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Isolation and Characterization of Rabbit Skeletal Muscle Protein Phosphatases C-I and C-II*

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Previous studies have shown that phosphorylase phosphatase can be isolated from rabbit liver and bovine heart as a form of M_r ~ 35,000 after an ethanol treatment of tissue extracts. This enzyme form was designated as protein phosphatase C. In the present study, reproducible methods for the isolation of two forms of protein phosphatase C from rabbit skeletal muscle to apparent homogeneity are described. Protein phosphatase C-I was obtained in yields of up to 20%, with specific activities toward phosphorylase *a* of 8,000–16,000 units/mg of protein. This enzyme represents the major phosphorylase phosphatase activity present in the ethanol-treated muscle extracts. The second enzyme, protein phosphatase C-II, had a much lower specific activity toward phosphorylase *a* (250–900 units/mg). Phosphatase C-I and phosphatase C-II had M_r = 32,000 and 33,500, respectively, as determined by sodium dodecyl sulfate disc gel electrophoresis. The two enzymes displayed distinct enzymatic properties. Phosphatase C-II was associated with a more active alkaline phosphatase activity toward *p*-nitrophenyl phosphate than was phosphatase C-I. Phosphatase C-II activities were activated by Mn^{2+}, whereas phosphatase C-I was inhibited. Phosphatase C-I was inhibited by rabbit skeletal muscle inhibitor 2 while phosphatase C-II was not inhibited. Both enzymes dephosphorylated glycogen synthase and phosphorylase kinase, but displayed different specificities toward the α- and β-subunit phosphates of phosphorylase kinase (Ganapathi, M. K., Silberman, S. R., Paris, H., and Lee, E. Y. C. (1980) *J. Biol. Chem.* 246, 3213–3217). The amino acid compositions of the two proteins were similar. Peptide mapping of the two proteins showed that they are distinct proteins and do not have a precursor-proteolytic product relationship.

Phosphorylase phosphatase was the first of the enzymes which are responsible for the reversible interconversion of proteins between modified and unmodified forms to be discovered (1). Despite their early history, the protein phosphatases involved in the interconversion of the enzymes of glycogen metabolism have proved to be difficult to isolate, and there exist a number of conflicting reports regarding the enzymology of these phosphatases (for reviews, see Refs. 2–6). Earlier studies from this laboratory had led to the first isolation of a phosphorylase phosphatase, as an enzyme of M_r ~ 35,000 from rabbit liver (7). The purification procedure utilized a treatment with ethanol which was shown to result in a size reduction of the enzyme activity in tissue extracts (7, 8). Similar enzymes could also be isolated from heart tissues (9–12). This enzyme has been termed protein phosphatase C (3, 9). It was recognized at the outset that the M_r ~ 35,000 enzyme form was not the native enzyme, and a number of studies have indicated that it represents either the catalytic subunit or a proteolytic derivative of a larger enzyme (2, 3, 13, 14).

The M_r ~ 35,000 type of phosphatase has been isolated by independent purification methods from rabbit liver (15) and from rabbit skeletal muscle (16). The rabbit liver phosphorylase phosphatases have been shown to be enzymes of broad specificity, and have been shown to dephosphorylate glycogen synthase and phosphorylase kinase (15, 17). These studies have been important in establishing the broad specificity of the protein phosphatases involved with the dephosphorylation of the enzymes of glycogen metabolism.

Since the rabbit skeletal muscle enzymes involved in the regulation of glycogen metabolism have been extensively studied, the properties of the phosphatases of this tissue are of particular interest. The rabbit skeletal muscle phosphorylase phosphatase of Gratecos *et al.* (16) was isolated as protein of M_r = 33,000. This enzyme preparation was studied as a phosphorylase phosphatase but it was reported not to act on phosphorylase kinase in the absence of divalent cations. Other studies of the specificity of the protein phosphatases of glycogen metabolism in rabbit muscle have largely been based on the study of partially purified enzymes. This includes the various enzyme preparations categorized as "protein phosphatase 1," which has been designated as the "multifunctional" phosphatase of glycogen metabolism (5, 6, 18). A homogeneous preparation of a M_r = 70,000 rabbit skeletal muscle enzyme, termed F_C, has been shown to act on glycogen synthase and phosphorylase and is dependent on ATP-Mg and a protein factor (13, 19, 20).

Our studies have been directed toward the isolation of the protein phosphatases by reproducible methods and the examination of the properties of the homogeneous proteins. In this paper, we report methods for the isolation of two such

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M_r ~ 35,000 rabbit skeletal muscle protein phosphatases which can be isolated from ethanol-treated rabbit skeletal muscle extracts as well as an investigation of the properties of the purified proteins.

EXPERIMENTAL PROCEDURES[1]

RESULTS

The Separation and Isolation of Two Forms of Rabbit Skeletal Muscle Protein Phosphatase C—The procedure developed for the isolation of phosphorylase phosphatase as the M_r = 35,000 form is based on that which was used for the isolation of the enzyme from rabbit liver (7) and heart muscle (9). The procedure reported here (see "Experimental Procedures") involves essentially four steps and provides reproducible methods for the isolation of two enzymes (see below) in the pure state. The crude rabbit muscle extract was precipitated with ammonium sulfate, and then subjected to ethanol treatment. The enzyme preparation was then chromatographed on DEAE-Sepharose, Sephadex G-75 (superfine), and DEAE-Sepharose (see "Experimental Procedures").

The elution of phosphorylase phosphatase activity during the initial DEAE-Sepharose chromatography is shown in Fig. 1. During this step, as in all the purification steps described, the activities toward phosphorylase *a* and histone, as well as an alkaline phosphatase activity were usually assayed. The alkaline phosphatase activity is one which dephosphorylated *p*-nitrophenyl phosphate at an alkaline pH in a Mg^{2+}-dependent reaction and was first reported in association with the M_r = 35,000 phosphorylase phosphatase isolated from canine heart (21). The phosphorylase phosphatase activity eluted as a single major peak (*I*, Fig. 1) which is clearly separated from the major alkaline phosphatase activity (*II*, Fig. 1) which eluted at a higher salt concentration. Histone phosphatase activity was associated with both peaks. (It was also occasionally observed that there appeared an earlier eluting fraction of histone phosphatase activity. The latter was not studied in detail.) These findings differ from those of Li *et al.* (21) who reported the close association of a single M_r = 35,000 form of phosphorylase phosphatase with alkaline phosphatase in heart.

Both peak I and peak II fractions can be purified to apparent homogeneity, and are designated as protein phosphatase C-I and protein phosphatase C-II, respectively. The data for a typical preparation of protein phosphatase C-I are shown in Table I. Subsequent to the initial DEAE-Sepharose chromatography the peak I enzyme fractions were subjected to gel filtration on Sephadex G-75 and a rechromatography on DEAE-Sepharose (see "Experimental Procedures"). The major phosphorylase phosphatase fraction was always associated with alkaline phosphatase and histone phosphatase activity. A typical purification yielded 0.6 mg of protein at a specific activity of 11,300 units/mg representing a 19% recovery and an apparent purification of 15,000-fold (Table I). Some loss of specific activity was occasionally sustained during the final step, as shown in the example given in Table I. The specific activity of this preparation of C-I toward histone was 490 units/mg, and toward *p*-nitrophenyl phosphate it was 3,740 units/mg. Relatively small increases in total activity were observed during the ethanol precipitation step, in contrast to previous experience with the liver enzyme preparations (7).

Phosphatase C-II was isolated by essentially the same procedures as for phosphatase C-I. This involved gel filtration on Sephadex G-75 (Fig. 9; "Experimental Procedures") and chromatography on DEAE-Sephadex. On Sephadex G-75, this activity eluted at the same volume as phosphatase C-I (Fig. 8; "Experimental Procedures"). Although phosphatase C-II displayed a high alkaline phosphatase activity (by comparison to phosphatase C-I), it was associated with protein phosphatase activities toward phosphorylase *a* and histone throughout purification. Purification data for a typical preparation of phosphatase C-II are shown in Table II. This preparation had a specific activity toward phosphorylase *a* of 555 units/mg of protein, toward histone of 580 units/mg of protein, and, as an alkaline phosphatase, of 30,000 units/mg of protein. Phosphatases C-I and C-II were very similar in their behavior during purification except for their clear separation on ion-

[1] Portions of this paper (including "Experimental Procedures" and Figs. 8–11) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-774, cite the authors, and include a check or money order for $5.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

