Comparative Study of Dogfish and Rabbit Muscle Phosphorylases†

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ABSTRACT: S-Carboxymethylated, $NaBH_4$-reduced, ^{32}P-labeled dogfish phosphorylase *a* was cleaved with cyanogen bromide and the ^{32}P and phosphopyridoxyl-containing fractions were separated by a combination of gel filtrations and ion-exchange chromatographies. The ^{32}P-labeled CNBr peptide was very similar to the corresponding peptide from rabbit phosphorylase and on digestion with chymotrypsin, gave a radioactive phosphopeptide, Glu-Arg-Arg-Lys-Gln-Ile-Ser(P)-Val-Arg-Gly-Leu, with a sequence identical with the phosphopeptide isolated from rabbit muscle phosphorylase except for the conservative substitution of an arginine for a lysine at position 2. Nine small CNBr peptides were isolated from the phosphopyridoxyl fraction. Two of these together yielded a unique sequence comprising the pyridoxal-5′-P binding site, Met-Lys(Pxy)-Phe-Met-Gly-Arg-Thr-Leu-Gln-Asn-Thr-Met, which is probably identical with the corresponding segment in rabbit muscle phosphorylase. Of the remaining 7 peptides, 5 (44 residues) were identical in composition and partial sequence with peptides derived from rabbit muscle phosphorylase; a sixth peptide was probably homologous with another rabbit octapeptide. The seventh peptide, which was absent from rabbit phosphorylase, had the sequence, *N*-acetyl-Ser-Lys-Pro-Lys-Ser-Asp-Met, and was confirmed as the NH_2-terminal sequence of the protein by isolation of the tetrapeptide *N*-acyl-Ser-Lys-Pro-Lys from a tryptic peptide map of native dogfish phosphorylase. Based on the partial or total sequence of eight homologous CNBr peptides, the distribution of methionine residues, the phosphopeptide sequence, the composition of the CNBr phosphopeptides and that of the whole protein (865 residues), the overall homology between dogfish and rabbit muscle phosphorylases appears to be 85–90%. From the known time of divergence of the dogfish from the main vertebrate line leading to mammals (*ca.* 450 million years), this corresponds to 1.1–1.7 amino acid mutations/100 residues per 100 million years, a slower rate than found thus far for other proteins with the exception of histones. The results provide a structural basis for the remarkable similarities in physical and regulatory properties between these two proteins described previously (Cohen, P., Duewer, T., and Fischer, E. H. (1971), *Biochemistry 10*, 2683).

The length of its polypeptide chain and multiplicity of interacting sites make glycogen phosphorylase one of the most complex of enzymes. Two of these sites, namely the serine phosphorylated when phosphorylase *b* is converted to phosphorylase *a*, and the lysine which binds pyridoxal-5′-P, can be monitored conveniently by the use of radioactivity and fluorescence, respectively. Nevertheless, the determination of peptide sequences surrounding the residues at these sites has been limited to the mammalian phosphorylases of rabbit, rat, and man (Hughes *et al.*, 1962; Nolan *et al.*, 1964; Sevilla and Fischer, 1969; Wolf *et al.*, 1970; Forrey *et al.*, 1971a). From an evolutionary standpoint, the sequence around the phosphoseryl residue is particularly important since it represents a region of the phosphorylase molecule with which both the control enzymes, kinase and phosphatase, must interact, and therefore should contain the elements necessary for this double recognition. Similarly, comparative studies of the pyridoxal-5′-P binding site should contribute to the understanding of the binding and mechanism of action of this cofactor in phosphorylase, since the presence of this molecule is a feature that all phosphorylases share in common (see review, Fischer *et al.*, 1970).

This manuscript describes the isolation and sequence determination of both these peptides from dogfish skeletal muscle phosphorylase, together with all other small CNBr peptides. A comparison with the corresponding peptides obtained from rabbit muscle phosphorylase has been carried out and the data obtained discussed in terms of the evolution of these two molecules. The results suggest that this comparative approach may be usefully applied to phosphorylases from other origins. This study constitutes the first step in the determination of the total sequence of both rabbit and dogfish muscle phosphorylases presently being carried out in collaboration with Drs. Neurath, Walsh, and Titani.

Materials and Methods

Materials. Dogfish phosphorylase *b* ($A^{1\%}_{280}$ = 12.9, subunit mol wt 100,000) was prepared by the method of Cohen *et al.* (1971) and rabbit muscle phosphorylase kinase according to DeLange *et al.* (1968). [γ-^{32}P]ATP was prepared according to Glynn and Chappell (1964) and purified as described previously (Cohen *et al.*, 1971). α-Chymotrypsin, trypsin (L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone treated), soybean trypsin inhibitor, diisopropyl phosphorofluoridate treated carboxypeptidases A and B, and *Escherichia coli* alkaline phosphatase were the best grades available from Worthington. Three-times-recrystallized thermolysin was

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obtained from Calbiochem. Dowex (Bio-Rad) was processed according to Schroeder (1967a,b). Urea (Mallinckrodt) and iodoacetate (Eastman) were recrystallized three times from water and petroleum ether (bp 30–60°), respectively. Nbs_2[1] (Sigma) and CNBr (Eastman) were used without further purification. Pyridine, phenyl isothiocyanate, and trifluoroacetic acid for Edman degradations were "Sequanal" reagent grade from Pierce. Anhydrous hydrazine (Eastman), *N*-ethylmorpholine, and α-picoline were distilled before use. *N*-Formylmethionine and *N*-acetylglutamic acid were obtained from Mann.

Amino Acid Analysis. Dogfish phosphorylase was dialyzed against 0.1 M KCl, then distilled water. Aliquots were lyophilized and hydrolyzed with 5.8 N HCl *in vacuo*, according to Moore and Stein (1963) for 24, 48, 72, and 96 hr at 109 ± 1°. Each time point was carried out in duplicate and included norleucine as an internal standard (Walsh and Brown, 1962). Peptides were analyzed after a single, 24-hr acid hydrolysis. Analysis was performed with a Spinco Model 120 automatic recording analyzer, equipped with an Infotronic integrator. The total cysteine content was determined on duplicate samples after oxidation with performic acid according to Moore (1963). Free sulfhydryl groups in the native and sodium dodecyl sulfate denatured protein were measured by titration with Nbs_2 (Ellman, 1959). Tryptophan was determined by the method of Bencze and Schmid (1957) and the procedure K of Spies and Chambers (1949); tryptophanyl peptides were detected by the Ehrlich stain on paper. $NaBH_4$ reduction and carboxymethylation of the protein, CNBr cleavage, separation of the CNBr fragments on G-75 Sephadex (Pharmacia), ion-exchange and paper chromatography, polyacrylamide gel electrophoresis, quantitative analysis of the column fractions by ninhydrin after alkaline hydrolysis, and of homoserine–homoserine lactone and ε-pyridoxyllysine were carried out as described in the preceding paper (Saari and Fischer, 1973). Procedures for the analysis of homoserine in CNBr peptide hydrolysates and for ε-pyridoxyllysine have been described (Forrey *et al.*, 1971b); destruction of ε-pyridoxyllysine on standard acid hydrolyses was usually between 30 and 40%.

