Amino Acid Sequence of Two Functional Sites in Yeast Glycogen Phosphorylase†

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ABSTRACT: The structure of two functional sites in baker's yeast (*Saccharomyces cerevisiae*) glycogen phosphorylase (EC 2.4.1.1) was determined as part of a study on the evolution of regulatory enzymes. S-Carboxymethylated, $NaBH_4$-reduced ^{32}P-labeled yeast phosphorylase *a* was cleaved with CNBr, thermolysin, and pepsin. Peptides labeled with ^{32}P or carrying the fluorescent pyridoxyl marker were isolated and purified using ion-exchange chromatography and gel filtration. CNBr cleavage yielded a single radioactive phosphopeptide (42 residues long) and one small fluorescent peptide with the unique sequence ε-Pxy-Lys-Phe-Val-Met. Thermolysin digestion gave rise to one radioactive octapeptide and two fluorescent peptides, 15 and 2 residues long, respectively. From a combination of subtractive Edman degradations and digestion with yeast protease C, the sequence of the ^{32}P-labeled octapeptide was established. Phosphothreonine was identified as the sole phosphorylated amino acid, giving the following structure for the site involved in the covalent regulation of yeast phosphorylase: Leu-Thr(P)-Gly-Phe-Leu-Pro-Gln-Glu. The two fluorescent thermolytic peptides, together with two additional pyridoxyl peptides isolated after peptic digestion of the enzyme yielded the following sequence around the site binding pyridoxal-5′-P, the cofactor essential for phosphorylase activity: Ile-Ser-Thr-Ala-Gly-Thr-Glu-Ala-Ser-Gly-Thr-Ser-Asn-Met-Lys(P-Pxy)-Phe-Val-Met. While the phosphorylated site bears no resemblance to the site of covalent control in vertebrate phosphorylases, the pyridoxal-P binding site in the yeast enzyme displays remarkable homologies with its animal counterparts; the finding that 14 out of 18 amino acids are identical strongly suggests that the cofactor must be directly involved in catalysis.

These studies were undertaken to investigate the evolution of intracellular control mechanisms. Glycogen phosphorylase seemed to be ideally suited for this purpose since it is essentially ubiquitous to all forms of life and its activity is subjected to both covalent and noncovalent regulation. Covalent (as opposed to allosteric or metabolic) regulation was thought initially to represent some sort of added sophistication in control processes possibly restricted to higher organisms; it has since been demonstrated in prokaryotes as well as in a variety of eukaryotes.

Yeast was selected because it is a unicellular eukaryote that has branched out from the main line of evolution leading to mammals more than a billion years ago. A previous publication (Fosset et al., 1971) reported the properties of purified yeast phosphorylase: the enzyme displays distinct similarities with mammalian phosphorylases in that it has a subunit molecular weight of ca. 100,000, and exists in two interconvertible forms that were designated as *b* and *a*, respectively, since one is partially active and the other, fully active. Conversion of yeast phosphorylase *b* to *a* was shown to require Mg–ATP and a specific phosphorylase kinase different from the mammalian phosphorylase kinases. Uptake of radioactivity from [γ-^{32}P]ATP was demonstrated but no further study of the site phosphorylated was carried out.

Yeast phosphorylase, like all glycogen phosphorylases so far investigated, contains 1 molecule of pyridoxal-5′-P[1]/subunit. In many other instances, resolution under mild conditions has led to total loss of activity that could be restored upon addition of the cofactor, but this has not yet been achieved with the yeast enzyme. However, the exact role of the cofactor has never been fully explained.

Since yeast phosphorylase is so far apart from its vertebrate or mammalian counterparts in terms of evolutionary distance, it appeared of particular interest to investigate the structure of some of its functional sites and determine which feature might have been conserved. This article reports on the primary structure of a segment of the phosphorylated site responsible for the covalent regulation of the enzyme and on the pyridoxal-5′-P binding site essential for its activity. This is the 12th publication of a series on the comparative properties of glycogen phosphorylases; for previous publication, see Cohen et al. (1973).

Materials and Methods

Fleischman's baker's yeast (*Saccharomyces cerevisiae*) was obtained in 1-lb pressed cakes as a generous gift of Standard Brands, Inc.