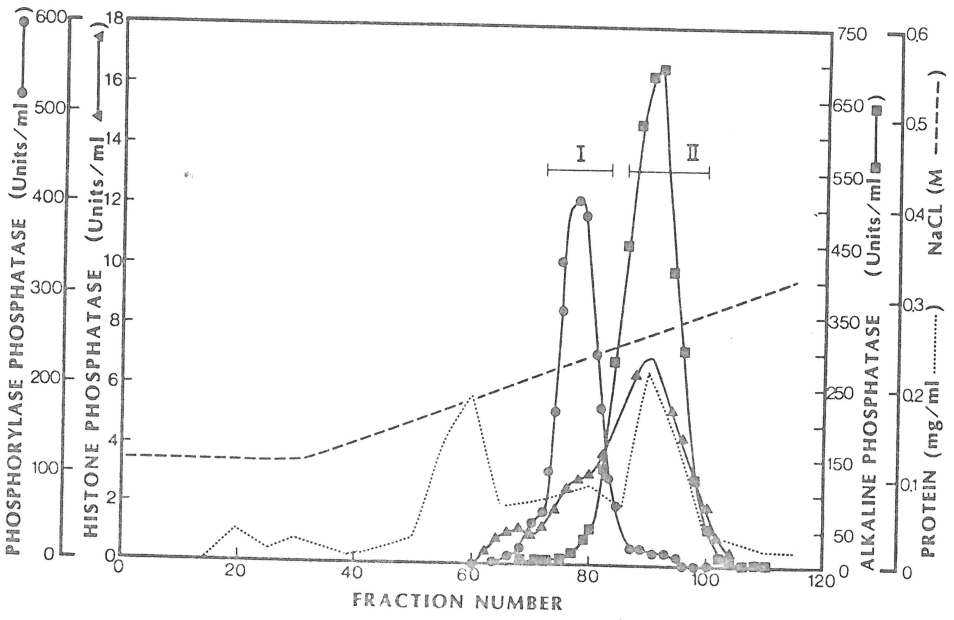

FIG. 1. **DEAE-Sepharose chromatography of ethanol-treated rabbit muscle extracts.** This shows a typical profile of protein phosphatase activities obtained after gradient elution on DEAE-Sepharose column chromatography. Conditions are as described under "Experimental Procedures." Fractions of 11 ml were collected and assayed for phosphorylase phosphatase (●), histone phosphatase (▲), and alkaline phosphatase (■) activity. Fractions 72–83 (peak I) and 86–100 (peak II) were pooled as indicated by the *horizontal bars*.

TABLE I

Purification of rabbit skeletal muscle protein phosphatase C-I

Rabbit skeletal muscle protein phosphatase C-I was purified as described under "Experimental Procedures." The table lists recoveries of protein and phosphorylase phosphatase activity for the five main steps in the procedure.

Purification step	Protein	Activity	Specific activity	Purification	Yield
	mg	*units*	*units/mg*	*-fold*	*%*
Crude extract	47,000	36,000	0.8		100
Ethanol precipitation	3,740	50,000	13	17	140
DEAE-Sepharose	10	26,700	2,700	3,400	74
Sephadex G-75	0.65	12,500	19,000	24,000	35
DEAE-Sepharose	0.62	7,000	11,300	15,000	19

TABLE II

Purification of rabbit skeletal muscle protein phosphatase C-II

Rabbit skeletal muscle protein phosphatase C-II was purified as described under "Experimental Procedures." The table lists recoveries of protein and phosphorylase phosphatase and alkaline phosphatase activities for the five main steps in the procedure. The first two listed steps are from the same preparation as described in Table I; the peak fractions of phosphatase C-II were obtained during the first DEAE-Sepharose chromatography step where they are separated from phosphatase C-I.

Purification step	Protein	Activity	Specific activity	Purification	Yield
	mg	*units*	*units/mg*	*-fold*	*%*
A. Assayed for phosphorylase phosphatase activity					
Crude extract	47,000	36,000	0.8		100
Ethanol precipitation	3,740	50,000	13	16	140
DEAE-Sepharose	23	2,700	115	144	8
Sephadex G-75	1.7	1,000	590	740	2.8
DEAE-Sepharose	1	580	580	740	1.6
B. Assayed for alkaline phosphatase activity					
Crude extract		840,000	18		100
Ethanol precipitation		210,000	56	3	25
DEAE-Sepharose		63,000	2,700	150	7.5
Sephadex G-75		52,000	30,000	1,700	6.2
DEAE-Sepharose		29,000	29,000	1,600	3.5

exchange chromatography. The recoveries of alkaline phosphatase activity indicated that it comprised only a small fraction of the total activity in the crude tissue extracts (Table II). Yields of this enzyme were about the same in terms of protein recovered as for protein phosphatase C-I.

The purification methods were reproducible. The recoveries were superior to those observed in our studies with the liver and heart muscle enzymes (7, 9), and fewer steps were required. Data obtained from nine preparations of C-I and C-II are as follows. The specific activities obtained for phosphatase C-I were 11,200 ± 2,500 units of phosphorylase phosphatase/mg of protein, 1,200 ± 1,300 units of alkaline phosphatase/mg of protein, and 410 ± 150 units of histone phosphatase/mg of protein. Values for phosphatase C-II were 600 ± 245 units of phosphorylase phosphatase/mg of protein, 15,000 ± 12,000 units of alkaline phosphatase/mg of protein, and 650 ± 260 units of histone phosphatase/mg of protein. The specific activities for the alkaline phosphatase were found to be highly variable and ranged from 4,000 to 39,000 units/mg for phosphatase C-II. A likely reason for this variability is that the alkaline phosphatase activity is relatively unstable, as will be described later. For any given set of preparations from the same tissue extract, however, the ratios of the alkaline phosphatase specific activities of the two enzymes were fairly constant (C-II/C-I = 10.3 ± 1.9). Preparations of the two

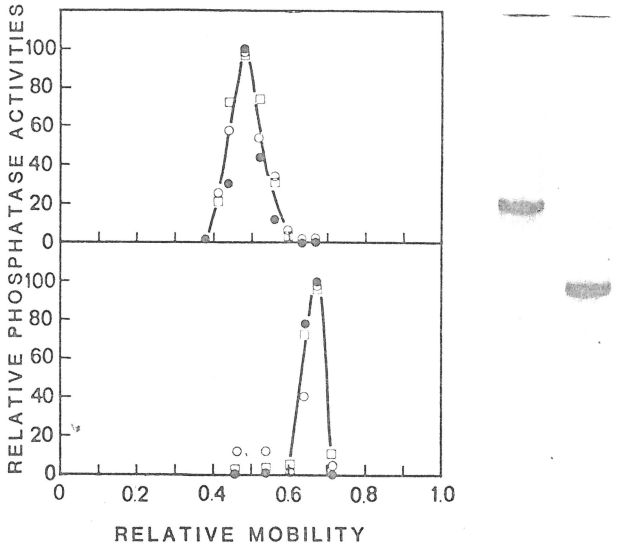

FIG. 2. **Nondenaturing polyacrylamide disc gel electrophoresis of phosphatases C-I and C-II.** Phosphatase C-I and phosphatase C-II were electrophoresed on 10% polyacrylamide disc gels in a Tris-phosphate buffer system as described under "Experimental Procedures." Gels were sliced into 2-mm sections and the activity was eluted by overnight extraction with buffer. Activity recovered from an electrophoresis of phosphatase C-I is shown at the *top*, and that for phosphatase C-II at the *bottom*. Activities are expressed as percentages of maximal activities, and the activities toward phosphorylase *a* (●), phosphorylae kinase (□), and glycogen synthase (○) were determined. A typical set of gels which was stained for protein is shown at the *right*, in the order of phosphatase C-I and phosphatase C-II.

enzymes were usually found to be essentially homogeneous by nondenaturing disc gel electrophoresis (Fig. 2) or by SDS[2] gel electrophoresis. In some preparations a slight cross-contamination of phosphatases C-I and C-II could be observed, but this could be eliminated by a further rechromatography on DEAE-Sepharose.

Physical Properties of Protein Phosphatases C-I and C-II—Phosphatases C-I and C-II were eluted on Sephadex G-75 with identical elution volumes, corresponding to M_r = 35,000 (Fig. 10; "Experimental Procedures"). The molecular weights of phosphatase C-I and C-II were estimated to be 32,000 and 33,500, respectively, by SDS disc gel electrophoresis (Fig. 11; "Experimental Procedures"). Phosphatases C-I and C-II were found to have sedimentation coefficients of 3.4 and 3.6, respectively (see "Experimental Procedures"), by sucrose density ultracentrifugation.

Enzymatic Properties of Protein Phosphatases C-I and C-II—As already discussed, phosphatases C-I and C-II are both associated with activities toward phosphorylase *a*, histone, and *p*-nitrophenyl phosphate during purification. Both enzymes also dephosphorylated glycogen synthase and phosphorylase kinase. The observed abilities of phosphatase C-I and C-II to dephosphorylate all three enzymes of glycogen metabolism were not due to cross-contamination. Examination of activities recovered from gel slices after nondenaturing disc gel electrophoresis showed that activities for all three substrates co-migrate at positions corresponding with the protein bands (Fig. 2). Moreover, the phosphatase C-I activities were inhibited by 5 mM Mn^{2+}, while the phosphatase C-II activities were activated (not shown). Both phosphatases C-I and C-II can also be shown to be associated with histone phosphatase and alkaline phosphatase activity in electrophoresis experi-

[2] The abbreviations used are: SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography.

ments (not shown). In addition, examination of the preparations used for these studies by SDS gel electrophoresis followed by the silver-staining procedure (see "Experimental Procedures") failed to reveal any cross-contamination.