Preparation of $NaBH_4$-reduced, ^{32}P-labeled phosphorylase *a* was carried out as described by Cohen *et al.* (1971). The carboxymethylation and CNBr reactions were essentially complete as judged by amino acid analysis; 8.8 mol of carboxymethylcysteine was found per subunit compared to 9.2 ± 0.1 by performic acid oxidation and Nbs_2 titration. The loss of methionine was 97%; no significant change was seen in any other amino acid from the values given in Table I.

Sequence analysis was carried out by the phenyl isothiocyanate method of Edman (1956) as described by Gray (1967). Diphenylthiourea and other by-products were removed with benzene. The amino acid released at each cycle was identified by the "subtractive" method of Konigsberg (1967); NH_2-terminal residues were also determined by the dansyl·Cl method of Gray and Hartley (1963) and Gray and Smith (1970) followed by thin-layer chromatography (Deyl and Rosmus, 1965). In tables listing these results, values were normalized to the amino acid given in bold face; impurities below 0.1 residue were omitted.

Enzymatic Digestions. Crystals of carboxypeptidase A were washed with water and dissolved in 2 M NH_4HCO_3 (Ambler, 1967); 0.01-ml aliquots of carboxypeptidase A and B (usually 1:100 molar ratio) were added to 0.2-ml peptide solutions in 0.2 M *N*-ethylmorpholine buffer (pH 8.5). Reactions were terminated by addition of 0.05 ml of glacial acetic acid, lyophilization, or both. Control experiments with peptide omitted were also carried out.

Trypsin and chymotrypsin were dissolved in 1.0 mM HCl and thermolysin in 10.0 mM calcium acetate. Soybean trypsin inhibitor was added to chymotrypsin at a 1:25 molar ratio; 0.01-ml aliquots of proteolytic enzyme (from 1:50 to 1:100 molar ratio) were added to peptide dissolved in 0.5 or 1.0 ml of 0.1 M NH_4HCO_3 (pH 8.0). Reactions were terminated by lyophilization. All enzymatic digestions were carried out at 35°.

For the isolation of the NH_2-terminal tetrapeptide from native dogfish phosphorylase *b*, the enzyme was dissolved in 50 mM sodium glycerophosphate (pH 7.0; 20 mg/ml), and the solution made 8 M in urea, incubated at 30° for 1 hr, and then dialyzed against 1.0 mM HCl. Ammonium bicarbonate was added to 0.1 M and the fine suspension was digested for 10 hr at 35° with a 1:50 molar ratio of trypsin to phosphorylase. The partially clarified solution was centrifuged and lyophilized; the residue was suspended in water and recentrifuged. Peptide mapping was carried out with aliquots of the supernatant.

N-Acetyl groups were detected by thin-layer chromatography following hydrazinolysis as described by Narita (1970). Formyl hydrazide and acetyl hydrazide standards were prepared from *N*-formylmethionine and *N*-acetylglutamic acid, respectively. These were well separated on thin-layer cellulose sheets (Eastman 6065) in collidine–water (10:2). The R_F values were: formyl hydrazide, 0.38; acetyl hydrazide, 0.48.

Results

The amino acid composition of dogfish phosphorylase is given in Table I. The number of cysteine residues obtained by performic acid oxidation and by Nbs_2 titration were in close agreement, showing the absence of disulfide bridge in the *b* form of the enzyme: 2.04 sulfhydryl groups reacted rapidly with Nbs_2 per subunit (Figure 1), as found in rabbit muscle phosphorylase *b* (Damjanovich and Kleppe, 1966; Buc and Buc, 1967). The overall compositions of the dogfish and rabbit enzymes are very similar, the most obvious differences being the higher lysine and lower arginine content of dogfish phosphorylase. The partial specific volume calculated from the composition (0.736) is 1.4% lower than the value of 0.746 obtained by pycnometric determination and calibrated density columns (Cohen *et al.*, 1971). Further comparison of the composition of these and other phosphorylases will be found in the Discussion.

Separation of the ^{32}P and Phosphopyridoxyl Peptides. Lyophilized, carboxymethylated, $NaBH_4$-reduced ^{32}P-labeled dogfish phosphorylase *a*, cleaved with CNBr, was dissolved in 5 ml of 1 M formic acid and chromatographed on G-75 Sephadex. The elution profile is shown in Figure 2, together with that obtained for rabbit muscle phosphorylase treated identically (Saari and Fischer, 1973). Although the ^{32}P and phosphopyridoxyl CNBr peptides eluted in essentially identical positions in the dogfish and rabbit enzymes, the 280-nm absorbance profiles differed considerably. The two marked fractions were pooled and treated separately.

Purification of the Phosphopyridoxyl and Other Small CNBr Peptides. The fluorescent fraction (F) containing the

[1] Abbreviations used are: Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); SE, sulfoethyl; Pxy, pyridoxyl; 5-P-Pxy, 5-phosphopyridoxyl; dansyl, dimethylaminonaphthalenesulfonyl; R-CB and D-CB, peptides generated by cyanogen bromide cleavage of rabbit and dogfish phosphorylase, respectively.

TABLE I: Amino Acid Composition of Dogfish Skeletal Muscle Glycogen Phosphorylase.

