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Characterization of Two Complementary Polypeptide Chains Obtained by Proteolysis of Rabbit Muscle Phosphorylase†

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ABSTRACT: A limited proteolysis of rabbit muscle phosphorylase by subtilisin results in a nick in the protomer which yields two reasonably homogeneous complementary fragments; one, of mol wt 30,000, containing the phosphoserine residue corresponds to the NH_2-terminal third of phosphorylase; the other, of mol wt 70,000, containing the lysine residue which binds pyridoxal 5'-phosphate, corresponds to the COOH-terminal two-thirds of phosphorylase. Information about the tertiary and quaternary structure of phosphorylase derived from the kinetics of this proteolytic treatment is discussed.

Several structural studies of proteins based on their sensitivity to proteolytic enzymes have been made. Linderström-Lang (1952) has proposed two models to describe the degradation by proteases: (a) the "one by one" model—the first break produces an overall destabilization of the protein structure and is followed by a fast and extensive degradation of the polypeptide chain; (b) the "zipper" model—the first cleavage in the protein is much faster than the other breaks.

Rabbit muscle phosphorylase *a* seems to be degraded by various proteases according to the zipper-type mechanism (Nolan *et al.*, 1964). From their observations, it can be concluded that one, or very few, limited parts of the protein backbone are sensitive to proteolysis and that the fragments thus formed are degraded very slowly. We were interested in further investigating the specific sensitivity of phosphorylase to limited proteolytic degradation to see if such an approach would provide information about the structure of this enzyme. We were mainly interested in the phosphorylase *b* to phosphorylase *a* conversion. The two forms of the enzyme were submitted to a mild proteolytic treatment and the degradation products examined. Subtilisin was chosen as the proteolytic enzyme for two reasons. First, it is a rather nonspecific protease (Harris and Roos, 1959) and the susceptible bonds depend more on their exposure than on the neighboring residues. Second, Graves *et al.* (1968) have extensively studied phosphorylase *b'*, a protein obtained by mild proteolytic treatment of phosphorylase *a* by trypsin, and it seemed interesting to compare the results obtained with trypsin and subtilisin.

Materials and Methods

Activity and Concentration of Phosphorylase. Phosphorylases *a* and *b* were assayed according to Helmreich and Cori (1966). Enzyme concentration was determined spectrophotometrically; the extinction coefficient at 280 nm used was $E_{1\,cm}^{1\%}$ 13.2 (Buc and Buc, 1968).

Phosphorylases. Rabbit muscle phosphorylase *b* was purified according to Fischer *et al.* (1958). Phosphorylase *a* labeled with ^{32}P was obtained by phosphorylation of phosphorylase *b* according to Krebs *et al.* (1964). Reduced phosphorylase *b* was prepared by reduction of the Schiff base between pyridoxal-P and the enzyme according to Strausbauch *et al.* (1967), except for the buffer which was replaced by 0.05 M Tris-acetate (pH 8.5). (All pH measurements were performed at room temperature.)

Carboxymethylation was performed in 0.1 M Tris-acetate (pH 8.1) and 6 M guanidine·HCl at room temperature. Phosphorylase *b* (*ca.* 10 mg/ml) was first denatured by incubation in this buffer for 20 min in the presence of dithiothreitol (2 mol/mol of cysteine residue). [^{14}C]Iodoacetic acid was then

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added (2–2.5 mol/mol of SH). The solution was then incubated for 30 min; mercaptoethanol was added to 1 M and the solution was dialyzed in the dark against 1 M formic acid with several buffer changes.

Polyacrylamide Gels. Gels in urea at pH 4.5 were prepared according to Reisfeld *et al.* (1962) except that 8 M urea was included in the 10% acrylamide gels as well as in the sample. Gels in sodium dodecyl sulfate were prepared according to Shapiro *et al.* (1967) and Weber and Osborn (1969). Staining was performed immediately after the run for 3 hr in 7.5% acetic acid containing 0.1% Coomassie Brilliant Blue. Gels were destained in 7.5% acetic acid. Sodium dodecyl sulfate was purchased from Serlabo, Paris; destaining problems were encountered when sodium dodecyl sulfate from another source was used.

For radioactivity measurements the gels were first cut into 1-mm thick slices which were individually dissolved by heating for a few hours at 70° in 0.5 ml of hydrogen peroxide (Saari, 1972). The dissolved gels were counted in scintillation vials containing 10 ml of Bray's solution using an Intertechnique SL 30 scintillation counter.

Subtilisin was purchased from The British Drug House. A stock solution containing 10 mg/ml of subtilisin in 0.05 M Tris-acetate (pH 8.5) was kept frozen until needed. Protease activity was assayed by a method modified from Kunitz (1947); 0.5 ml of the subtilisin solution in 0.05 M Tris-acetate (pH 8.5) was added to 0.5 ml of a 10 mg/ml casein (Difco) solution in the same buffer. The mixture was incubated for 30 min at 37°; 1.5 ml of 5% CCl_3COOH was then added. The precipitate was eliminated and the optical density at 275 nm of the supernatant was measured. A standard curve for activity was obtained by performing the assay with various amounts of subtilisin and plotting the optical density of the supernatant *vs.* enzyme concentration. The curve obtained is not a straight line but is quite smooth and highly reproducible. The stock solution, when diluted 4000-fold, gave an optical density of 0.450 under these conditions.