The specific activities of a set of phosphatase C-I and C-II preparations toward several substrates were determined within the same time period to avoid possible differences due to loss of enzymatic activity during storage. The values obtained, given as units per mg of protein, in the order of phosphatase C-I and then phosphatase C-II, were as follows: phosphorylase *a*, 11,300 and 555 (640); rabbit skeletal muscle phosphorylase kinase, 3,100 and 130 (450); rabbit skeletal muscle glycogen synthase, 1,100 and 280 (850); lysine-rich histone, 490 and 580. The values in parentheses for the specific activities of phosphatase C-II toward the enzymes of glycogen metabolism are those assayed in the presence of 5 mM Mn^{2+}. Phosphatase C-II activity toward these three substrates was generally stimulated by Mn^{2+}, whereas the activities of phosphatase C-I were inhibited. In a typical experiment, the percentages of control activities of phosphatase C-I toward phosphorylase *a* at 1, 5, and 10 mM Mn^{2+} were 49, 11, and 4%, respectively, as compared to 160, 160, and 140%, respectively, for phosphatase C-II. For both C-I and C-II, however, the activities toward histone were stimulated by Mn^{2+}, and in both cases the alkaline phosphatase activities toward *p*-nitrophenyl phosphate required the presence of Mg^{2+}.

Phosphatases C-I and C-II possess a high degree of specificity toward the β- and α-subunit phosphates, respectively, of both rabbit skeletal (22) and bovine heart (23) phosphorylase kinase. The β-subunit phosphate of rabbit skeletal muscle phosphorylase kinase is hydrolyzed by phosphatase C-I approximately 100 times more rapidly than the α-subunit phosphate, while phosphatase C-II removes the α-subunit phosphate 3–5-fold more rapidly than the β-subunit phosphate (22). For glycogen synthase, only the phosphate release was determined, and the site specificity of these two phosphatases toward this multiphosphorylated protein (24) remains to be investigated.

The pH optimum of the alkaline phosphatase activity was about pH 8.5 for both phosphatases C-I and C-II, and was dependent on divalent cations (not shown), as described by Li *et al.* (21) for the M_r = 35,000 canine heart protein phosphatase. The K_m values for *p*-nitrophenyl phosphate of phosphatase C-I and C-II were 9 and 7 mM, respectively. Phosphatase C-I and C-II had K_m values for phosphorylase *a* of 2 and 6 μM, respectively. It can be estimated, based on these values and the average specific activities quoted earlier, that the V_{max} values for C-I and C-II are approximately 67,000 and 8,000 nmol of P_i released/min/mg of enzyme, respectively. These values, expressed as turnover numbers, are equivalent to about 2,200 mol of P_i released/min/mol of phosphatase C-I and 280 mol of P_i released/min/mol of phosphatase C-II.

Inhibitor 2 was prepared from rabbit skeletal muscle by the method of Foulkes and Cohen (25). The phosphorylase phosphatase activity of phosphatase C-I was inhibited by inhibitor 2 but phosphatase C-II was not inhibited (Fig. 3). Phosphatase C-II, assayed as a histone phosphatase in the presence of Mn^{2+}, was also not inhibited (not shown).

The heat stabilities of both phosphatase C-I and phosphatase C-II were examined (Fig. 4). In both cases, the alkaline phosphatase activities were relatively unstable at 50 °C in 50% glycerol, with half-lives of about 3–6 min. The phosphorylase phosphatase activities of both enzymes, on the other hand, were stable. The histone phosphatase activities were found to have an intermediate stability. These findings are

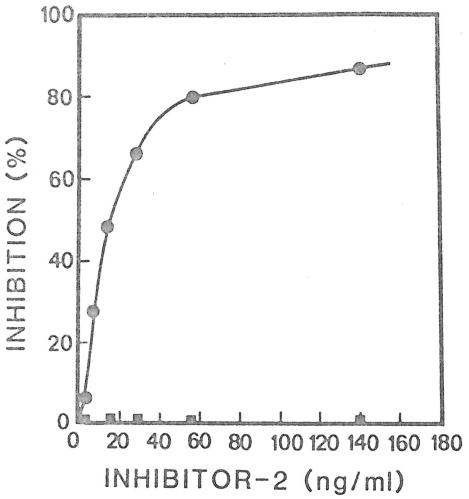

FIG. 3. **Effects of inhibitor 2 on protein phosphatases C-I and C-II.** Inhibitor 2 was added to the assay mixture for phosphorylase phosphatase activity at the concentrations shown. The inhibition of phosphorylase phosphatase activity observed for phosphatase C-I is shown by ●, and that for phosphatase C-II is shown by ■.

surprising since these activities appear to be intrinsic to each protein by the criteria of association by several separation methods, although a possible explanation may lie in the fact that the three phosphatase assays are carried out under different conditions. This phenomenon of preferential heat lability has also been observed for the alkaline phosphatase activity of a purified heart protein phosphatase (21). Additionally, partially purified preparations of skeletal muscle phosphorylase phosphatase ("phosphatase-III") have been shown to preferentially lose the ability to dephosphorylate (and inactivate) inhibitor 1 during incubation in the absence of divalent cations, and this property is in fact utilized to pretreat the phosphatase in the assay for inhibitor 1 (26).

Protein phosphatases C-I and C-II, although apparently homogeneous preparations, might each still consist of mixtures of proteins of similar size and charge, a possibility that is suggested by the heat stability data. Preparations were therefore also examined by two-dimensional gel electrophoresis (electrophoresis in urea in the first dimension, followed by electrophoresis in SDS in the second dimension) by the procedure of Rubin and Leonardi (27). Both phosphatase C-I and C-II preparations were found to consist of a single major component by this procedure following staining by the silver method (data not shown). The question of the intrinsic nature of the *p*-nitrophenyl phosphatase activity of phosphatase C-II was also examined by the use of a monoclonal antibody raised against phosphatase C-II (28). The antibody used (IB5) was found to inhibit all three phosphatase activities of phosphatase C-II in parallel as a function of time (Fig. 5). These data provide additional evidence that these activities are intrinsic to phosphatase C-II, although it may be noted that since antibody IB5 cross-reacts with phosphatase C-I (which is absent in these preparations of C-II), the possibility of a third cross-reactive enzyme activity is not totally eliminated. Similar findings (not shown) were obtained when the phosphorylase phosphatase and histone phosphatase activities of phosphatase C-I were examined.

Structural Comparisons of Phosphatases C-I and C-II—The amino acid compositions of preparations of phosphatases C-I and C-II were determined, and are shown in Table III. The amino acid compositions of the two proteins are very similar and this similarity is suggestive of the possibility that the proteins are structurally related, as is also indicated by the

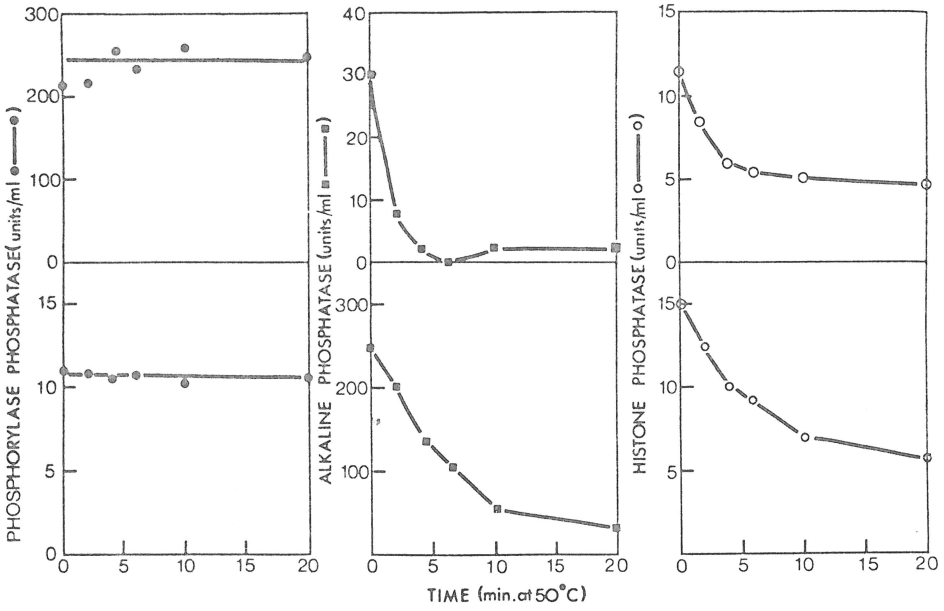

FIG. 4. **Heat stabilities of rabbit muscle phosphatases C-I and C-II.** Purified phosphatase C-I and phosphatase C-II, in storage buffer containing 50% glycerol, were incubated at 50 °C. After incubation for the times indicated, the samples were withdrawn, placed on ice, and then appropriately diluted with 1% bovine serum albumin in 50 mM imidazole chloride, 5 mM EDTA, 0.5 mM dithiothreitol, pH 7.45, and immediately assayed. *Left, center,* and *right* depict the phosphorylase phosphatase, alkaline phosphatase, and histone phosphatase activities, respectively. In each case, the data for phosphatase C-I is given at the *top* and that for phosphatase C-II at the *bottom.*

