Amino Acid Sequence of the Phosphorylated Site in Rabbit Liver Glycogen Phosphorylase*

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ABSTRACT: Rabbit liver glycogen phosphorylase (α-1,4-glucan:orthophosphate glucosyl transferase, EC 2.4.1.1) has been purified in its inactive (dephosphorylated) and active (phosphorylated) forms. A molecular weight of 185,000 was calculated from sedimentation equilibrium measurements for both forms of the enzyme in contrast with values of 185,000 and 370,000 reported for the inactive and active forms of rabbit muscle phosphorylase, respectively (Seery, V. L., Fischer, E. H., and Teller, D. C. (1967), *Biochemistry 6*, 3315). Amino acid analyses carried out on rabbit liver phosphorylase showed a distinct degree of similarity between the liver and the skeletal muscle enzyme. Phosphorylation of inactive liver phosphorylase with rabbit muscle phosphorylase kinase in the presence of [γ-^{32}P]adenosine triphosphate resulted in the incorporation of covalently bound phosphate into the molecule with a concomitant appearance of enzymatic activity as measured in the presence or absence of adenosine monophosphate. A hexapeptide was isolated from the phosphorylated site and its sequence determined as: Arg-Gln-Ile-(P)Ser-Ile-Arg. This sequence is identical with that obtained for the phosphorylated hexapeptide isolated from rabbit muscle phosphorylase (Fischer, E. H., Graves, D. G., Snyder Crittenden, E. R., and Krebs, E. G. (1959), *J. Biol. Chem. 234*, 1698) except for the conservative substitutions of an arginyl for a lysyl residue and an isoleucyl for a valyl residue.

These findings confirm the structure proposed previously for the liver hexapeptide on the basis of its amino acid composition (Appleman, M. M., Krebs, E. G., and Fischer, E. H. (1966), *Biochemistry 5*, 2101).

Liver glycogen phosphorylase was first shown to exist in phosphorylated and dephosphorylated forms by Wosilait and Sutherland (1956). The dephospho or inactive form of the enzyme, in contrast to muscle phosphorylase *b*, was found to be essentially inactive even in the presence of AMP; however, partial activity was observed when high concentrations of sodium sulfate were included in the assay (Appleman *et al.*, 1966). The enzymatic conversion of the inactive enzyme to the active form resulted in the incorporation of covalently bound phosphate with the concomitant appearance of enzymatic activity measurable in the presence or absence of AMP (Rall *et al.*, 1956).

Structural studies conducted on the phosphorylated site of phosphorylases from rabbit and human muscle (Fischer *et al.*, 1959; Hughes *et al.*, 1962) have led to the isolation of a hexapeptide with the amino acid sequence: Lys-Gln-Ile-(P)Ser-Val-Arg. The sequence for the rabbit enzyme has been extended to include 14 amino acids (Nolan *et al.*, 1964) and recently (Sevilla and Fischer, 1969) a similar sequence (except for the substitution of an aspartyl for a glutamyl residue) was elucidated for rat muscle phosphorylase. Larner and Sanger (1965) isolated a phosphohexapeptide from rabbit skeletal muscle glycogen synthetase and reported a very similar, if not identical, sequence to that found in muscle phosphorylase. The amino acid composition of a phosphopeptide isolated from rabbit liver phosphorylase was determined by Appleman *et al.* (1966) from which a sequence was proposed, but not actually determined. The present publication describes some properties of rabbit liver phosphorylase including its molecular weight and amino acid composition, and the determination of the amino acid sequence of its phosphorylated site.

Materials and Methods

Rabbit liver dephosphophosphorylase was purified according to the procedure of Appleman *et al.* (1966) through the DEAE-cellulose column chromatography step. When more purified enzyme was required, dephosphophosphorylase was first converted into the active form (see below) and then subjected to a second DEAE-cellulose chromatography (Davis *et al.*, 1967). Higher concentrations of salt were required to elute the phosphorylated form of the enzyme so that it emerged later than the accompanying impurities (Figure 1). Rabbit liver phosphophosphorylase was also prepared according to the procedure of Maddaiah and Madsen (1966); since in this latter purification procedure, some of the enzyme was dephosphorylated, fractions obtained after the Sephadex G-200 step were reconverted into the active form.