Amino Acid Residue	Extrapolated or Av Value[a] (μmol)	Calcd No. of Residues/ Subunit Mol Wt of 100,000	Rabbit Muscle Phosphorylase
Lysine	0.186 ± 0.002	58.0	47
Histidine	0.070 ± 0.002	21.8	21
Arginine	0.175 ± 0.003	54.6	64
Aspartic acid	0.327 ± 0.003	101.4	98
Threonine	0.125	39.0	34
Serine	0.116	36.2	30
Glutamic acid	0.302 ± 0.003	94.2	102
Proline	0.116 ± 0.004	36.2	43
Glycine	0.166 ± 0.001	51.8	50
Alanine	0.192 ± 0.002	59.9	65
Valine	0.193 ± 0.001	60.2	61
Methionine	0.078 ± 0.001	24.3	22
Isoleucine	0.158 ± 0.001	49.3	49
Leucine	0.244 ± 0.002	76.1	81
Tyrosine	0.117 ± 0.002	36.5	37
Phenylalanine	0.117 ± 0.003	36.5	38
Cysteine	0.029	9.1,[b] 9.3[c]	9[g]
Tryptophan		14,[d] 15[e]	14[h]
		859.2	865
Ammonia	0.215	67	77

[a] Average of duplicate determinations on four times of hydrolysis (24–96 hr). [b] From performic acid oxidation (two samples). [c] From Nbs_2 titration of sodium dodecyl sulfate denatured protein. [d] Average of five determinations by the method of Spies and Chambers (1949). [e] Determined in duplicate by the method of Bencze and Schmid (1957). [f] Recalculated from Sevilla and Fischer (1969) for a subunit molecular weight of 100,000 (Cohen *et al.*, 1971). [g] The data of Sevilla and Fischer (1969) yield 8.5 cysteine residues/100,000 g. Zarkadas *et al.* (1968, 1970) have isolated and sequenced nine cysteinyl peptides after digestion with pepsin. [h] Recalculated from Sevilla and Fischer (1969) using $A_{280}^{1\%} = 12.9$ (Cohen *et al.*, 1971) and a subunit molecular weight of 100,000.

phosphopyridoxyl group was lyophilized, and the residue was dissolved in pyridine acetate buffer (pH 3.1) (Schroeder, 1967a), adjusted to pH 2.5 with glacial acetic acid, and chromatographed on Dowex 50-X2 as described under Methods. At the completion of the gradient (pH 4.4), the column was further washed with pH 5.0 pyridine acetate buffer; the elution profile is shown in Figure 3. From the nine fractions obtained, nine peptides were isolated and, wherever possible, named according to the analogous CNBr peptides isolated from rabbit muscle phosphorylase (Saari and Fischer, 1973) using the terminology R-CB and D-CB for peptides generated from CNBr cleavage of rabbit and dogfish phosphorylase, respectively. The compositions are given in Table II and partial or complete sequences in Table III. In characterizing peptides by chromatography or electrophoresis, R_F values have been expressed relative to phenol red (chromatography) or lysine (electrophoresis at pH 3.6). Most of the CNBr peptides chromatographed in two positions as expected from the slow equilibrium between homoserine and homoserine lactone (Armstrong, 1949).

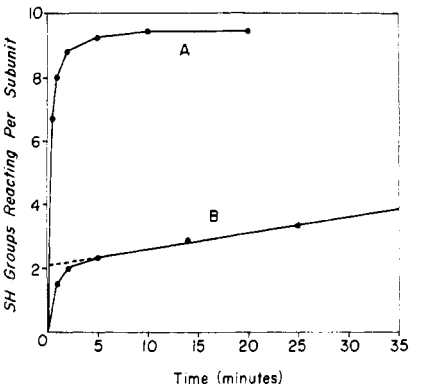

FIGURE 1: Titration of dogfish phosphorylase *b* with Nbs_2 (A) in the presence, and (B) absence of 1% sodium dodecyl sulfate. The reaction mixture consisted of 1.7 ml of Tris-HCl (0.1 M, pH 8.0, 25°), 1.0 ml of enzyme, and 0.3 ml of Nbs_2 (10^{-2} M, adjusted to pH 7.0 with NaOH). The final enzyme concentration was 0.46 mg/ml. Immediately prior to the reaction, the enzyme was incubated for 30 min with 50 mM mercaptoethanol, then passed through Sephadex G-25 equilibrated in 50 mM sodium glycerophosphate (pH 7.0) to remove this reagent.

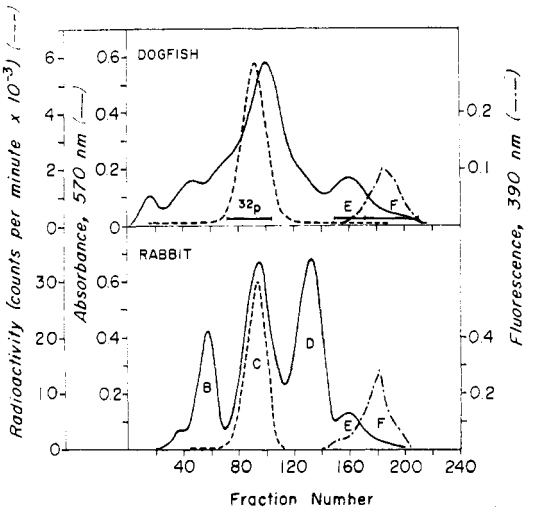

FIGURE 2: Sephadex G-75 elution profiles of cyanogen bromide digests of [^{32}P]S-carboxymethylated, $NaBH_4$-reduced phosphorylase *a* from dogfish (*ca.* 150 mg) and rabbit (*ca.* 200 mg) skeletal muscles. Conditions are identical with those described in Saari and Fischer (1973). Horizontal lines indicate the fractions pooled after elution. The flow rates were 25 ml/hr and 4-ml fractions were collected.

Fraction 1. A peptide map showed the presence of four major and one minor ninhydrin spots corresponding to five peptides that were eluted with pyridine acetate buffer (pH 6.5). Their R_F values were 0.20, 0.38, 0.52, 0.62, 0.72, respectively (Phenol Red = 1.00). The first two had identical compositions and partial sequences[2] indicating that they were identical with R-CB 4. The peptides with R_F 0.52 and 0.72 also gave an identical analysis and their composition suggested that they might be homologous with R-CB 6; if this is so, however, the partial sequence shows that there must be at least four changes. The minor spot with an R_F of 0.62 only contained small amounts of glutamic acid and glycine.

Fraction 2 contained two peptides, one of which crystallized on standing for *ca.* 1 week. After washing the crystals twice with 1 M formic acid, the composition and partial sequence

[2] The detailed analyses of subtractive Edman degradations not reported here can be obtained by writing directly to the authors.

TABLE II: Composition of CNBr Peptides Isolated from Dogfish Phosphorylase.

