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Peptide Sequences and Relative Reactivity of the Reactive Sulfhydryl Groups of Rabbit Muscle Phosphorylase*

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ABSTRACT: Rabbit muscle phosphorylase *b* contains four cysteine residues which are exceptionally reactive with 2,4-dinitrochlorobenzene. These have been characterized by preparing phosphorylase *b* with an average of three groups substituted as *S*-[^{14}C]DNP-cysteine, digesting with trypsin, and isolating two unique radioactive peptides. The following sequences were found: peptide A, Ile-DNP-Cys-Gly-Gly-Trp-Gln-Met-Glu-Glu-Ala-Asp-Asp-Trp-Leu-Arg; peptide B, Phe-Gly-DNP-Cys-Arg-Asp-Pro-Val-Arg. Peptide B does not overlap with the sequence Lys-Phe, which is one of the points of attachment of pyridoxal phosphate in native phosphorylase. Phosphorylase *a* also yields these two peptides. The reactive sulfhydryl groups were found to react with 2,4-dinitrochlorobenzene at nearly equal rates in both phosphorylases *a* and *b;* AMP, however, selectively protects the sulfhydryl group giving rise to peptide B in both forms. It has been found possible to maintain the reactive sulfhydryl groups of phosphorylase *b* in the fully reduced state by frequently recrystallizing the protein in the presence of dithiothreitol. Otherwise, the number of reactive sulfhydryl groups is less than four, even in freshly prepared enzyme, and gradually decreases over a period of weeks.

Dinitrophenylating agents, such as FDNB[1] and CDNB, appear to react rapidly with four sulfhydryl groups of phosphorylase *b* (α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) from rabbit muscle at pH 8.0 (Gold, 1968). The precise number of reactive groups observed varies with different preparations of enzyme up to a maximum of 3.6, but is significantly lower in many cases. Since the enzyme is a dimer of apparently identical subunits of 92,500 molecular weight, it is probable that each subunit contains two especially reactive cysteine residues out of a total of nine cysteine residues (Zarkadas *et al.*, 1968). Dinitrophenylation of the four reactive sulfhydryl groups results in an increase in the kinetic constants of the enzyme without greatly changing the maximum velocity or causing dissociation of subunits. The remaining sulfhydryl groups react slowly with FDNB and CDNB.

Kleppe and Damjanovich (1969) have reported that phosphorylase *b* contains two sulfhydryl groups that are highly reactive with DTNB in disulfide interchange at pH 6.8, and another four cysteine residues that react more slowly. Substitution of the reactive groups results in no loss of activity, measured in a standard assay, while substitution of the less reactive sulfhydryl groups produces inactivation with concomitant dissociation into subunits. In investigating the reaction of phosphorylase *b* with DTNB, Kastenschmidt *et al.* (1968) isolated a derivative containing up to 3.2 mixed disulfide groups which retained dimeric structure and essentially full enzymic activity at high substrate concentration.

Another reagent that has proven valuable in the study of phosphorylase is iodoacetamide. Battell *et al.* (1968a) found that two sulfhydryl groups of phosphorylase *b* react rapidly with iodoacetamide at pH 7.5 without inactivation, while another four sulfhydryls react more slowly and their substitution results in inactivation and dissociation into subunits. The two classes of cysteine residues were identified by isolation of three unique peptides containing alkylated cysteine (Battell *et al.*, 1968b); the reactive groups occur in the sequence Gly-Cys-Arg-Asp, while the four less reactive sulfhydryls occur in the sequences Ala-Cys-Ala-Phe and Asn-Ala-Cys-Asp. More recently this group reported that freshly prepared phosphorylase *b* contains 3.1 highly reactive sulfhydryl groups (Zarkadas *et al.*, 1970). They also succeeded in isolating a new alkylated cysteine peptide, Asn-Gln-Lys-Ile-Cys-Gly-Gly-Trp-Gln-Ser, and extending the sequence of the other reactive cysteine residue to Gly-Cys-Arg-Asp-Pro-Val-Arg-Thr-Asn-Phe.

