Biochemistry

Volume 12, Number 26 December 18, 1973

Cyanogen Bromide Peptides of Rabbit Muscle Glycogen Phosphorylase†

John C. Saari‡ and Edmond H. Fischer*

ABSTRACT: As the initial step in the total sequence determination of rabbit muscle glycogen phosphorylase (EC 2.4.1.1), the enzyme was cleaved with cyanogen bromide and the resulting peptides were separated. Carboxymethylation with [^{14}C]-iodoacetate, fixation of pyridoxal-5′-P with $NaBH_4$ and labeling of the enzyme with [^{32}P]ATP and phosphorylase kinase provided the specific markers required for the development of appropriate separation procedures. The complex peptide mixture was resolved into six fractions from which 18 CNBr peptides were isolated and characterized. These include an 88-residue fragment containing the phosphorylated site, fragments containing the cofactor binding site, and a 32-residue peptide which lacked homoserine and is presumed to represent the blocked COOH-terminus of the enzyme. Chromatographic separation and polyacrylamide gel electrophoresis provided evidence for 4 additional CNBr peptides which have not yet been isolated, bringing the total to 22, a value consistent with that expected for a protein with identical subunits of mol wt 100,000.

Glycogen phosphorylase (EC 2.4.1.1) is involved in one of the most complex regulatory mechanisms yet described, in which a cascade of enzymatic reactions, allosteric transitions, and covalent modifications all focus to control its activity (for review, see Fischer *et al.*, 1970). The number of binding sites on the enzyme alone is indicative of the complexity of this regulatory process: in addition to the three substrates (glycogen, P_i, and Glc-1-P)[1] phosphorylase binds several effectors (nucleotides, Glc-6-P) and the cofactor pyridoxal-5′-P. It is reversibly phosphorylated and therefore possesses binding sites for both phosphorylase kinase and phosphatase, the two enzymes responsible for its covalent modification. Finally, it also possesses two distinct subunit binding regions to account for the formation of dimeric and tetrameric species. Recently, all enzymes involved have been shown to be associated with glycogen particles suggesting that an even higher level of organization exists *in vivo* (Meyer *et al.*, 1970). The overall picture of phosphorylase that emerges is that of an enzyme in which recognition sites cover much of its surface, a concept that has interesting evolutionary implications (see accompanying paper, Cohen *et al.*, 1973).

The large body of knowledge that has accumulated on the control and structure of phosphorylase demands some explanation in molecular terms that can come only from a detailed structural analysis of the protein. This paper describes the first steps toward the elucidation of the total sequence of the enzyme. The following approach was used. An initial investigation of the purity and subunit homogeneity of the enzyme was carried out. This was done in view of earlier reports that several glycolytic enzymes are heterogeneous when examined by isoelectric focusing chromatography (Susor *et al.*, 1969) and that phosphorylase itself might be composed of nonidentical subunits (Valentine and Chignell, 1968). Then maximal use was made of marker peptides to assist in the development of suitable fractionation procedures; starting material included [^{14}C]carboxymethylated phosphorylase *a*, ^{32}P-labeled phosphorylase *a* containing one radioactive seryl phosphate residue per monomer, and $NaBH_4$-reduced phosphorylase *b* in which a unique fluorescent phosphopyridoxyl group is fixed covalently to the protein (Fischer and Krebs, 1958). The methodology developed has been applied to a comparative study of dogfish and rabbit muscle phosphorylase described in the following paper (Cohen *et al.*, 1973). Another recent paper describes the initial ordering of some of the CNBr peptides of rabbit muscle phosphorylase (Raibaud and Goldberg, 1973). This is the tenth publication on the comparative properties of

† From the Department of Biochemistry, University of Washington, Seattle, Washington 98195. *Received August 9, 1973.* This work was supported from grants from the National Institutes of Arthritis and Metabolic Diseases (AM 07902), National Institutes of Health, U. S. Public Health Service, the National Science Foundation (GB 20482), and the Muscular Dystrophy Association of America.

‡ Recipient of a National Institutes of Health U. S. Public Health Service Predoctoral Traineeship. Present address: The Rockefeller University, New York, New York 10021.

[1] Abbreviations used are: CM, carboxymethyl; SE, sulfoethyl; Glc, glucose.

glycogen phosphorylases; for the previous publication, see Fosset *et al.* (1971).

Materials and Methods

CNBr (Eastman) was stored *in vacuo* at −20° and used without further purification; iodoacetic acid (Eastman) was recrystallized three times from petroleum ether (bp 30–60°) and stored at −20° in an amber bottle to minimize photodecomposition. Urea was recrystallized from water; pyridine, α-picoline, and *N*-ethylmorpholine were distilled before use. Dowex 1-X2, Dowex 50-X2 (Bio-Rad Laboratories), and Dowex 50-X8 (Beckman, Spinco Division) were processed according to Schroeder (1967a,b). Dialysis tubing (Union Carbide) was boiled three times in 1 mM EDTA, then in 50% ethanol.

Rabbit muscle phosphorylase *b* was prepared by the method of Fischer and Krebs (1958) and reduced with sodium borohydride by the low pH–high salt procedure of Strausbauch *et al.* (1967). Separation of pyridoxyl peptides was carried out in the dark as far as possible to prevent photochemical destruction of the phosphopyridoxyl derivative. ^{32}P-Labeled phosphorylase *a* was prepared from both native and $NaBH_4$-reduced phosphorylase *b* using purified phosphorylase kinase (DeLange *et al.*, 1968). [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell (1964). The labeled enzyme was dialyzed and its specific radioactivity was determined after acid hydrolysis by liquid scintillation counting and amino acid analysis (Cohen *et al.*, 1971). Typical specific radioactivities were 65–70 × 10^6 cpm/μmol of enzyme monomer.