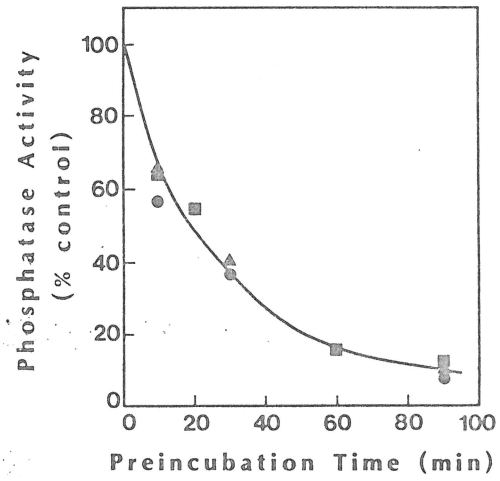

FIG. 5. **Inhibition of the phosphorylase phosphatase, histone phosphatase, and alkaline phosphatase activities of protein phosphatase C-II by a monoclonal antibody.** Phosphatase C-II (10 μl, 2.5 μg/ml) was mixed with 100 μl of monoclonal antibody IB5 (100 μg/ml) and incubated at 30 °C for the indicated times. Samples were then appropriately diluted and assayed for phosphorylase phosphatase (●), histone phosphatase (▲) and alkaline phosphatase (■) activities as described under "Experimental Procedures."

finding of cross-reactive monoclonal antibodies (28). However, both the latter findings would also be consistent with phosphatase C-I being a proteolytic cleavage product of phosphatase C-II, *i.e.* that there is a complete sequence overlap. This issue of the possible derivation of phosphatase C-I from C-II was of serious concern, as it has been suggested that the M_r = 35,000 phosphatases may be derived by proteolytic cleavage of a larger precursor (13), and also that multiple M_r ~ 35,000 forms can be derived from a M_r = 38,000 subunit (29). The two proteins were therefore compared by peptide mapping of proteolytic digests of radioiodinated samples of both phosphatases C-I and C-II. The enzymes were treated with either trypsin (Fig. 6) or *Staphylococcus aureus* V-8 protease (Fig. 7), and then analyzed by reverse phase HPLC on a C_{18} column. In each case, phosphatases C-I and C-II gave distinct profiles. These data establish that phosphatase C-I is not a cleavage product of phosphatase C-II, but do not eliminate the possibility that these two proteins possess structural

TABLE III

Amino acid compositions of protein phosphatases C-I and C-II

Amino acid compositions were determined as described under "Experimental Procedures."

Amino acid	C-I	C-II	Phosphatase C-I	Phosphatase C-II
	residues/100		*residues/32,000*	*residues/33,500*
Asp	10.3	11.3	29.8	33.8
Thr	4.3	5.4	12.4	16.2
Ser	6.6	6.2	19.1	18.6
Glu	11.5	13.8	33.4	41.4
Pro	5.5	4.5	15.9	13.4
Gly	9.3	7.1	27.0	21.4
Ala	6.1	6.8	17.8	20.5
Cys	3.7	2.4	10.7	7.2
Val	5.6	6.1	16.2	18.4
Met	1.2	1.4	3.5	4.1
Ile	5.1	4.5	14.8	13.4
Leu	9.9	9.5	28.8	28.5
Tyr	3.3	3.8	9.6	11.5
Phe	4.7	3.4	13.6	10.2
His	1.8	2.3	5.2	6.8
Lys	5.9	6.2	17.0	18.7
Arg	5.3	5.3	15.4	15.9

homologies. Attempts to determine the NH_2-terminal amino acid sequences using a Beckman 890C Sequencer in experiments kindly performed by Dr. K. Brew (University of Miami) were unsuccessful and indicated that the NH_2 termini of both phosphatase C-I and C-II are blocked. This argues against the possibility that phosphatase C-I is a proteolytic derivative arising from cleavages of phosphatase C-II in the region of its NH_2 terminus.

DISCUSSION

In the present work, we have demonstrated that two low molecular weight protein phosphatases can be isolated from rabbit skeletal muscle. The purification procedures reported are relatively simple and provide good recovery of the two proteins. Both enzymes exhibit a broad specificity, but have distinct differences. The first enzyme, protein phosphatase C-I, is similar to other M_r ~ 35,000 phosphorylase phosphatases which we have isolated from liver (7) and bovine heart (9), and is highly active toward phosphorylase *a*. The second enzyme is one which was initially detected by its high activity

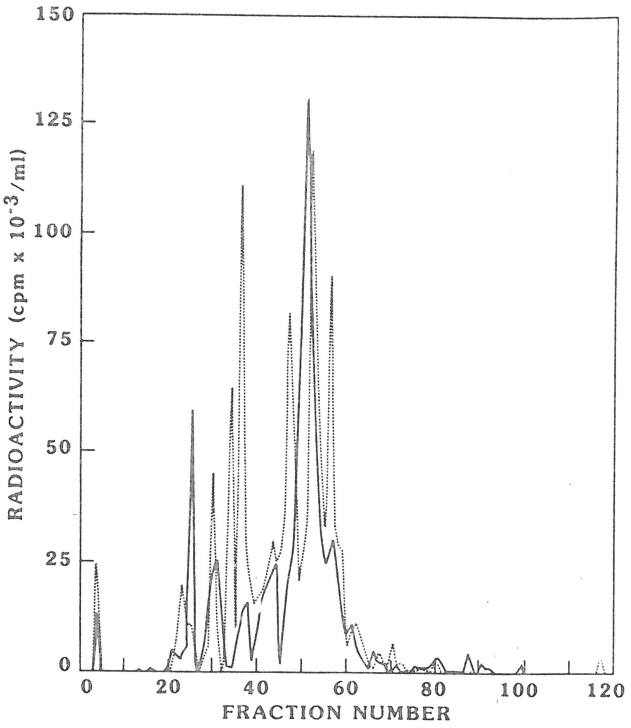

FIG. 6. **HPLC peptide mapping of tryptic digests of ^{125}I-labeled protein phosphatases C-I and C-II.** Phosphatases C-I and C-II were iodinated with ^{125}I with chloramine-T and then digested with trypsin. The tryptic digests were then chromatographed by HPLC on a Waters μBondapak C_{18} column using a gradient of 0–80% acetonitrile in 0.1% phosphoric acid. Fractions of 1 ml each were collected and counted on a γ-counter. Details are given under "Experimental Procedures." Data for the tryptic digest of phosphatase C-I are shown by —, and data for the tryptic digest of C-II are shown by · · · · · . (Individual data points have been omitted for clarity of presentation).

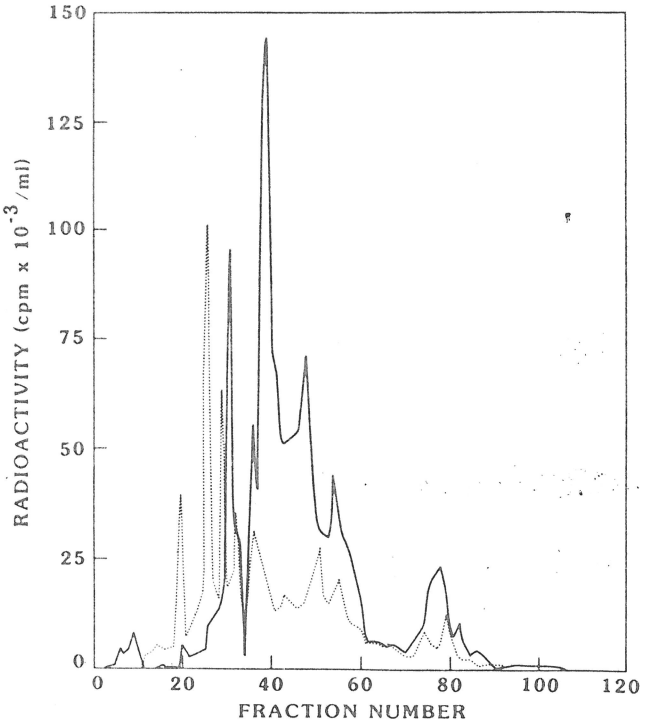

FIG. 7. **HPLC peptide mapping of *S. aureus* V-8 protease digests of ^{125}I-labeled protein phosphatases C-I and C-II.** Phosphatases C-I and C-II were iodinated with ^{125}I with chloramine-T and then digested with *S. aureus* V-8 protease. The *S. aureus* V-8 protease digests were then chromatographed by HPLC on a Waters μBondapak C_{18} column using a gradient of 0–80% acetonitrile in 0.1% phosphoric acid. Fractions of 1 ml each were collected and counted on a γ-counter. Details are given under "Experimental Procedures." Data for the digest of phosphatase C-I are shown by —, and data for the digest of C-II are shown by · · · · · . (Individual data points have been omitted for clarity of presentation).