In the present work we have isolated two tryptic peptides

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[1] Abbreviations used are: FDNB, 2,4-dinitrofluorobenzene; CDNB, 2,4-dinitrochlorobenzene; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PTH, phenylthiohydantoin; DNS or dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

containing the cysteine residues that react rapidly with CDNB. The sequences of the peptides are Phe-Gly-DNP-Cys-Arg-Asp-Pro-Val-Arg and Ile-DNP-Cys-Gly-Gly-Trp-Gln-Met-Glu-Glu-Ala-Asp-Asp-Trp-Leu-Arg, in general, but not complete, agreement with the sequences determined by Zarkadas *et al.* (1970). Rates of dinitrophenylation of these two cysteine sulfhydryl groups have been found to be nearly equal in phosphorylase *a* as well as in phosphorylase *b;* AMP affords significant protection to the first group, relative to the second. Evidence has been obtained that the N-terminal phenylalanine in the first peptide is not the same as the phenylalanine in the sequence Lys-Phe that is known to be one of the points of attachment of pyridoxal phosphate (Nolan *et al.*, 1964). In addition, we describe a procedure by which phosphorylase *b* can be prepared routinely with nearly 4.0 reactive sulfhydryl groups.

Materials

Phosphorylase *b* was prepared by the method of Fischer and Krebs (1962) modified as described by Gold (1968). Phosphorylase *a* was prepared according to Krebs and Fischer (1962) using a crude preparation of phosphorylase *b* kinase. Both enzymes were recrystallized at least three times before use. Trypsin, treated with diphenylcarbamyl chloride to reduce its chymotrypsin content, and pronase were obtained from Calbiochem, while α-chymotrypsin and carboxypeptidases A and B were products of Worthington Biochemical Corp. Aminopeptidase M was obtained from Henley and Co. All ion-exchange resins and Bio-Gel P-10 were obtained from Bio-Rad Labs. Dithiothreitol was a product of Calbiochem and 97% hydrazine was obtained from Fairmount Chemical Co.

Methods

High-voltage electrophoresis was carried out with a Savant flat-plate apparatus using Whatman No. 3MM paper. The pH 1.85 buffer consisted of 5% formic acid and 15% acetic acid, while the pH 6.4 buffer was 10% pyridine plus sufficient acetic acid to give the correct pH. Paper strips were scanned for radioactivity with a Nuclear Chicago Actigraph II scanner or stained with cadmium ninhydrin reagent. High concentrations of DNP peptides could be observed directly in ultraviolet light. After preparative electrophoresis peptides were eluted from the paper with 5% acetic acid by downward capillary flow. Pyridine–acetic acid buffers used in column chromatography were prepared as follows: pH 6.4 buffer was adjusted to the proper pH with acetic acid; pH 5.6 buffer contained four parts by volume of pyridine to one of acetic acid; pH 4.6 buffer contained two parts of pyridine to three parts of acetic acid. Buffer concentrations are expressed as the molar concentration of pyridine.

Acid hydrolysis of peptides was carried out for 22 hr at 112° with glass-distilled, 5.7 N HCl in glass tubes sealed at a pressure below 0.1 mm. Basic hydrolysis was performed by heating the peptide with 76 mg of $Ba(OH)_2 \cdot H_2O$ in 0.5 ml of water at 112° for 22 hr in evacuated sealed tubes. The barium was precipitated with CO_2 and the precipitate was washed with a small volume of water. Amino acid analysis was carried out with a Beckman amino acid analyzer under standard conditions for accelerated analysis. Enzymic digests were analyzed for glutamine and asparagine using the lithium citrate buffer described by Benson *et al.* (1967). Since no peptides contained serine, or glutamine and asparagine together, it was possible to use the normal buffer system in subsequent experiments. Liquid scintillation counting was done as described previously (Gold, 1968).

Edman degradation of peptides was carried out as described by Schroeder (1967); the PTH derivatives were identified by thin-layer chromatography (Pataki, 1968). Dansyl–Edman degradation was done following the procedure of Gray (1967) using thin-layer chromatography to identify the DNS amino acids. Hydrazinolysis was routinely carried out by heating the peptide at 112° for 5 hr with 0.2 ml of 97% hydrazine (anhydrous) in an evacuated sealed tube. After evaporating the hydrazine *in vacuo* over H_2SO_4 the residue was used for amino acid analysis without further treatment. For determining C-terminal aspartic acid, 25 mg of hydrazine sulfate was included and the heating was carried out at 60° for 16 hr. Cyanogen bromide cleavage was done by allowing the peptide to react with 0.025 M CNBr in 70% formic acid for 22 hr at room temperature. Solvent was then removed by evaporation *in vacuo*.

Enzymatic digestion of peptides was carried out in 0.1 M *N*-ethylmorpholine–acetic acid buffer at 30°. Concentrations of enzyme and times of digestion had to be worked out for each experiment. The following pH values were used: α-chymotrypsin and pronase, pH 8.0, carboxypeptidases A and B, and aminopeptidase M, pH 7.5. Reaction mixtures were applied directly to paper for electrophoresis or diluted with sample diluting buffer for amino acid analysis.