Proteolytic action was stopped by phenylmethylsulfonyl fluoride (stored as a 10^{-1} M solution in dioxane) as suggested by Barrel and Glazer (1968). This treatment is essential before performing the sodium dodecyl sulfate gels since this detergent does not inactivate subtilisin (Gounaris and Ottesen, 1965) but denatures phosphorylase *b*, thus permitting further proteolysis.

Insoluble Subtilisin. Subtilisin was insolubilized by covalent bonding on Sepharose 4B (Pharmacia) activated with cyanogen bromide as described by Cuatrecasas *et al.* (1968). The buffer used during fixation was 0.1 M potassium phosphate (pH 7.8). After fixation the gel was thoroughly washed with 0.05 M Tris-acetate (pH 8.5) containing 1 M potassium chloride, rinsed with 0.05 M Tris-acetate (pH 8.5), and stored at 4° in the same buffer. The gel was washed again just before each use to eliminate subtilisin molecules which were released. Activity of the insolubilized preparation was measured as described above, but with continuous shaking of the suspension.

Gel Filtration in 6 M Guanidine·HCl. The method of Fish *et al.* (1969) was followed, using Sepharose 4B (Pharmacia). Guanidine·HCl was purchased from Carlo Erba.

Analytical centrifugation was performed with a Centriscan centrifuge (Measuring and Scientific Equipment); 2-cm thick single-sector cells were used. Sedimentation was followed with the schlieren-type scanning system. Sedimentation speed was 55,000 ± 20 rpm. Protein concentration was 3 mg/ml. The temperature was 20 ± 0.1°.

Tryptic Peptides. [^{14}C]Carboxymethylated protein was digested with 2% (weight by weight of phosphorylase) bovine pancreatic trypsin (Worthington) in 0.1 M ammonium bicarbonate (pH 8.0) at 37° for 4 hr. Peptides were chromatographed on Whatman 3MM paper using the solvent described by Brown and Hartley (1966): 1-butanol–acetic acid–water–pyridine (30:10:6:24, v/v). Radioactive peptides were revealed by autoradiography. Tryptophan containing peptides were colored according to Easley (1965).

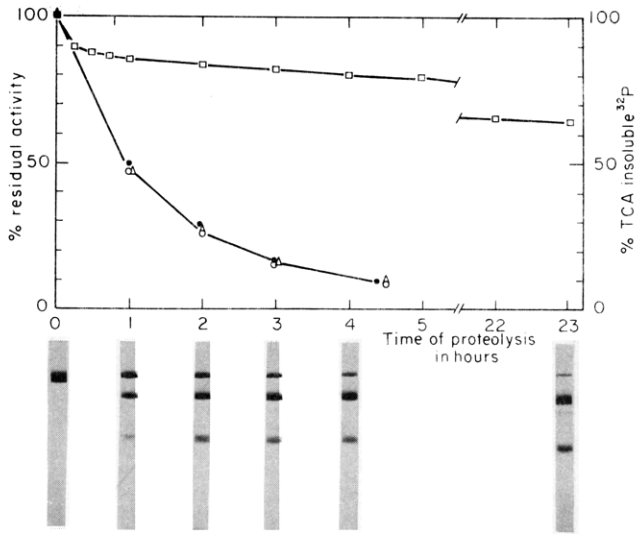

FIGURE 1: Kinetics of proteolysis. Crystals of [^{32}P]phosphorylase *a* and phosphorylase *b* were centrifuged. The pellet was dissolved and dialyzed overnight at 4° in 0.05 M Tris-acetate (pH 8.5)–0.01 M β-mercaptoethanol. The proteins were diluted to 3 mg/ml; phosphorylase *b* was made 10^{-3} M in AMP. Proteolysis was performed at 4° with 4 μg/ml of subtilisin. At time indicated in abscissa, 2 μl of the stock solution of phenylmethylsulfonyl fluoride was added to a 200-μl aliquot of treated phosphorylase. The aliquot was incubated for 15 min at 4°; it was then assayed for activity and *ca.* 20 μg of protein was analyzed on sodium dodecyl sulfate gels (see Methods). Phosphorylase *a* was precipitated with trichloroacetic acid (5% final concentration) and the radioactivity of the supernatant was measured; quenching by trichloroacetic acid was negligible; (●) per cent residual activity of phosphorylase *b*; (○) per cent residual activity of phosphorylase *a* assayed without AMP; (△) per cent residual activity of phosphorylase *a* assayed with AMP; (□) per cent radioactivity in the precipitate.

Amino Acid Composition. The protein sample was extensively dialyzed against 1 M formic acid, lyophilized, then hydrolyzed (in tubes sealed under vacuum) with 6 M HCl at 110° for 24 hr. Analysis of the amino acids was performed on a JLC-5AH automatic amino analyzer.

