography on Sephadex G-200*

The dialysed protein from the previous DEAE-cellulose treatment (10ml) was applied to a column (2cm×80cm) of Sephadex G-200, and fractions (4.5ml) collected. Fractions were assayed as described in the Materials and Methods section with (●) 30μM-troponin I, (■) 20μM-phosphorylase *a*, (▲) 18μM-histone. Fractions were pooled as indicated.

Table 1. *Fractionation of phosphoprotein phosphatases from rat skeletal muscle*

Phosphatase fractions were prepared as described in the Results and Discussion section from 100g of rat skeletal muscle. The total enzyme activity towards each phosphoprotein was either assayed at, or has been calculated to be at, saturating concentration of substrate. The values quoted are means of four separate preparations.

Protein fraction	Total protein (mg)	Sp. activity (nmol/min per mg)			Total activity (μmol/min)			Phosphatase activity ratios		
		Phosphorylase *a*	Troponin I	Histone	Phosphorylase *a*	Troponin I	Histone	Phosphorylase / Histone	Troponin / Histone	Troponin / Phosphorylase
100000*g* supernatant	3200	2.02	13.81	0.472	6.5	44.2	1.52	4.3	29	6.8
100000*g* pellet	230	18.7	23.4	1.03	4.3	5.4	0.24	18	23	1.3
Pooled fractions from DEAE-cellulose	178	13.66	128.8	4.52	2.4	23.0	0.81	3.0	29	9.4
Sephadex G-200 fraction										
I	28	0.363	19.5	0.325	0.01	0.54	0.009	1.1	58	54
II	77	6.14	130	5.14	0.47	10.0	0.40	1.2	25	21
III	17	20.2	37.9	1.95	0.35	0.64	0.033	10.3	20	1.9
Polylysine column fractions										
Ia	9.0	1.25	29.7	0.838	0.011	0.27	0.008	1.5	36	24
IIa	22	0.192	2.73	0.101	0.007	0.06	0.003	1.8	14	27
IIb	12	11.0	50.7	3.24	0.13	0.60	0.039	3.34	16	4.6
IIc	21	20.5	339	12.0	0.44	7.2	0.25	1.7	28	17
IIIa	8.5	16.3	27.9	1.67	0.14	0.23	0.014	9.8	17	1.7
IIIb	1.7	47.4	88.7	3.04	0.08	0.015	0.005	16	29	1.9

activities with molecular weights distributed over a wide range. The three fractions could represent different aggregation states (Brandt *et al.*, 1974) although their different substrate specificities suggest more fundamental differences, as do results presented below.

Polylysine–Sepharose 4B chromatography. The dialysed fractions from step 3 were each applied to columns (1 cm × 25 cm) of polylysine–Sepharose 4B equilibrated with 50 mM-Tris/HCl (pH 7.5)/30 mM-2-mercaptoethanol. The columns were washed with 1 bed-vol. of the same buffer, and phosphatase activity eluted with a linear gradient to 1.2 M-NaCl in the same buffer. Fig. 3 shows the distribution of phosphatase activities for each of the three fractions from step 3. Fraction I gave a single peak of phosphatase activity, predominantly towards troponin I (Fraction Ia). The phosphatase activities towards phosphorylase *a* and histone coincided with this, but were at least 20-fold lower. Fraction II showed a more complex profile of activity. An initial shoulder had phosphatase activity predominantly towards troponin I (fraction IIa), followed by two peaks of activity towards all three substrates (fractions IIb and IIc). Fraction III showed two peaks of activity (fractions IIIa and IIIb), mainly towards troponin I and phosphorylase. The pooled fractions were dialysed and stored frozen in the presence of 0.3 M-sucrose.