toward *p*-nitrophenyl phosphate, and has a lower specific activity toward phosphorylase *a*. Phosphatases C-I and C-II can also be differentiated in terms of their respective specificities toward the subunits of phosphorylase kinase, their relative specificities toward phosphorylase *a* and *p*-nitrophenyl phosphate, their response to Mn^{2+} cations, and inhibition by protein phosphatase inhibitor 2. They therefore display distinct characteristics, although they are similar in size and chromatographic behavior. Our data indicate that the smaller enzyme, phosphatase C-I, is not a proteolytic derivative of phosphatase C-II. Their similar amino acid compositions suggest the possibility that these proteins may be structurally related. Calculations based on the amino acid compositions of the difference index by the method of Metzger *et al.* (30) or statistical analysis of compositional differences by the method of Marchalonis *et al.* (31) both provided values which are comparable to those of proteins having a close structural relationship. Studies using monoclonal antibodies to the two proteins have shown that these are capable of cross-reacting, and provide more direct evidence that these two proteins have common structural feature(s) (28).

The present studies provide a general parallel with the studies of the rabbit liver enzymes (15) which provided the only previous rigorous description of two phosphatases from the same tissue which dephosphorylate the enzymes of glycogen metabolism. In this case two proteins of $M_r \sim 35{,}000$ were also isolated, and which had $M_r = 34{,}000$ and 30,500, respectively, as determined by SDS gel electrophoresis. The phosphorylase kinase phosphatase activity of phosphatase C-I is highly specific for the β-subunit phosphate but does dephosphorylate the α-subunit, albeit at a much slower rate (22). Phosphatase C-II, on the other hand, dephosphorylates the α-subunit phosphate 3–5 times more rapidly than the β-subunit phosphate (22). Both enzymes display comparable activities toward glycogen synthase. Given the specific activities of the two enzymes, and given that comparable amounts of enzyme protein were recovered from the ethanol-treated extracts, it can be reasonably argued that, if anything, phosphatase C-I contributes the major activity toward phosphorylase *a* and the β-subunit of phosphorylase kinase, but that both C-I and C-II could provide comparable activities toward glycogen synthase. The concept of a single, "multifunctional" phosphatase (5, 6) is clearly inadequate for the system, as has been previously noted (3), if only because of the existence of multiple enzyme forms of overlapping specificity. A more rigorous assessment of the possible physiological functions of phosphatases C-I and C-II must await the determination of whether and how their properties differ from the native holoenzymes from which they are presumed to be derived.

Specificity toward the subunit phosphates of phosphorylase kinase as well as sensitivity toward the protein phosphatase inhibitors 1 and 2 have been used by Cohen's laboratory to identify various skeletal muscle protein phosphatases. Rabbit skeletal muscle was proposed to have two phosphatases, "phosphatase-1" and "phosphatase-2" (5, 6, 18, 32, 33); these had been reported to be relatively specific for the α- and β-subunits of phosphorylase kinase, respectively. Only phosphatase 1 is inhibited by the heat-stable protein phosphatase inhibitors (5, 6, 26, 34). These enzymes activities have not been fully characterized, and they have been attributed to

have a variety of physical properties. An enzyme preparation, originally termed protein phosphatase-III (18), had been shown to act on phosphorylase *a*, glycogen synthase, and phosphorylase kinase (β-subunit). This preparation had activity forms of M_r = 75,000 and 45,000. A homogeneous preparation of M_r = 125,000 which had subunits of M_r = 35,000 was reported (6), and this had the same specificity as that described for protein phosphatase-III preparations (18). A partially purified preparation containing enzyme activities of M_r = 62,000 and 34,000 and an ATP-Mg-dependent form were both shown to have similar specificities to protein phosphatase-III (33). All these preparations have been referred to as "protein phosphatase-1," an inappropriate nomenclature because of the inference of a singular entity which clearly is not the case, although these may possibly be derived from a single precursor.

More recent studies of the specificities of the protein phosphatases have led to the extension of this nomenclature (35–37). Thus, phosphatase 2 phosphatase activities have been extended to cover five enzyme forms, only one of which (phosphatase 2B) has been isolated to homogeneity (38). It has also been suggested that phosphatase 1 has a major role in glycogen metabolism, and that phosphatase 2 (now called phosphatase 2A) has a role in glucogenesis and lipogenesis (37, 39), based on their observed specificities and apparent relative distributions in skeletal muscle and liver. Despite the categorizations of the relationships, physiological functions, and regulation of phosphatase 1 and phosphatase 2 (5, 6, 35, 37, 39), a careful distinction between a convenient nomenclature and specific identity must be exercised until the enzymology of these proteins is more carefully defined. The relationship of phosphatase C-I and C-II to the various skeletal muscle preparations of phosphatases 1 and 2 is a matter of conjecture, although it is likely that phosphatase C-I and C-II may have been components of some of the skeletal muscle phosphatase 1 and 2 preparations that have been reported. From the perspective of phosphatase 1 and phosphatase 2 activities, our findings may be considered significant in establishing that proteins with activities corresponding to these two types can be isolated as forms of $M_r \sim$ 35,000 from rabbit skeletal muscle, and in providing a first study of these proteins with rigorous attention to their purity.

Another group of rabbit skeletal muscle protein phosphatases of $M_r \sim$ 70,000 to 80,000 have been studied in purified forms (13, 19, 20, 29, 33). These include an essentially inactive form of phosphorylase phosphatase (F_C) which is activated by ATP-Mg and a protein factor, F_A. This form was first isolated from rabbit skeletal muscle by Vandenheede *et al.* (20). This enzyme had a M_r = 70,000, but was inactive unless it was activated by ATP-Mg and a protein factor, F_A (20), which is a protein kinase, in a reaction in which the presence of inhibitor 2 is required. More recently, a $M_r \sim$ 35,000 skeletal muscle phosphatase prepared by essentially the methods reported here, and which presumably is phosphatase C-I, has been shown to form a complex(es) with inhibitor 2 (40), comfirming an earlier observation that liver phosphatase C will form stable complexes with heat-stable protein inhibitors (41). The complex was found to exhibit ATP-Mg sensitivity, similar to that of F_C and had the substrate specificity of phosphatase 1 (40). An ATP-Mg-sensitive phosphorylase phosphatase has also been purified to homogeneity by Ballou *et al.* (29) from skeletal muscle, and shown to consist of a M_r = 38,000 catalytic subunit complexed with a M_r = 31,000 subunit thought to be inhibitor 2. Proteolytic digestion with trypsin led to loss of the inhibitor moiety and generation of multiple polypeptide bands of M_r = 33,000 to 38,000 (29), and these authors raised the possibility that multiple M_r = 35,000 forms may be generated by proteolysis from the 38,000 subunit. Both this enzyme preparation and the reconstituted enzyme preparation (29, 40) exhibited $M_r \sim$ 70,000 under nondenaturing conditions. These studies suggest the $M_r \sim$ 35,000 forms of phosphorylase phosphatase can exist as complexes with inhibitor 2. However, these findings are inconsistent with the original description of the ATP-Mg-dependent enzyme as having a single subunit of M_r = 70,000 on SDS disc gel electrophoresis (19). It has been proposed that the M_r = 35,000 forms may be derived from F_C by proteolysis which occurs during the ethanol treatment step (13, 42). It is thus possible that phosphatase C-I is identical with the catalytic subunit of the preparation reported by Ballou *et al.* (29), a subfragment of that subunit (29) or of the M_r = 70,000 subunit of F_C. Based on the observed substrate specificities of these preparations, they are unlikely to be related to phosphatase C-II. However, there remains the uncertainty whether these ATP-Mg-dependent enzymes represent native forms, or enzymes derived by a posthomogenization process. Since it has been shown that phosphatase C-I will complex with inhibitor 2, it is conceivable that such complexes can be formed during the purification procedures, due to the release of phosphatase C-I from its putative precursor of $M_r \sim$ 250,000. Relevant to this issue is the fact that the preparation of Ballou *et al.* (29) involved a treatment with 50% room temperature acetone, and the fact that the levels of the ATP-Mg-dependent enzyme described as F_C increases during its purification (42).