Results

Preparation of DNP Phosphorylase b. Phosphorylase *b* was treated with dithiothreitol and [*U*-^{14}C]CDNB as described previously (Gold, 1968). After incubation with 0.5 mM [*U*-^{14}C]-CDNB for 90 min the protein was freed of excess reagent by gel filtration on Bio-Gel P-10 that had been preequilibrated with 0.01 M ammonium acetate, pH 8.0. Products contained an average of 3 DNP groups based on a molecular weight of 185,000.

Tryptic Digestion of [^{14}C]*DNP Phosphorylase b.* Numerous experiments were carried out on the digestion of [^{14}C]DNP phosphorylase *b* with trypsin, but the usual result was formation of an insoluble "core" material that contained substantial amounts of the isotope. Analysis indicated that the precipitate was rich in hydrophobic amino acids relative to native phosphorylase. Satisfactory digestion was finally achieved with diphenylcarbamyl chloride treated trypsin in 2 M urea at 28° (Smyth, 1967) using *ca.* 5 mg/ml of protein and 0.09 mg/ml of trypsin. The digestion was continued for 2 hr while the pH was maintained at 8 by periodic addition of dilute ammonia. Under these conditions at least 80% of the isotope was found in soluble peptides and the large bulk of this occurred in two peptides formed in comparable amounts. High-voltage electrophoresis at pH 6.4 revealed an acidic peptide (A) and a highly basic peptide (B) with only traces of other radioactive materials.

Purification of Tryptic Peptides. Preliminary separation of the radiopeptides was carried out by column chromatography. Pyridine was added to the digest from 1 g of protein to a final concentration of 0.5 M and the pH was adjusted to

TABLE I: Amino Acid Compositions of the Radiopeptides Isolated after Tryptic Digestion of [^{14}C]DNP Phosphorylase *b* and Their Derivatives.

Amino Acid[a]	A	A-1	A-1[b]	A-2	A-2[b]	A-3[b]	B	B-1	B-2	B-3
Arg	1.03			1.01	0.99		2.02	1.69		
Asp	2.08			1.92	1.81		1.04	1.10		0.95
Glu	3.14	1.25		2.05	2.09					
Gln[b]			1.29							
Pro							1.03	0.92		1.08
Gly	1.99	1.94	1.99			1.70	0.97	1.23	0.89	
Ala	1.08			1.08	1.15					
Val							1.00	1.00		0.99
Met	0.73									
Homoser		+	0.56							
Ile	0.92	0.81	1.07			1.17				
Leu	1.05			0.95	0.93					
Phe							0.95			
Trp	1.3[c]	0.6[c]	1.08	0.6[c]	1.07	1.14				
DNP-Cys[d]	1.0	0.86	+			+	1.1	0.9	1.1	

[a] From acid hydrolysis unless noted otherwise. [b] From aminopeptidase M hydrolysis. [c] From alkaline hydrolysis. [d] From radioactivity.

6.4 with acetic acid. After removing a small amount of precipitate, the solution was passed through a short column (0.9 cm × 20 cm) of Bio-Rex 70 (100–200 mesh), a carboxylic acid resin, that had been equilibrated with 0.5 M pyridine–acetic acid, pH 6.4. The acidic peptide (A) was not adsorbed and could be washed through with fresh buffer. Elution of the basic peptide (B) was carried out with 3 M pyridine–acetic acid, pH 6.4.

Peptide B from the preliminary separation was purified by chromatography on a column of Bio-Rex 70, 200–400 mesh, using a linear gradient of 1–3 M pyridine–acetic acid, pH 6.4. Material from digestion of several grams of protein, amounting to 21 μmoles of peptide B, was chromatographed on a 1.9 cm × 66 cm column with a total of 2000 ml of buffer at 40° and a flow rate of 80 ml/hr. A single radioactive component appeared in a sharp peak at 2.4 M buffer. Final purification was accomplished by preparative electrophoresis at pH 1.85. The ultraviolet spectrum of the peptide in 1% acetic acid had a maximum at 333 nm and no band near 280 nm.