Carboxymethylation was carried out according to Gurd (1967). Prior to reaction, a dialyzed suspension of the enzyme was adjusted to the following final concentrations: phosphorylase, 10 mg/ml; ammonium bicarbonate, 0.1 M; and urea, 8 M. Bicarbonate was added before urea to prevent isoelectric precipitation of the enzyme. Even though the enzyme contains no disulfide bond (Damjanovich and Kleppe, 1967; Battell *et al.*, 1968a,b), dithiothreitol was added at twice the molar concentration of protein cysteinyl residues. After 15 min, a 1.2 molar excess of ^{14}C or cold iodoacetate over total SH content was added. The pH of the reaction was maintained at 8.2 with 6 N KOH. After 10 min, the solution was made 0.1 M in mercaptoethanol and dialyzed against 1 M formic acid, lyophilized, and stored at −20°. Light was excluded throughout to minimize photooxidation of the iodide ion. Amino acid analysis confirmed that alkylation was confined to cysteinyl residues and as expected 8–9 residues of CM-cysteine were recovered per mole of enzyme subunit.

CNBr cleavage of phosphorylase was performed essentially as described by Steers *et al.* (1965) and Gross (1967). Lyophilized, carboxymethylated phosphorylase was dissolved in 70% formic acid (10-mg/ml final concentration) and allowed to react with a 50-fold molar excess of CNBr over methionine. After 16–20 hr at room temperature, the solution was diluted 10-fold with water and lyophilized, and the residue stored at −20°.

Gel isoelectric focusing experiments were performed according to Wrigley (1968a,b); glucose (1.0%), urea (8 M), and Triton X-100 (0.25%) were included in some experiments.

High-voltage paper electrophoresis was run at pH 3.6 and 6.5 (Ryle *et al.*, 1955; Bennett, 1967). For preparative separations, peptide solutions were applied over a 5–8-cm strip; marker spots included on either side were stained with ninhydrin to locate the peptides. Descending paper chromatography was carried out in the 1-butanol–pyridine–glacial acetic acid–H_2O (90:60:18:72, v/v) solvent of Bennett (1967); Whatman 3MM paper washed by paper chromatography with developer was used throughout.

Gel filtration on Sephadex G-75, equilibrated in 1 M formic acid, was carried out in two 2.5 × 120 cm columns connected with flow adapters (Pharmacia); flow proceeded down one column and up the other giving an effective length of 240 cm. Chromatographies on Dowex 1-X2 and 50-X2 were carried out in 0.6 × 60 cm columns using the volatile buffer systems of Schroeder (1967a,b). Dowex 50-X8 (1 × 20 cm column) was developed at 55° with a double linear gradient of pyridine acetate (Vanaman *et al.*, 1968). Peptides were monitored automatically by absorption at 280 nm, and by reaction with ninhydrin after alkaline hydrolysis (Hirs, 1967) except when urea was present. Phosphopyridoxyl-containing fractions were located by their characteristic fluorescence (Strausbauch and Fischer, 1970; Forrey *et al.*, 1971a), and radioactive fractions by liquid scintillation counting using the dioxane scintillant of Bray (1960); addition of 0.5 ml of 88% formic acid to the scintillation vial gave uniform counting efficiencies and prevented precipitation of the sample. Urea and nonvolatile buffers were removed from the larger (>60 residue) CNBr peptides by dialysis against 1 M formic acid in acetylated dialysis membranes (Vanaman *et al.*, 1968).

Polyacrylamide gel electrophoresis was carried out in 10% gels in 8 M urea at pH 4.5 using β-alanine as the running buffer or, for molecular weight determinations, in sodium dodecyl sulfate according to Shapiro *et al.* (1967) and Weber and Osborn (1969). Gels were stained with Coomassie Brilliant Blue. [^{32}P]CNBr peptides were located by slicing the gel and dissolving each segment in 1.0 ml of 30% H_2O_2 for 30 min at 100°; 0.5 ml of 88% formic acid was added and the solutions were counted as above.

The molecular weight of the large CNBr peptides was determined in a Spinco Model E analytical ultracentrifuge by the meniscus depletion method of Yphantis (1964), following dialysis against 10 mM sodium acetate and 8 M urea at pH 4.5. Runs were carried out at *ca.* 0.8 mg/ml in 4 mm solution columns at 20°. Data reduction was performed according to the computer program of Teller *et al.* (1969), using partial specific volumes calculated from the amino acid compositions of the peptides.

Amino acid analysis was performed by the procedure of Spackman *et al.* (1958) using a Spinco Model 120C amino acid analyzer equipped with an Infotronic integrator. Homoserine lactone was converted to homoserine by dissolving the dried hydrolysate in 0.5 ml of 0.05 N NaOH and warming at 30° for 15 min. Citrate buffer (1.6 ml, pH 3.22, 0.2 M) was added and 1.0 ml of the sample was applied to each column of the analyzer. The initial pH of the long column buffer was lowered from pH 3.25 to 3.22 to resolve homoserine from glutamic acid. The color value of homoserine was taken as the average of the threonine and serine values (Tang and Hartley, 1967).

Amino-terminal analysis was performed as described by Stark (1967). Samples of peptide were carbamylated in urea, dialyzed, and added to an hydrolysis tube containing a known quantity (usually 0.5 μmol) of carbamylnorleucine. After cyclization, the concentration of peptides was obtained either by counting an aliquot by liquid scintillation (radioactive peptides) or by amino acid analysis following hydrolysis of an aliquot. Correlation for mechanical losses of the acidic and neutral fractions was based on the recovery of norleucine. The dansyl chloride amino-terminal group procedure of