Iodoacetic acid (Eastman) was recrystallized three times from petroleum ether. Urea was purified by treating a saturated solution with a mixed-bed resin (AG 501-X8 from Bio-Rad) and subsequently crystallized from 50% ethanol–water. [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell (1964). CNBr (Eastman) and pyridine (Baker) were used without further purification. Dowex 50-X2 was processed according to Schroeder (1967) and Dowex 50-X8 according to Schaffer (1967). Three-times

† From the Department of Biochemistry, University of Washington, Seattle, Washington 98195. *Received December 11, 1974.* This work was supported by grants from the National Science Foundation (GB 3249), the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health (AM 07902), and the Muscular Dystrophy Association of America. This is the 12th publication of a series on the Comparative Properties of Glycogen Phosphorylases; for previous publication see Cohen et al., 1973.

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[1] Abbreviations used are: Pxy, pyridoxyl; P-Pxy, pyridoxyl-5′-P; dansyl, dimethylaminonaphthalenesulfonyl.

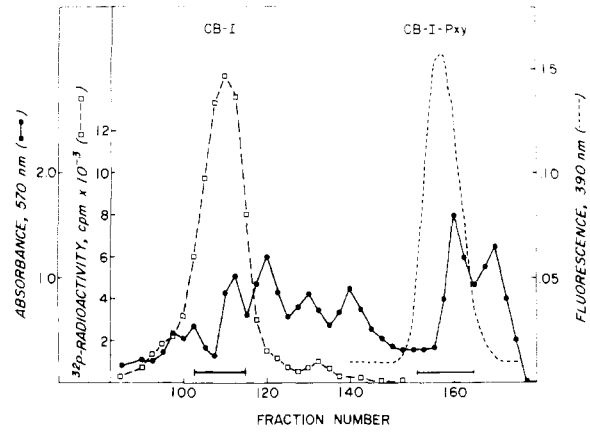

FIGURE 1: Sephadex G-50 elution profile of the soluble fraction from a CNBr digest of S-carboxymethylated, $NaBH_4$-reduced [^{32}P]phosphorylase *a* from yeast. Horizontal lines indicate fractions pooled after elution. The flow rate was 20 ml/hr; 3-ml fractions were collected.

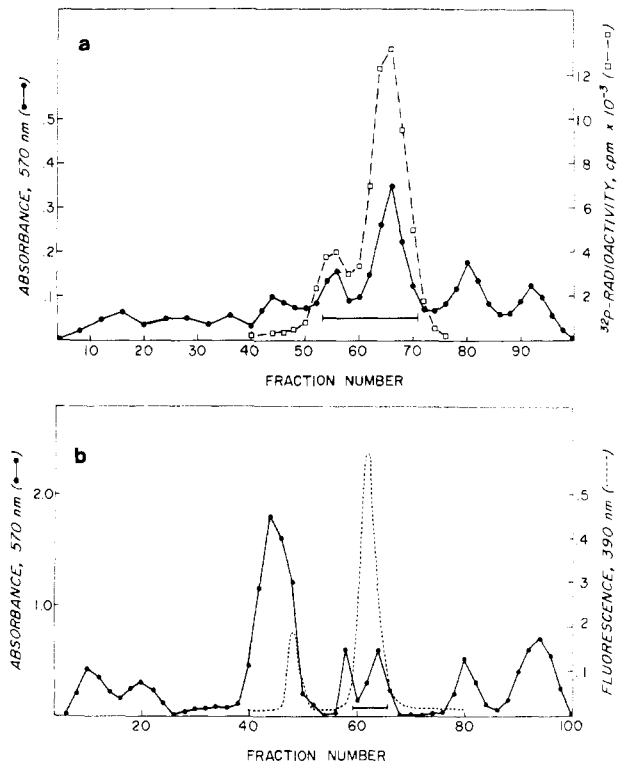

FIGURE 2: (A) Dowex 50-X2 chromatography of the radioactive fraction from Sephadex G-50. Fractions (2 ml) were collected at a flow rate of 15 ml/hr; those designated by the horizontal bar were pooled. (B) The flow rate was 15 ml/hr; 2-ml fractions were collected. Fractions marked by the horizontal bar were pooled.

recrystallized thermolysin was obtained from Calbiochem; pepsin (two times crystallized), diisopropyl phosphorofluoridate treated carboxypeptidase A, and *Escherichia coli* alkaline phosphatase were from Worthington. Yeast protease C was a generous gift from Dr. P. Levi of this Department.