Table 1 shows the specific activities and ratios of activities towards the substrates for the phosphatase fractions obtained from polylysine–Sepharase chromatography. Recoveries of phosphatase activity from this step were at least 60%. Fraction IIc contained the highest proportion of activity towards all three substrates, and also the highest specific activities towards troponin I and histone. Fraction IIIb had the highest specific activity towards phosphorylase *a*. The fractions showing activity predominantly towards troponin I (Ia and IIa) were relatively minor fractions containing appreciable quantities of contaminating protein.

The fractionation using polylysine–Sepharose was undertaken because Gratecos *et al.* (1973) had reported that phosphorylase phosphatase had a high affinity for this material. This appeared to involve ion-exchange chromatography, although for elution of phosphatase activity, a higher ionic strength buffer was required than for DEAE-cellulose. By using separation procedures based on molecular size and affinity for anion-exchange material, six separate phosphatase activities were separated, showing different substrate specificities. Overall the recovery of phosphatase activity was approximately 20%, with the largest losses occurring during the chromatography on DEAE-cellulose and Sephadex G-200. It is therefore possible that very labile phosphatase activities were lost during this procedure, and that the six fractions represent only part of the total activity originally extracted.

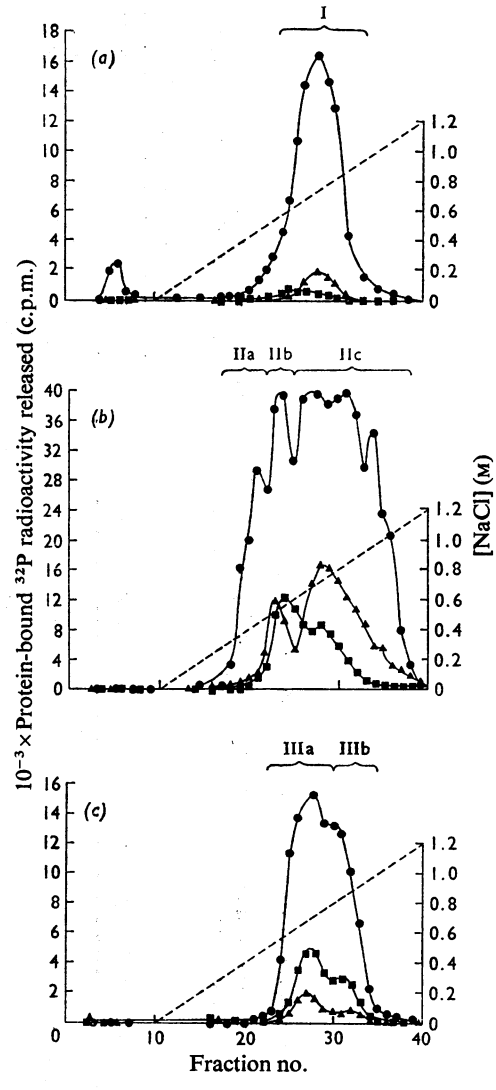


Fig. 3. *Separation of phosphoprotein phosphatase fractions by chromatography on polylysine–Sepharose*

Each pooled fraction separated by Sephadex G-200 chromatography was applied to a column (1 cm × 25 cm) of polylysine–Sepharose. After an initial wash of 1 column-volume, phosphatase activity was eluted with a linear gradient to 1.2 M-NaCl. Fractions (4 ml) were collected and pooled as indicated. Phosphatase activities were assayed as described in the Materials and Methods section with (●) 30 μM-troponin I, (■) 20 μM-phosphorylase *a*, (▲) 18 μM-histone; ----, NaCl concn. (*a*) Sephadex G-200 fraction I; (*b*) fraction II; (*c*) fraction III.