In previous studies, we have proposed that the M_r = 35,000 protein phosphatase obtained after dissociative treatments of tissue extracts is derived from a larger precursor, and may represent the catalytic subunit of a larger protein. Significant supporting evidence for a catalytic subunit-holoenzyme hypothesis has emerged. This is based on studies of the dissociation of the enzyme in relatively crude tissue extracts (2, 3, 8, 14, 43, 44), on the reassociation of low molecular weight fractions with noncatalytic fractions (41, 45), and on the isolation of multisubunit high molecular weight protein phosphatases first reported from rat liver (2). These enzymes can generally be shown to contain a $M_r \sim$ 35,000 subunit, as well as subunits of $M_r \sim$ 70,000 and sometimes of ~55,000 (2, 4, 46–53). In terms of subunit structure, protein phosphatases isolated from several sources using different protein substrates appear to fall into two groups, those with subunits of $M_r \sim$ 70,000 and 35,000 (4, 46–51) and those with all three sizes of subunits (2, 52–54). Imaoka *et al.* (54) have isolated a pig heart phosphorylase phosphatase to homogeneity. This enzyme possessed subunits of M_r = 69,000, 55,000, and 35,000. The catalytic activity was associated with the M_r = 35,000 component, and these investigators were able to partially reconstitute a larger enzyme which contained the M_r = 69,000 and 35,000 subunits. Only two of these preparations described above are from rabbit skeletal muscle (4, 50), and one of these was reported to have two catalytic subunits of different molecular weights (50, 51).

Despite these findings, the exact status of phosphatase C-I and C-II as catalytic subunits of holoenzymes of this type remains open, because of the lack of examples of homogeneous preparations from rabbit skeletal muscle. We have shown that phosphorylase phosphatase activity in crude muscle extracts behaves as a form of $M_r \sim$ 260,000 during gel filtration, and that this can be dissociated by the action of Ca^{2+}-dependent proteases to forms of $M_r \sim$ 70,000 and 35,000 (14). We have also reported in preliminary form the properties of an isolated high molecular weight rabbit skeletal muscle protein phosphatase which has subunits of M_r = 70,000 and 35,000 and

enzymatic properties very similar to phosphatase C-II (4), including an identical relative specificity toward the α-subunit phosphates of phosphorylase kinase and the ability to bind to monoclonal antibodies against phosphatase C-II.[3] However, its catalytic subunit is nonidentical with phosphatase C-II as it is slightly larger (M_r = 35,000) than phosphatase C-II (M_r = 33,500).[3] The latter findings add a cautionary note to the use of enzymatic properties for establishing molecular identity in this area of research. While the general hypothesis that C-I and C-II are the catalytic subunits of larger holoenzymes remains a good working hypothesis, we would caution against the uncritical categorization of these as such.

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[3] M. K. Ganapathi, M. Speth, and E. Y. C. Lee, unpublished results.

SUPPLEMENTARY MATERIAL TO
ISOLATION AND CHARACTERIZATION OF RABBIT
SKELETAL MUSCLE PROTEIN PHOSPHATASES C-I AND C-II

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EXPERIMENTAL PROCEDURES

Materials - Imidazole (crystalline, Grade 1), DL-dithiothreitol, EDTA, p-nitrophenyl phosphate, bovine serum albumin, bovine heart cAMP-dependent protein kinase (specific activity, 2,000 units/mg protein towards casein), myoglobin (equine), lactate dehydrogenase (type II, rabbit muscle), ovalbumin (grade V), theophylline (1, 3-dimethylxanthine), alpha-chymotrypsin (type 1, bovine pancreas) were obtained from Sigma Chemical Co. Imidazole was recrystallized from acetone before use. DEAE-Sepharose CL-6B, Sephadex G-75 (superfine), and Sephacryl S-200, were purchased from Pharmacia Fine Chemicals. Human erythrocyte carbonic anhydrase C was a gift of Dr. P.L. Whitney of the Department of Biochemistry, University of Miami. Calf thymus lysine-rich histone, trypsin (TPCK-treated), and soybean trypsin inhibitor were obtained from Worthington Biochemical Company. [γ-^{32}P]ATP was purchased from ICN Pharmaceuticals, Incorporated, or New England Nuclear Company (10-40 Ci/mmole). [^{125}I]Sodium iodide (17.4 Ci/μg) was purchases from New England Nuclear Company. Ammonium sulfate (Enzyme Grade) was purchased from Schwarz-Mann. Frozen rabbit muscle (used for the preparation of phosphorylase b) was bought from Pel-Freez Biologicals, Incorporated.

Protein determinations - Protein was determined by the method of Bradford (54). Bovine serum albumin was used as a standard.

Preparation of ^{32}P-labelled substrates - Phosphorylase b was isolated from frozen rabbit muscle by the method of Fischer and Krebs (56), and recrystallized three times. It was converted to the a form with rabbit muscle phosphorylase kinase as described by Krebs and Fischer (57) using [γ-^{32}P]ATP (specific radioactivity, ca. 200 Ci/mole). After two Norit treatments to remove AMP and three recrystallizations, the phosphorylase a crystals were resuspended in a 1:1 (v/v) solution of glycerol and buffer (50 mM imidazole chloride, 5 mM theophylline, 0.5 mM dithiothreitol, pH 7.2) and stored at -20°C.

Phosphorylated lysine-rich histone was prepared as described previously (9), using partially purified beef heart cAMP-dependent protein kinase.

Phosphorylase kinase was prepared according to the procedure of Cohen (58), and further purified on DEAE-Sepharose (22). Phosphorylated phosphorylase kinase was prepared (22) using a homogeneous preparation of the catalytic subunit of bovine heart cAMP-dependent protein kinase which was a generous gift of Drs. D.L. Brautigan and E.H. Fischer of the University of Washington, Seattle. The phosphorylated rabbit muscle phosphorylase kinase used in these experiments contained approximately 1 mole ^{32}Pi/312,000 g protein, and the distribution of label was 35% in the α-subunit and 65% in the β-subunit, determined after SDS disc gel electrophoresis as described previously (22).

Glycogen synthase was prepared from rabbit skeletal muscle according to the procedure of Nimmo et al. (59). The enzyme was phosphorylated with the catalytic subunit of cAMP-dependent protein kinase, approximately one mole of phosphate being incorporated per mole of subunit.

A monoclonal antibody (IB5) against phosphatase C-II was prepared and characterized as described elsewhere (28).

Assay of protein phosphatase activities - The method used to assay phosphorylase phosphatase activity was essentially as previously described (9,14). Reactions were initiated by the addition of 90 µl of a warmed (30°C) [^{32}P]phosphorylase a assay mixture to 10 µl of sample. The final assay mixture (0.1 ml) which contained 0.2 mg/ml [^{32}P]phosphorylase a, 50 mM imidazole chloride, 0.5 mM dithiothreitol, 5 mM theophylline, pH 7.2, was incubated for 5 to 20 min at 30°C. Reactions were terminated by the addition of 10 µl of 100% trichloroacetic acid. After centrifugation of the precipitated protein for 10 minutes in a bench-top centrifuge, 50 µl of the supernatant were withdrawn, and dried on filter paper (Whatman 31 ET) and the amount of radioactivity present was determined by liquid scintillation counting. The amount of radioactivity released was used to determine the amount of phosphorylase a converted to phosphorylase b. One unit of activity was defined as the amount of enzyme which dephosphorylated 0.2 mg (1 nmole) of phosphorylase a (dimer) per minute.

Histone phosphatase activity was assayed by the release of ^{32}Pi from phosphorylated lysine-rich histone as described previously (9). The final mixture (50 µl) contained 40 mM imidazole chloride, 5 mM $MnCl_2$, 0.2 M NaCl, 0.8 mM dithiothreitol, pH 7.2 and 10 µM [^{32}P]histone (based on incorporated ^{32}Pi). After 5-20 minutes of incubation at 30°C, the reactions were stopped by the addition of 20 µl of 40 mM H_2SO_4, 80 mM silicotungstic acid containing 0.5 mM inorganic phosphate. To this solution 40 µl of 1 mg/ml bovine serum albumin were added to facilitate precipitation. After centrifugation for 5 minutes in a bench top centrifuge, 40 µl of the supernatant were transferred to filter paper discs (Whatman 31 ET), dried under a heat lamp, and radioactivity present determined by liquid scintillation counting. One unit of activity was defined as that which released 1 nmole of ^{32}Pi per min.

Glycogen synthase phosphatase activity was assayed under similar conditions as for phosphorylase phosphatase. Assays contained 0.25 mg/ml of glycogen synthase. One unit of activity was defined as the amount of enzyme which released 1 nmole of ^{32}Pi per minute.