	CB-1	CB 3′	CB 4	CN 5	CB 6	CB 7	CB 8	CB 10	CB A	CB 14[a]
Pxy-Lys		0.70 (1)								
Lys				0.13			1.01 (1)		2.05 (2)	6
His							0.80 (1)			4
Arg				0.87						10
Asp			2.20 (2)	1.00 (1)	2.07 (2)	1.22 (1)		1.29 (1)	1.00 (1)	10
Thr				1.86 (2)		1.78 (2)		0.17		5
Ser	0.10	0.16				0.16	0.35	0.21	1.51 (2)	4
Hse	1.06 (1)	1.00 (1)	0.89 (1)	0.86 (1)	0.81 (1)	1.00 (1)	1.00 (1)	0.94 (1)	0.93 (1)	1
Glu			1.12 (1)	1.03 (1)	2.12 (2)	0.29		4.13 (4)		8
Pro							1.13 (1)		1.07 (1)	2
Gly	0.12	0.13	1.00 (1)	0.93 (1)	0.26	1.98	3.08 (3)	2.10 (2)		4
Ala			1.00 (1)		0.14	0.92 (1)	2.00 (2)	2.00 (2)		3
Val			0.98 (1)		1.80 (2)	0.21				7
Ile				0.12		0.88 (1)	0.86 (1)	1.05 (1)		4
Leu	1.00 (1)			0.97 (1)	1.03 (1)	1.82 (2)				8
Tyr							0.81 (1)			7
Phe		1.09 (1)				0.13		2.70 (3)		3
Trp										2[b]
Residues	2	3	7	8	8	10	11	14	7	88

[a] The composition is normalized to $^{32}P = 1.0$ taking the subunit molecular weight of the protein as 100,000 (Cohen *et al.*, 1971) and $A_{280}^{1\%}$ as 12.9. The peptide was hydrolyzed for 24 hr at 110° and losses of serine and threonine were assumed to be 10 and 5%, respectively. [b] Determined by the method of Bencze and Schmid (1957).

were identical with R-CB 10, which also crystallized at acid pH (Saari and Fischer, 1973). D-CB 10 was subsequently crystallized in larger amounts from fraction E of Figure 2. The second peptide remaining in solution was identical in composition and partial sequence with R-CB 7, with only *ca.* 5% contamination by D-CB 10.

Fraction 3 contained only trace amounts of peptides predominantly found in other fractions.

Fraction 4 consisted mainly of a dipeptide, which, upon further purification by paper chromatography, yielded a single ninhydrin spot (R_F 0.72); this peptide was identical with R-CB 1.

Fraction 5 yielded a single pure peptide; it was termed D-CB A since no analogous peptide could be found in rabbit muscle phosphorylase. Successive Edman degradations failed to release any amino acid, suggesting that the amino terminus was blocked. Digestion with a 1:100 molar ratio of trypsin for 3 hr and electrophoresis at pH 3.6 gave three ninhydrin-positive spots corresponding to two peptides which accounted for the composition of D-CB A. Edman degradation and carboxypeptidase A digestion of the second peptide (T-2, R_F 0.11 and 0.36) yielded the sequence Ser-Asx-Hse. A charge of −1 determined from its electrophoretic migration at pH 6.5 indicated that Asx was present as aspartic acid; electrophoresis was carried out after pretreatment with 2 M ammonia at 25° to promote the conversion of homoserine lactone to homoserine. The first peptide (T-1: Lys_2, Ser, Pro) was not a mixture of Ser-Lys and Pro-Lys because its R_F (0.64 at pH 3.6) differed from that of an authentic sample of Ser-Lys (R_F 0.86). The low R_F corresponding to a +1 charge for the tetrapeptide again suggested the presence of a blocked NH_2-terminus.

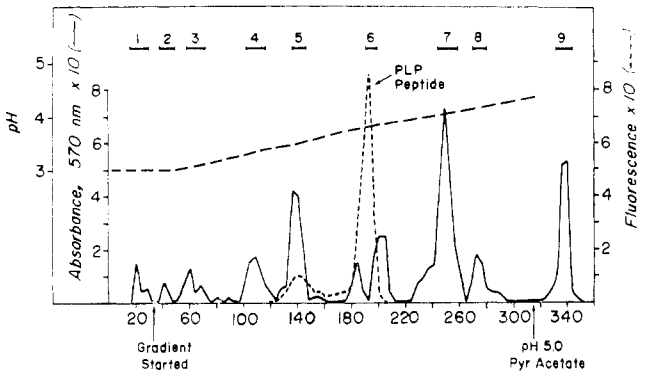

FIGURE 3: Dowex 50-X2 chromatography of the fluorescent fraction (F) from Sephadex G-75. Absorbance at 570 nm was determined after reaction with ninhydrin. The horizontal bars indicate the fractions pooled after elution.

To unmask the amino terminus, T-1 was heated with 6 N HCl for 2.5 min at 100°. Electrophoresis at pH 3.6 gave only one major ninhydrin spot (T-1′) with a composition identical with that of T-1 but an R_F of 1.03. Two successive Edman degradations released serine, then lysine, indicating a Ser-Lys-Pro-Lys sequence if one assumes that tryptic digestion had cleaved opposite the second lysyl residue. The failure of bovine trypsin to cleave a Lys–Pro bond is well documented (Hill, 1965). The above data are summarized in Table IV.

D-CB A was also isolated in high yield from three preparations of dogfish phosphorylase. The finding that its NH_2-terminal seryl residue is blocked suggested that it was derived from the amino-terminus of the enzyme itself and therefore amenable to isolation from tryptic digests of the whole protein. By contrast, if serine were preceded by methionine and blockage had occurred during the work-up (*e.g.*, by formylation or lyophilization from formic acid), this would not be feasible. Figure 4 shows the peptide pattern of dogfish phosphorylase following tryptic digestion as described under Methods. Only one ninhydrin-positive peptide out of the 60 observed migrated with an R_F of 0.65 on pH 3.6 electrophoresis and 0.23 on paper chromatography, *i.e.*, in the required position for T-1. After elution, this peptide had a composition identical

TABLE III: Partial or Total Sequences of Cyanogen Bromide Peptides Obtained from Dogfish and Rabbit Muscle Phosphorylase.