Results

Kinetics of Proteolysis. The proteolysis of phosphorylase by subtilisin has been followed by the loss of phosphorylase activity and by the molecular weight distribution of the polypeptides observed on sodium dodecyl sulfate gels. The results are shown in Figure 1. It can be seen that the kinetics of inactivation of phosphorylase *a* and *b* are identical and that the loss of activity quantitatively corresponds to the loss of material migrating like intact protomers. Moreover, two more rapidly migrating bands appear on sodium dodecyl sulfate gels; the larger band will be called "fragment I" and the smaller "fragment II." Their molecular weights as determined

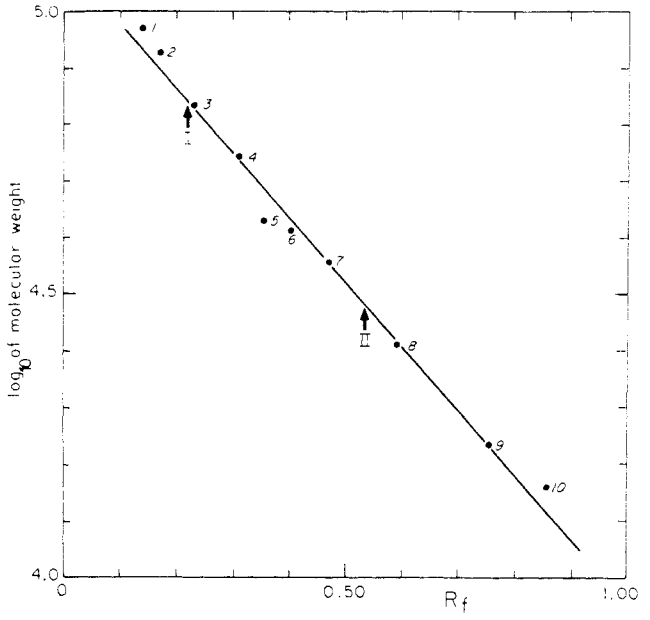

FIGURE 2: Molecular weight of the fragments. Polyacrylamide gels (10%) in sodium dodecyl sulfate were used as described under Methods. The marker proteins were: (1) phosphorylase *b*; (2) aspartokinase; (3) bovine serum albumin; (4) tryptophanase; (5) ovalbumin; (6) alcohol dehydrogenase; (7) lactate dehydrogenase; (8) chymotrypsinogen; (9) myoglobin; (10) lysozyme. Migration is compared to that of Bromophenol Blue.

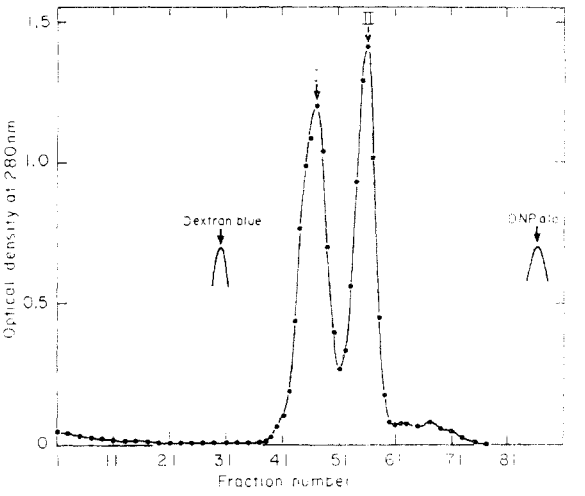

FIGURE 3: Separation of the fragments. The separation was performed as described in the text. Fractions 46, 47, and 48 were mixed to give pool I; fractions 55, 56, and 57 were mixed to give pool II (see Plate 1).

by sodium dodecyl sulfate polyacrylamide gels (Figure 2) were 70,000 for fragment I and 30,000 for fragment II. The release of ^{32}P from phosphorylase *a* during proteolysis is shown in Figure 1. It can be seen that the percentage of release is fairly small and therefore that degradation by subtilisin differs considerably from the degradation by trypsin as reported by Nolan *et al.* (1964).

After 24-hr proteolysis one obtains essentially two large polypeptide chains, fragments I and II. That these fragments remain tightly associated before denaturation has been shown by sedimentation velocity measurements. Native phosphorylase *b* sedimented in 0.05 M Tris-acetate (pH 8.5)–10^{-3} M AMP with an $s_{20,w}$ of 8.4 S. The species obtained after 24-hr proteolytic treatment, when centrifuged in the same buffer, showed two boundaries. One, containing 75% of the material, looked homogeneous and had an $s_{20,w}$ of 8.3 S, corresponding to the native dimer while the other, containing 25% of the material, had an $s_{20,w}$ of 4.5 S, corresponding to the monomer. It can be concluded therefore that the break in the polypeptide chain does not considerably modify the tertiary and quaternary structures of the protein; the fragments remain associated in a structure somehow similar to the native enzyme. Nevertheless, the structure is not identical since a tendency toward monomerization has been observed and the activity is lost. Moreover, while phosphorylase *b* is soluble at pH 7.0, the treated protein aggregates and slowly precipitates at this pH.

The results obtained suggested that the phosphorylase protomers were cleaved by subtilisin into two fragments by a specific cut in the polypeptide chain. It therefore appeared of interest to isolate and characterize these fragments.