Phenyl isothiocyanate, pyridine, ethyl acetate, and trifluoroacetic acid for Edman degradation were Pierce "sequenal grade."

Yeast phosphorylase b and *a* were prepared by the method of Fosset et al. (1971). The initial DEAE-Sephadex chromatography was replaced by a DE-52 (Whatman) chromatography followed by a heat treatment for 1 hr at 30°. ^{32}P-labeled phosphorylase *a* was prepared by phosphorylation of phosphorylase *b* with [γ-^{32}P]ATP and a partially purified preparation of yeast phosphorylase kinase (Lerch et al., 1975).

Cyanogen bromide cleavage of phosphorylase (10 mg/ml) was performed in 70% formic acid with a 100-fold molar excess of CNBr over methionine content (Steers et al., 1965; Gross, 1967) at room temperature and in the dark for 20 hr. The reaction mixture was diluted tenfold with water and subsequently lyophilized.

Enzymatic Digestions. Thermolysin was dissolved in 5 m*M* $CaCl_2$ and the substrate (2.0 μM) digested at 40° in 40 ml of 0.1 *M* NH_4HCO_3 buffer (pH 8.0) for 24 hr at a 1:100 molar ratio. Pepsin digestion of phosphorylase (2 mg/ml) was carried out in 5% formic acid at 25° for 18 hr by adding 1% (w/w) pepsin. Carboxypeptidase A (1 mg/ml) in 2 *M* NH_4HCO_3 was added to 0.4 ml of peptide solutions (1:50 molar ratio) in 0.2 *M* *N*-ethylmorpholine buffer (pH 8.5) at 30°. Aliquots were removed and diluted into 1.0 ml of sodium citrate buffer (pH 2.2) and applied immediately to the amino acid analyzer. Yeast protease C (1 mg/ml) in distilled water was added to 0.4 ml of peptide solutions (1:50 molar ratio) in 0.02 *M* pyridine-acetate buffer (pH 5.0) at 30°.

Amino acid analyses were performed on a Beckman Model 120 C automatic analyzer according to Moore and Stein (1963). N^{ϵ}-(Pxy)-lysine was determined according to Forrey et al. (1971); its destruction during 22-hr hydrolysis in 5.7 *N* HCl was approximately 40%. In protease C digests, glutamine was distinguished from glutamic acid on the 60-cm column of the amino acid analyzer by running the pH 3.22 buffer at 33° instead of 55°. Peptides were hydrolyzed in 1 ml of constant boiling 5.7 *N* HCl for 22 hr in nitrogen-flushed then evacuated sealed tubes at 108°. Homoserine lactone was converted to homoserine and analyzed according to Saari and Fischer (1973).

High voltage paper electrophoresis was carried out at pH 3.5 (Ryle et al., 1955). Phosphoserine and phosphothreonine were separated on a Dowex 50-X8 column according to Rask et al. (1970). Chromatography on Dowex 50-X2 was carried out either on 1 × 100 or 0.6 × 60 cm columns using the volatile buffer systems of Schroeder (1967). Gel filtration on Sephadex G-50 equilibrated in 1 *M* acetic acid was carried out in a 2.5 × 120 cm column. Peptides were analyzed by reaction with ninhydrin after alkaline hydrolysis (Hirs, 1967). Radioactive fractions were measured by liquid scintillation counting according to Saari and Fischer (1973); phosphopyridoxyl-containing fractions were detected by diluting 20-μl aliquots into 0.2 ml of 0.1 *M* phosphate buffer (pH 7.0) and measuring the characteristic fluorescence emission at 390 nm following excitation at 330 nm in a Farrand spectrofluorometer.

Sequence determinations were carried out by substractive Edman degradations according to Gray (1967) with slight modifications.[2] Peptides were dissolved in 0.2 ml of 50% pyridine to which 0.1 ml of 5% phenyl isothiocyanate in pyridine was added. Coupling was allowed to proceed at 45° for 60 min and the reaction mixture was flash-evaporated at 40°. The phenylthiocarbamyl derivative was cleaved with 0.2 ml of trifluoroacetic acid at 45° for 15 min and trifluoroacetic acid was removed under a stream of nitrogen. The residue was taken up in 150 μl of H_2O and ex-

[2] Detailed analyses of subtractive Edman degradations not reported here have been submitted for examination to the reviewers and can be obtained by writing directly to the authors.