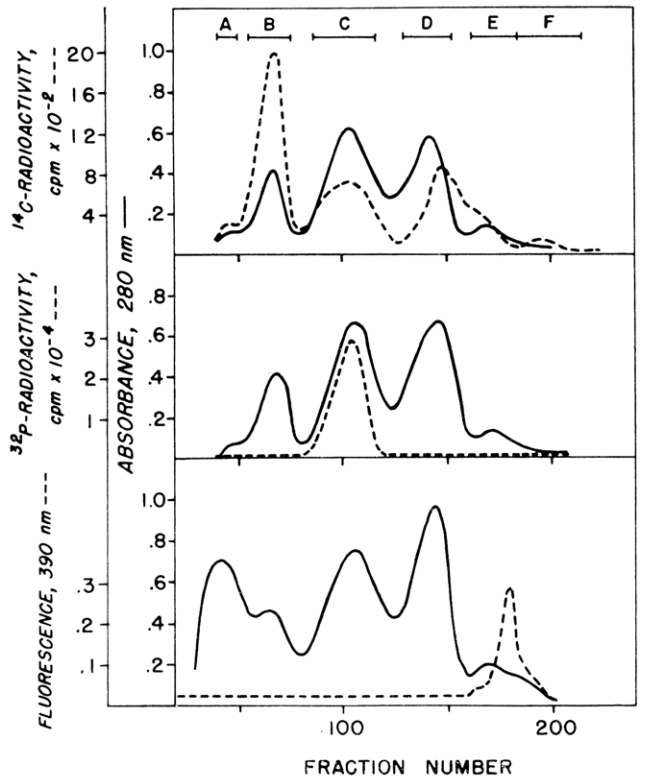

FIGURE 1: G-75 Sephadex gel filtration of CNBr peptides obtained from [^{14}C]carboxymethylated phosphorylase *a* (upper frame), [^{32}P]carboxymethylated phosphorylase *a* (middle frame), and $NaBH_4$-reduced phosphorylase *b* (lower frame). Approximately 200 mg of peptides was applied to the column in a volume of 10 ml. The column (2.5 × 240 cm) was developed at room temperature with 1 M formic acid at a flow rate of 30 ml/hr; 3-ml fractions were collected and analyzed for radioactivity, for absorption at 280 nm, and for fluorescence at 390 nm after excitation at 330 nm. Fractions A–F were collected separately.

Gray (1967) was also applied except that the final derivative was determined by thin-layer chromatography as described by Forrey *et al.* (1971b).

Results

Homogeneity of the Starting Material. The recent finding by Susor *et al.* (1969) that several proteins previously considered homogeneous demonstrated microheterogeneity when subjected to isoelectric focusing prompted a reinvestigation of phosphorylase by this approach. Application of this technique seemed all the more relevant in view of the report by Valentine and Chignell (1968) based on electron microscopy that phosphorylase might be made up of different subunits.

Phosphorylase *a* initially displayed three bands on gel isoelectric focusing; however, a single band was observed when glucose was included. Since this carbohydrate is known to displace the dimer–tetramer equilibrium almost entirely toward the dimer (Wang *et al.*, 1965; Metzger *et al.*, 1967), it was concluded that the multiple band pattern resulted from the presence of various stable aggregates of the enzyme. Several other gel isoelectric focusing experiments were conducted in the presence of 8 M urea and 0.25% Triton X-100. Again, only one major band could be seen suggesting that the subunits were identical by this criterion (see Saari, 1970).

Cyanogen bromide cleavage was investigated in a number of solvents, but as noted previously (Hofmann, 1964; Steers *et al.*, 1965), 70% formic acid gave the best results. In this solvent, cleavage proceeded essentially to completion in 4 hr at room temperature with a 50-fold molar excess of CNBr over the methionine concentration; the reaction was conveniently carried out overnight (16–20 hr) yielding preparations with less than 3% residual methionine. Homoserine recovery was somewhat variable due to problems associated with lactone formation but approached 95% in most cases. Reproducibility of CNBr cleavage was excellent judging from the gel electrophoretic patterns of many peptide preparations.

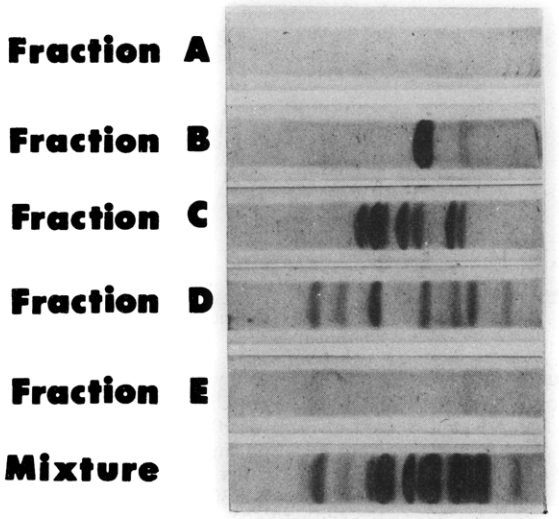

FIGURE 2: Polyacrylamide gel electrophoresis in 8 M urea of samples from the peak tubes of fractions A–E obtained after G-75 Sephadex chromatography. Migration was from right to left; conditions are given under Methods.

Gel Filtration of the CNBr Peptides. Formic acid (1 M) proved to be an excellent solvent for gel filtration, eliminating completely the severe aggregation discussed earlier (Forrey *et al.*, 1971b). Figure 1 illustrates the elution patterns of CNBr peptides obtained from [^{14}C]carboxymethylated phosphorylase *a*, [^{32}P]carboxymethylated phosphorylase *a*, and $NaBH_4$-reduced phosphorylase *b*, respectively. The ninhydrin profile was essentially identical with that of the 280-nm absorbance pattern except that an additional peak appeared in the last fraction (F). As will be seen below, this fraction contains several small peptides devoid of tyrosine and tryptophan. In Figure 1, bottom frame, the large amount of material eluting at the void volume most probably results from aggregation due to disulfide bond formation.[2]

Figure 2 illustrates the disc gel electrophoresis patterns of material removed from the peak tubes of fractions A–E obtained by G-75 Sephadex chromatography. Fractions A, E, and F (not illustrated) contained no peptide detectable by this procedure, although many ninhydrin-positive spots appeared on paper electrophoresis of fractions E and F; presumably, the small peptides involved either diffused rapidly from the gel or did not stain with Coomassie Blue. As expected from the slow equilibrium between homoserine and homoserine lactone (Armstrong, 1949), the CNBr peptides generally migrated in two positions (doublets) on disc gel electrophoresis as well as on ion-exchange chromatography or paper electrophoresis as shown below.

Resolution of the G-75 Sephadex Fractions A to F. Since the

[2] In initial studies the $NaBH_4$-reduced enzyme was not carboxymethylated for fear of substituting the pyridoxyl ring; subsequently (Cohen *et al.*, 1973) no evidence for this reaction was obtained.