Separation of the Fragments. After attempts to dissociate the fragments by mild denaturing treatments proved totally unsuccessful, ionic detergents or 6 M guanidine·HCl were used. However, under such conditions, the phenylmethylsulfonyl fluoride treatment of subtilisin was not expected to be totally efficient since it has been shown that in denaturing media the blocking effect of phenylmethylsulfonyl fluoride can be reversed (Gold and Fahrney, 1964). For complete blocking of the proteolytic enzyme, two procedures can be followed: irreversible inactivation of subtilisin at pH 2 as described by Adkins and Foster (1966) or use of insoluble subtilisin. The latter method was selected. Phosphorylase *b* was dialyzed overnight at 4° against 0.05 M Tris-acetate (pH 8.5) and 10^{-2} M β-mercaptoethanol, then diluted to 20 mg/ml, and supplemented with 10^{-3} M AMP. Sepharose-bound subtilisin was added to a final concentration equivalent to 20 μg/ml of soluble subtilisin. The mixture was gently shaken at 4° for 24 hr and filtered on Whatman GF/B glass paper to remove the insoluble protease. Phenylmethylsulfonyl fluoride was then added to the filtrate in order to block the subtilisin which might have been released from the gel. The proteins contained in the filtrate were carboxymethylated (see Methods), dialyzed against 1 M formic acid, lyophilized, and redissolved in 6 M guanidine·HCl at pH 6.5. The preparation was stored at −25°; 1 ml of this solution (*ca.* 20 mg/ml of protein) was injected by density onto the Sepharose column (90 × 1.5 cm) previously equilibrated with guanidine·HCl. Fractions (2 ml) were collected and the optical density at 280 nm was monitored. Figure 3[1] shows the elution pattern obtained. Fractions containing each fragment were pooled as indicated in Figure 3 and the pools were submitted to electrophoresis on sodium dodecyl sulfate gels (Plate 1). It can be seen that the fragments have been satisfactorily separated on the column.

Amino Acid Analysis of the Two Fragments. From the kinetics of appearance of the two fragments (Figure 1) and from their molecular weights, it could be inferred that fragments I and II are complementary. If this were true, one would expect to obtain after separation on Sepharose twice as much material (by weight) in peak I as in peak II. A quantitative amino acid analysis of the two pools was performed to test this hypothesis.

The method of Edelhoch (1967) allows one to determine, from the optical densities at 280 and 288 nm, the tryptophan content of pools I and II. The amino acid composition of each

[1] Abbreviation used is: Dnp-Ala, dinitrophenylalanine.

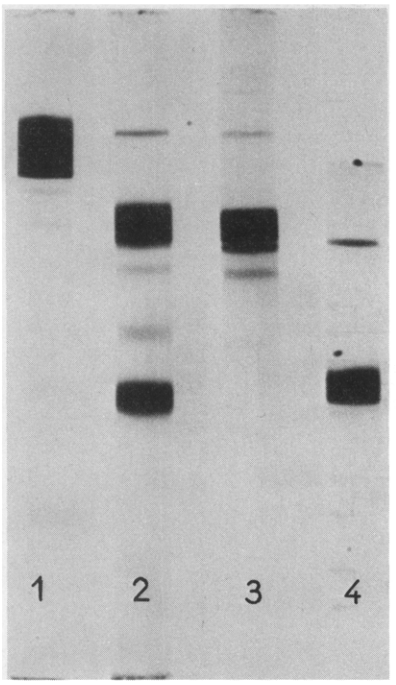

PLATE 1: Homogeneity of the isolated fragments. The protein before proteolysis (1), after proteolysis or 24 hr (2), and pool I (3) were analyzed on 7.5% polyacrylamide gels in sodium dodecyl sulfate. Pool II (4) was analyzed on a 10% polyacrylamide gel in sodium dodecyl sulfate.

fragment was determined as described under Methods and the results are shown in Table I.

Comparison of these results with the composition of the intact enzyme described by Sevilla and Fischer (1969) further supports the assumption that the fragments are complementary. The extinction coefficients at 280 nm were determined in 6 M guanidine·HCl (pH 6.5). They were found to be $A^{1\%}_{1\,cm} = 9.5$ for fragment I and $A^{1\%}_{1\,cm} = 17.3$ for fragment II. This allowed us to estimate that peak I contained 16.3 mg of protein while peak II contained 8.1 mg. It was assumed that the two fragments were completely separated and that no cross-contamination occurred. It should be noted that fragment I is comparatively rich in polar residues (lysine, arginine, glutamate, aspartate) while fragment II is rich in nonpolar residues (tryptophan, tyrosine, glycine).

Cyanogen Bromide Peptides of the Fragments. The results obtained up to this point strongly supported the idea that both fragments were complementary but conclusive evidence was lacking. Therefore chemical studies were undertaken. Since the NH_2- and COOH-terminal residues of phosphorylase cannot be detected by conventional methods (Sevilla and Fischer, 1969), no attempt was made to use these residues as markers. On the other hand, cyanogen bromide peptides have been extensively studied by Saari and Fischer (1973) who showed that peptide CB13 is the COOH-terminal and CB14 is the NH_2-terminal peptide of phosphorylase (Fischer *et al.*, 1972). These peptides can be identified on urea polyacrylamide gels.

Pools I and II (see Figure 3) were dialyzed against 10% formic acid, lyophilized, dissolved in 70% formic acid, and submitted to cleavage by cyanogen bromide as described by Saari (1972) and Saari and Fischer (1973). The resulting peptides were analyzed on urea gels (Plate 2). The nomenclature of the peptides is that of Saari and Fischer (1973).

Fragment II yields two peptides which are visible on the gel. Starting from the top the first peptide, which we call D1, is a doublet which is absent in fragment I and might be one of the

TABLE I: Amino Acid Composition of Phosphorylase *b* Fragments.