Kinetic properties of phosphatase fractions

Distribution of phosphatase activity. During the initial stages of fractionation, a 100000*g* centrifugation step was included. The pellet from this step contained appreciable quantities of glycogen, and was probably similar to the 'glycogen particle' of Meyer *et al.* (1970). As this had a relatively high phosphorylase phosphatase activity, the 100000*g* pellet of the present study was also examined. The pellet was resuspended in 12vol. of buffer and diluted a further 40–400 times for assay. This dilution was sufficient to dissociate phosphorylase phosphatase from the glycogen (Haschke *et al.*, 1970), and hence allowed total activities to be measured. The amount of phosphatase activity is shown in Table 1. Approx. 40% of the activity towards phosphorylase *a* was present in the pellet, whereas only 12% of the activities towards histone and troponin I were sedimented. Thus most of the latter two activities were retained for further fractionation. This result also indicates the presence in skeletal muscle of a phosphatase with activity towards troponin I separate from phosphorylase phosphatase.

Determination of Michaelis constants. K_m values for the phosphatase fractions were determined by varying the substrate concentrations from $0.25\times K_m$ to at least $2\times K_m$. Data were fitted to the Michaelis equation by weighted linear regression as described by Cleland (1967). Visual inspection of double-reciprocal plots of the data showed no departure from linearity except for fractions IIIa and IIIb with histone as the substrate. In these cases substrate inhibition occurred at concentrations of protein-bound phosphate greater than 10μM ($>2\times K_m$), and in calculation of K_m values these concentrations were ignored. The effect was previously noted by Kato & Sato (1974). Table 2 shows the K_m values for the fractions from polylysine–Sepharose chromatography. The values are expressed in terms of protein-bound [^{32}P]phosphate, and not on the basis of total-phosphate concentration. There appeared to be little variation in the K_m values between fractions for any of the substrates, suggesting that if the various fractions are different phosphatases rather than different forms of the same phosphatase, this is not reflected in the K_m values. The K_m values obtained with histone as substrate were similar to those reported in rabbit skeletal muscle (Kato & Bishop, 1972) and liver (Kato & Sato, 1975), and bovine heart (Nakai & Thomas, 1974). The K_m values with troponin I were lower than those previously reported for rabbit skeletal-muscle phosphorylase phosphatase (England *et al.*, 1972). In this previous study the K_m with troponin was higher than that with phosphorylase *a*, whereas the reverse was true in the present study. This is possibly due to the use of the 'glycogen particle' as a source of phosphatase by England *et al.* (1972), whereas the results reported in the present paper were obtained with soluble phosphatase. The K_m values with phosphorylase *a* were similar to those previously reported (Kato *et al.*, 1974).

Table 2. *K_m values for phosphatase fractions from polylysine–Sepharose chromatography*

K_m values were determined as described in the Results and Discussion section. Results from at least three different preparations were combined for the calculation of these values. K_m values are given as means ± S.D. with a minimum of 15 substrate concentrations for each determination.

Fraction	Phosphatase K_m (μM) with as substrate: Phosphorylase *a*	Troponin I	Histone F1
Ia	12.7±8.5	4.1±0.8	4.9±0.7
IIa	19.9±5.4	6.8±1.4	7.5±2.1
IIb	12.4±1.5	5.4±1.5	4.7±1.8
IIc	12.3±2.3	3.9±0.7	1.3±0.5
IIIa	6.8±0.7	5.7±1.2	6.5±0.8
IIIb	8.9±1.7	4.3±0.7	2.2±1.0

Table 3. *Effect of Mn^{2+} on the activities of phosphatase fractions*

Fractions were assayed with the various substrates in the presence of EDTA (1 mM) or $MnCl_2$ (1 mM) as described in the text. Results are given as the ratio of activity with Mn^{2+} to that with EDTA, and are the mean results of two separate phosphatase preparations.