The combined eluate containing crude peptide A from the preliminary Bio-Rex 70 column was adjusted to pH 5.6 with acetic acid and adsorbed on a column of AG 1-X2, 200–400 mesh, that had been equilibrated with 0.5 M pyridine–acetic acid, pH 5.6. Material from 1 g of protein was chromatographed on a 1.9 cm × 70 cm column at 45° using a 4000-ml linear gradient of 0.5–2.5 M pyridine–acetic acid, pH 5.6. A single radioactive band eluted at 1.9 M buffer. After evaporating to dryness, this product was rechromatographed on a 0.9 cm × 50 cm column of AG 50-X2, 200–400 mesh, using a 400-ml linear gradient of 1–2.5 M pyridine–acetic acid, pH 4.6, at 50°. Since the peptide, at this stage, was insoluble in the initial buffer it was dissolved in a small volume of 50% acetic acid and a small amount of the ion-exchange resin was added. After evaporation to dryness the resin was added to the top of the column and elution was begun with the first buffer. Again, a single radioactive peak was obtained at 1.9 M buffer. Attempts to purify the peptide by paper electrophoresis were fruitless because of its low solubility; however, the peptide is usually pure, as judged by amino acid analysis, after the two chromatograms described. The ultraviolet spectrum of peptide A in 20% formic acid has maxima at 334 and 272 nm, suggestive of tyrosine or tryptophan in addition to *S*-DNP-cysteine.

Amino acid analyses of these peptides and their degradation products are given in Table I. Peptide A consistently shows a low methionine content and appears to contain more than one tryptophan residue. Peptide B, surprisingly for a tryptic peptide, contains two arginine residues.

Amino Acid Sequences. The basic peptide (B) readily loses 1 equiv of free phenylalanine on treatment with either aminopeptidase M (Pfleiderer *et al.*, 1964) or α-chymotrypsin. A heptapeptide (B-1) was isolated by means of high-voltage electrophoresis at pH 1.85 after chymotryptic digestion. B-1 contains the same amino acids as B with the exception of phenylalanine and was shown to have N-terminal glycine by dinitrophenylation and analysis of the free amino acids liberated on acid hydrolysis. Edman degradation of B confirmed the N-terminal sequence Phe-Gly. Carboxypeptidase B liberated one of the two arginine residues from peptide B. When the crude carboxypeptidase B digest was subjected to hydrazinolysis good yields of ornithine and valine were obtained. Since ornithine must have come from the free arginine the penultimate residue is valine and the sequence must be Phe-Gly-(DNP-Cys, Arg, Asp, Pro)-Val-Arg.

Peptide B is completely inert toward trypsin but it is readily digested with pronase which liberates both arginine residues and the phenylalanine residue as free amino acids. Two peptides can be isolated by high-voltage electrophoresis at pH 1.85 and 3.5: B-2 and B-3, respectively. B-2 contains only glycine and DNP-cysteine and gave PTH-glycine on

Edman degradation. B-3 is a tripeptide of aspartic acid, proline, and valine. Hydrazinolysis of B-3 liberated free valine while Edman degradation gave PTH-aspartic acid and PTH-proline on successive stages. This establishes the sequence Asp-Pro-Val for B-3 and proves that the peptide contains aspartic acid rather than asparagine. The evidence is unequivocal that B is Phe-Gly-DNP-Cys-Arg-Asp-Pro-Val-Arg and this agrees with the sequence found by Zarkadas *et al.* (1970) for one of the cysteine residues that react rapidly with iodoacetamide.

Peptide A is quite large but fortunately it could be cleanly split by cyanogen bromide into two peptides (A-1 and A-2) of nearly equal length. These were purified by high-voltage electrophoresis at pH 1.85. The peptides contain glutamic and aspartic acid residues and the question whether they exist as such or as glutamine or asparagine was of immediate concern. This was settled when it was discovered that both peptides could be completely digested with aminopeptidase M. Amino acid analysis of the digests showed that A-1 contains only glutamine, while A-2 contains only glutamic and aspartic acids. The enzymic digests also clearly revealed one tryptophan residue in each peptide and a homoserine residue in A-1.

The N-terminal amino acid of A-1 was found to be isoleucine by the dansyl method and by observing the loss of isoleucine after treating the peptide with FDNB. α-Chymotrypsin does not release any free amino acids from A-1 but a new DNP cysteine peptide (A-3) can be isolated by high-voltage electrophoresis at pH 1.85. A-3 lacks glutamine and homoserine, which must therefore be at the C terminus. Carboxypeptidase A causes liberation of only tryptophan from A-3; thus the C-terminal sequence of A-1 is Try-Gln-Homoser. A-1 must, therefore, correspond to the peptide sequence for the second reactive cysteine residue found by Zarkadas *et al.* (1970), and no further work was done on it. The sequence deduced for A-1, Ile-DNP-Cys-Gly-Gly-Trp-Gln-Homoser, disagrees with the one reported by Zarkadas *et al.* in that a methionine residue is indicated for a position which those authors assigned to serine. Since the sequences around all the cysteine residues are known (Zarkadas *et al.*, 1968) there is no possibility that the peptides arise from different regions of the protein. The difference probably reflects an error in sequence determination on the part of Zarkadas *et al.* (1970), since that part of their sequence rests upon relatively weak evidence.