Amino Acid Residue	Calcd No. of Residues/Fragment I (Mol Wt 70,000)	Calcd No. of Residues/Fragment II (Mol Wt 30,000)	Calcd No. of Residues for Stoichiometric Mix. of Fragments I + II (Mol Wt 100,000)	Rabbit Muscle Phosphorylase[a]
Lysine	36.8	11.0	47.8	47.0
Histidine	16.4	6.8	23.2	20.9
Arginine	45.0	14.3	59.3	63.8
Aspartic acid	71.8	31.1	102.9	98.1
Threonine	24.2	14.2	38.4	34.1
Serine	24.6	5.4	30.0	29.5
Glutamic acid	76.2	30.3	106.3	102.0
Proline	28.3	15.6	43.9	42.5
Glycine	31.1	23.2	54.3	49.6
Alanine	47.8	20.6	68.4	65.2
Valine	37.0	14.0	51.0	61.3
Methionine	16.7	8.7	25.4	21.8
Isoleucine	28.5	6.8	35.3	40.5
Leucine	54.1	24.6	78.7	81.4
Tyrosine	20.8	14.6	35.4	36.6
Phenylalanine	27.2	10.7	37.9	38.5
Cysteine	8.3	3.0	11.3	8.5
Tryptophan	6.6	5.5	12.1	12.8

[a] The amino acid composition of phosphorylase *b* is that given by Sevilla and Fischer (1969) normalized for 100,000 g of phosphorylase *b*.

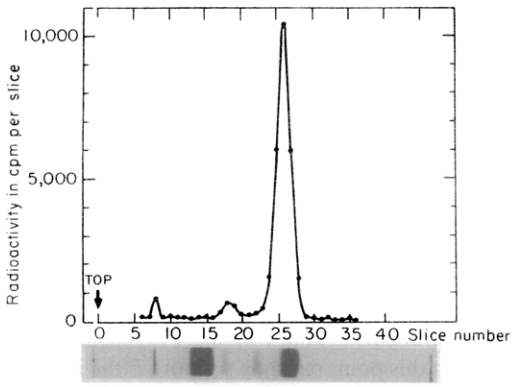

FIGURE 4: Localization of the phosphoserine residue. [^{32}P]Phosphorylase (100 μg) was proteolyzed for 24 hr as described in Figure 1 and layered on a sodium dodecyl sulfate gel. After migration, the radioactivity distribution was analyzed as described under Methods.

peptides of the fraction D described by Saari and Fischer (1973). This peptide has not been purified at the present time. Below it comes a triplet which looks like CB14 (a doublet plus a faint band just above), but which migrates a little further. This is to be expected if fragment II were on the NH_2-terminal side of phosphorylase and were slightly and homogeneously degraded by subtilisin at its NH_2 terminus.

With fragment I we can locate with little ambiguity peptides CB15, CB18, and a third one we call D2 (CB20 of Saari, 1972). A new peptide appears while CB17 disappears. Finally a peptide similar to the COOH-terminal peptide of phosphorylase (CB13) can be seen. It thus appears quite likely that fragment I is derived from the COOH-terminal part of phosphorylase while fragment II is derived from the NH_2-terminal part.

Localization of the Serine Phosphate. Recent results (Fischer *et al.*, 1972) have shown that the seryl phosphate residue of phosphorylase *a* is located some 14 residues away from the NH_2 terminal; moreover only 30% of the peptides carrying the serine phosphate obtained after 24-hr proteolysis become trichloroacetic acid soluble (see Figure 1). Thus it appears likely that one of the fragments should still carry the serine phosphate. ^{32}P-Labeled phosphorylase *a* was submitted to proteolysis and the resulting fragments were analyzed on sodium dodecyl sulfate gels. The distribution of radioactivity was examined as described under Methods. The results are shown in Figure 4. Nearly all the label migrates with fragment II and only a small fraction of the radioactivity appears to be associated with a faint contaminating band.

This result confirms the location of fragment II at the NH_2-terminal side of the phosphorylase protomer. However, it seems to contradict the results obtained with cyanogen bromide peptides. If the serine phosphate, which is located very near the NH_2 terminus, is still present on fragment II, there should be practically no degradation of the NH_2-terminal extremity by subtilisin. Yet the CNBr peptide distribution on urea gels indicates that peptide CB14 (the NH_2 terminus) has been extensively degraded. A possible explanation for this apparent contradiction may be that the serine phosphate location was determined with phosphorylase *a* while the cyanogen bromide peptides were obtained from subtilisin treated phosphorylase *b*. In spite of the fact that the kinetics of inactivation of the two kinds of phosphorylases are identical (see Figure 1), the susceptibility to proteolytic cleavage of the NH_2-terminal extremity of the two species might be different.