Phosphorylase kinase phosphatase activity was assayed as described previously (22). The reaction mixture (0.05 ml) contained 1 µM phosphorylase kinase (0.33 mg/ml), 1 mg/ml bovine serum albumin, 50 mM imidazole chloride, 2 mM EDTA and 1 mM dithiothreitol, pH 7.2. One unit of activity was defined as the amount of enzyme which released 1 nmole ^{32}Pi per minute.

Except where otherwise stated, phosphorylase phosphatase, phosphorylase kinase phosphatase and glycogen synthase phosphatase activities were assayed in the absence of added divalent cations, while histone phosphatase was assayed in the presence of 5 mM Mn^{++}.

Assay of alkaline phosphatase activity - Alkaline phosphatase activity was determined essentially as by Li et al. (21). To a 20 µl sample, 180 µl of a solution containing 20 mM p-nitrophenyl phosphate, 50 mM Tris-HCl, 20 mM $MgCl_2$, 1 mM dithiothreitol, pH 8.5 were added to initiate the reaction. After 10-20 minutes of incubation at 30°C, the reaction was stopped by the addition of 1.8 ml of 0.5 M Na_2CO_3. The release of p-nitrophenol was measured by absorbance at 410 nanometers. One unit of alkaline phosphatase activity was defined as the amount of enzyme which released 1 nmole of p-nitrophenol per min.

Purification of rabbit muscle phosphatase C-I - Step 1. Preparation of crude muscle extract. Four female New Zealand rabbits were given lethal doses of Nembutal and exsanguinated. Muscle from the hind limbs and back was excised and placed on ice. All further procedures were done at 4°C unless otherwise specified. A typical preparation consisted of 2.15 kg of muscle, which was ground in a meat grinder and homogenized with 3 volumes (6,450 ml) of cold Buffer A (50 mM imidazole chloride, 5 mM EDTA, 0.5 mM dithiothreitol, pH 7.45) in a Waring blendor for 30 seconds at medium speed. The homogenate was then centrifuged at 10,000 x g for 15 min, and the resulting supernatant was filtered through glass wool.

Step 2. Ammonium Sulfate Precipitation - The supernatant was brought to 70% saturation with ammonium sulfate by the slow addition of the solid salt to the stirred solution. The pH was maintained near neutrality by the addition of small amounts of potassium bicarbonate. After standing for 1 hour the precipitate was collected by centrifugation (10,000 X g, 20 min) and the pellet redissolved in a minimal volume of Buffer A (final volume approximately 0.5 ml per 1 g of the initial weight of tissue).

Step 3. Ethanol Precipitation - Five volumes of room temperature, 95% ethanol were rapidly added to the redissolved pellets with mixing. The mixture was then immediately centrifuged (5,000 X g, 5 minutes, 4°C). The hard pellet was extracted by homogenization with Buffer A (about 500 ml) in a blendor at low speed for 30 seconds. This suspension was centrifuged (16,000 x g, 15 min) and the pellet discarded. The supernatant was dialyzed overnight against three changes of Buffer A to remove ammonium sulfate and ethanol. Particulate material was removed by centrifugation (10,000 x g, 15 min).

Step 4. Concentration by DEAE-Sepharose - The enzyme solution from the preceding step (1,500 ml) was applied to a column (5 x 4 cm) of DEAE-Sepharose, pre-equilibrated with 0.1 M NaCl/Buffer A at a rapid flow rate of 300 ml/h. The column was washed with 250 ml of 0.1 M NaCl/Buffer A. The enzyme activity was eluted with 0.5 M NaCl/Buffer A. The active fractions were pooled (210 ml), and then dialyzed for 2-3 hours against Buffer A.

Step 5. DEAE-Sepharose Chromatography - The dialyzed material was applied to a DEAE-Sepharose column (2.5 X 80 cm) equilibrated with 0.1 M NaCl/Buffer A. A linear gradient of 0.1 M NaCl to 0.4 M NaCl in Buffer A (total volume, 1350 ml) was applied at a flow-rate of 80-100 ml/h. Fractions of 10 ml were collected, and assayed for protein and phosphatase activities toward phosphorylase a, histone and p-nitrophenyl phosphate. A typical elution profile is shown in Fig. 1 (see "Results"). Two major activity peaks were identified. The first was a major phosphorylase phosphatase peak ("I", Fig. 1) which was pooled and further purified as described below as protein phosphatase C-I. The second was a major alkaline phosphatase activity peak which was pooled and dialyzed against 50% glycerol/50% Buffer A and stored at -20°C before further purification as phosphatase C-II. On occasion a histone phosphatase fraction which eluted before the phosphatase C-I fractions was also detected, but was not further studied. The pooled phosphatase C-I fractions were diluted with 2.5 volumes of Buffer A or desalted by a brief dialysis against Buffer A, and concentrated by application to a small (1.5 cm diameter x 2 cm) DEAE-Sepharose column equilibrated with 0.1 M NaCl/20% glycerol/Buffer A. The enzyme activity was eluted with 0.6 M NaCl/20% glycerol/Buffer A in a volume of 18 ml.

Step 6. Sephadex G-75 Gel Filtration - The concentrated enzyme solution was chromatographed on a Sephadex G-75 Superfine column (2.5 x 85 cm) at a flow rate of 5-10 ml/h in 0.1 M NaCl/20% glycerol/Buffer A. The fractions were assayed for protein and phosphatase activities using phosphorylase a, histone and p-nitrophenyl phosphate. A typical elution profile is shown in Fig. 8. The major phosphorylase phosphatase peak was pooled and further purified by rechromatography on DEAE-Sepharose. It may be noted that with histone as a substrate, a higher molecular weight fraction was also occasionally observed, as shown in the example in Fig. 8. This fraction displayed very little phosphorylase phosphatase activity assayed in the absence of Mn^{++}. It is possibly the same fraction observed on the initial DEAE-Sepharose chromatography step, but was not further investigated in this work.

Step 7. Re-chromatography on DEAE-Sepharose - The active fractions of Step 6 were applied to a DEAE-Sepharose column (1.5 x 50 cm) equilibrated with 0.1 M NaCl/20% glycerol/Buffer A, at a flow rate of 50 ml/h. The column was eluted with a linear gradient (400 ml) from 0.1 M to 0.4 M NaCl in 20% glycerol/Buffer A at a flow rate of 13 ml/h. The fractions were assayed for protein, protein phosphatase and alkaline phosphatase activities (not shown). The active protein phosphatase fractions were dialyzed against 50% glycerol/Buffer A and stored at -20°C. Under these conditions the phosphorylase phosphatase activity was stable for at least 3 months.

Purification of protein phosphatase C-II - The major alkaline phosphatase fractions obtained by DEAE-Sepharose chromatography (step 5) were purified to homogeneity by essentially the same procedures as for protein phosphatase C-I. The pooled fractions obtained in Step 5 were chromatographed on Sephadex G-75 (superfine) as described for phosphatase C-I (see Step 6). The enzyme activities eluted at an identical elution volume as for phosphatase C-I, and a typical elution profile is shown in Fig. 9. Following the gel filtration step the phosphatase C-II fractions were rechromatographed on DEAE-Sepharose (not shown). The active fractions were pooled and stored as described for phosphatase C-I.

Non-denaturing polyacrylamide gel electrophoresis - This procedure was performed using 10% polyacrylamide Tris-phosphate disc gels (60). Samples were concentrated by dialysis for two hours against a solution of 25 mM potassium phosphate, pH 7.4, 1 mM EDTA, 20% ethylene glycol, 0.9 mM beta-mercaptoacetic acid, 1 mM mercaptoethanol, 30% sucrose. The concentrated sample (250 µl) was

applied to a pre-electrophoresed disc gel and run at a constant current of 1 mA/tube, for approximately four hours. All procedures were done at 0-4°C. Gels were then either stained with Coomassie Brilliant Blue or sliced, and the activity eluted from the 2 mm gel slices in 200 µl of 20% glycerol/1% bovine serum albumin/50 mM imidazole, 5mM EDTA, 0.5mM dithiothreitol, pH 7.45. After an overnight incubation in this buffer, enzyme activities were determined as described above. Results are shown in Fig. 2 (see "Results").

Analytical gel filtration - Purified protein phosphatases C-I and C-II were chromatographed on a Sephadex G-75 (superfine) column (1.5 x 60 cm). A 1 ml sample was applied to a column that was equilibrated and run in 0.1 M NaCl/Buffer A at 9 ml/h. Fractions of 1.3 ml were collected and assayed for phosphatase activities. Calibration of the column for molecular weight estimation was done using 1 mg/ml solutions of bovine serum albumin (M_r = 68,000), ovalbumin (M_r = 43,000), carbonic anhydrase (M_r = 29,000), and myoglobin (M_r = 17,500). An apparent molecular weight of 35,000 was found for both phosphatases C-I and C-II (Fig. 10).