Peptide A-2 contains N-terminal glutamic acid, determined by the dansyl and FDNB methods. Carboxypeptidase B acts on A-2 to release arginine quantitatively and when the crude digest is dried and treated with hydrazine, free ornithine and leucine are found. Since ornithine must come from the free arginine the penultimate residue is leucine. When the peptide is treated first with carboxypeptidase B and then with carboxypeptidase A, free arginine, leucine, and tryptophan are found; if the crude digest is evaporated to dryness and treated with hydrazine and hydrazine sulfate at 60° for 16 hr, free ornithine, leucine, tryptophan, and aspartic acid are observed. More vigorous conditions of hydrazinolysis cause destruction of the aspartic acid. The sequence, from the evidence presented so far, is Glu-(Glu, Ala, Asp)-Asp-Trp-Leu-Arg, and this is consistent with the fact that digestion with pronase produces arginine and leucine as the only free amino acids.

Sequencing A-2 by the dansyl–Edman procedure leads to the partial sequence Glu-Glu-Ala-Asp. This is partially confirmed by the fact that conditions were found for digestion of A-2 with aminopeptidase M that produce 57% glutamic acid, 19% alanine, and 8% aspartic acid. Alanine, therefore, must be released before either aspartic acid residue can be released.

The sequence of peptide A must be Ile-DNP-Cys-Gly-Gly-Trp-Gln-Met-Glu-Glu-Ala-Asp-Asp-Trp-Leu-Arg.

Peptides from Reduced DNP Phosphorylase b. Since peptide B is produced by tryptic digestion, it is probable that the N-terminal phenylalanine residue is preceded in the protein sequence by a lysine or arginine residue. A possibility exists, therefore, that this is the same phenylalanine residue that occurs in the sequence Lys-Phe that was found by Fischer *et al.* (1958) and by Nolan *et al.* (1964) to be the site at which pyridoxal phosphate is bound. Pyridoxal phosphate is completely split out of the polypeptide during tryptic digestion, since denaturing conditions cause release of the coenzyme, and the lysine residue becomes susceptible to cleavage by trypsin. This possibility was tested by reducing [^{14}C]DNP phosphorylase *b* according to Fischer *et al.* (1958) and comparing the digest of reduced protein to that of nonreduced protein.

Reduction of phosphorylase with sodium borohydride at pH 4.5 converts the substituted aldimine form of the coenzyme (Kent *et al.*, 1958), through the Schiff's base form, into an ϵ-*N*-phosphopyridoxyllysine derivative which retains enzymic activity. The substituted lysine residue is not susceptible to tryptic cleavage, but trypsin is capable of splitting at the phenylalanine residue adjacent to the substituted lysine. This has been shown also to be true for reduced glutamate decarboxylase, which yields a tryptic peptide having the C-terminal sequence (ϵ-*N*-phosphopyridoxyl)-Lys-Phe (Strausbauch and Fischer, 1970). If the phenylalanine residue is indeed common to the two sequences in phosphorylase *b*, then reduced DNP phosphorylase *b* should fail to yield peptide B on tryptic digestion but should give a corresponding peptide lacking the N-terminal phenylalanine residue. A peptide of this structure (B-1) has been prepared by digestion of peptide B with α-chymotrypsin and was found to be distinguishable from B by high-voltage electrophoresis at pH 1.85, having a slightly greater mobility than B. An alternate possibility, that neither the substituted lysine nor phenylalanine peptide bonds in this particular phosphorylase derivative are split by trypsin, would lead to the disappearance of B and appearance of a new peptide of unknown characteristics.

[^{14}C]DNP phosphorylase *b* containing an average of 2.4 DNP groups was divided into two portions. One fraction was digested with trypsin as previously described and the crude digest was treated with two volumes of 100% formic acid and 6 volumes of 0.075 M cyanogen bromide in 70% formic acid. After standing overnight at room temperature, the solution was evaporated to dryness *in vacuo*. Cyanogen bromide treatment converts peptide A into A-1, which gives a sharper band on electrophoresis. The remaining DNP phosphorylase *b* was reduced with sodium borohydride (Fischer *et al.*, 1958); no pyridoxal phosphate could be detected spectrophotometrically after treating this derivative with 0.3 M perchloric acid (Kent *et al.*, 1958). After digestion of the reduced protein with trypsin and cyanogen bromide the two digests (reduced and unreduced) were subjected to high-voltage electro-

TABLE II: Radioassay of Peptides Obtained by Trypsin and Cyanogen Bromide Digestion of Reduced and Nonreduced [^{14}C]DNP Phosphorylase *b*.[a]

Source of Peptides	A-1[b]	B[b]	A-1/B
Nonreduced DNP-ph *b*	3221	2029	1.59
Reduced DNP-ph *b*	2403	1643	1.46

[a] Direct liquid scintillation counting of paper areas containing peptide after electrophoresis at pH 1.85. [b] Disintegrations per minute.