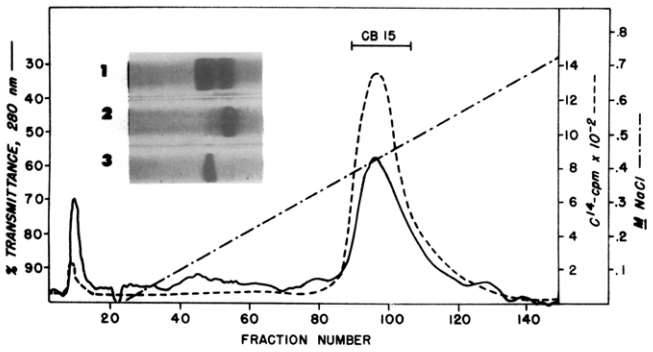

FIGURE 3: CM-Sephadex chromatography of fraction B. Lyophilized fraction B derived from 2 μmol of [^{14}C]carboxymethylated phosphorylase *a* was dissolved in 3 ml of 10 mM sodium acetate–8 M urea (pH 4.5) and applied to a 1 × 30 cm column of CM-Sephadex C-25, previously equilibrated with this buffer. The column was developed at room temperature with a 300-ml NaCl gradient from 0 to 1.0 M with a flow rate of 48 ml/hr; 3-ml fractions were collected. The insert shows the gel electrophoretic pattern of components of fraction B before chromatography (1), in the breakthrough (2), and in the main peak corresponding to CB 15 (3). Migration is from right (anode) to left.

order of CNBr peptides along the protein backbone is not known, no rational numbering system could be used. Initial ordering of CNBr peptides is described in this issue by Raibaud and Goldberg (1973). Attempts, not altogether successful, were made to assign the smallest CB numbers to the smallest peptides.

FRACTION A. Little peptide material could be detected by ninhydrin analysis or radioactivity. Gel electrophoresis of large amounts of this fraction revealed several bands (not illustrated) corresponding to contamination by the major component of fraction B (CB 15) and perhaps peptides derived from nonquantitative CNBr cleavage. Fraction A was not further analyzed.

FRACTION B contained ^{14}C but no ^{32}P radioactivity (Figure 1, upper frame); disc gel electrophoresis (Figure 2) disclosed one major and one minor doublet band. The latter was removed on CM-Sephadex chromatography in 8 M urea yielding pure CB 15 (Figure 3).

The amino acid composition of CB 15 is shown in Table I. Its minimum molecular weight calculated on the basis of CM-cysteine content is approximately 9000; however, high-

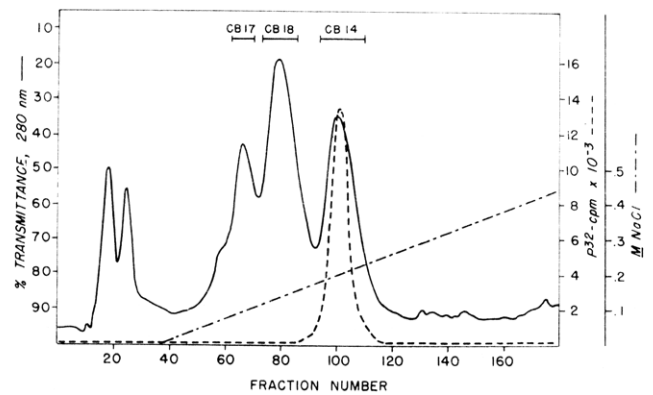

FIGURE 4: SE-Sephadex chromatography of fraction C. Fraction C, derived from 2 μmol of [^{32}P]carboxymethylated phosphorylase *a*, was dissolved in 10 mM pyridine formate–8 M urea buffer (pH 2.4) and applied to a jacketed, 1 × 20 cm column of SE-Sephadex G-25 previously equilibrated in this buffer at 55°. The column was developed with a 300-ml linear NaCl gradient from 0 to 0.5 M; 3-ml fractions were collected.

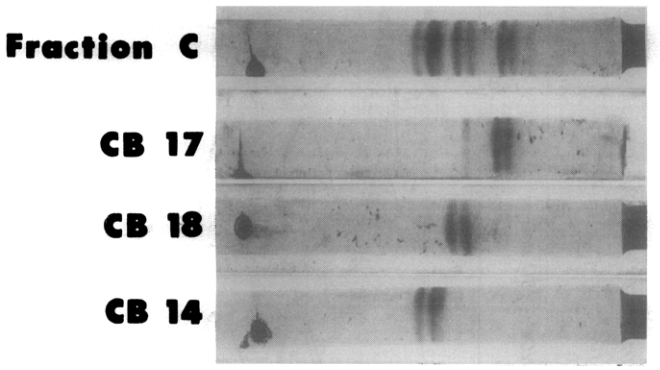

FIGURE 5: Polyacrylamide gel electrophoresis in 8 M urea of the pooled fractions from Figure 4. Electrophoresis was from right to left at pH 4.5.

speed sedimentation equilibrium centrifugation in 8 M urea gave number- and weight-average molecular weights of 17,643 and 17,040, respectively; a value close to 18,000 was confirmed by sodium dodecyl sulfate gel electrophoresis, while quantitative amino-terminal group analysis yielded 1.0 equiv of alanine/17,700 g of protein.

FRACTION C contained some of the cysteinyl residues (as indicated by the ^{14}C radioactivity distribution seen in Figure 1, upper frame) and all the ^{32}P (middle frame) of the parent enzyme. Polyacrylamide gel electrophoresis (Figure 2) revealed the presence of six major bands appearing in three doublets, and a seventh minor band migrating just behind the most basic doublet; ^{32}P radioactivity was entirely associated with this latter doublet. As expected from their size, none of the larger peptides migrated on paper electrophoresis or chromatography. Fraction C was resolved into 4 major peaks on SE-Sephadex chromatography in 8 M urea (Figure 4). The breakthrough fraction only contained traces of a number of peptides and was not further analyzed; its 280-nm absorbance is exaggerated probably due to differences in urea concentration between the sample and equilibrating buffers. The other 3 peaks each corresponded to one doublet (CB 17, 18, and 14). Pooled fractions of CB 14 and 17 were homogeneous as judged by disc gel electrophoresis (Figure 5) and automatic sequenator analysis (M. Hermodson, unpublished results). The latter technique revealed CB 18 to be contaminated with 15–20% of CB 17. The amino acid compositions, amino-terminal residues and yields of these peptides are given in Table I. The size of the peptides was confirmed by sedimentation equilibrium giving molecular weight values of 9125, 10,368, and 9342 for CB 14, 17, and 18, respectively. These values were further supported by gel electrophoresis in sodium dodecyl sulfate.