Phosphorylase activity was determined in the presence of 10^{-3} M AMP by the procedure of Hedrick and Fischer

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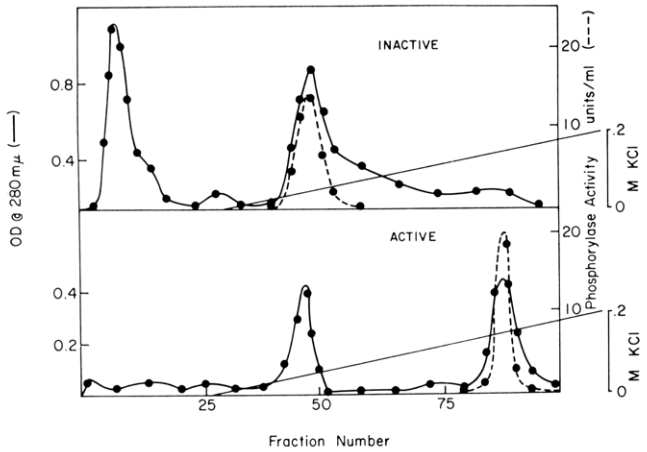

FIGURE 1: Elution pattern of rabbit liver glycogen phosphorylases from DEAE-cellulose column chromatography. Columns were equilibrated with 2 mM Tris·HCl–2 mM EDTA at pH 7.0 and 10-ml fractions were collected. Upper profile: a dephosphophosphorylase fraction containing 5000 "high-salt" units (4.9 units/mg of protein) was applied to a 2 × 60 cm column and 1500 units with a specific activity of 12 were eluted. Lower profile: a phosphophosphorylase fraction containing 4000 low-salt units (30 units/mg of protein) was applied to a 1 × 30 cm column and 2000 units with a specific activity of 48 were eluted. The activity of dephosphophosphorylase was determined in the presence of 0.7 M sodium sulfate while phosphophosphorylase activity was measured in the usual low-salt assay.

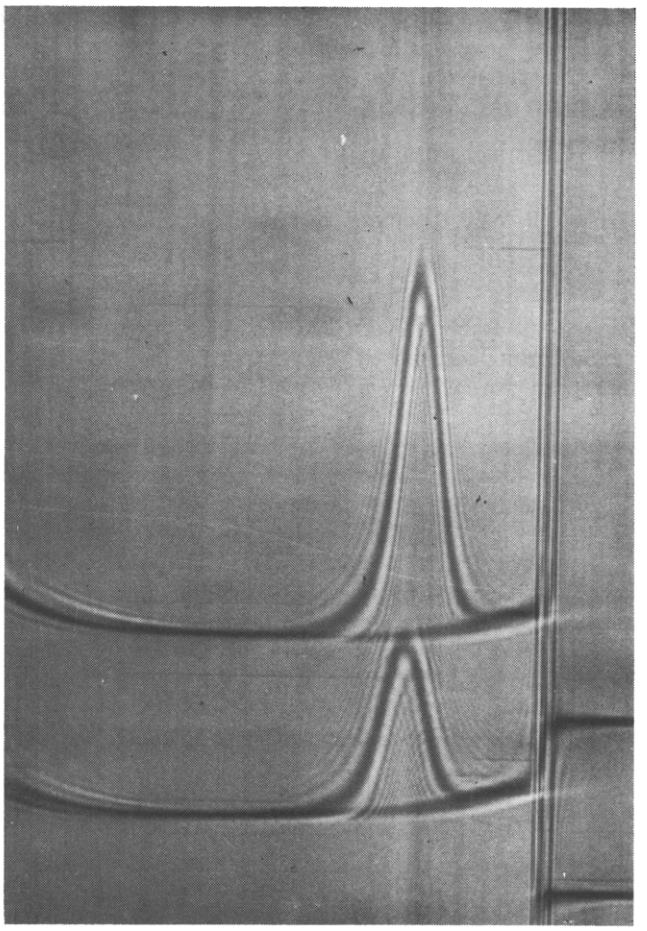

FIGURE 2: Sedimentation velocity patterns of rabbit liver glycogen phosphophosphorylase at 11.0 mg/ml (upper pattern) and 5.5 mg/ml (lower pattern) in 50 mM sodium glycerophosphate, 50 mM KCl, 2 mM EDTA, and 15 mM 2-mercaptoethanol (pH 6.9). Sedimentation from right to left was carried out for 40 min at 52,100 using a bar angle of 70°, $s_{20,w} = 8.3$ S.