TABLE III: Radioassay of Peptides Obtained by Trypsin and Cyanogen Bromide Digestion of Phosphorylase Samples Containing Varying Numbers of DNP Groups.

Phosphorylase	AMP[a]	DNP/Subunit[b]	A-1[c]	B[c]	A-1/B
		0.73	1035	547	1.89
a		1.46	1967	1000	1.97
		1.73	2285	1225	1.87
	+	0.38	1193	205	5.83
a	+	0.68	2188	475	4.60
	+	0.99	2730	650	4.20
		0.57	840	485	1.73
b		1.05	1580	912	1.73
		1.40	2018	1327	1.52
	+	0.23	1158	282	4.10
b	+	0.42	1615	488	3.31
	+	0.59	2515	968	2.60

[a] 1 mM AMP present during reaction with CDNB. [b] Incubation times with CDNB were 10, 30, and 65 min. [c] Disintegrations per minute.

phoresis side by side at pH 1.85 and 6.4. Radioautography revealed no significant difference between the two digests at either pH. Quantitative estimates of the peptides were made by excising the radioactive areas from the pH 1.85 electropherogram and counting the paper directly by liquid scintillation counting (Table II). The great bulk of the isotope was in peptides A-1 and B which occurred in virtually the same ratio in both digests. We must conclude that the Lys-Phe sequence from the pyridoxal phosphate binding site does not overlap with peptide B.

Relative Rates of Reaction of the Two Sulfhydryl Groups with CDNB. In order to estimate the relative reactivity of the two sulfhydryl groups, samples of phosphorylase were allowed to react with 0.5 mM [^{14}C]CDNB for various time periods in order to introduce varying numbers of DNP groups. The relative degree of substitution of the two sulfhydryls was estimated by digesting the samples with trypsin and cyanogen bromide as described above and subjecting the digests to electrophoresis at pH 1.85. After radioautography the areas of paper containing the peptides were excised and counted.

This procedure suffers from two drawbacks: it is not known what proportion of each of the sulfhydryl groups in question is initially in the reduced state and available for substitution, and the losses occurring during digestion and electrophoresis are uncertain. If we assume that the losses are constant from sample to sample a rough estimate of the relative reactivities can be made by considering the ratio of peptides A-1 and B from protein samples containing increasing numbers of DNP groups. If the two sulfhydryl groups react at equal rates the ratio of peptides should be constant up to complete substitution. If one is more reactive than the other, the ratio should reflect this in samples having low degrees of substitution while the ratio should change significantly in samples having a high degree of substitution.

Data from experiments with phosphorylase *a* and *b* in the absence and presence of AMP are shown in Table III. The great bulk of isotope in each sample is found in peptides A-1 and B and the same peptides appear to be formed from both phosphorylase *a* and *b*. The data are inadequate to permit calculation of the rate constants for reaction of the individual sulfhydryl groups. In the absence of AMP the two sulfhydryl groups appear to react at nearly equal rates in both phosphorylase *a* and *b*, as evidenced by the constancy in the ratios of peptides as substitution progresses. The content of the free sulfhydryl group giving rise to peptide A-1 is considerably greater, in both phosphorylases, than that giving rise to the basic peptide. When reaction with CDNB is carried out in the presence of AMP it appears that the sulfhydryl group giving rise to peptide B is protected to a significantly greater extent than the other group. AMP reduces the overall rate of reaction of phosphorylase *b* with CDNB by only 33% under these conditions (Gold, 1968). Therefore, AMP probably exerts its protective effect nearly exclusively on the sulfhydryl group giving rise to peptide B.