Fraction	Ratios of phosphatase activities (Mn^{2+}/EDTA) with as substrate: Phosphorylase *a*	Troponin I	Histone F1
Ia	1.2	1.1	0.9
IIa	1.6	1.7	1.4
IIb	0.9	1.2	1.3
IIc	1.0	1.3	1.2
IIIa	1.9	2.7	65.0
IIIb	0.6	1.1	3.1

Effects of Mn^{2+}. Several previous studies of phosphoprotein phosphatase have indicated either a total or partial dependence on Mn^{2+} (Kato & Bishop, 1972; Kato & Sato, 1974; Nakai & Thomas, 1974). This effect was noted when either glycogen synthase I or histone was the substrate, whereas with phosphorylase *a*, Mn^{2+} at high concentrations caused an inhibition of phosphatase activity. The effects of Mn^{2+} and EDTA on the phosphatase activities from the polylysine–Sepharose chromatography were therefore investigated. Table 3 shows the ratios of activities for each fraction when incubated with 1 mM-Mn^{2+} or 1 mM-EDTA. With both troponin I and phosphorylase *a* as substrates, Mn^{2+} caused a small activation only with fractions IIa and IIIa, and no significant inhibition was seen. When histone was

the substrate, Mn^{2+} caused an activation with fractions IIIa and IIIb only; fraction IIIa showed a very large activation, mainly because there was negligible activity without the metal ion. However, even after activation, the activities of fractions IIIa and IIIb towards histone were low compared with the other substrates. The major phosphatase activities towards all three substrates did not therefore show any significant requirement for Mn^{2+}, or for any other metal ion, since the presence of EDTA did not inhibit the activity (results not shown). These results do not preclude the possibility of the phosphatases containing a tightly bound metal ion.

Polyacrylamide-gel electrophoresis of phosphatase fractions

Several of the fractions from the polylysine–Sepharose chromatography showed substantial activity towards all three protein substrates. To test whether each fraction contained phosphatase(s) with activity towards each substrate, or separate specific phosphatases, electrophoretic studies were carried out. The phosphatase fractions were subjected to discontinuous polyacrylamide-gel electrophoresis, the gels sliced transversely, and the sections assayed for phosphatase activity as described in the Materials and Methods section. The patterns of phosphatase