The phosphopeptide CB 14 was also purified by column isofocusing in 6 M urea and was isoelectric near pH 10.[3] The peptides isolated by both methods had almost identical compositions; both contained the minor band mentioned earlier (see Figure 5).

FRACTION D proved to be extremely difficult to resolve because (a) most peptides emerged as doublet peaks due to a charge difference of one between homoserine and homoserine lactone; (b) they were too large to be separated on paper; and (c) they failed to elute from ion-exchange columns in the absence of urea. The system described here resulted in the isolation of 2 peptides, and allows an estimation to be made

[3] Further experimental details and results may be obtained by writing directly to the authors. See also Saari (1970).

TABLE 1: Amino Acid Compositions of the CNBr Peptides of Rabbit Muscle Glycogen Phosphorylase.[a]

Amino Acid	CB 1	CB 2	CB 3	CB 4	CB 5	CB 6	CB 7	CB 8	CB 9	CB 10	CB 11	CB 12	CB 13	CB 14	CB 15	CB 16	CB 17	CB 18
Lysine		1.09	1.01					0.96	0.91		0.15		1.51	7.24	15.45		7.16	3.10
Histidine								0.83					0.24	4.90	5.67		1.06	2.21
Arginine					0.81				0.94		0.95		3.37	10.06	10.26	0.93	8.44	8.80
CM-Cysteine												0.93			2.00	0.70	1.08	1.01
Aspartic acid				2.02	1.21	1.00	0.98		0.89	1.12	3.22	4.52	2.76	9.16	16.75	4.08	19.84	10.00
Threonine					1.85	0.99	1.84				1.74	1.15	1.52	4.99	4.50	0.35	3.56	4.53
Serine									1.04		0.11	0.13	2.43	3.74	4.44	1.09	5.10	1.20
Homoserine	0.94	0.97	0.99	0.99	1.00	1.10	0.95	0.91	1.01	0.90	1.00	1.00	0.12	0.62	0.88	0.81	0.95	0.87
Glutamic acid				0.99	1.13	0.91			2.18	4.00	0.32	3.66	4.24	8.95	17.23	7.36	10.14	8.87
Proline								0.97			2.22		4.79	2.25	8.19		5.15	3.43
Glycine				1.03	1.08	0.18	1.95	2.94	1.16	2.00	1.00	1.79	1.82	3.14	4.92	5.71	4.65	2.05
Alanine		1.00		1.00		0.99	1.00	2.00	1.00	2.00	0.18	3.49	3.03	4.30	9.69	3.70	10.00	6.76
Valine				1.01		2.48			1.94		1.97	1.04	1.55	7.22	11.94	0.23	8.12	8.27
Isoleucine							1.00		1.03	1.00	0.14	0.26	2.70	2.81	12.78	1.09	6.06	3.33
Leucine	1.00				1.00	1.02	1.99	1.04	1.05		0.29	4.51	1.24	8.39	15.85	5.64	11.90	11.83
Tyrosine								0.89			1.43	0.76	0.82	5.59	6.07	0.11	3.12	2.29
Phenylalanine			1.00							2.88			1.00	3.74	6.46	1.00	9.22	3.34
Tryptophan[b]									—		—	—	—	2.00	1.91	—	1.33	2.92
^{32}P														1.00				
Total	2	3	3	7	8	8	10	11	13	14	14	24	34	89	155	33	116	84
Yield (%)	41	29	17	21	12	14	13	28	18	14	20	23	27	79	65	13	38	52
NH_2-Terminal group[c]	Leu	Ala	Lys	Asx	Gly	Val	Leu	Ile	Ser	Ala	—	—	—	Block	Ala	—	Arg	Arg
Fraction	F	F	F	E, F	E, F	F	E	E	E	E	E	E	D	C	B	D	C	C

[a] All values were obtained after 24-hr hydrolysis except for CB 14, 15, 17, and 18; for these, average values after four times of hydrolysis (24, 48, 72, and 96 hr) were used, with the usual extrapolation for valine and isoleucine, and threonine and serine, which increase and decrease during hydrolysis, respectively; values less than 0.10 equiv have been omitted. [b] Tryptophan was analyzed by the spectrophotometric method of Bencze and Schmid (1957). A horizontal bar indicates that the tryptophan content is not known. [c] A horizontal bar indicates that no amino-terminal residue could be detected by the dansyl·Cl technique. [d] Amino-terminal arginine in CB 17 and 18 was detected by automatic sequencer analysis (M. Hermodson, unpublished).

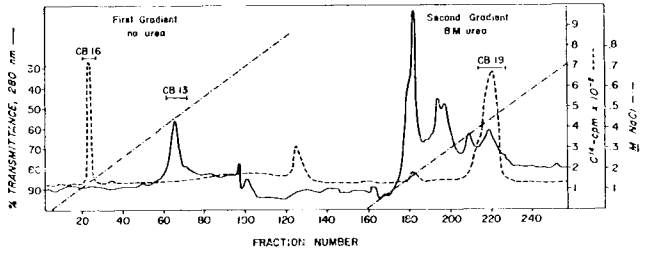

FIGURE 6: Fraction D, originating from 2 μmol of [^{14}C]carboxymethylated phosphorylase *a*, was dissolved in 3 ml of 10 mM pyridine formate buffer (pH 2.4) and applied to a 1 × 30 cm column of SE-Sephadex equilibrated with this buffer at 55°. After development of the column with 300 ml of a linear NaCl gradient from 0 to 1 M, the column was re-equilibrated with 10 mM pyridine formate containing 8 M urea. A second 300-ml linear NaCl gradient from 0 to 1 M completed development of the column.