Preparation of Phosphorylase b Containing Reactive Sulfhydryl Groups in the Fully Reduced State. In the course of the experiments described above numerous preparations of phosphorylase *b* were subjected to reaction with CDNB. Enzyme was always pretreated with dithiothreitol at pH 8.0 and freed of DTT by means of rapid gel filtration, yet the incorporation of DNP groups was quite variable. Similar variability was observed previously in work with FDNB (Gold, 1968). As indicated in the introduction, other workers have found between one and two reactive sulfhydryl groups in the phosphorylase *b* subunit by use of several reagents. We have found that the best method of preparing phosphorylase *b* containing the reactive sulfhydryl groups intact is to recrystallize the enzyme frequently as described by Fischer and Krebs (1962) but substituting 0.015 M dithiothreitol for 0.03 M cysteine. After dissolving the crystalline enzyme in 0.05 M sodium barbital, pH 8.0, containing 5 mM dithiothreitol and 1 mM EDTA, the solution is subjected to rapid gel filtration on Bio-Gel P-10 equilibrated with whatever buffer is desired. Enzyme prepared in this way usually contains at least 3.8 sulfhydryl groups that react rapidly with DTNB under the conditions described by Kleppe and Damjanovich (1969) or at pH 8.0. In contrast, those workers observed approxi-

mately two reactive sulfhydryl groups. The reactive sulfhydryl titer of a particular enzyme preparation remains high over reasonable periods of time. One sample, recrystallized eight times over a period of 6 weeks, still contained 3.88 reactive sulfhydryls per dimer. In comparison, the reactive sulfhydryl titer of enzyme recrystallized from mercaptoethanol had dropped to 1.64 per dimer after 3 weeks.

Discussion

Clearly, the sulfhydryl groups of phosphorylase that react rapidly with CDNB are the same as those that are exceptionally reactive with iodoacetamide (Zarkadas *et al.*, 1970). The one difference in sequence, the occurrence of methionine in a position that Zarkadas *et al.* (1970) found to be occupied by serine, is not sufficient to alter this conclusion. Although Kleppe and Damjanovich (1969) found only one sulfhydryl group (SH I) highly reactive with DTNB in each phosphorylase *b* subunit, there appear to be two such groups when special care is taken to keep the sulfhydryls in a reduced state. Sulfhydryls of the SH I type are undoubtedly the same as those indicated to be especially reactive with CDNB and iodoacetamide; this is based upon the fact that substitution of these sulfhydryl groups by any of the reagents results in an enzyme that has full or nearly full activity at high substrate concentrations. Kastenschmidt *et al.* (1968) isolated a phosphorylase *b* dimer containing up to 1.6 sulfhydryls per subunit substituted by mixed disulfide formation with DTNB, and this too was fully active at high substrate concentrations. A significant point of disagreement is whether substitution of these two reactive sulfhydryls results in changes in the kinetic parameters of phosphorylase *b*. Substitution by dinitrophenyl groups appears to have a profound effect upon the kinetic constants (Gold, 1968), while substitution with DTNB abolishes homotropic cooperativity for AMP (Kastenschmidt *et al.*, 1968). Alkylation with iodocetamide appears to have no effect upon any of the kinetic parameters (Battell *et al.*, 1968b). It is possible that these kinetic differences are real and reflect differences in the nature of the substituting groups.

By a similar argument, it is likely that the two sulfhydryl groups designated SH II by Kleppe and Damjanovich (1969) correspond to those that react slowly with iodoacetamide and occur in the sequences Ala-Cys-Ala-Phe and Asn-Ala-Cys-Asp (Battell *et al.*, 1968b). The effect of substitution of these sulfhydryl groups in phosphorylase *b* is complete inactivation and dissociation into subunits.

Some protection against CDNB appears to be afforded to the sulfhydryl group occurring in the partial sequence Gly-Cys-Arg-Asp by AMP. This is seen both in the gross rate of dinitrophenylation (Gold, 1968) and in the ratios of peptides A-1 and B formed. Kleppe and Damjanovich (1969) report no detectable protection of the SH I sulfhydryl groups by AMP when DTNB is the modifying reagent. However, their methods would not detect a moderate level of protection but only a very high degree of protection. This effect of AMP may be attributed to the presence of the sulfhydryl group in the vicinity of the AMP binding site or to a conformational change in the protein induced by AMP binding; no decision can be reached on the basis of evidence presently in hand.

An effect of "ageing" upon the kinetics of phosphorylase has been reported by Helmreich and Cori (1964) who observed that the K_m of phosphorylase *b* for AMP depended upon the age of the enzyme solution. Similarly, Helmreich *et al.* (1967) found that the dissociation constant of AMP and phosphorylase *a* increased with the age of the enzyme preparation and that the kinetics changed in a way that suggest an increase in K_m for AMP. We believe that these changes may be attributed to irreversible destruction of the reactive sulfhydryl groups. When these sulfhydryl groups are substituted with at least two types of reagent the effect is to increase the K_m for AMP; K_m for other substrates may also be increased. We have already pointed out the difficulty of maintaining the sulfhydryl groups in the reduced state. Another effect related to this phenomenon is a gradual decrease in the solubility of phosphorylase *b* in the course of repeated recrystallization at pH 6.8 in the presence of mercaptoethanol. In fact, phosphorylase *b* containing an average of three DNP groups on the reactive sulfhydryls has very poor solubility at pH 6.8 and had to be handled at pH 8.0 to avoid precipitation.