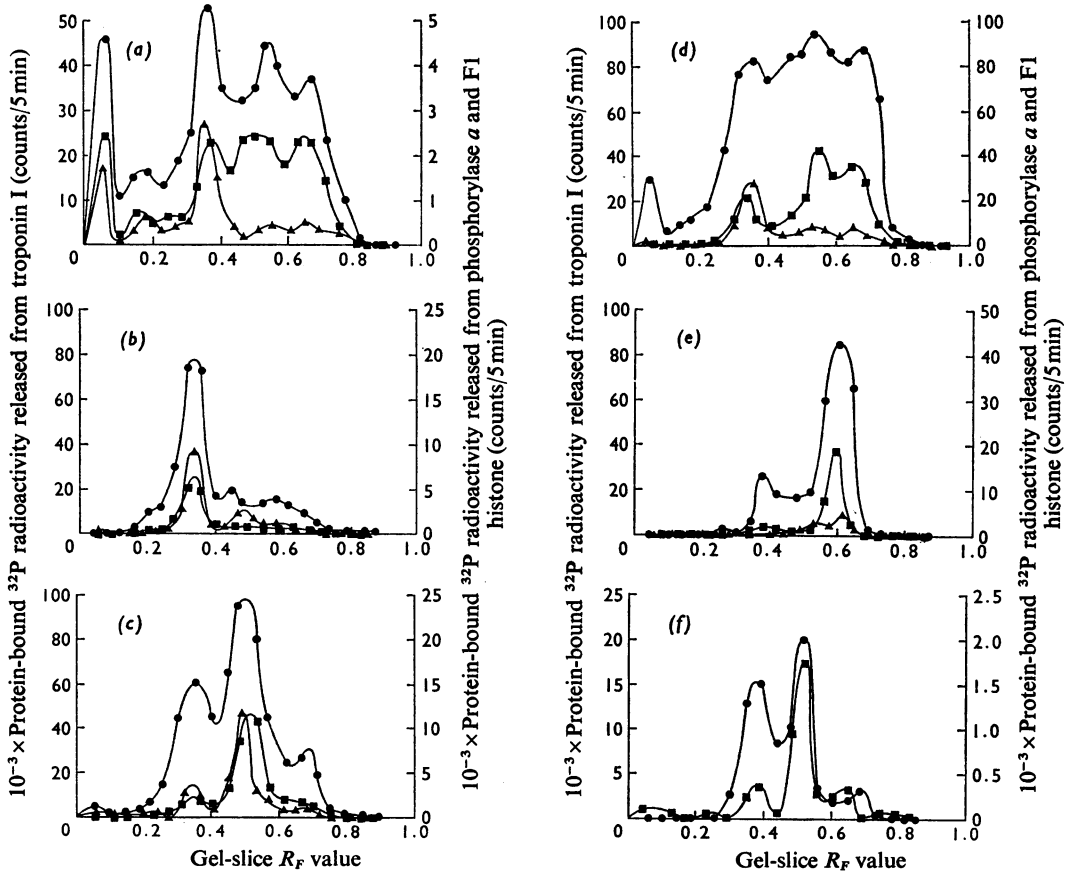


Fig. 4. *Discontinuous polyacrylamide-gel electrophoresis of phosphatase fractions from polylysine-Sepharose chromatography*

A portion (100μl) of each phosphatase fraction separated by polylysine–Sepharose chromatography was applied to 7.5% (w/v) polyacrylamide gels (0.6cm × 10cm). Electrophoresis was carried out at 4°C for 3h at 1.5mA per tube. Gels were sliced into 3mm sections and assayed as described in the Materials and Methods section with (●) 2μM-troponin I, (■) 2μM-phosphorylase *a*, (▲) 3μM-histone. (*a*) Fraction Ia from polylysine–Sepharose chromatography; (*b*) fraction 2a; (*c*) fraction 2b; (*d*) fraction 2c; (*e*) fraction 3a; (*f*) fraction 3b.

activities are shown in Fig. 4, and it can be seen that several of the fractions show several areas of activity.

Fractions Ia and IIa were found to contain a phosphatase that was active towards troponin I only. However, as Figs. 4(*a*) and 4(*b*) show, there were considerable differences between these fractions on electrophoresis. Fraction Ia showed several bands of activity, whereas fraction IIa showed one major band which migrated with the same R_F value as the main band of fraction Ia. It is therefore possible that these two fractions contained the same phosphatase in different states of aggregation, although these results could be explained if fraction Ia contained a phosphatase additional to that in fraction IIa. Previous reports (Brandt *et al.*, 1974; Huang & Glinsmann, 1975) have shown that phosphatases can exist in different aggregation states, and evidence to this effect is also presented below.

Fraction IIb showed two peaks of activity towards all three substrates. There was an initial peak, which on the basis of mobility and ratio of activities towards the substrates appeared to be the same as that identified in fractions Ia and IIa. The second peak (R_F value 0.5) had considerable activity towards all three substrates. Fraction IIc gave a complex pattern of activity on electrophoresis. Apart from an initial peak of activity (R_F 0.05) which was probably aggregated material, there was a peak of activity with R_F value 0.36, which had activity towards all three substrates. This showed similar ratios of phosphatase activities towards the substrates as the peak of fraction IIb with R_F value 0.5, and could possibly have been a different aggregation state of the same general phosphatase. The other two peaks of fraction IIc (R_F values 0.5 and 0.7) had no activity towards histone.

Fraction IIIa had a major peak of activity towards troponin I and phosphorylase *a* with an R_F value of 0.6, plus a small activity towards troponin I with a lower mobility (R_F value 0.36). The latter was also seen with fraction IIIb, which in addition had a major peak of activity with an R_F value of 0.5. The peak with R_F value 0.36 appeared to be similar in both fractions to the peak with the same mobility as fraction IIa. However, it is unlikely that fraction IIIa is a low-molecular-weight form of fraction IIa, as when fraction IIa was converted into a molecule of molecular weight similar to that of fraction IIIa (see below), its R_F value on electrophoresis was then 0.65. Also, fractions IIIa and IIIb had little or no activity towards histone, whereas fraction IIa had a small, but detectable, amount of this activity. The peak of fraction IIIa with an R_F value of 0.6 was similar in ratios of activities towards phosphorylase *a* and troponin I as the region of fraction IIc with R_F values of 0.5–0.7.