(1965) where 1 unit of activity represents the release of 1 μmole of P_i from glucose 1-phosphate per min at 30°. Assays of the dephosphorylated enzyme were carried out with the addition of 10^{-3} M AMP and sodium sulfate at a final concentration of 0.7 M and have been referred to as "high-salt" activities, in contrast to "low-salt" activities determined in the absence of sodium sulfate (Appleman *et al.*, 1966). The low-salt assay measures only the phosphorylated or active form of the enzyme. The following maximum specific activities were obtained for the two forms of the enzyme under the four possible sets of assay conditions: phosphophosphorylase high-salt assay +AMP 36, −AMP 19; low-salt assay +AMP 70, −AMP 50. For dephosphophosphorylase, the following values were obtained: high-salt assay +AMP 25, −AMP 13; low-salt assay +AMP 2, −AMP 0.

Protein concentration of phosphorylase solution was determined spectrophotometrically arbitrarily using the absorbancy index $A_{280}^{1\%}$ 11.9 obtained for the rabbit muscle enzyme (Appleman *et al.*, 1963).

Sedimentation equilibrium studies were performed in the Spinco Model E analytical ultracentrifuge according to the procedure of Yphantis (1964) equipped with interference optics. Triplicate sedimentation equilibrium runs were carried out on 0.05–0.1% solutions of phosphorylase in 50 mM sodium glycerophosphate, 50 mM KCl, 2 mM EDTA, and 0.1 M 2-mercaptoethanol at pH 7.0. Data reduction was performed on the IBM 7090–7094 IBSYS system using computer programs developed by Teller *et al.* (1969).

Amino acid composition was performed by the procedure outlined by Moore and Stein (1963) using norleucine (0.1 μmole/ml) as an internal standard (Walsh and Brown, 1962). Phosphorylase (5–10 mg/ml) was dissolved in and extensively dialyzed against 20 mM Tris·HCl (pH 7.0). Suitable aliquots of the protein–norleucine solution containing 1 mg of protein were lyophilized and hydrolyzed *in vacuo* (after repeated flushing with nitrogen) with 1 ml of twice redistilled 5.7 N HCl. Hydrolyses were carried out on duplicate samples at 108° for 24, 48, 72, and 96 hr. Peptides were hydrolyzed for 24 hr only. After removal of HCl in a vacuum desiccator over NaOH, the samples were analyzed on a Spinco Model 120C analyzer. "Half-cystine" was determined as cysteic acid after performic acid oxidation of the protein according to the procedure of Moore (1963). Tryptophan was determined spectrophotometrically by the procedures of Bencze and Schmid (1957) and Edelhoch (1967).

Conversion of liver dephosphophosphorylase into the phosphorylated form was carried out according to the method of Krebs (1966) using rabbit muscle phosphorylase kinase. When ^{32}P-labeled phosphorylase was required, [γ-^{32}P]ATP, prepared according to Glynn and Chappell (1964), was employed. The specific radioactivity was usually in the range of 10^7–10^8 cpm/μmole. Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer using a scintillant containing 125 g of naphthalene, 7.5 g of 2,5-diphenyloxazole, and 375 mg of 1,4-bis[2-(5-phenyloxazole)]benzene dissolved in 1 l. of dioxane.

TABLE I: Amino Acid Composition of Rabbit Liver Glycogen Phosphorylase.

Amino Acid	Liver Av (g/100 g)[b]	Residues/ 92,500 g of Protein	Skeletal Muscle[a] Phosphorylase Residues/ 92,500 g of Protein
Lysine	8.60 ± 0.17	55	43
Histidine	3.62 ± 0.14	22	19
Arginine	8.25 ± 0.17	44	59
Aspartic acid	13.15 ± 0.18	92	91
Threonine[c]	4.31 ± 0.12	33	32
Serine[c]	4.42 ± 0.10	39	27
Glutamic acid	13.39 ± 0.11	84	94
Proline	4.54 ± 0.10	36	39
Glycine	3.99 ± 0.04	49	46
Alanine	5.98 ± 0.18	62	60
Valine[d]	7.40 ± 0.17	58	57
Methionine	3.55 ± 0.14	23	20
Isoleucine[d]	6.85 ± 0.27	48	45
Leucine[d]	11.30 ± 0.13	80	75
Tyrosine	4.90 ± 0.12	25	34
Phenylalanine	6.97 ± 0.06	39	36
Half-cystine[e]	1.57 ± 0.12	12	9
Tryptophan[e]	2.77 ± 0.09	12	12

[a] Data from Sevilla and Fischer (1969). [b] Average of chromatographic data reported here; includes standard deviation of the mean. [c] Standard deviation of the intercept following extrapolation of least-squares plots to zero time of hydrolysis. [d] Extrapolated to maximum time of hydrolysis. [e] Determined as described in text.