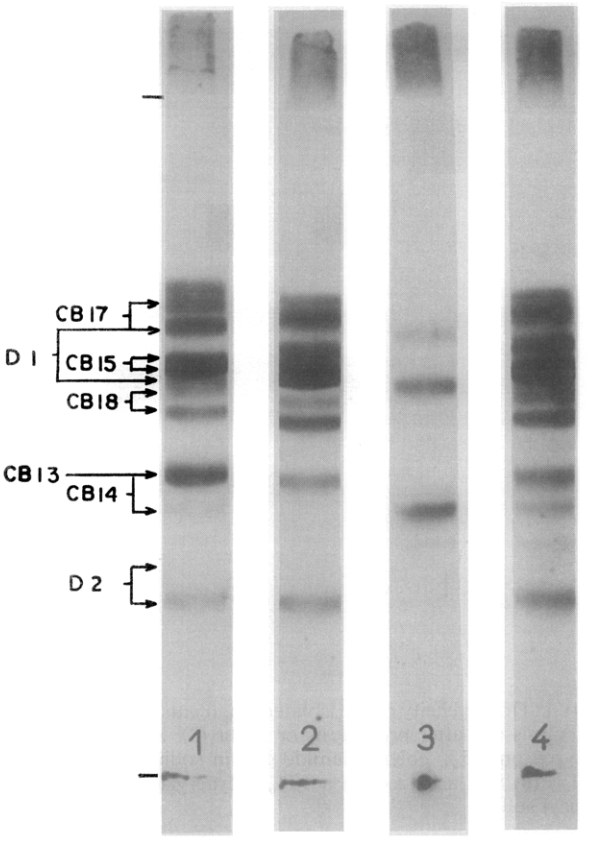

PLATE 2: Urea gels of the cyanogen bromide peptides: (1) 20 μg of peptides obtained from intact phosphorylase *b*; (2) 20 μg of peptides obtained from pool I; (3) 20 μg of peptides obtained from pool II; (4) 20 μg of peptides obtained from pool I and 10 μg of peptides obtained from pool II.

This latter possibility was tested by submitting both kinds of phosphorylases to proteolytic cleavage for 24 hr as described in Figure 1, and analyzing the CNBr peptides of the resulting fragments on urea gels. For phosphorylase *b*, the results were similar to those shown on Plate 2 for the mixture of fragments I and II. In the case of phosphorylase *a*, the pattern is identical except for the disappearance of the peptide which in proteolyzed phosphorylase *b* migrated further than CB14. In its place a peptide which migrates at the same position as authentic CB14 was observed. This observation confirms that the proteolytic treatment produces the same break on the two kinds of phosphorylases, except that the NH_2-terminal extremity is probably protected from degradation in phosphorylase *a*.

Tryptic Peptides of the Fragments. Another simple method to study the complementarity of the fragments was to locate some tryptic peptides of the intact protomer in one or the other of fragments I and II.

After separation of the fragments the tryptic peptides were obtained and analyzed by paper chromatography as described under Methods. Plate 3 shows the location of the peptides containing [^{14}C]carboxymethylated cysteine or tryptophan as well as the most distinct ninhydrin-positive peptides. The complementarity of the fragments is again confirmed.

Location of the Pyridoxal-5'-P Binding Site. In order to further characterize the fragments it was of interest to locate other ligand binding sites. However, since the fragments can be separated only after denaturation, only covalent irreversible binding sites could be examined. Two such sites exist on phosphorylase: the phosphorylation site studied above and the site

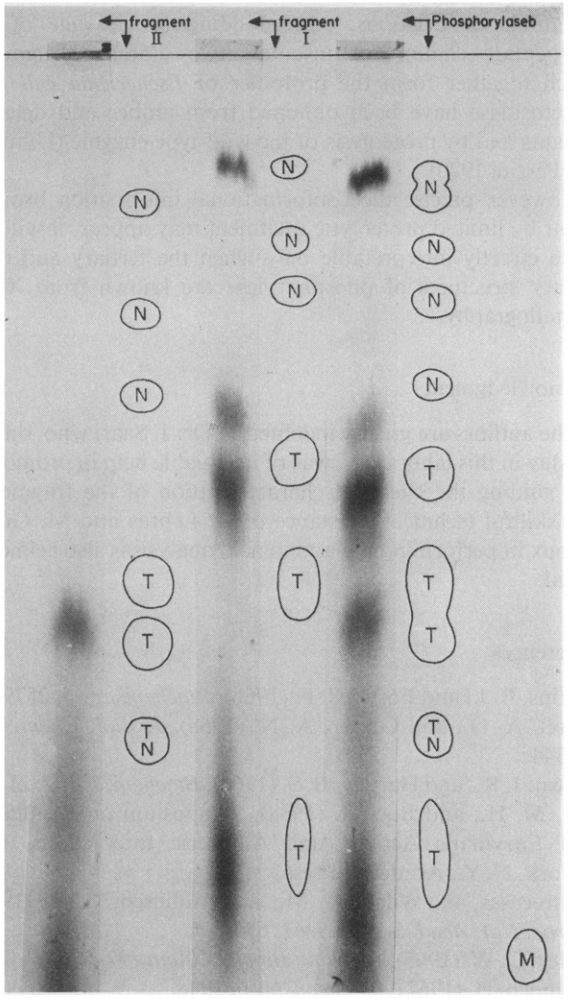

PLATE 3: Tryptic peptides of the fragments. The tryptic peptides were obtained and analyzed by chromatography as described in the text: T, tryptophan-containing peptides; N, ninhydrin-stained peptides; M, migration marker Phenol Red; dark spot, ^{14}C-acetylated cysteine-containing peptides as revealed by autoradiography.

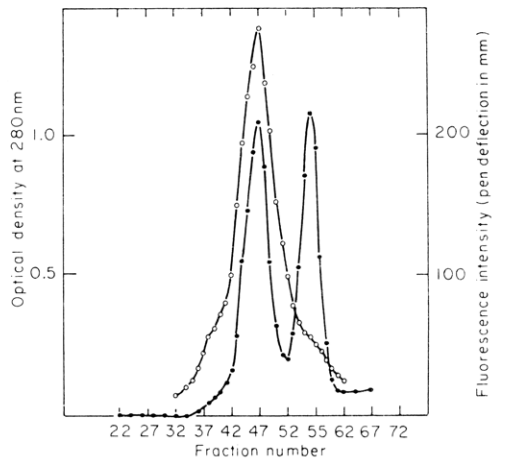

FIGURE 5: Localization of the phosphopyridoxyllysine residue. The fragments were separated on a Sepharose column and the fluorescence of the reduced Schiff base was monitored as described in the text: (○) fluorescence intensity ($\lambda_{excitation}$ = 335 nm; $\lambda_{emission}$ = 405 nm); (●) optical density at 280 nm.