Table I: Composition of the CNBr, Thermolysin, and Pepsin Peptides from Yeast Phosphorylase.

	CB-I	CB-I-Pxy	Th-I	Th-I-Pxy	Th-II-Pxy	P-I-Pxy	P-II-Pxy
ε-Pxy-lysine		0.62 (1)		0.87 (1)	0.78 (1)	0.69 (1)	0.67 (1)
Lysine	2.00 (2)						
Histidine	1.00 (1)						
Arginine	3.41 (3–4)						
Aspartic acid	1.75 (2)				1.21 (1)		1.26 (1)
Threonine	4.95 (5)		0.84 (1)		2.60 (3)		1.05 (1)
Serine	1.84 (2)				2.43 (3)		1.79 (2)
Homoserine	0.94 (1)	1.00 (1)					
Glutamic acid	7.13 (7)		2.10 (2)		1.14 (1)		
Proline	4.53 (4–5)		0.92 (1)				
Glycine	1.54 (1–2)		1.00 (1)		2.22 (2)		1.34 (1)
Alanine	1.02 (1)				2.12 (2)		0.89 (1)
Valine		0.94 (1)					
Methionine				1.00 (1)	1.03 (1)	1.00 (1)	0.94 (1)
Isoleucine	4.56 (4–5)				0.80 (1)		
Leucine	3.81 (4)		2.02 (2)				
Tyrosine							
Phenylalanine	1.07 (1)	1.00 (1)	0.98 (1)			0.97 (1)	1.00 (1)
Tryptophan							
Residues	38–42	4	8	2	15	3	9
Yield (%)	55	45	80	30	28	25	35
NH_2-terminal group	Ile	ε(Pxy)Lys	Leu	Met	Ile	Met	Ala

tracted three times with 0.5 ml of ethyl acetate; aliquots of the aqueous layer (5–20 nmol) were used for amino acid analysis.

Direct Edman degradation was carried out by the same procedure. Phenylthiohydantoin amino acids were identified directly by gas chromatography as the silylated derivatives (Hermodson et al., 1972). Automatic sequence analysis was carried out in the Beckman Model 890A automatic protein sequencer according to Hermodson et al. (1973).

Results

Yeast phosphorylase does not undergo $NaBH_4$ reduction under the mild conditions developed for the rabbit muscle enzyme (Strausbauch et al., 1967). Therefore, a new procedure was devised which takes advantage of the good stability of $NaBH_4$ at high pH and the change in conformation induced in the enzyme by low concentrations of urea (Fischer et al., 1958).

^{32}P-labeled phosphorylase *a* (200 mg, 10 mg/ml) was dissolved in 0.1 *M* ammonium bicarbonate buffer (pH 8.0) at 0°, and 10 mg of solid $NaBH_4$ was added; a saturated urea solution was then introduced drop by drop. Reduction was completed when the urea concentration reached approximately 2 *M*. The solution was brought to 8 *M* by adding solid urea; the enzyme was carboxymethylated according to Saari and Fischer (1973), extensively dialyzed against 0.1 *M* ammonium bicarbonate and lyophilized.

Isolation of the ^{32}P and Phosphopyridoxyl CNBr Peptides. A lyophilized CNBr digest of S-carboxymethylated, $NaBH_4$-reduced ^{32}P-labeled yeast phosphorylase *a* (200 mg) was dissolved in 20 ml of 30% acetic acid and the pH adjusted to 4.0 with 5 *N* ammonia. The resulting suspension was centrifuged and the supernatant containing 70–80% of the original 390 nm fluorescence and radioactivity was applied to a Sephadex G-50 column in 1 *M* acetic acid. The elution profile is shown in Figure 1. The ^{32}P- and P-Pxy-labeled CNBr peptides were pooled separately and lyophilized. The residues were dissolved in 1 *M* acetic acid and chromatographed on Dowex 50-X2. The elution profiles for both peptides are given in Figure 2a and b, respectively. In each case, doublet peaks appeared due to the homoserine–homoserine lactone equilibrium (Saari and Fischer, 1973). The phosphopyridoxyl peptide was further purified by rechromatography under identical conditions on the same column after treatment with alkaline phosphatase (5 μg/0.5 μmol of peptide) at pH 8.0 for 6 hr at 37° according to the differential procedure of Strausbauch and Fischer (1970); see also Forrey et al. (1971).