Protein-bound ^{32}P was determined after precipitating samples with trichloroacetic acid in the presence of carrier bovine serum albumin according to DeLange *et al.* (1968).

Isolation of phosphopeptides and amino acid sequence determinations were carried out using methods similar to those described by Fischer *et al.* (1959) and Nolan *et al.* (1964) for the characterization of the phosphorylated site of rabbit muscle phosphorylase *a*. Twice-recrystallized, 1-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone treated trypsin and diisopropylphosphorofluoridate-treated leucine aminopeptidase (Worthington) were used in these procedures. Amino-terminal groups were determined by the dansyl chloride[1] method of Gray (1964); the substituted amino acids were identified by comparison with dansyl amino acid standards following two-dimensional, thin layer chromatography in chloroform–methanol–acetic acid (90:10:1) and 1-propanol–concentrated ammonia (80:20) on prepared 20 × 20 cm plates of silica gel G purchased from Brinkmann Instruments.

[1] Abbreviation used is: dansyl-Cl, 5-dimethylaminonaphthalene-1-sulfonyl chloride.

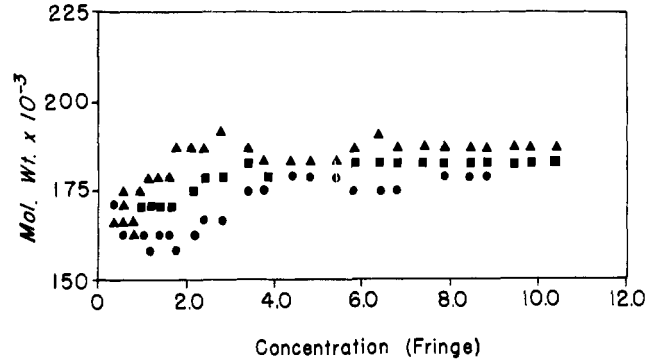

FIGURE 3: High-speed equilibrium run of active rabbit liver phosphorylase (phosphoenzyme); representative distribution of molecular weight moments as obtained from the computer program of Teller *et al.* (1969). ■ represents $M_{n,x}$, ▲ represents $M_{w,x}$, and ● represents $2M_{n,x} - M_{w,x}$. Phosphorylase (0.5 mg/ml) was centrifuged in 50 mM sodium glycerophosphate, 50 mM KCl, 2 mM EDTA, and 15 mM 2-mercaptoethanol buffer (pH 6.9). After 5-hr overspeeding at 20,000 rpm, the rotor speed was adjusted to 13,000 rpm until equilibrium was reached (13 hr).

Results

Molecular Weight of Liver Phosphophosphorylase. Phosphophosphorylase isolated according to Maddaiah and Madsen (1966) was subjected to rechromatography on DEAE-cellulose. The peak tubes, containing maximum enzyme activities, were pooled and dialyzed against neutral saturated ammonium sulfate until precipitation occurred. The precipitate, collected by centrifugation, was suspended in 50 mM KCl–2 mM EDTA–50 mM sodium glycerophosphate–1.5 mM 2-mercaptoethanol (pH 7.0) and this solution was dialyzed overnight against the same buffer. The resulting preparation produced a single band when subjected to acrylamide gel electrophoresis (Davis *et al.*, 1967) and sedimented as a single component in the analytical ultracentrifuge (Figure 2). The purified enzyme displayed a specific activity of 50 units/mg (1500 units/mg measured by the method of Illingworth and Cori, 1953) when assayed in the presence of AMP.

High-speed equilibrium centrifugation was carried out by the procedure of Yphantis (1964). The molecular weight was determined using a partial specific volume of 0.741 cc gm^{-1}, calculated from the amino acid composition. Number- ($M_{n,r}$ = 180,994), weight- ($M_{w,r}$ = 188,430), and z-average ($M_{z,r}$ = 193,000) molecular weights were obtained as an average of five determinations with initial enzyme concentrations ranging from 0.5 to 1 mg per ml (Figure 3). Very similar values were reported for the dimeric forms of other phosphorylases (Seery *et al.*, 1967; Sevilla and Fischer, 1969). Identical sedimentation constants of 8.3 were also obtained for the two forms of rabbit liver phosphorylase confirming earlier reports for this enzyme (Appleman *et al.*, 1966) and for dog liver phosphorylase (Wosilait and Sutherland, 1956).