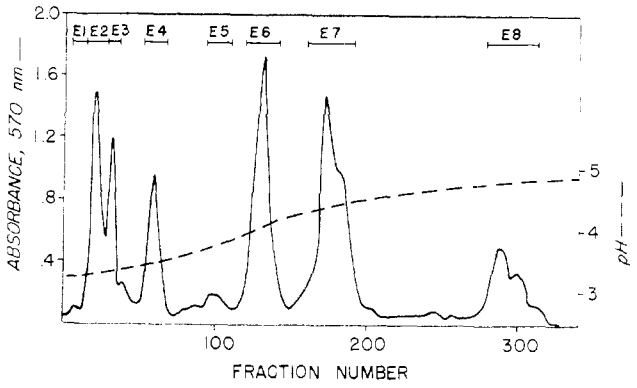

FIGURE 7: Chromatography of fraction E. The soluble portion of fraction E, derived from 11.2 μmol of carboxymethylated phosphorylase *a*, was applied to a 0.6 × 60 cm column of Dowex 50-X2; development and analysis are described under Methods: 1-ml fractions were collected at a flow rate of 15 ml/hr.

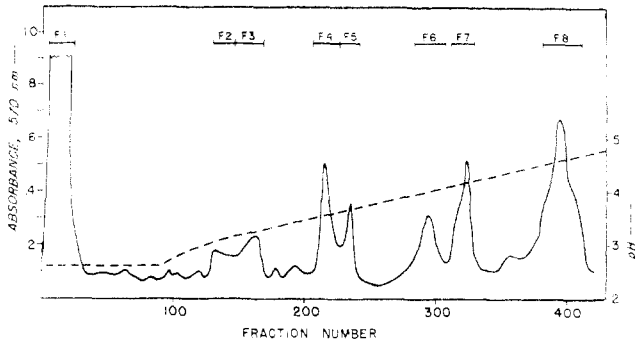

FIGURE 8: Chromatography of fraction F. Lyophilized fraction F derived from 6 μmol of carboxymethylated phosphorylase *a* was dissolved in pH 2.75, 0.1 M pyridine acetate buffer and applied to a 1 × 20 cm column of Dowex 50-X8. A 600-ml double linear gradient of pyridine acetate buffers (Vanaman *et al.*, 1968) at 55° was used to develop the column; 1-ml fractions were collected at a flow rate of 30 ml/hr.

of the total number of fragments present. Peptides CB 13 and 16 were isolated by SE-Sephadex chromatography of fraction D using a double elution system first with volatile buffers, then with buffers containing 8 M urea (see Figure 6). Yields and compositions are given in Table I. The fraction marked CB 19 in Figure 6 contained one major peptide but was not suitable for compositional analysis. Repeated gel electrophoresis of CB 13 gave only a single band with no evidence for a doublet as seen with CB 14, 15, 17, and 18, suggesting that it lacks a homoseryl residue; this was confirmed by amino acid analysis. Both techniques indicated a purity of *ca.* 90%. The absence of homoserine strongly suggests that CB 13 is the COOH-terminal CNBr peptide of rabbit phosphorylase. Polyacrylamide gel patterns of fraction D (Figure 2) suggests that *ca.* 3 (doublet) peptides remain to be isolated.

FRACTIONS E AND F. Again, the homoserine–homoserine lactone equilibrium producing peptide pairs considerably complicated the isolation of these low molecular weight peptides. Attempts to convert the lactone to the free acid by base treatment prior to chromatography were unsuccessful, presumably because the equilibrium was rapidly re-established at the higher temperature maintained during chromatography.

Fractions E and F were isolated by two procedures, namely, (I) pH 4 precipitation followed by G-50 Sephadex chromatography in acetic acid (Forrey *et al.*, 1971b)[4] and (II) G-75 Sephadex chromatography. The composition of the two fractions appeared to differ only slightly. In both instances, lyophilized fraction E failed to dissolve completely in 1 M formic acid. After washing the insoluble material twice with water, amino acid analysis (Table I) indicated the presence of a single peptide (CB 10). CB 10 also crystallized spontaneously from fraction E upon standing for 1 week in the cold; its low solubility at acidic pH might result from a high content of aromatic and acidic amino acids.

The soluble portion of fraction E derived from procedure I (pH 4 Sephadex G-50) was further chromatographed on Dowex 50-X2 and 8 subfractions (E-1 to E-8) were collected (Figure 7). Only E-4 and E-8 consisted of pure peptides (CB 7 and CB 8, respectively) while the other subfractions had to be further purified by high-voltage paper electrophoresis at pH 3.6 and 6.5; compositon, yields and amino-terminal residues are listed in Table I. Subfractions E-6 and E-7 were never completely resolved and probably contained one or possibly two unidentified peptides. Fraction E obtained from procedure II was further fractionated on Dowex 50-X8, yielding a somewhat less complex set of subfractions. Procedures I and II differed in one important respect, however: whereas peptides CB 2, 4, 8, 9, and 12 could be isolated from both, only procedure I yielded CB 7 and procedure II, CB 5.

FRACTION F was also chromatographed on Dowex 50-X8 as shown in Figure 8. Eight subfractions were obtained, 5 of which (F-2, -3, -4, -5, and -7) consisted of pure peptides as judged by compositional and chromatographic analysis, while subfractions F-1, -6, and -8 had to be further purified by high-voltage paper electrophoresis at pH 3.6; amino acid compositions, yields, and amino-terminal residues are listed in Table I.

The phosphopyridoxyl CNBr peptide (obtained after CNBr cleavage of $NaBH_4$-reduced phosphorylase) was primarily located in fraction F and corresponds to the ε-pyridoxyllysine derivative of CB 3 (Forrey *et al.*, 1971b). A peptide, CB 3-5, having the additive amino acid composition of CB 3 plus CB 5 including two homoseryl residues (and apparently resulting from the conversion of methionine to homoserine without peptide-bond cleavage) was also isolated though it was found primarily in fraction E (Forrey *et al.*, 1971b).