The pyridoxyl peptide (CB-I-Pxy) was pure as judged by amino acid composition (Table I); three successive turns of the Edman degradation gave the sequence ε-(Pxy)-Lys-Phe-Val-Hse as summarized in Table II. This sequence was confirmed by digestion of peptide CB-I-Pxy with carboxypeptidase A as described under Materials and Methods.

ε-(Pxy)-lysine was only determined from an aliquot removed after 2-hr digestion and, as expected, was obtained in the same amount as phenylalanine. The sequence given above is identical with those reported for the pyridoxyl peptides isolated from rabbit and dogfish phosphorylases (CB-3′ of Saari and Fischer, 1973 and Cohen et al., 1973), with the exception that a valyl residue is inserted between phenylalanine and the COOH-terminal homoserine.

The ^{32}P-labeled phosphopeptide (CB-I) was sufficiently pure as judged by its amino acid composition (Table I) and NH_2-terminal analysis by dansylation which yielded exclusively isoleucine. Isoleucine was also identified as the sole NH_2-terminal group when 400 nmol of CB-I was subjected to automatic sequence analysis. Since much of the peptide was lost in the course of successive extractions, only the sequence of the first 12 residues could be determined (Table III). There is no homology between this partial sequence and the sequences obtained from the corresponding phosphopeptides isolated from rabbit muscle phosphorylase (R-CB-14, Saari and Fischer, 1973), or dogfish muscle phosphorylase (D-CB-14, Cohen et al., 1973). Furthermore, CB-I differs in size from the corresponding CNBr phosphopeptides obtained from vertebrate phosphorylases, with 42 residues as opposed to 89 and 88 for R-CB-14 and D-CB-14, respectively.

Isolation and Sequence of the ^{32}P-labeled Peptide after Thermolysin Digestion. Figure 3 illustrates the elution profile of a thermolytic digest of S-carboxymethylated, $NaBH_4$-

Table II: Alignment of Peptides and Sequence of the Pyridoxal-P Binding Site in Yeast Glycogen Phosphorylase.[a]

CB-I-Pxy	P-Pxy Lys-Phe-Val–Hse[b]
Th-I-Pxy	⇒ P-Pxy Met(Lys)
Th-II-Pxy	P-Pxy Ile-Ser-Thr-Ala-Gly-Thr-Glu-Ala(Ser,Gly,Thr,Ser,Asn,Met,Lys)
Pr-I-Pxy	P-Pxy (Met,Lys,Phe)
P-II-Pxy	P-Pxy Ala-Ser-Gly-Thr-Ser-Asn-Met(Lys,Phe)
Total sequence:	P-Pxy Ile-Ser-Thr-Ala-Gly-Thr-Glu-Ala-Ser-Gly-Thr-Ser-Asn-Met-Lys-Phe-Val-Met

[a] (⇒) direct Edman; (→) subtractive Edman; (←) carboxypeptidase A. [b] An aliquot of the aqueous phase after turn 3 was applied to the amino acid analyzer and yielded 90% of the expected amount of homoserine.

Table III: Sequences of the Phosphopeptides from Yeast Glycogen Phosphorylase.[a]

CB-I	⇒ ⇒ ⇒ ⇒ ⇒ ⇒ ⇒ ⇒ ⇒ ⇒ ⇒ ⇒ ⇒ Ile-Thr-Glu-Glu-Pro-Thr-Arg-Pro-His-Gln-Ile-Pro (. . . .
TH-I	→ → ← ← ← ← ← ← . . . . Leu(P)-Thr-Gly-Phe-Leu-Pro-Gln-Glu

[a] (⇒) Direct Edman (automatic sequencer analysis); (→) subtractive Edman; (←) yeast protease C.