Activation of phosphatase fractions

Kato *et al.* (1974) and Brandt *et al.* (1974) have reported that certain forms of phosphoprotein phosphatases could be activated by treatment with high concentrations of 2-mercaptoethanol or ethanol-plus-ammonium sulphate. This was associated with a decrease in molecular weight of the phosphatase. The phosphatase fractions prepared above were tested in a similar manner to compare their behaviour with that observed previously, and also to show differences between the fractions. In addition, the effect of incubation at elevated temperatures was investigated.

The effect of 2-mercaptoethanol was tested by freezing the various phosphatase fractions at −20°C overnight in the presence of 200mM-2-mercaptoethanol (Kato *et al.*, 1974). After thawing, the fractions were diluted and assayed for activity; the maximum concentration of 2-mercaptoethanol present during the assay was 50mM. All fractions except IIa showed no change in activity with all three substrates after treatment. Fraction IIa showed an increase in activity from three- to seven-fold with all three substrates. The activated fraction IIa was subjected to discontinuous polyacrylamide-gel electrophoresis, and showed a major peak of activity (R_F 0.65) plus a small amount of activity of low mobility which was probably polymerized material (Fig. 5*b*). The untreated fraction IIa had a single peak of activity with an R_F value of 0.35 (Fig. 5*a*). This suggested a decrease in molecular weight which was investigated further by chromatography on Sephadex G-200. The untreated fraction showed a single peak with a mol.wt. of approx. 150000, whereas the treated fraction gave a peak with mol.wt. 25000. This change in molecular weight was not associated with any change in the ratios of phosphatase activities towards the three substrates. Kato & Sato (1974) also reported a decrease in molecular weight on treatment of phosphatase from muscle with 2-mercaptoethanol, but this only resulted in a decrease in mol.wt. from 300000 to 150000.

The effect of elevated temperature on the phosphatase activities were investigated by incubating undiluted fractions at 55°C for up to 16min, rapidly diluting into cold buffer, and assaying for phosphatase activity at 30°C. All fractions except fraction IIa showed an inactivation of phosphatase with all substrates, whereas fraction IIa showed a three- to eight-fold increase in activity towards troponin I and phosphorylase *a*, but a slight inactivation of activity towards histone. With all fractions, the activity towards histone was more labile than that towards the other substrates, suggesting that this activity could be distinct from the other phosphatases. Detailed analysis of the results of heat treatment was not feasible because of the possibility of some fractions containing proteinases which could have contributed to the heat lability. Electrophoresis of the activated fraction IIa (Fig. 5*c*) again showed the presence of a peak of activity with an R_F value of 0.65, and gave a mol.wt. of 25000 by Sephadex G-200 chromato-