When fraction F was isolated through the pH 4 G-50 Sephadex procedure (I) mentioned earlier, an additional peptide (CB 6) was obtained, perhaps corresponding to one of the unresolved components of fraction E.

Discussion

All available evidence obtained here and elsewhere by sedimentation analysis (velocity and equilibrium, Seery *et al.*, 1970), free boundary or gel electrophoresis, or by isoelectric focusing indicates, that rabbit phosphorylase is made up of identical subunits. Polyacrylamide gel electrophoreses carried out in sodium dodecyl sulfate (Cohen *et al.*, 1971) or urea (Hedrick *et al.*, 1969) have consistently confirmed that the subunits are of a single size (mol wt 100,000 ± 2000) and charge. This was also supported by hybridization experiments involving heart muscle phosphorylase isozyme I (Yunis *et al.*, 1962) and skeletal muscle phosphorylase (isozyme III) which yielded a single hybrid molecule (Davis *et al.*, 1967),

[4] Fractions E and F correspond to the low molecular weight fractions of Figure 1b, Forrey *et al.* (1971b).

and not two as expected if nonidentical subunits were present. There is only a single pyridoxal-5′-P and seryl phosphate site per subunit (Nolan *et al.*, 1964) and sequence studies at these sites as well as around the 9 cysteinyl groups of the enzyme (Zarkadas *et al.*, 1968, 1970) provided no evidence for nonidentical subunits (for review, see Fischer *et al.*, 1970). Finally, data obtained from the 18 CNBr peptides isolated here are consistent with the existence of a single peptide chain.

It was originally feared that insolubility might present a major obstacle to the resolution of the CNBr fragments. Surprisingly this was not the case and the peptide mixture was readily soluble in several solvents; however, many of these preparations proved to be unsuitable for further purification. For example, sequential addition of glacial acetic acid and water which yields a clear peptide solution led to smearing and aggregation upon gel filtration. As described in Forrey *et al.* (1971b), this problem was initially avoided by introducing a precipitation step at pH 4, though it was found subsequenty that 1 M formic acid gave excellent resolution with no trace of aggregation. In this system, all electrophoretic components present in the original peptide mixture could be accounted for after gel filtration and the marker peptides (*i.e.*, the radioactive phosphopeptide and the fluorescent phosphopyridoxyl peptide) were exclusively confined to fractions C and F, respectively.

Prolonged exposure of the protein to formic acid during the purification originally caused some concern because of the possibility of unspecific peptide-bond cleavage or deamidation (Schultz, 1967). Although Humbel *et al.* (1968) found no evidence for peptide-bond hydrolysis in insulin dissolved in concentrated formic acid, Brown *et al.* (1967) and Hofmann (1964) reported bond cleavage in β-galactosidase, transacetylase and trypsin, respectively, following exposure to pH's between 1 and 3. No evidence for such reactions was found in this study. First, gel electrophoretic mobility of CM-phosphorylase was unchanged following exposure of the native protein to 70% formic acid for 20 hr at room temperature. Second, quantitative end group analysis (Stark, 1967) revealed no new amino-terminal residue.[3] Finally, all the isolated peptides contained homoserine in close to stoichiometric amounts except CB 13 which presumably originates from the COOH-terminus of the enzyme.

Chromatography in urea containing buffers was particularly valuable for the separation of the large CNBr peptides encountered here. Although these were usually soluble below pH 4, they failed to elute from ion-exchange columns at low pH unless urea was present. Carbamylation of the peptides in urea (Stark *et al.*, 1960) did not occur, probably because of the low pH at which the chromatographies were run.

At the start of this study, the nature of the blocked amino-terminus of rabbit muscle phosphorylase (Appleman *et al.*, 1963) was totally unknown. Since then, the NH_2-terminus of both rabbit and dogfish muscle phosphorylase commencing with *N*-acetylserine has been determined (unpublished work carried out in collaboration with Drs. Neurath, Walsh, and Titani; see also Cohen *et al.*, 1973). On the other hand, no further information has been gathered as to the nature of the blocked COOH-terminus (Appleman *et al.*, 1963), though an additional procedure was applied, namely, selective tritiation according to Holcomb *et al.* (1968). This procedure readily revealed the identity of the COOH-terminal residue in rat phosphorylase, a molecule of equal size found to terminate with a Lys-Ile sequence (Sevilla and Fischer, 1969), and insulin with two COOH-terminal groups.[3] While the occurrence of a blocked COOH-terminal is unusual, several examples have been reported (for review, see Narita, 1970).

The yield of the larger peptides (CB 14, 15, 17, and 18) were excellent since the single charge difference introduced by the homoserine–homoserine lactone equilibrium was not sufficient to noticeably affect their chromatographic behavior. This was unfortunately not the case with the smaller peptides in fractions E and F; each of these migrated as doublets on ion-exchange chromatography or paper electrophoresis. Along with the overlap of fractions E and F, this led, for instance, to the distribution of a single peptide (CB 4) in more than four separate fractions; such behavior was undoubtedly responsible for the low yield in which the smaller peptides were obtained.

From the evidence here, based on polyacrylamide gel electrophoresis and chromatographic patterns, *ca.* 4 CNBr peptides remain to be isolated, bringing the total estimated number of CNBr peptides to 22, a value consistent with a subunit molecular weight of 100,000 for this enzyme (Cohen *et al.*, 1971). Partial or total sequences of some of the small CNBr peptides are given in the accompanying manuscript (Cohen *et al.*, 1973); the total sequence of the molecule is presently being determined in collaboration with Drs. Neurath, Walsh, and Titani.

Acknowledgments

We acknowledge the excellent technical assistance of Mrs. Theresa Duewer and Mr. Richard Olsgaard. Frequent discussions with Drs. Hans Neurath, Kenneth Walsh, and Ralph Bradshaw were extremely helpful during the course of this work.