for Schiff base formation between pyridoxal-P and the protein. The lysine implicated in this Schiff base was located after phosphorylase *b* was submitted to subtilisin treatment for 24 hr as described above. Spectral examination confirmed that pyridoxal-P was still bound on the proteolyzed protein. Borohydride reduction was then performed as described under Methods and the fragments were separated on Sepharose in 6 M guanidine·HCl. Elution was monitored by measuring optical density at 280 nm (for detecting the fragments) and fluorescence (excitation at 355 nm; emission at 405 nm) of the reduced Schiff base. Figure 5 shows that the lysine which binds pyridoxal-P is located on fragment I.

NH_2-Terminal Residues of the Fragments. The fragments obtained from phosphorylase *b* were dansylated as described under Methods. Fragment I yielded three main residues: alanine, leucine, and some isoleucine; fragment II yielded glycine and a small amount of aspartic acid These results, though qualitative, indicate a reasonable degree of homogeneity of the two fragments.

Discussion

The observations reported herein lead to the conclusion that the proteolytic treatment described results in the cleavage of rabbit muscle phosphorylase in two complementary polypeptide chains of mol wt 70,000 and 30,000 respectively. That these peptides are complementary can be shown by discriminating among the three following possibilities which could account for the production of two fragments with these molecular weights. (a) Fragment II is a degradation product of fragment I. Localization of the serine phosphate exclusively on fragment II and recovery of tryptic and CNBr peptides from fragment II and the intact protein, but not from fragment I, exclude this assumption. (b) Fragments I and II originate from different protomers of phosphorylase—some being degraded from the COOH-terminal end to yield fragment II, others being degraded from the NH_2 terminus to yield fragment I. With such a mechanism, the recovery of material in the fragments should be less than 50% whereas the recovery was always more than 60%. (c) Fragments I and II are complementary. All the results reported in the present paper are compatible with this model. The sum of the molecular weights of the fragments is equal to the molecular weight of the intact protomer. The combined amino acid composition of the fragments compares favorably with that of the intact enzyme (Table I). The weight ratio of the material contained in the two peaks during separation of the fragments on the Sepharose column is 2:1 as expected (Figure 3). The tryptic peptides containing cysteine and tryptophan (Plate 3) and the cyanogen bromide peptides (Plate 2) are complementary. Fragment I is on the COOH-terminal side (it contains CB13) while fragment II is on the NH_2-terminal side (it contains CB14 and the serine phosphate).

The two fragments have been characterized in the following way. After isolation, they have been shown to be fairly homogeneous; on sodium dodecyl sulfate gels, at either high or low protein concentration, the band obtained for each fragment is unique and as thin as that obtained for a pure protein. NH_2-Terminal residues were not too numerous (2 for fragment II, 3 for fragment I). Moreover the cyanogen bromide peptides obtained indicate that essentially no degradation of the COOH-terminal of fragment I occurs (CB13 is not affected) whereas some degradation by cleavage of approximately the same number of residues occurs at the NH_2-terminal end of fragment I. This degradation appears extremely limited with phosphorylase *a*. Finally the cleavage between the two fragments seems to occur in peptide CB17, the single peptide which does not migrate identically in the fragment mixture

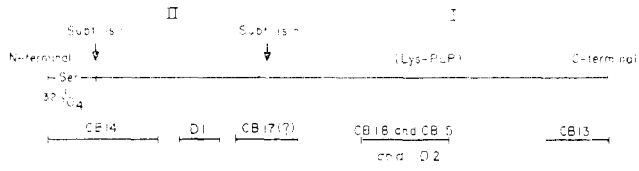

FIGURE 6: Recapitulative diagram.

and in the original protein (Plate 2). It thus can be concluded that the cleavage affects only one or very few neighboring bonds located in CB17 since the band derived from CB17 remains very thin on urea gels.

On these two complementary and relatively homogeneous fragments several "markers" surrounded by a known sequence of amino acids have been located: phosphopyridoxyllysine (Fischer *et al.*, 1970; Forrey *et al.*, 1971), serine phosphate (Nolan *et al.*, 1964), and cysteine (Zarkadas *et al.*, 1968, 1970; Gold and Blockman, 1970).

Figure 6 summarizes some of the results which may be of interest for establishing the primary structure of phosphorylase.

Another type of information can be obtained from these results, which deals with the tertiary and quaternary structures of the enzyme. It has been shown that a region of the polypeptide chain about one-third of the distance from the NH_2 terminal is highly sensitive to proteolysis. Therefore this region must be on the surface of the molecule. The kinetics of inactivation suggest that a break in this segment causes complete loss of activity while neither the tertiary nor the quaternary structures appear to undergo a dramatic modification. The cleavage takes place at the same point and with the same kinetics in phosphorylases *a* and *b*. The sensitive segment must therefore be in a region of the molecule which is not affected by the change in conformation promoted by phosphorylation of phosphorylase *b*. Thus it is particularly unlikely that this region is located in the association areas between the dimers. On the contrary, phosphorylase *b*, which is dimeric under the conditions of proteolysis, is degraded at its NH_2 terminal, while phosphorylase *a*, which is tetrameric (Wang and Graves, 1963, 1964), is not degraded at this end. This finding supports the assumption that the NH_2 terminal of phosphorylase is involved in the association of dimers into tetramers (Graves *et al.*, 1968).