SDS polyacrylamide disc gel electrophoresis - Gels of 10% polyacrylamide and 6 cm in length were used. Electrophoresis was carried out as by Weber and Osborn (61) using a continuous buffer system of 0.1 M $NaPO_4$, pH 7.1. Samples were prepared by heating at 100°C for 3 min in 1% mercaptoethanol and 0.4% SDS. The final glycerol concentrations of all samples were adjusted to 25% and bromophenol blue was added as a tracking dye. Myoglobin (3 µg was included as an internal marker. Mobilities were determined by densitometric scanning (700 nm) of the Coomassie Brilliant Blue stained gels, using a Gilford 240 spectrophotometer. The mobilities relative to myoglobin as an internal standard were determined, and the data plotted as shown in Fig. 11. The subunit molecular weights of protein phosphatase C-I and C-II were determined to be 32,000 and 33,500, respectively. For routine evaluation of the purity of the phosphatase preparations they were electrophoresed in slab gels by the method of Laemmli (62) and stained by the silver stain procedure (63).

Sucrose density gradient ultracentrifugation - Ultracentrifugation of phosphatase C-I and phosphatase C-II was performed using 5-20% sucrose gradients (prepared in 50 mM imidazole chloride, 0.5 mM dithiothreitol, 5 mM EDTA, pH 7.45). The enzymes were first dialyzed against the buffer and 100 µl samples applied to the gradients. Centrifugation was for 15 hours at 50,000 rpm in a SW 56 rotor, using a Beckman L2-65B ultracentrifuge at 4°C. Fractions of 5 drops each were collected from the bottom of the tubes, and assayed for phosphatase activities. The standards, bovine serum albumin (M.W. 68,000, 4.3 S) or catalase (M.W. 260,000, 11.3 S) were included in each sample as internal markers. Sedimentation coefficients were determined by the method of Martin and Ames (64). Both phosphatase C-I and C-II eluted as single peaks of activity, each associated with phosphorylase phosphatase, histone phosphatase, and alkaline phosphatase activities (not shown), corresponding to sedimentation coefficients of 3.4 S and 3.6 S, respectively.

Amino acid analysis - Samples (50 - 60 µg protein) for analysis were hydrolysed in 200 µl of 6N hydrochloric acid containing 1% phenol at 110°C for 24, 48 and 72 hours in sealed evacuated tubes. Analyses were performed with a JEOL SAH amino acid analyser. The content of cysteine plus cystine was determined as carboxymethyl cysteine after reduction and carboxymethylation (65). Values for serine and threonine were determined by extrapolation to zero time of hydrolysis.

Peptide Mapping of Radioiodinated Protein Phosphatases C-I and C-II - Phosphatases C-I and C-II (100 µg each) were dialyzed against distilled water, lyophylized and resuspended in 0.5 ml of 50 mM sodium phosphate pH 7.4. The proteins were then iodinated (66) by addition of Choramine-T (1.8 mg/ml) and 2 mCi of [^{125}]NaI for 5 min at room temperature. The reactions were stopped by the addition of sodium metabisulfite to a final concentration of 2.3 mg/ml. The iodinated proteins were chromatographed on a Sephadex G-75 column (0.5 x 8 cm) equilibrated with 50 mM sodium bicarbonate, pH 8. The radioiodinated enzymes were recovered as single peaks; recovery was approximately 2 x 10^8 cpm of radioactivity for both proteins. Samples of the iodinated proteins (4 x 10^6 cpm) were digested with either trypsin (10 µg) or S. aureus V-8 protease (10 µg) in a total volume of 110 µl of 50 mM sodium bicarbonate, pH 8.0, at 30°C for 24 h. Samples of the digests (50 µl) were then subjected to HPLC chromatography (67) on a Waters µBondapak C_{18} column (10µm, 0.4 x 30 cm) on a Waters HPLC. A linear gradient of 0.1% phosphoric acid (pH 2.2) to 80% acetonitrile/0.1% phosphoric acid was used. The total gradient volume was 120 ml and the flow rate was 2 ml/min. Fractions of 1 ml were collected and counted in a gamma counter.

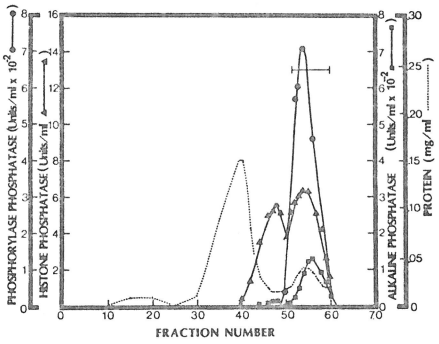

Fig. 8. Sephadex G-75 chromatography of protein phosphatase C-I. This figure shows a typical elution profile obtained after Sephadex G-75 chromatography of protein phosphatase C-I in the purification procedure. A Sephadex G-75 (superfine) column (2.5 x 85 cm) was pre-equilibrated in 0.1 M NaCl/20% glycerol/Buffer A, and run at a flow rate of 5 ml/h. Fractions of 4 ml each were collected, and assayed for phosphorylase phosphatase (circles), histone phosphatase (triangles) and alkaline phosphatase (squares) activities. The major phosphorylase phosphatase activity peak (fractions 52-60) was pooled for further purification as described in the text.

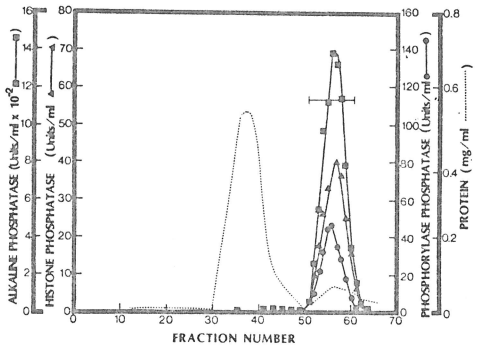

Fig. 9. Sephadex G-75 chromatography of protein phosphatase C-II. This figure shows a typical elution profile obtained for the purification of phosphatase C-II on Sephadex G-75. Those fractions from the initial DEAE-Sepharose separation corresponding to phosphatase C-II were chromatographed on the same Sephadex G-75 column under identical conditions as that used for phosphatase C-I (see Fig. 8). The alkaline phosphatase peak with coeluting histone phosphatase and phosphorylase phosphatase activities was pooled (as indicated by bar) for further purification as described in the text. Symbols for activities are as for Fig. 8.

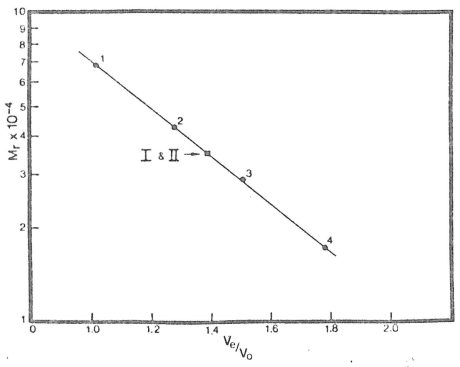

Fig. 10. Analytical gel filtration of phosphatases C-I and C-II. The molecular weights estimated by analytical gel filtration of phosphatase C-I and phosphatase C-II on Sephadex G-75 are shown here. Conditions are as described in the text. The standards used were 1, bovine serum albumin; 2, ovalbumin; 3, carbonic anhydrase; and 4, myoglobin. Phosphatase C-I and C-II coeluted at a position shown ("I,II") by the solid square, corresponding to M_r = 35,000.

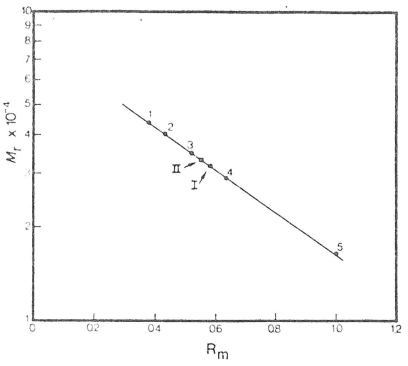

Fig. 11. **SDS disc gel electrophoresis of phosphatases C-I and C-II.** Phosphatases C-I and C-II were subjected to SDS disc gel electrophoresis as described in the text. Purified phosphatases C-I and C-II were run on 10% polyacrylamide disc gels with myoglobin as an internal marker. The mobilities relative to myoglobin, taken as 1 (R_m), are the average of four separate determinations. Phosphatase C-I ("I") had a M_r = 32,000 and phosphatase C-II ("II") had a M_r = 33,500. In all determinations only one Coomassie Brilliant Blue staining band was observed besides the myoglobin standard. The standards used were 1, ovalbumin; 2, alcohol dehydrogenase; 3, lactate dehydrogenase; 4, carbonic anhydrase; 5, myoglobin.