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Cross-Linking of Collagen and Elastin. Properties of Lysyl Oxidase*

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ABSTRACT: Lysyl oxidase catalyzes the formation of the lysine-derived aldehyde, allysine. This is the first step in the cross-linking reaction of collagen and elastin. The present study describes the partial purification and some of the properties of this enzyme.

The specific activity of the enzyme extracted from chick embryo cartilage was increased 440-fold by $(NH_4)_2SO_4$ precipitation, acid precipitation, and gel filtration. Its molecular weight was approximately 170,000 as judged by gel filtration. Elevated oxygen tension was found to stimulate activity. A metal cofactor is required for activity since dialysis against solutions with α,α-dipyridyl abolished activity. Cupric ion restored full enzymatic activity and cobaltous and ferrous ions restored some activity. It is likely that copper is the naturally occurring cofactor since enzyme activity was absent in extracts from copper-deficient chicks. The lathyrogen β-aminopropionitrile acts as an irreversible inhibitor of lysyl oxidase *in vitro* and *in vivo*.

Lysyl oxidase converts specific lysyl residues in collagen and elastin into the corresponding δ-semialdehyde, allysine[1] (Pinnell and Martin, 1968; Martin *et al.*, 1970; Siegel and Martin, 1970a). These aldehyde residues then condense, probably by a spontaneous reaction, to form covalent cross-links (Gross, 1969; Deshmukh and Nimni, 1969; Schiffmann and Martin, 1970). When this normal biosynthetic process is impaired in either experimental lathyrism (Martin *et al.*, 1961) or copper deficiency (Miller *et al.*, 1965; Shields *et al.*, 1962; O'Dell *et al.*, 1961; Kimball *et al.*, 1964), the collagen and elastin from these animals have increased numbers of lysyl residues and decreased amounts of allysine (Piez, 1968; Chou *et al.*, 1969).

Lysyl oxidase activity was initially detected in extracts of embryonic chick bone (Pinnell and Martin, 1968). In one assay elastin biosynthetically labeled with [6-^3H]lysine was prepared as a substrate. As allysine was formed during the incubation with the lysyl oxidase preparation, tritium was released from the substrate, isolated by distillation, and used as a measure of the reaction. In a second assay, elastin biosynthetically labeled with [^{14}C]lysine was used as a substrate. The allysine formed during incubation with lysyl oxidase was subsequently converted by oxidation into α-aminoadipic acid. The radioactivity in this compound served as a measure of the enzymatic reaction. With these assays it was possible to demonstrate that lysyl oxidase activity was inhibited by low levels of the lathyrogen (BAPN)[2] (Pinnell and Martin, 1968). This compound inhibits the cross-linking of collagen and elastin *in vivo*.

In a separate study (Siegel and Martin, 1970a), the action of lysyl oxidase on collagen was examined. Enzyme catalyzed formation of allysine was observed in both the α1 and α2 chains. In addition, the formation of the β_{12} component of collagen, a dimer composed of an α1 and α2 chain covalently linked, was shown to occur. The formation of allysine and of the cross-linked dimer was inhibited by BAPN.

The purpose of the present paper is to report on the partial purification and further characterization of lysyl oxidase. The enzyme has been purified 440-fold and found to have a tightly bound metal cofactor. The relationship between experimental lathyrism or copper deficiency and lysyl oxidase activity has also been studied.

Materials and Methods

Preparation of Enzyme from Chick Embryos. The 105,000*g* supernatant fluid obtained by homogenizing the cartilaginous ends of the tibiae and femora from 6 dozen 17-day-old chick embryos (approximately 25 g of tissue) in 50 ml of 0.1 M NaH_2PO_4–0.15 M NaCl, pH 7.7, was prepared as described previously (Pinnell and Martin, 1968). A saturated solution of $(NH_4)_2SO_4$ adjusted to pH 7.5 at 4° with concentrated NH_4OH was added to the fluid to give a final concentration of $(NH_4)_2SO_4$ of 277 g/l. or 45% saturation. The precipitate that formed was collected by centrifugation at 17,300*g* for 10 min. This pellet was suspended in 8–10 ml of 0.01 M NaH_2PO_4–0.015 M NaCl which had been adjusted to pH 7.7 with NaOH and

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[1] The trivial name used is: allysine, α-aminoadipic acid δ-semialdehyde.

[2] The abbreviation used is: BAPN, β-aminopropionitrile fumarate.