	Dogfish Phosphorylase	Rabbit Phosphorylase	No. of Residues (Dogfish)	Residues In Common
CB 1	Leu-Hse	Leu-Hse	2	2
CB 2	Absent	Ala-Lys-Hse		
CB 3′	ε-(Pxy)Lys-Phe-Hse	ε-(Pxy)Lys-Phe-Hse	3	3
CB 4	Asx-Gly-Ala(*Asx,Glx,Val*)Hse	Asx-Gly-Ala(*Asx,Glx,Val*)Hse	7	7
CB 5	Gly-Arg-Thr-Leu-Gln-Asn-Thr-Hse	Gly-Arg-Thr-Leu(*Gln,Asn,Thr*)Hse	8	8
CB 6	Leu-Val-Asx(*Asx,Glx,Glx,Val*)Hse	Val(*Leu,Asx,Thr,Ala,Glx,Val*)Hse	8	4
CB 7	Leu-Asx-Gly-Ala(*Leu,Thr_2,Gly,Ile*)Hse	Leu-Asx-Gly-Ala-Leu(*Thr_2,Gly,Ile*)Hse	10	10
CB 8	Ile(*Gly,Gly,Lys,Ala,Ala,Pro,His,Gly,Tyr*)Hse	Ile-Gly-Gly-Lys-Ala-Ala-Pro(*His,Gly,Tyr*)Hse	11	11
CB 10	Ala-Glx-Glx-Ala(*Asx,Glx_2,Gly_2,Ile,Phe_3*)Hse	Ala-Glx-Glx-Ala(*Asx,Glx_2,Gly_2,Ile,Phe_3*)Hse	14	14
CB A	*N*-Acetyl-Ser-Lys-Pro-Lys-Ser-Asp-Hse	Absent		
Total			63	59

TABLE IV: NH_2-Terminal Peptide of Dogfish Phosphorylase (CB-A).[c]

	N-Acetyl-Ser-Lys-Pro-Lys-Ser-Asp-Hse							
	Composition	R_F 0.65 T1	R_F 1.03 T1*[a]	T1* First Edman	T1* Second Edman	R_F 0.11, 0.27 T2	First Edman T2	Peptide-X[b]
Lys	2.05 (2)	**2.00** (2)	**2.00** (2)	1.86 (2)	1.19 (1)			**2.00** (2)
Asp	**1.00** (1)		0.16			**1.00** (1)	**1.00** (1)	0.32
Ser	1.50 (2)	0.98 (1)	1.08 (1)	0.17	0.10	0.86 (1)	0.29	1.07 (1)
Hse	0.93 (1)					0.74 (1)	0.84 (1)	
Glu								0.33
Pro	1.07 (1)	0.99 (1)	0.86 (1)	**1.00** (1)	**1.00** (1)			0.94 (1)
Gly			0.34	0.14	0.15			0.20
Val								0.14
Leu								0.13

[a] T1* is the major spot on pH 3.6 electrophoresis after deacylation of T1 with 6 N HCl for 2.5 min at 100°. [b] X is T-1 isolated from a peptide map after tryptic digestion of the whole enzyme. [c] Values normalized to amino acids are in bold face.

with that of T-1, except for minor contamination (Table IV). Following a 2-min exposure to 6 N HCl at 110°, only serine and lysine were released on successive Edman degradations (not shown in Table IV). This confirms that CB A constitutes the amino-terminal segment of dogfish phosphorylase.

Following hydrazinolysis of CB A, a spot staining strongly with $AgNO_3$–alkaline KOH reagent was observed on thin-layer chromatography, eluting in exactly the same position as acetyl hydrazide. As described under Methods this was well resolved from formyl hydrazide. As controls, peptides CB 1 and 6 were also treated with 6 N HCl for 2 min at 110° and neither displayed a spot in the acetyl hydrazide position. Cyclized glutaminyl peptides also give a positive reaction in the position of acetyl hydrazide. However, D-CB A was devoid of glutamic acid or glutamine and pyrrolidonecarboxylic acid. Moreover, pyrrolidonecarboxylic acid hydrazide stains many times less strongly than acetyl hydrazide. As little as 10 nmol of acetyl hydrazide yielded a strongly staining spot with the $AgNO_3$–KOH reagent, whereas pyrrolidonecarboxylic acid and amino acid hydrazides hardly appeared at this concentration.

Fraction 6, containing *ca.* 85% of the initial fluorescence, was only 50% pure. After lyophilization, the residue was dissolved in 1.0 ml of water; the solution was adjusted to pH 8.0, incubated with 50 μg of alkaline phosphatase for 24 hr at room temperature, then rechromatographed on Dowex 50-X2 as before; the fluorescent peptide emerged at pH 4.1 as compared to 3.8 before reaction. The pooled fractions were *ca.* 90% pure as judged by amino acid analysis. Pyridoxyllysine and phenylalanine were released by successive turns of the Edman cycle, yielding a sequence (ε-(Pxy)Lys-Phe-Hse) identical to that of R-CB 3′ (see Table III and Forrey *et al.*, 1971a). Elution of the fluorescent material essentially as one peak from both the Sephadex G-75 and Dowex 50 columns (Figures 2 and 3) provided evidence, additional to the stoichiometry data (Cohen *et al.*, 1971), that pyridoxal-5′-P is bound to a unique lysyl residue of the protein. The minor fluorescent peak (Figure 3) most likely consisted of the same peptide with homoserine rather than homoserine lactone.

Fraction 7 containing one major peptide, *ca.* 90% pure, gave a yellow color with ninhydrin on paper, suggestive of an NH_2-terminal glycine; this was confirmed by dansylation. Successive Edman degradation gave the sequence Gly-Arg-Thr-Leu-Glx. The loss of glutamic acid after turn five was low (0.27 of a residue) suggesting that a glutaminyl residue had cyclized; nothing was released after turn 6. Digestion with carboxypeptidase A at a 1:100 molar ratio for 3 hr confirmed that homoserine occupied the COOH-terminal position; threonine, leucine, and some material emerging in the position of serine was produced but no free glutamic or aspartic

TABLE V: Sequence of Dogfish Muscle Phosphorylase Peptide CB-5.