Amino acid analysis was carried out on the same phosphorylase sample used for the molecular weight studies; results are presented in Table I. The values obtained for serine, threonine, and proline decreased with the time of hydrolysis; as usual extrapolation to zero time was carried out. The intercept, obtained from a least-squares plot of the data, and

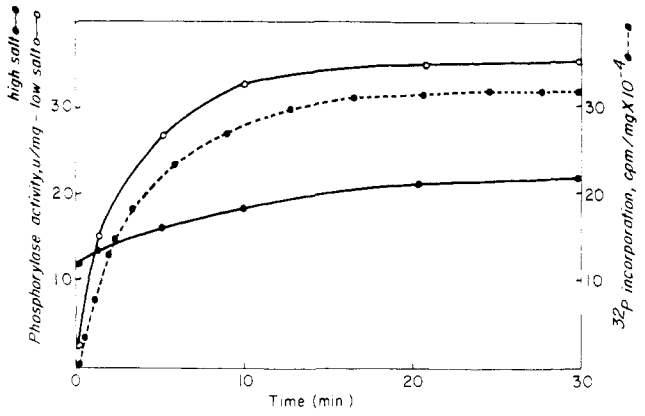

FIGURE 4: Phosphorylation of inactive liver glycogen phosphorylase with purified rabbit muscle phosphorylase kinase. The reaction mixture consisted of 9.6 ml of a partially purified phosphorylase fraction obtained from a DEAE-cellulose chromatography, 0.05 M magnesium acetate–0.016 M [γ-^{32}P]ATP (5×10^7 cpm/μmole), and 0.4 ml (0.24 mg/ml containing 32,600 units/mg at pH 8.2) of activated kinase. The final phosphorylase concentration of the reaction mixture was 7.7 mg/ml.

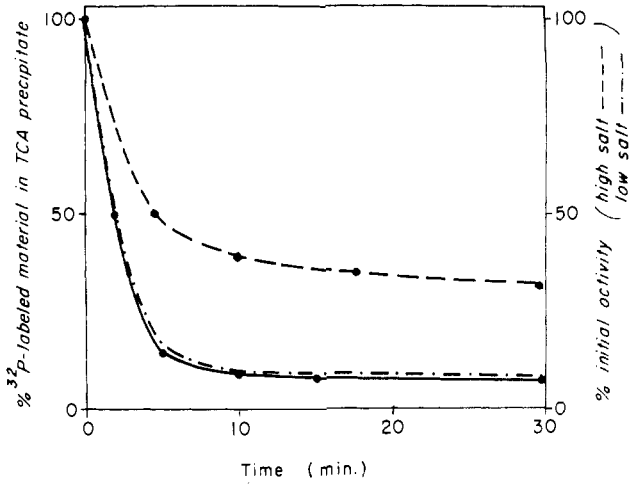

FIGURE 6: Time course of a typical tryptic release of ^{32}P from ^{32}P-labeled rabbit liver glycogen phosphorylase. The reaction mixture consisted of 38 ml of enzyme (1.7×10^6 cpm, 170 mg of protein) and 1 mg of trypsin. The reaction was carried out at room temperature.

its standard deviation are presented in Table I. Values for valine, leucine, and isoleucine, were obtained following extrapolation to maximum time of hydrolysis; all other amino acids gave values independent of the hydrolysis time and are presented as the mean of all points. The amino acid composition of muscle phosphorylase *a* has been included for comparison (Sevilla and Fischer, 1969).

Phosphorylation of Liver Phosphorylase Using [γ-^{32}*P*]*ATP.* Rabbit liver dephosphophosphorylase preparations (5–15 mg/ml) were dialyzed against 50 mM sodium glycerophosphate–15 mM 2-mercaptoethanol (pH 8.2) prior to phosphorylation with purified rabbit muscle phosphorylase kinase and [γ-^{32}P]ATP. Figure 4 shows the correlation between the appearance of phosphorylase activity (as measured in the low-salt assay) and phosphate uptake. Activity measured in the high salt assay is also shown. After 30 min, radioactivity incorporation reached 0.65 mole of phosphate/92,500 g of protein.