The results we have obtained show that mild proteolytic treatment of phosphorylase is a valuable tool for investigating protein structure and will probably be useful in establishing its amino acid sequence. It should be pointed out that in the method used the specificity of the proteolytic cleavage depends essentially on the conformation of the protein substrate and not on the specificity of the protease. From a more general point of view, the nature of the information given by such limited proteolysis is comparable to that given by specific chemical modifications of amino acid side chains of proteins. For example, the importance of the integrity of a given peptide bond for maintaining the activity and structure of an enzyme, the localization of such a bond within the protein or on its surface, or in the association areas can be demonstrated by the two methods. The same restrictions must also be taken into account for both. What are the specificity and the number of modifications performed? Is the effect associated with the modification a direct or an indirect one? These two types of modifications closely resemble two classical types of genetic modifications of proteins: missense mutations corresponding to the chemical modifications of side chains and nonsense mutations or deletions, corresponding to cleavage of the polypeptide chain. For instance, two globular fragments which together form the protomer of *Escherichia coli* β-D-galactosidase have been obtained from amber and deletion mutants and by proteolysis of the wild-type enzyme (Ullmann and Perrin, 1970).

However precise the conformational information brought about by limited proteolytic treatment may appear, it will become directly interpretable only when the tertiary and quaternary structures of phosphorylase are known from X-ray crystallography.

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Deuterium Isotope Effects and Chemically Modified Coenzymes as Mechanism Probes of Yeast Glyoxalase-I†

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ABSTRACT: The previously reported observation that the rates of disproportionation of the hemimercaptals of glutathione and substituted phenylglyoxals, catalyzed by yeast glyoxalase-I, are insensitive to substituents raised the question of whether or not the intramolecular hydride migration step is rate determining. This question has been investigated using deuterated α-ketoaldehydes. The disproportionation of methylglyoxal and perdeuteriomethylglyoxal shows an isotope effect on V_{max} ($V_{max,H}/V_{max,D} = 2.9$). This is comparable to the isotope effect observed in the hydroxide ion catalyzed disproportionation of methylglyoxal and perdeuteriomethylglyoxal, $k_H/k_D = 3.8$. Likewise, the glyoxalase-I-catalyzed disproportionation of phenylglyoxal and α-deuteriophenylglyoxal shows an isotope effect ($V_{max,H}/V_{max,D} = 3.2$) comparable to the hydroxide reaction ($k_H/k_D = 5.0$), leading to the conclusion that hydride migration is the rate-determining step for the glyoxalase-I reaction. For both pairs of α-ketoaldehydes, this isotope effect is also reflected in K_M, suggesting that the catalytic rate constant (k_3) is larger than the rate constant for dissociation of the enzyme–substrate complex (k_2) and that $K_M \simeq k_3/k_1$. Using purified preparations of glyoxalase-I, k_1 and k_3 were determined. The coenzyme role of glutathione in the glyoxalase-I reaction was evaluated using pH–rate profiles and chemically modified coenzymes. In the pH range 4.5–9, V_{max} shows no pH sensitivity; K_M values, however, increase at high and low pH suggesting that dissociable groups of pK values of about 5 and 8.5 are involved in binding the substrate to the enzyme. These apparent pK values are sensitive to the apolar character of the α-ketoaldehydes. V_{max} values are not affected if *N*-acetylglutathione is used in place of glutathione. K_M values, however, increase. Methylation of the glycyl residue of glutathione prevents binding of the hemimercaptals to the enzyme. Thus, the dissociable groups on glutathione appear to be involved primarily in enzyme–substrate formation rather than in the catalytic reaction.

The glyoxalase system (Scheme I) which catalyzes the disproportionation of α-ketoaldehydes into the corresponding α-hydroxycarboxylic acids has been known for many years, although its biological role remains unclear. Several aspects of the mechanism of glyoxalase-I (*S*-lactoylglutathione methylglyoxal-lyase (isomerizing), EC4.4.1.5) which catalyzes the actual disproportionation reaction have been of interest, including the fact that the substrate for glyoxalase-I is a hemimercaptal formed in the preenzymic reaction between α-ketoaldehyde and coenzyme glutathione (Cliffe and Waley, 1961). Recent suggestions that the kinetic data may equally well be interpreted as indicating an ordered reaction requiring glutathione to bind before α-ketoaldehyde binds to the active site have been made (Bartfai *et al.*, 1973), but considerable other data are available which support Cliffe and Waley's original suggestion of Scheme I[1] (Davis and Williams, 1969; Vander Jagt *et al.*, 1972a). In our earlier studies on the substrate specificity of yeast glyoxalase-I, we reported that the reaction shows extremely broad specificity for the hemimercaptals of both aliphatic and aromatic α-ketoaldehydes and reported that V_{max} values are quite insensitive to variations in the α-ketoaldehyde (Vander Jagt *et al.*, 1972a). In particular, a series of substituted phenylglyoxals was examined for substituent effects on V_{max}. It was anticipated that the glyoxalase-I reaction, known to involve intramolecular hydride migration (Franzen, 1956; Rose, 1957), might be sensitive to the polarity of the α-ketone group and that the polarity of this group would be variable for a series of substituted phenylglyoxals. This was supported by studies of the disproportionation of

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