Rabbit CB-5 peptide: Gly-Arg-Thr-Leu(*Gln,Asn,Thr*)Hse
Dogfish CB-5 peptide: Gly-Arg-Thr-Leu-Gln-Asn-Thr-Hse

	Composition of CB-5	First Edman	Second Edman	Third Edman	Fourth Edman	Fifth Edman	Carboxypeptidase A Digestion	Composition of C-1	First Edman C-1	Second Edman C-1
Lys	0.12									
Arg	0.87 (1)	0.80 (1)	*0.10*							
Asp	**1.00** (1)	**1.00** (1)	**1.00** (1)	**1.00** (1)	**1.00** (1)	**1.00** (1)		1.10 (1)	0.92 (1)	*0.24*
Thr	1.86 (2)	1.76 (2)	1.85 (2)	*1.21* (1)	1.25 (1)	1.20 (1)	0.94	**1.00** (1)	**1.00** (1)	**1.00** (1)
Ser							0.57[a]			
Hse	0.86 (1)	0.86 (1)	0.68 (1)	0.90 (1)	0.82 (1)	0.26[b]	1.00	0.94 (1)	0.81 (1)	0.83 (1)
Glu	1.03 (1)	1.03 (1)	1.12 (1)	0.97 (1)	1.00 (1)	*0.73*[c]		0.85 (1)	*0.29*	0.20
Gly	0.93 (1)	*0.37*	0.22	0.16	0.15	0.14		0.27	0.14	0.11
Ala								0.15		
Ile	0.12									
Leu	0.97 (1)	0.88 (1)	0.77 (1)	0.85 (1)	*0.36*	0.32	0.39			

[a] Asn and Gln coelute with serine in the buffer system of Spackman *et al.* (1958). The low value suggests almost complete cyclization of Gln. [b] Low value results from insufficient base treatment resulting from incomplete removal of HCl following hydrolysis. [c] Loss of only 0.27 residue suggests Gln. The presence of Gln and Asn was confirmed by carboxypeptidase A attack.

acid. Since significant amounts of leucine (0.39 mol, see Table VI) were released, carboxypeptidase A attack must have passed through Asx and Glx, strongly suggesting that these residue were present as asparagine and glutamine. The peptide was then digested with chymotrypsin at a 1:50 molar ratio for 5 hr; pH 3.6 electrophoresis gave two ninhydrin spots with R_F values of 0.30 (C-1) and 0.72 (C-2), which were eluted with pyridine acetate (pH 6.5). C-2 stained yellow with ninydrin like the original peptide and therefore corresponded to the amino-terminal region; C-1 accounted for the four residues at the COOH-terminus. Edman degradations removed a glutamyl, then an aspartyl residue, yielding the sequence Gln-Asn-Thr-Hse for this fragment. The composition and sequence of this octapeptide are identical with R-CB 5.[3] Since work on rabbit muscle phosphorylase has indicated that R-CB 3′ and R-CB 5 are adjacent in the primary sequence of the molecule (Forrey *et al.*, 1971a), this gives the sequence of 12 residues around the phosphopyridoxyllysine residue in dogfish phosphorylase. The data used to derive the sequence of dogfish D-CB 5 are given in Table V.

Fractions 8 and 9 consisted mainly (*ca.* 80%) of the same peptide; paper chromatography gave two major ninhydrin spots (R_F 0.20 and 0.36), with identical compositions and amino-terminal isoleucyl residues as R-CB 8.

Purifications of the CNBr Phosphopeptide (D-CB 14). The pooled ^{32}P fraction was lyophilized, and the residue was dissolved in a pyridine–formate buffer (200 ml of 98% formic acid and 16 ml of pyridine per l., apparent pH 2.1 at 25°, containing 0.1 M NaCl and 8 M urea). The solution was chromatographed at 55° on a 1 × 20 cm column of SE-Sephadex equilibrated in the same buffer, and eluted with a linear gradient from 0.1 to 0.3 M NaCl (150 ml/chamber), after the large 280-nm breakthrough peak had been eliminated. The elution profile (not illustrated) showed two peaks; material from the second peak emerging at 0.2 M NaCl, and coinciding with radioactivity, was collected and dialyzed against 1 M formic acid, using acetylated dialysis tubing (Vanaman *et al.*, 1968), then lyophilized. Total recovery of radioactivity was 50–55% in several preparations.

As previously noted for the rabbit phosphopeptide, gel electrophoresis showed two major peptide bands (2 and 3 in Figure 5) plus a third minor tailing band (1). Bands 2 and 3 were clearly radioactive, and originated, as expected, from the slow equilibrium between homoserine and homoserine lactone. The proximity of band 1 and 2, however, made it difficult to ascertain whether the first one was also radioactive. It is not known whether band 1 is an impurity or is derived from D-CB 14. The amino acid composition of D-CB 14 based on ^{32}P = 1:00 (see Table II) was very similar to that of R-CB 14 (Saari and Fischer, 1973).

Chymotryptic Phosphopeptides of D-CB 14. D-CB 14 was dissolved in a small volume of 1.0 mM HCl, whereupon addition of 0.1 M NH_4HCO_3 produced a fine suspension that was rapidly attacked by chymotrypsin; following a 20-hr digestion with two successive additions of protease (1:50 final molar ratio) the digest was chromatographed on Dowex 1 and one major radioactive peak emerged at pH 8.4, that was resolved into two radioactive, ninhydrin-positive spots on paper chromatography: C-1 (*ca.* 80%, R_F 0.16) and C-2 (*ca.* 20%, R_F 0.08).[4] Their compositions were identical except that C-1 (11 residues) contained glycine and leucine, both absent from C-2. Since the COOH-terminal sequence of C-1 was established as Gly-Leu from carboxypeptidase A digests (Table VI), this indicated that both peptides were derived from the same 11-residue segment of the protein.

[3] Residues 5–7 in R-CB 5 have not yet been ordered. However, carboxypeptidase A digestion showed that as in the dogfish peptide, asparagine and glutamine are present (Forrey *et al.*, 1971a). Furthermore, glutamine is indicated as the amino acid at position 5 from the failure of Edman degradations to release any amino acid at the fifth cycle.

[4] A third minor radioactive peptide C 3 (R_F 0.30) was also obtained in one digest. It lacked the amino-terminal two residues (Glu-Arg); generation of C 2 and 3 suggests trace contamination of the purified chymotrypsin by trypsin.