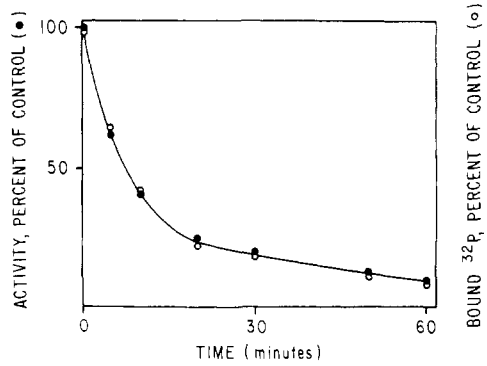

FIGURE 5: Conversion of active liver phosphorylase (phosphophosphorylase) into its inactive form with purified muscle phosphorylase phosphatase. A phosphorylase solution (0.9 ml) containing 55 low-salt units/ml and 6250 protein bound cpm/ml was dialyzed against 50 mM Tris–30 mM 2-mercaptoethanol (pH 7.5) prior to addition of 0.1 ml (*ca.* 60 units) phosphorylase phosphatase.

The phosphate incorporated into liver phosphorylase was characterized by subjecting aliquots of the phosphorylated enzyme to the action of purified rabbit muscle phosphorylase phosphatase (Hurd, 1967). In a typical experiment this enzyme catalyzed the release of 90% of the protein-bound radioactivity, with a parallel loss in low salt activity (Figure 5). Further evidence indicating that radioactivity was being incorporated only into phosphorylase was obtained by electrophoresis of the reaction mixture on 5% acrylamide gels: radioactivity appeared exclusively in the major protein band also displaying phosphorylase activity when measured according to the procedure of Davis *et al.* (1967).

Trypsin Hydrolysis of ^{32}*P-Labeled Liver Phosphorylase.* ^{32}P-labeled liver phosphorylase was isolated from the phosphorylation reaction mixture by repeated precipitation with 1.5 volumes of saturated ammonium sulfate followed by dialysis against distilled water. In a typical tryptic attack, 1 mg of crystalline trypsin was added to 38 ml of the dialyzed phosphorylase preparation containing 170 mg of protein. Aliquots (0.1 ml) of the reaction mixture were removed at intervals and measured for trichloroacetic acid precipitable radioactivity. Aliquots were also removed, diluted approximately 100-fold in 0.1 M maleate buffer (pH 6.5), and assayed for phosphorylase activity which was determined in the presence and absence of 0.7 M sodium sulfate. A rapid release of protein-bound radioactivity paralleled by the simultaneous loss of low-salt activity was observed (Figure 6). High salt activity decreased to 30% of its initial value during proteolysis; this residual activity presumably is equivalent to that seen with muscle phosphorylase *b'* following tryptic attack of phosphorylase *a* (Cori and Cori, 1945; Keller, 1955; Nolan *et al.*, 1964). After a 30-min incubation, one-tenth volume of cold 50% trichloroacetic acid was added and the resulting suspension was cooled in ice for 15 min. The suspension was centrifuged and the pellet washed with two 10-ml portions of cold 5% trichloroacetic acid. The supernatant solution and washings obtained from several tryptic digestions involving a total of 390 mg of phosphorylase were combined,

TABLE II: Properties of Purified Rabbit Liver Phosphopeptides A, B, and C.

Property	Peptide A	Peptide B	Peptide C
Ninhydrin reaction	None	+	+
Electrophoretic migration on paper[a]			
At pH 3.6 (in centimeters)	+2.5	−3.0	−8.0
At pH 6.5 (in centimeters)	+6.0	0	−2.5
Approximate R_F values in butanol–acetic acid–10^{-3} M EDTA (4:1:5)	0.4	0.2	0.1

[a] 2000 V, 35 mA for 1 hr; not corrected for migration due to electroosmosis.

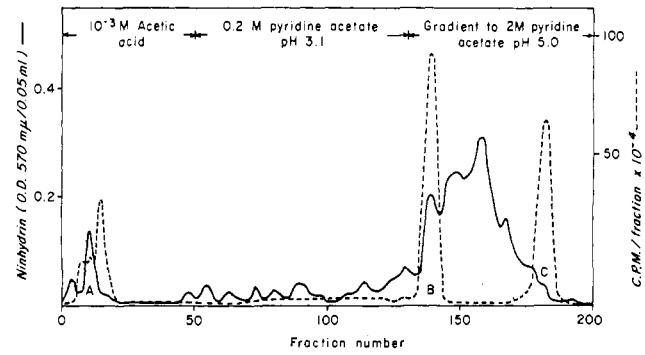

FIGURE 7: Chromatographic separation of ^{32}P-labeled peptides from rabbit liver glycogen phosphorylase. Phosphopeptide (2.65 μmoles) (1.4×10^7 cpm) was applied to a 0.9×120 cm column of Dowex 50-X2 equilibrated with dilute acetic acid at 37°. Ten-ml fractions were collected and 90% of the applied counts were eluted from the column in peaks A, B, and C.

extracted five times with equal volumes of diethyl ether, and lyophilized. A total of 2.65 μmoles of phosphopeptide containing 1.4×10^7 cpm was obtained.