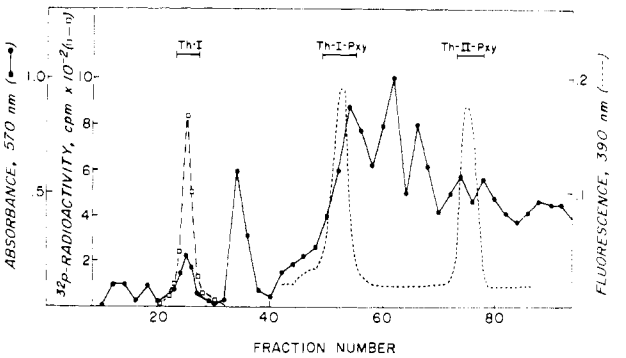

FIGURE 3: Dowex 50-X2 chromatography of a thermolysin digest of S-carboxymethylated, $NaBH_4$-reduced [^{32}P]phosphorylase *a* from yeast. Fractions (3 ml) were collected at a flow rate of 30 ml/hr. Horizontal lines indicate fractions which were pooled.

reduced ^{32}P-labeled phosphorylase *a* from a Dowex 50-X2 column. The phosphopeptide (Th-I) was slightly retarded and emerged after the breakthrough volume, which appears to be typical for other phosphopeptides such as those from α_{S1B} and β_A-caseins (Ribadeau-Dumas et al., 1971). It was further purified on a Sephadex G-25 column (1.0 × 120 cm) and shown to be pure as judged by both amino acid analysis and paper chromatography at pH 3.5; in this system, it moved toward the anode, in agreement with its elution pattern from the Dowex 50-X2 column. The amino acid composition (Th-I) is given in Table I. Seven successive turns of the Edman degradation yielded the sequence Leu-Thr-Gly-Phe-Leu-Pro-Glx. From the amino acid composition, the eighth and last residue must be glutamic acid or glutamine; this was subsequently confirmed by yeast protease C digestion as illustrated in Figure 4. The above data are consistent with the action pattern of yeast protease C which is known to proceed readily through prolyl residues and to stop at glycine.

Previous studies on yeast phosphorylase *a* (Fosset et al., 1971) had shown that the phosphate introduced in the enzyme is acid stable and base labile, suggesting that phosphorylation had occurred at a seryl or threonyl residue.

Since the phosphopeptide contains only one threonine and no serine, there was a strong presumption that the phosphate group is bound to threonine. This was confirmed by Dowex 50-X8 chromatography of a sample after mild acid hydrolysis (16 hr at 100° in 2 *N* HCl) as indicated under Materials and Methods; pure unlabeled phosphoserine and phosphothreonine were included as markers (Figure 5). The elution profile clearly shows that all the radioactivity cochromatographs with phosphothreonine with none found with the phosphoserine marker. Therefore, the total sequence of the phosphopeptide from yeast phosphorylase is Leu-Thr(*P*)-Gly-Phe-Leu-Pro-Gln-Glu, as summarized in Table III.

Isolation of Two Pyridoxyl Peptides after Thermolysin Digestion. The two fluorescent peptides isolated from the Dowex 50-X2 column (see Figure 3) were also purified by the "differential" procedure of Strausbauch and Fischer (1970). The two peptides (Th-I-Pxy and Th-II-Pxy) were pure as judged by their amino acid composition reported in Table I. The dipeptide (Th-I-Pxy) yielded Met-ε-(Pxy)Lys after one turn of direct Edman degradation; the second pentadecapeptide (Th-II-Pxy) was subjected to Edman degradation and yielded the following partial sequence Ile-Ser-Thr-Ala-Gly-Thr-Glu-Ala . . . for its first seven residues. These data are summarized in Table II.

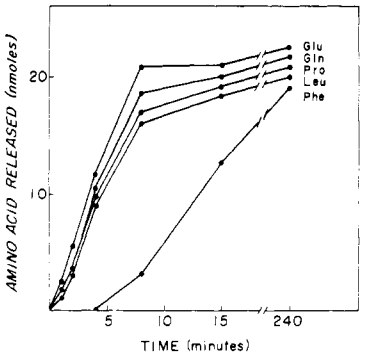

FIGURE 4: Yeast protease C digestion of the thermolytic ^{32}P-labeled phosphopeptide (Th-I). Conditions as indicated under Materials and Methods.