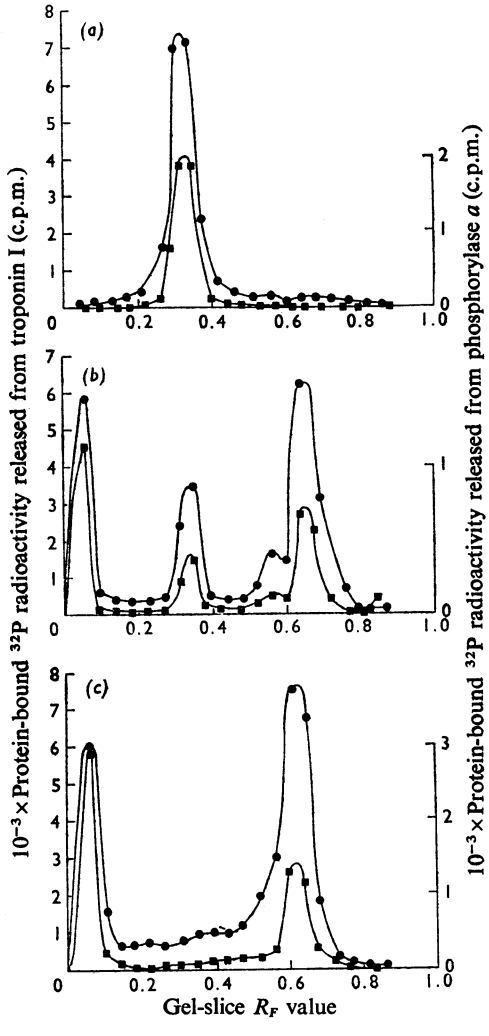


Fig. 5. *Polyacrylamide-gel electrophoresis of fraction IIa after activation by elevated temperature or treatment with 2-mercaptoethanol*

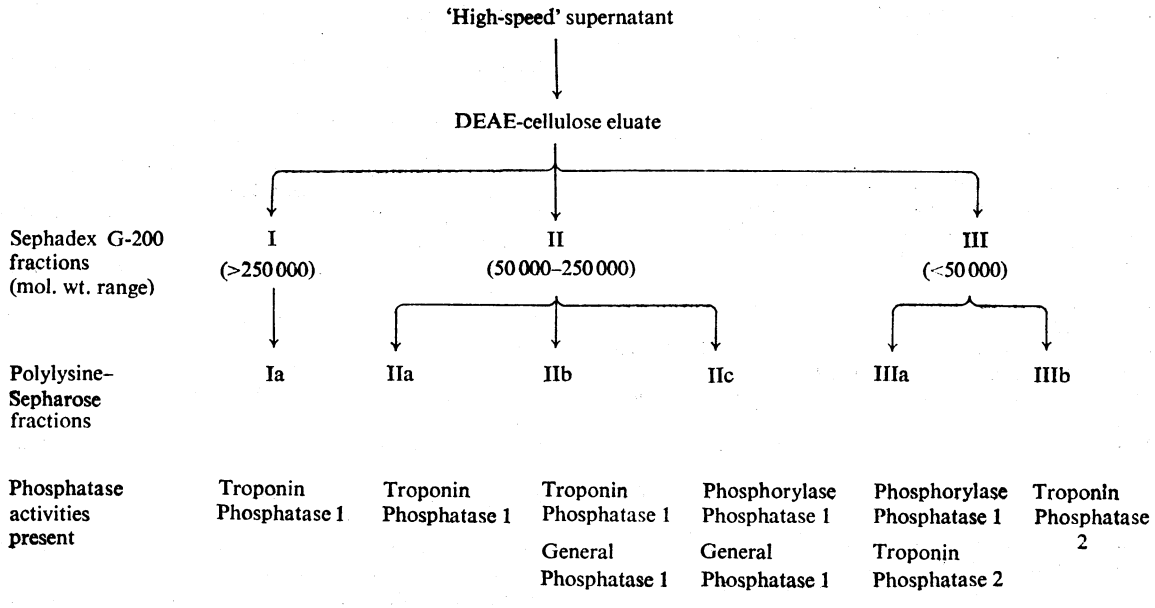
Fraction IIa from polylysine–Sepharose chromatography (*a*) was activated by heating for 16min at 55°C (*b*), or by freezing in 0.2M-2-mercaptoethanol (*c*). Samples (50μl) were subjected to electrophoresis on polyacrylamide gels as described in the legend to Fig. 3. Sections were assayed with (●) 2μM-troponin I, (■) 3μM-phosphorylase *a*.

graphy. It thus appeared that fraction IIb could be activated by treatment with 2-mercaptoethanol or at elevated temperatures, both of these causing a decrease in mol.wt. to an active unit of 25000. Brandt *et al.* (1974) and Huang & Glinsmann (1975) have found an active phosphatase unit of similar molecular weight, and have interpreted their results as the association of this unit with an inhibitory unit, this complex being dissociated on activation. The results presented above are consistent with this hypothesis, although the effect was seen only with fraction IIa.