TABLE VI: Summary of Data for the Phosphopeptide Sequence of Dogfish Muscle Phosphorylase.[i]

Rabbit phosphopeptide[a]:

P

Ser-Asp-Gln-Glu-Lys-Arg-Lys-Gln-Ile-Ser-Val-Arg-Gly-Leu

Dogfish phosphopeptide:

P

Glu-Arg-Arg-Lys-Gln-Ile-Ser-Val-Arg-Gly-Leu

Peptide	b	C-1	d	c
C-1	⇀ → →		←	←
C-2		C-2		◁ e
Th-1[g]	Th-1	f ← ←		
Th-2[h]		Th-2		
Th-3		Th-3		

[a] Nolan *et al.* (1964). [b] Direct Edman; identified as the trimethylsilyl derivative by gas chromatography. [c] CpA 1:100, 1 hr. [d] CpA 1:25, 20 hr. [e] CpB 1:100, 3 hr. [f] CpA 1:100, 3 hr. [f] CpA 1:100, 3 hr. [g] Thermolytic digestion at 1:100, 22°, 6 hr. [h] 1:50, 20 hr. Unless indicated, enzymatic digestions were carried out at 35°. [i] C = chymotrypsin; CpA, CpB = carboxypeptidase A and B; Th = thermolysin. Code: ⇀ direct Edman → subtraction Edman ← Carboxypeptidase A ◁ Carboxypeptidase B.

The complete sequence was elucidated by carboxypeptidase B attack on C-2 and by Edman degradation and thermolytic attack of C-1 as summarized in Table VI. The peptide bonds Gln–Ile and Gly–Leu were cleaved most rapidly by thermolysin as expected (Bradshaw, 1969). However, extensive cleavage unexpectedly occurred at Arg–Gly (Table VI) and Arg–Arg bonds (not illustrated), to about 70 and 100%, respectively, during the 20-hr digestion. By contrast, the phosphoseryl–valine bond, which was anticipated to be susceptible to thermolysin, was only cleaved to 20% under the same conditions. It is unknown whether the anomalous cleavages result from the presence of a trypsin-like activity as a trace contaminant.

Discussion

A comparative study of the physical and catalytic properties of dogfish and rabbit skeletal muscle phosphorylases (Cohen *et al.*, 1971) showed that these proteins share many properties in common, although distinct differences do exist, particularly in association–dissociation behavior and solubility at low temperature. Suggestions of structural homology are now confirmed by the present data.

The amino acids surrounding the *phosphopyridoxyllysyl* residue presumably constitute part of the binding site of this coenzyme; identity of sequence in the dogfish and rabbit proteins is therefore consistent with the apparently identical mode of binding and release of this cofactor, spectral properties, reduction with $NaBH_4$, etc., previously noted (Cohen *et al.*, 1971). A sequence of 11 residues around the *phosphoseryl* residue showed only a single conservative amino acid substitution, with an arginine at position 2 in the dogfish

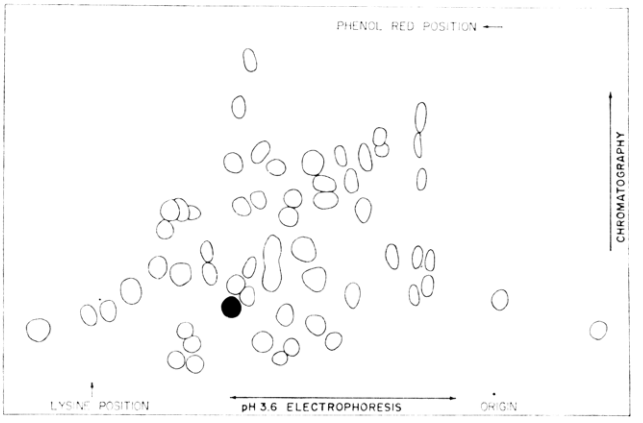

FIGURE 4: Peptide map of a tryptic digest of dogfish phosphorylase *b*. The shaded spot is the NH_2-terminal tetrapeptide of the molecule.

phosphopeptide replacing a lysine in the rabbit peptide. The five phosphorylases whose phosphopeptide sequences are known (Fischer *et al.*, 1970) show a total of four such replacements, each explainable by a single base change. These enzymes can all be phosphorylated and dephosphorylated by *rabbit* muscle phosphorylase kinase and phosphatase, respectively; furthermore, the interconversion reactions proceed at an identical rate with dogfish and rabbit phosphorylases *b* and *a* (Cohen *et al.*, 1971), even though the two species are far removed in terms of evolutionary distance. The excellent stoichiometry obtained for the dogfish phosphopeptide composition normalized on $^{32}P = 1.00$ confirmed the absorbance index (12.9) and subunit molecular weight (100,000), determined previously for the native enzyme (Cohen *et al.*, 1971).

The identity in 22 of the 23 amino acids surrounding the phosphopyridoxyllysyl and phosphoseryl residues in dogfish and rabbit muscle phosphorylases suggests that these are strongly conserved regions. This is perhaps not surprising since they were selected in this study by virtue of their catalytic and regulatory functions; active-site peptides of other glycolytic enzymes from fish and mammalian sources have also demonstrated a similar extent of homology, *e.g.*, the SH group in glyceraldehyde-3-phosphate dehydrogenases (Allison, 1968) or the phosphoseryl residue of phosphoglucomutase (Hashimoto *et al.*, 1966; Milstein and Milstein, 1968). But

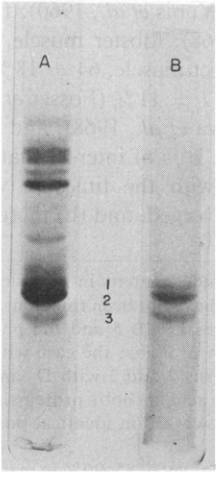

FIGURE 5: Polyacrylamide gel electrophoresis in 8 M urea of the [^{32}P]CNBr peptide fraction (A) before and (B) after SE-Sephadex chromatography. Migration is from top to bottom. The gels were stained with Coomassie Blue.

are these areas more highly conserved than the primary sequence as a whole? While an exact appreciation of this problem must of course await elucidation of complete sequences, an attempt has been made to estimate the overall extent of homology from the present evidence. Analysis of the CNBr peptides from rabbit muscle phosphorylase (Saari and Fischer, 1973) has shown that 11 out of 22 are less that 15 residues long, and therefore, should provide a convenient point for starting such a comparative study. While these peptides are unusual in possessing two methionyl residues in close proximity (on the average, only 1 methionine/38 residues is expected), there is no other *a priori* reason to suspect that they should represent invariant regions, apart from CB 3′ and CB 5 which together comprise the sequence around the pyridoxyllysyl residue. Nevertheless, of the 9 peptides isolated from fraction F (D-CB 1, 3′, 4, 5, 7, 8, and 10), 7 (55 residues) were identical in composition and partial or complete sequence with peptides derived from rabbit phosphorylase, and an eighth (D-CB 6) was tentatively assumed to be homologous with R-CB 6.[5] Considering these eight peptides, 59 out of 63 residues are identical, corresponding to 94% homology. Considering specifically methionyl residues, and including CB 14, 14 ± 4 methionines are in identical positions depending whether all or none of these peptides are linked together in the primary sequence. On the other hand, two changes involving methionine have been found. First, the NH_2-terminal peptide CB A is present only in the dogfish enzyme; second, R-CB 2 seems to be present only in rabbit phosphorylase (Table III) since duplicate isolates of all the fraction F peptides from the dogfish failed to reveal this component.[6] Although the CNBr phosphopeptides D-CB14 and R-CB 14 are quite similar in composition and size, recent evidence suggests that the former is seven residues shorter, and that D-CB A and 14 are the first and second peptides respectively from the amino-terminus of the protein (Titani *et al.*, unpublished results).