References

Appleman, M. M., Yunis, A. A., Krebs, E. G., and Fischer, E. H. (1963), *J. Biol. Chem. 238*, 1358.

Armstrong, M. D. (1949), *J. Amer. Chem. Soc. 71*, 3399.

Battell, M. L., Smillie, L. B., and Madsen, N. B. (1968a), *Can. J. Biochem. 46*, 609.

Battell, M. L., Zarkadas, C. G., Smillie, L. B., and Madsen, N. B. (1968b), *J. Biol. Chem. 243*, 6202.

Bencze, W. L., and Schmid, K. (1957), *Anal. Chem. 29*, 1193.

Bennett, J. C. (1967), *Methods Enzymol. 11*, 330.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Brown, J. L., Brown, D. M., and Zabin, I. (1967), *J. Biol. Chem. 242*, 4254.

Cohen, P., Duewer, T., and Fischer, E. H. (1971), *Biochemistry 10*, 2683.

Cohen, P., Saari, J. C., and Fischer, E. H. (1973), *Biochemistry 12*, 5233.

Damjanovich, S., and Kleppe, K. (1967), *Biochem. Biophys. Res. Commun. 26*, 65.

Davis, C. H., Schliselfeld, L. H., Wolf, D. P., Leavitt, C. A., and Krebs, E. G. (1967), *J. Biol. Chem. 242*, 4824.

DeLange, R. J., Kemp, R. G., Riley, W. D., Cooper, R. A., and Krebs, E. G. (1968), *J. Biol. Chem. 243*, 2200.

Fischer, E. H., and Krebs, E. G. (1958), *J. Biol. Chem. 231*, 65.

Fischer, E. H., Pocker, A., and Saari. J. C. (1970), *Essays Biochem. 6*, 23.

Forrey, A. W., Olsgaard, R. B., Nolan, C., and Fischer, E. H. (1971a), *Biochimie 53*, 269.

Forrey, A. W., Sevilla, C. L., Saari, J. C., and Fischer, E. H. (1971b), *Biochemistry 10*, 3132.

Fosset, M., Muir, L. W., Nielsen, L. D., and Fischer, E. H. (1971), *Biochemistry 10*, 4105.
Glynn, I. M., and Chappell, J. B. (1964), *Biochem. J. 90*, 147.
Gray, W. R. (1967), *Methods Enzymol. 11*, 139.
Gross, E. (1967), *Methods Enzymol. 11*, 238.
Gurd, F. R. N. (1967), *Methods Enzymol. 11*, 532.
Hedrick, J. L., Smith, A. J., and Bruening, G. E. (1969), *Biochemistry 8*, 4012.
Hirs, C. H. W. (1967), *Methods Enzymol. 11*, 199, 325.
Hofmann, T. (1964). *Biochemistry 3*, 356.
Holcomb, G. N., James, S. A., and Ward, D. N. (1968), *Biochemistry 7*, 1291.
Humbel, R. E., Derron, R., and Neumann, P. (1968), *Biochemistry 7*, 621.
Metzger, B., Helmreich, E., and Glaser, L. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 994.
Meyer, F., Heilmeyer, L. M. G., Haschke, R. H., and Fischer, E. H. (1970), *J. Biol. Chem. 245*, 6642.
Narita, K. (1970), *Mol. Biol. Biochem. Biophys. 8*, 70.
Nolan, C., Novoa, W. B., Krebs, E. G., and Fischer, E. H. (1964), *Biochemistry 3*, 542.
Raibaud, O., and Goldberg, M. E. (1973), *Biochemistry 12*, 5154.
Ryle, A. P., Sanger, F., Smith, L. F., and Kitai, R. (1955), *Biochem. J. 60*, 541.
Saari, J. C. (1970), Ph.D. Thesis, University of Washington, Seattle, Wash.
Schroeder, W. A. (1967a), *Methods Enzymol. 11*, 351.
Schroeder, W. A. (1967b), *Methods Enzymol. 11*, 361.
Schultz, J. (1967), *Methods Enzymol. 11*, 255.
Seery, V. L., Fischer, E. H., and Teller, D. C. (1970), *Biochemistry 9*, 3591.
Sevilla, C. L., and Fischer, E. H. (1969), *Biochemistry 8*, 2161.
Shapiro, A. L., Viñuela, E., and Maizel, J. V. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Stark, G. R. (1967), *Methods Enzymol. 11*, 125.
Stark, G. R., Stein, W. H., and Moore, S. (1960), *J. Biol. Chem. 235*, 3177.
Steers, E., Jr., Craven, G. R., and Anfinsen, C. B. (1965), *J. Biol. Chem. 240*, 2478.
Strausbauch, P. H., and Fischer, E. H. (1970), *Biochemistry 9*, 233.
Strausbauch, P. H., Kent, A. B., Hedrick, J. L., and Fischer, E. H. (1967), *Methods Enzymol. 11*, 671.
Susor, W. A., Kochman, M., and Rutter, W. J. (1969), *Science 165*, 1260.
Tang, J., and Hartley, B. S. (1967), *Biochem. J. 102*, 593.
Teller, D. C., Horbett, T. A., Richards, E. G., and Schachman, H. K. (1969), *Ann. N. Y. Acad. Sci. 164*, 66.
Valentine, R. C., and Chignell, D. A. (1968), *Nature (London) 218*, 953.
Vanaman, T. C., Wakil, S. J., and Hill, R. L. (1968), *J. Biol. Chem. 243*, 6409.
Wang, J. H., Shonka, M. L., and Graves, D. J. (1965), *Biochem. Biophys. Res. Commun. 18*, 131.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Wrigley, C. W. (1968a), *J. Chromatogr. 36*, 362.
Wrigley, C. W. (1968b), *Sci. Tools 15*, 17.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.
Yunis, A. A., Fischer, E. H., and Krebs, E. G. (1962), *J. Biol. Chem. 237*, 2809.
Zarkadas, C. G., Smillie, L. B., and Madsen, N. B. (1968), *J. Mol. Biol. 38*, 245.
Zarkadas, C. G., Smillie, L. B., and Madsen, N. B. (1970), *Can. J. Biochem. 48*, 763.