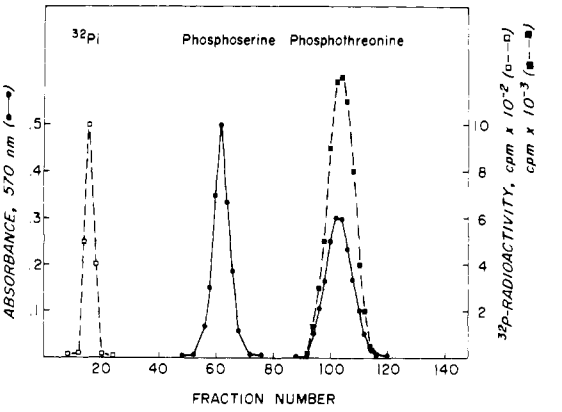

FIGURE 5: Dowex 50-X8 chromatography of phosphothreonine, phosphoserine, and thermolytic phosphopeptide (Th-I) after acid hydrolysis. The column (2.5 × 20 cm) was developed with 10 m*M* HCl at a flow rate of 30 ml/hr; 3-ml fractions were collected.

Isolation and Sequence of Two Pyridoxyl Peptides after Peptic Digestion. S-Carboxymethylated, $NaBH_4$-reduced phosphorylase *a* was cleaved with pepsin as indicated under Materials and Methods. Two fluorescent fractions were obtained upon chromatography on Dowex 50-X2 (not illustrated); they were further purified once more by the differential procedure of Strausbauch and Fischer (1970). Both, designated as P-I-Pxy and P-II-Pxy, respectively, were pure as judged from their amino acid composition (Table I).

The tripeptide (P-I-Pxy) containing methionine, ϵ-(Pxy)lysine, and phenylalanine obviously represents the overlap peptide for the CNBr fragment CB-I-Pxy; it was not further sequenced. The second pyridoxyl peptide (P-II-Pxy) was subjected to seven successive turns of the subtractive Edman degradation and yielded the sequence Ala-Ser-Gly-Thr-Ser-Asn-Met. In view of the composition of Th-II-Pxy and its partial sequence, peptide P-II-Pxy can only be positioned at the carboxyl end of Th-II-Pxy as indicated in Table II in which these data are summarized.

Table IV lists the sequences of the phospho- and the pyridoxyl peptides obtained from yeast phosphorylase and compares them to similar sequences isolated from dogfish and rabbit muscle phosphorylase.

Discussion

Conversion of phosphorylase *b* to *a* by phosphorylation of the protein is accompanied by distinct changes in quaternary structure: the inactive *b* species is a dimer while the active *a* form exists predominantly as a tetramer. Yet, the physiological significance of this association process is unclear since phosphorylase *a* itself can exist in both dimeric and tetrameric states, the former being the most active (Wang and Graves, 1964). This situation seems more straightforward in yeast where phosphorylation of the protein converts the less active phosphorylase *b* tetramer into an active dimer.

The phosphorylated site responsible for the covalent control of phosphorylase activity has been highly conserved in the chordata. For example, in muscle phosphorylase from the Pacific dogfish, a very primitive vertebrate, a sequence of 11 residues around the phosphoserine group showed a single conservative substitution, with an arginine replacing lysine found in the rabbit muscle enzyme (Cohen et al.,

Table IV: Sequences of the Phospho- and Pyridoxal-P Peptides from Yeast Phosphorylase and Comparison with the Dogfish and Rabbit Muscle Enzymes.[a]

Source	
	Phosphopeptides
Rabbit muscle[a]	Ser-Asp-Glu-Glu-Lys-Arg-Lys-Glu-Ile-Ser(P)-Val-Arg-Gly-Leu
Dogfish muscle[b]	Ser-Asp-*Met*-Glu-*Arg*-Arg-Lys-Glu-Ile-Ser(P)-Val-Arg-Gly-Leu
Yeast	*Leu-Thr-Gly-Phe-Leu-Pro-Gln-Glu*(P)
	Pyridoxyl-P Peptides
Rabbit muscle[c]	Ile-Ser-Thr-Ala-Gly-Thr-Gln-Ala-Ser-Gly-Thr-Gly-Asp-Met-Lys-Phe(P-Pxy)- -Met-Arg-Thr-Leu
Dogfish muscle[b]	Met-Lys-Phe(P-Pxy)- -Met-Gly-Arg-Thr-Leu
Yeast	Ile-Ser-Thr-Ala-Gly-Thr-*Glu*-Ala-Ser-Gly-Thr-*Ser*-*Asn*-Met-Lys-Phe(P-Pxy)-*Val*-Met

[a] Nolan et al. (1964). [b] Cohen et al. (1973). [c] Forrey et al. (1971). [d] Differences from the rabbit muscle sequences are italicized.