General discussion

The fractionation of phosphatase activities described in the present paper lead to the separation of six fractions which were distinct on the basis of molecular size, affinity for anion-exchange material, and substrate specificity. A summary of this separation is given in Scheme 1. The results of the electrophoretic studies, however, showed that several of these fractions appeared to contain more than one phosphatase activity. In addition, it appeared that the phosphatases could exist in multiple aggregation states, possibly in combination with other proteins (Brandt *et al.*, 1974; Huang & Glinsmann, 1975; Brandt *et al.*, 1975). These complications have made it difficult to identify the actual number of different phosphatases present, but the classification below and shown in Scheme 1 is the simplest that is consistent with the data presented above. Fractions Ia and IIa appeared to contain a phosphatase with activity predominantly towards troponin I, but with only minor activity towards the other two substrates. This is designated 'phosphatase TP-1'. Fractions IIb and IIc contained a general phosphatase with substantial activity towards all three substrates ('phosphatase GP-1'). It is possible that more than one phosphatase was present in this activity, although the experiments above failed to separate or distinguish them. Fraction IIb also contained phosphatase TP-1. In addition, fraction IIc contained a phosphatase with a much higher ratio of activity towards phosphorylase *a* compared with troponin I than the other phosphatases ('phosphatase PP-1'). This activity was probably phosphorylase phosphatase, which is known to have substantial activity towards troponin I (England *et al.*, 1972). Fraction IIIa also contained an appreciable amount of phosphatase PP-1. Fractions IIIa and IIIb also contained an activity similar to phosphatase TP-1, although showing a lower electrophoretic mobility in spite of a lower molecular weight. This activity has therefore been designated 'phosphatase TP-2'. The above classification assumes that some phosphatases exist in multiple forms, so that electrophoretically distinct peaks with very similar ratios of activities to the substrates have been assumed to represent the same phosphatase.

Previous reports have suggested the presence of a phosphatase with a broad substrate specificity (Kato & Bishop, 1972; Zieve & Glinsmann, 1973; Nakai & Thomas, 1973; Kato & Sato, 1974; Nakai & Thomas, 1974). The presence of this phosphatase in skeletal muscle has been confirmed in the present study, but, in addition, other phosphatases of greater specificity have also been identified. The discrepancy between



Scheme 1. *Fractionation of phosphatase activities of skeletal muscle*

reports of specific and non-specific phosphatases could then be explained as the isolation of different phosphatases with different properties. It appears that muscle contains both high- and low-specificity phosphatases, which could have distinct roles in the control of protein dephosphorylation in the cell.

The present study has shown the appreciable activity and general distribution of phosphatase activity towards troponin I. This included both specific and non-specific activities. The total measured activity towards troponin I in crude muscle 'low-speed' supernatants was approximately 0.5 μmol of phosphate removed/min per g of fresh muscle. The calculated concentration of troponin I in skeletal muscle is 50–80 μmol/g, which is equivalent to a concentration of approximately 0.2 mM. Therefore if all the troponin I were phosphorylated with 1 mol of phosphate/mol of troponin I, dephosphorylation could occur in 10 s. This calculation assumes that all the phosphatase was active at the same time, and that access to the troponin I was not restricted. Although neither of these assumptions can be proved at present, this calculation gives a minimum time for troponin I dephosphorylation *in vivo*, and suggests that it could occur on a physiologically realistic timescale. It is not clear at present whether phosphorylation of troponin I occurs physiologically in skeletal muscle (J. T. Stull, personal communication), although the discovery of phosphatases active towards troponin I suggests a possible role in this tissue. Phosphorylation of troponin I in cardiac muscle under physiological conditions has, however, been demonstrated (England, 1975).

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