Purification of ^{32}P-Labeled Phosphopeptides. The radioactive peptide mixture was purified by column chromatography on Dowex 50-X2 (Figure 7). Three radioactive fractions were collected and designated A, B, and C in analogy to the phos hopeptides isolated from muscle phosphorylase *a* (Fischer *et al.*, 1959); each was pooled and concentrated by rotary evaporation under reduced pressure at 25–35° and purified further on paper by high-voltage electrophoresis and chromatography.

Chromatography of fraction C resulted in the separation of one major and one minor radioactive band from several nonradioactive peptides. The R_F of the minor band corresponded to that obtained for purified peptide B in the same solvent system. Several ninhydrin positive, nonradioactive bands were also present and three combinations of chromatography and electrophoresis on paper were required before a single radioactive, ninhydrin positive spot resulted. Of 0.88 μmole of fraction C (based on radioactivity) eluted from Dowex 50-X2, 0.34 μmole was obtained in pure form. Fractions A and B also further purified by paper electrophoreses and chromatographies; however, the purification of peptide B was hampered by its spontaneous conversion to a peptide which had the chromatographic and electrophoretic characteristics of peptide A. The properties of the purified peptides A, B, and C from liver phosphorylase are listed in Table II.

Determination of the Amino Acid Sequence of Peptide C. Quantitative amino acid analyses of four preparations of purified peptide C gave the following average values in micromoles: serine, 0.063 (1); glutamic acid, 0.077 (1); isoleucine, 0.123 (2); and arginine, 0.113 (2). The concentration of Ser-P based on radioactivity measurements was 0.077 (1) μmole. Peptides B and A gave similar compositions except for the presence of only one arginyl residue instead of two.

The amino acid sequence of radioactive hexapeptide C was determined by the following series of experiments. Peptide C (0.01 μmole) was treated with dansyl-Cl and hydrolyzed, and the resulting dansyl-amino acids were identified as described under Methods; only dansylarginine was detected. Likewise the amino-terminal amino acid in peptide B was identified as glutamic acid. No amino-terminal residue was detected for peptide A by this method, as would have been expected if the amino-terminal glutaminyl residue had cyclized to a pyrrolidonecarboxylic derivative as found for the phosphopeptide derived from rabbit muscle phosphorylase (Fischer *et al.*, 1959). Incubation of peptide C (0.02 μmole) with 0.6 mg of trypsin for 24 hr at 25° followed by separation of the reaction components resulted in the detection of radioactivity in those spots corresponding to peptides B and A. Only the amino-terminal arginyl residue was released, and glutamic acid appeared as the new amino-terminal group. Peptide C (0.1 μmole) in 3 ml was incubated with 20 ml of leucine aminopeptidase for 24 hr at 25° in the presence of 0.02 M Tris–0.08 M $MnCl_2$ (pH 8.0). The resulting mixture was separated by high-voltage paper electrophoresis. One major radioactive peptide was eluted and its amino-terminal group identified as isoleucine by its dansyl derivative.

This radioactive peptide was subjected to one turn of the Edman procedure upon which the amino-terminal isoleucyl residue was released as its phenylthiohydantoin derivative. Dansylation of the remaining radioactive tripeptide followed by acid hydrolysis yielded dansylserine (amino terminal) and free isoleucine and arginine, indicating the sequence (P)Ser-Ile-Arg for the carboxyl-terminal end of hexapeptide C.

Two sets of evidence indicated that glutamic acid in the peptide existed in the amide form. (a) Considering the charge of the six amino acids and that of the phosphate group in peptide C, peptide C should have behaved as a neutral compound at pH 6 whereas it clearly migrated toward the negative pole (Table II). Similarly, peptide B should have been acidic at this pH, whereas its expected acidic character became apparent only after conversion into peptide A. (b) The ability of an amino-terminal glutaminyl residue to undergo cyclization to the pyrrolidone derivative has been well documented (Hirs *et al.*, 1956) and would account for the loss in reactivity of peptide A toward ninhydrin and dansyl-Cl.