The homology between the two enzymes was also estimated from their amino acid compositions by the divergence factor of Harris and Teller (1973). A value of $D = 0.026$ was obtained. From the calibration curves constructed from families of related proteins of known sequence (Harris and Teller, 1973), this corresponds to an estimated sequence homology of 88 ± 6%. From similar data obtained for other phosphorylases the following degree of homology was estimated when each enzyme was compared to rabbit muscle phosphorylase: rat muscle, 95 ± 3% (Sevilla and Fischer, 1969); human muscle, 92 ± 4% (Yunis *et al.*, 1960); frog muscle, 91 ± 4% (Metzger *et al.*, 1968); lobster muscle, 84 ± 8% (Assaf and Graves, 1969); insect muscle, 64 ± 18% (Childress and Sacktor, 1970); yeast, 77 ± 11% (Fosset *et al.*, 1971); potato, 72 ± 13% (Kamogawa *et al.*, 1968); and rabbit liver, 85 ± 8% (Wolf *et al.*, 1970). It is of interest that (a) these values show some correlation with the times at which the species are thought to have diverged, and (b) there is a greater degree of homology between vertebrate muscle phosphorylases than between the rabbit muscle and liver enzymes, as also indicated by their phosphopeptide sequences and marked differences in AMP activation (Wolf *et al.*, 1970; Fischer *et al.*, 1972).

Taken together these results suggest an overall identity of the order of 85–90% of the residues. Since the dogfish diverged from the main vertebrate line leading to mammals about 450 million years ago, this corresponds to 1.1–1.7 amino acid changes/100 residues per 100 million years. With the exception of histones, this is a slower rate of mutation than found thus far for other proteins; the slowly evolving glyceraldehyde 3-phosphate dehydrogenase and cytochrome *c* display 2 and 3 amino acid mutations/100 million years, respectively (Dayhoff, 1972).

The large size of the phosphorylase subunit and the high degree of invariance of its primary structure are perhaps a reflection of the multiplicity of functional sites found in this molecule. This enzyme interacts with three substrates, effectors such as AMP and glucose-6-P, the coenzyme pyridoxal-5′-P and two enzymes involved in its phosphorylation and dephosphorylation. In addition, two types of subunit contacts have been identified in vertebrate muscle phosphorylases, allowing for the formation of the phosphorylase *b* dimer and *a* tetramer species; in hemoglobin, such contact regions were found to be rather invariant (Perutz *et al.*, 1968). Whether or not some of these sites overlap, a substantial portion of the surface of phosphorylase must be devoted to specific recognitions providing a distinct requirement for conserving such regions throughout evolution.

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[5] Differences in amide content in peptides D-CB 4, 6, 7, and 10, though very unlikely, have not been ruled out. The four residues investigated for such changes in CB 5 and the phosphopeptide (see below) were identical in both proteins; the ease with which Edman degradations proceeded at turns 2 and 3 with D- and R-CB 10 suggested the presence of glutamic acid in both proteins. Finally, D- and R-CB 4 and 7 eluted from Dowex 50 in identical positions (see also Saari and Fischer, 1973).

[6] The probable homology of 85–90% in methionine residues is initially surprising in view of the different elution profiles of CNBr digests of the two proteins on Sephadex G-75 (Figure 2). However, such a result could easily be accounted for by slight differences in methionine and tryptophan distribution, particularly in fractions B and D.

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Formation of Enolpyruvate in the Phosphoenolpyruvate Carboxytransphosphorylase Reaction†

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ABSTRACT: Carboxytransphosphorylase converts phosphoenolpyruvate and P_i to pyruvate and PP_i. In the presence of CO_2, oxalacetate and PP_i are formed. The addition of a proton to yield pyruvate is nonstereospecific whereas the addition of CO_2 is only to the *si* face of phosphoenolpyruvate to yield oxalacetate. These results are explained by a mechanism in which phosphoryl transfer to P_i gives rise to enzyme-bound enolpyruvate which is specifically carboxylated. Pyruvate is formed from enolpyruvate after it has dissociated from the enzyme and the keto form of pyruvate never occurs on the enzyme. That the formation of the keto form of pyruvate is nonenzymatic is shown by the nonstereospecificity and the failure of carboxytransphosphorylase to detritiate [3-^{3}H]-pyruvate. According to this mechanism the requirement for pyrophosphate for $^{14}CO_2$–oxalacetate exchange should not involve phosphoryl transfer but may be indirect or synergistic. Contrary to expectation, however, neither methylene diphosphonate nor imidodiphosphate which were competitive inhibitors was effective in replacing pyrophosphate for this reaction, and are presumed not to contribute the degree of fit required for the synergistic role. A mechanism in which one phosphorus of pyrophosphate forms a pentacovalent adduct with the C-2 oxygen of enolpyruvate in the decarboxylation step might be much less favorable with the pyrophosphate analogs due to the polarity rules that govern the stereochemistry of such compounds. The nonstereoselectivity of pyruvate formation could then result from the decomposition of this metastable intermediate by a partially nonenzymatic route.

Phosphoenolpyruvate carboxytransphosphorylase (EC 4.1.1.38) is known to catalyze reactions 1 and 2 in which CO_2 and proton appear to act as alternate electrophiles (Davis *et al.*, 1969; Wood *et al.*, 1969a,b) for the activated carbon 3 of phosphoenolpyruvate[1] (P-enolpyruvate). The stereochemistry of CO_2 addition has been examined (Rose *et al.*, 1969)

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[1] The abbreviations used are: PCP, methylene diphosphonate; PNP, imidodiphosphate; P-enolpyruvate, phosphoenolpyruvate.