1973); in both instances, phosphoserine occupies the 14th position from the NH_2-terminus of the protein (Titani et al., unpublished results). Because of this considerable degree of homology, both enzymes can be acted upon by either rabbit or dogfish muscle phosphorylase kinase and phosphatase; furthermore, these interconversions proceed at precisely the same rate no matter what the origin of the substrate (Cohen et al., 1973). In rabbit muscle phosphorylase, the seryl-P site is in a very basic region of the molecule: CNBr cleavage releases an 89 amino acid fragment with an isoelectric point around pH 10 which contains, on the average, a lysine or arginine every five residues (Saari and Fischer, 1973). The seryl residue is inserted between two hydrophobic amino acids followed by an arginyl residue on the distal side, which is essential for phosphorylase phosphatase activity (Graves et al., 1960; Nolan et al., 1964). The overall basicity of the phosphorylated site is consistent with the fact that phosphorylase phosphatase is an acidic protein (i.p., ca. pH 5) which is strongly inhibited by polylysine (K_i ca. 4 μM, Gratecos, Detwiler and Fischer, unpublished results).

By contrast, the phosphorylated site in yeast phosphorylase is very acidic and a threonyl rather than a seryl residue is phosphorylated. While phosphorylation of a threonyl residue in the I subunit of troponin by either rabbit muscle protein kinase or phosphorylase kinase has recently been reported (E. G. Krebs, personal communication), this is the first example of metabolic interconversion of an enzyme in which this amino acid is invoved. As expected, none of the animal converter enzymes (phosphorylase kinase or phosphatase) will act on yeast phosphorylase and, conversely, the yeast protein phosphokinases so far investigated (Lerch et al., 1975) do not touch dogfish or rabbit muscle phosphorylase. Therefore, aside from the fact that in all these covalent modifications, two negative charges are introduced into the protein which brings about a change in quaternary structure and enzymatic activity, little analogy can be seen between the phosphorylated site in yeast and animal phosphorylases.

By contrast, the extent of structural homology around the coenzyme binding site is truly remarkable. In this instance, 14 out of 18 residues are identical in the yeast and rabbit muscle enzymes. Of the four differences observed, glutamic acid and asparagine in the rabbit are replaced by glutamine and aspartic acid, respectively. These represent two conservative substitutions involving a single base change in the phosphorylase genome. The charge of the peptide has not even been altered since the amide group has been shifted from one position to another. In the third mutation, serine has replaced glycine and, in the fourth, a valyl residue has been inserted between the highly conserved phenylalanine and methionine adjacent to the lysyl residue carrying the pyridoxal-5′-P group.

Of course, functional sites in proteins can be expected to be highly conserved, and this has been well documented in a comparative study of rabbit and dogfish phosphorylase (Cohen et al., 1973). But then, the role of pyridoxal-P in phosphorylase has never been fully elucidated. On the one hand, it has been found in stoichiometric amounts in all phosphorylases so far investigated and its removal leads to total inactivation. On the other hand, there is clear evidence that if it is involved in direct catalysis, it must function in a way different than accepted for all other classical pyridoxal-P requiring enzymes. Therefore, both a structural and catalytic role have been postulated (for review, see Fischer et al., 1970). The present finding that the pyridoxal-P binding site in yeast phosphorylase has been highly conserved during the approximately 1.5 billion years during which *Saccharomyces* have diverged from the main line of evolution leading to mammals lends strong support for a catalytic role for the cofactor.

Acknowledgments

We express our thanks to Standard Brands, Inc., for generous gifts of baker's yeast, to Mr. Richard Olsgaard for the amino acid analyses, and to Mr. Lowell Ericsson for the automatic sequencer runs and gas chromatographic identification of phenylthiohydrantoin derivatives of amino acids. We are also very grateful to Miss Eileen Wright for the typing of this manuscript.

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