TABLE III: Summary of Enzymatic Degradation and End-Group Analysis of Phosphopeptides *from Active Liver Phosphorylase.*

Treatment	Material Attacked	Radioactive Peptide Formed	Amino-Terminal Group	Amino Acid Released
1. Tryptin digestion	[^{32}P]Phosphophosphorylase	C	Arg	
		B	Glu	
		A	None	
2. Spontaneous conversion	B	A	None	
3. Trypsin	C	B, A	Glu	Arg
4. Leucine aminopeptidase	C	Major peptide	Ile	Arg, Gln (Ile)[a]
5. Edman procedure	Major peptide from step 4	Tripeptide	Ser	Phenylthiohydantoin-Ile derivative

[a] Trace amounts of isoleucine were produced due to further degradation of the remaining tripeptide by leucine aminopeptidase.

The above results, summarized in Table III, in conjunction with the known specificity of trypsin (Neurath, 1957) clearly indicate that the hexapeptide isolated from rabbit liver phosphorylase has the sequence: Arg-Gln-Ile-Ser(P)-Ile-Arg.

Discussion

Obvious structural similarities in the isozymes of phosphorylase can be seen when the amino acid sequence of the phosphate incorporating sites are compared. Identical sequences of Lys-Gln-Ile-Ser(P)-Val-Arg have been reported for the hexapeptides isolated from human (Hughes *et al.*, 1962), rabbit (Fischer *et al.*, 1959; Nolan *et al.*, 1964), and rat skeletal muscle phosphorylase (Sevilla and Fischer, 1959), while a tetradecapeptide from the latter two enzymes differs by only one conservative substitution. The sequence of the liver hexapeptide differs from its skeletal muscle counterpart by two conservative substitutions, an arginyl for a lysyl residue and an isoleucyl for a valyl residue. That the structural differences observed in these phosphopeptides does not markedly affect their charge or chemical properties is substantiated by the ability of muscle phosphorylase phosphatase and kinase to react readily with the phosphorylases from both liver and muscle. Structural similarities between these enzymes are also indicated in the remarkable selectivity of trypsin for the phosphorylated site. With the muscle enzyme, limited tryptic attack results in the production of phosphorylase *b'*, a form of the enzyme that is still enzymatically active but requires AMP for activity (Cori and Cori, 1945; Keller, 1955; Nolan *et al.*, 1964). Consistent with this observation is the production of a liver enzyme that is active when assayed in the presence of high salt concentration.

A molecular weight of 185,000 for rabbit liver phosphorylase is lower than the value of 237,000 proposed by Wosilait and Sutherland (1956), but compares well with values of 185,000–200,000 determined for the dimeric forms of skeletal muscle and heart phosphorylases. The original observations concerning the identical size of the dephosphorylated and phosphorylated forms of dog liver phosphorylase have been confirmed for the rabbit enzyme. Based on molecular weight considerations similarities in the oligomeric structures of the liver and skeletal muscle enzymes have been assumed, even though relatively little is known concerning the subunit structure of the liver enzyme. The existence of a monomer has never been demonstrated although limited association–dissociation of the enzyme has been observed upon exposure to *p*-mercuribenzoate (Appleman *et al.*, 1966).

A comparison of the amino acid analyses of rabbit liver and skeletal muscle phosphorylases listed in Table I indicates that approximately one-third of the residues differ by less than 2 residues/mole of enzyme subunit (92,500*g* protein), another third by 3–5 residues/mole, and the rest by 9–15 residues/mole. However, even in this latter category, differences might not be as significant as they may seem in terms of the overall chemical characteristics of the two enzymes. For instance, lysine and arginine differ by 12 and 15 residues respectively. However, the sum of both (99 in liver phosphorylase *vs.* 102 in the muscle enzyme) differs by only 3 residues out of a total of approximately 800 residues. The same can be said if one groups together all hydrophobic amino acids; the sum of alanine, leucine, isoleucine, valine, methionine, tyrosine, and tryptophan is essentially the same, namely, 308 *vs.* 303. Nevertheless the two enzymes differ in their overall net charge (there is a 10-residue difference in the content of glutamic acid + glutamine) and, consequently, have different mobilities on acrylamide gel electrophoresis (Davis *et al.*, 1967).

Finally, when the overall amino acid composition of rabbit liver phosphorylase is compared with that of other known phosphorylases, in terms of the composition divergence factor of Harris (1969), one can see a greater difference between the rabbit liver and muscle phosphorylase than between the muscle enzymes from widely different species (rabbit, rat, man, frog, and dogfish[2]). The same conclusion was reached over 12 years ago by Henion and Sutherland (1957): using the criterion of inhibition by antibodies, these authors concluded that there was more "similarity" among the liver phosphorylases of various species than between muscle and liver phosphorylases of the same animal.

[2] Philip Cohen, unpublished results from this laboratory.

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