ACTIVE SITE PEPTIDES FROM GLYCOGEN PHOSPHORYLASE

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Summary. The isolation and sequence of active site peptides from glycogen phosphorylase b is described. These cysteine peptides become exposed to preferential labeling upon removal of pyridoxal 5'-phosphate (PLP) from the enzyme and they have the sequence Ala-Cys, Asx-Ala-Cys-Asp and Asx-Glx-Lys-Cys-Gly-Gly. The first two of these peptides appear to be derived from the PLP binding site of phosphorylase.

Glycogen phosphorylase is a multisite enzyme. Besides its substrates (glycogen, inorganic phosphate and glucose-1-phosphate), this enzyme specifically recognizes PLP† and AMP. In the absence of either one of these effectors, the b form of the enzyme is devoid of catalytic activity (Cori and Green, 1943; Illingworth et al., 1958; Shaltiel et al., 1969) indicating that PLP and AMP have a dominant influence on the structure of the catalytic site. Recent studies even suggest that PLP may be located at the catalytic site of the enzyme (Bresler and Firsov, 1968; Cortijo and Shaltiel, 1970).

Regarding the mode of binding of PLP, Fischer and his associates (1958) suggested that the cofactor is covalently bound to the protein through an ϵ-amino group of a lysine and another group (X) which was not identified. We have recently reported (Zaidenzaig and Shaltiel, 1969) the existence of cysteine residues in phosphorylase which become exposed to preferential labeling with FDNB only upon removal of PLP from the enzyme. S-dinitrophenylation of these cysteines inactivated the enzyme and prevented the binding of PLP at its native site, as indicated by fluorescence studies (Zaidenzaig et al., 1970). The above findings suggest that the labeled cysteines are at the PLP site and may therefore provide group X (a sulfhydryl) for the binding of PLP to the protein. We wish to report here the isolation and sequence determination of three peptides containing the labeled cysteine residues and summarize the evidence for assigning two of these to the PLP binding site.

† Abbreviations: DNP, 2,4-dinitrophenyl; FDNB, 1-fluoro-2,4-dinitrobenzene; PLP, pyridoxal 5'-phosphate.

MATERIALS AND METHODS

Glycogen apophosphorylase b from rabbit skeletal muscle was prepared and characterized as described previously (Shaltiel et al., 1966). It was freed from excess thiols by gel filtration on a Sephadex G-25 column. Freshly prepared solutions of apoenzyme were used for labeling with FDNB. A molecular weight of 185000 was used for phosphorylase b or the apoenzyme (Seery et al., 1967; De Vincenzi and Hedrick, 1967). Uniformly labeled [^{14}C] FDNB was purchased from the Radiochemical Centre, England. The specific radioactivity of [^{14}C] FDNB solutions was determined as described previously (Shaltiel and Soria, 1969). Radioactivity was measured in a Packard Model 3003 Tri-Carb liquid scintillation spectrometer. Amino acid composition and sequence were determined by the methods described in Methods in Enzymology, Vol. XI (Hirs, 1967).

RESULTS AND DISCUSSION

Labeling of the Apoenzyme. Glycogen apophosphorylase b (185 mg, 1 μMole) was reacted with [^{14}C] FDNB (2 μMoles, specific radioactivity 2.45 x 10^5 cpm/μMole) in a buffer composed of sodium glycerophosphate (5 x 10^{-2} M) and EDTA (10^{-3} M), pH 7.5. The reaction was conducted at a protein concentration of 8.8 mg/ml and allowed to proceed at 22° for 1 hour. The solution of the labeled protein was dialyzed overnight against 6 liters of 0.01 N HCl to remove excess FDNB (if any) and to lower the pH

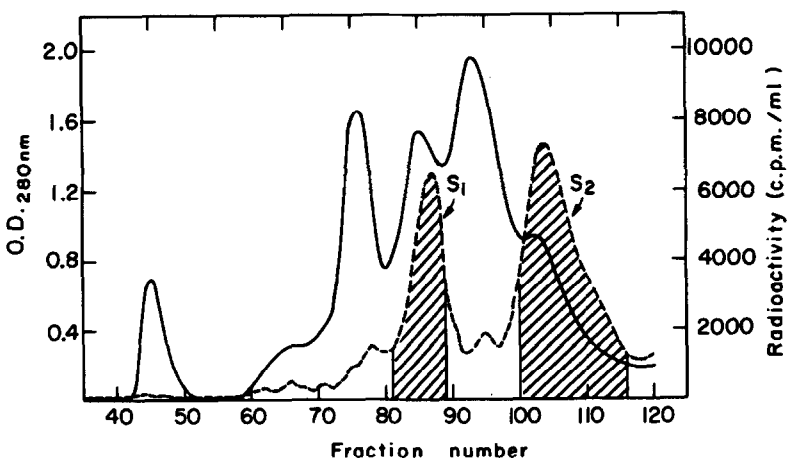

Fig. 1: Elution pattern of the peptic digest of DNP-apophosphorylase b. The column (Sephadex G-25 fine, 1.5 x 180 cm) was equilibrated at 22° with 2% acetic acid. Fractions of 3.2 ml were collected and their absorbancy at 280 nm (——) as well as their radioactivity (---) were monitored.

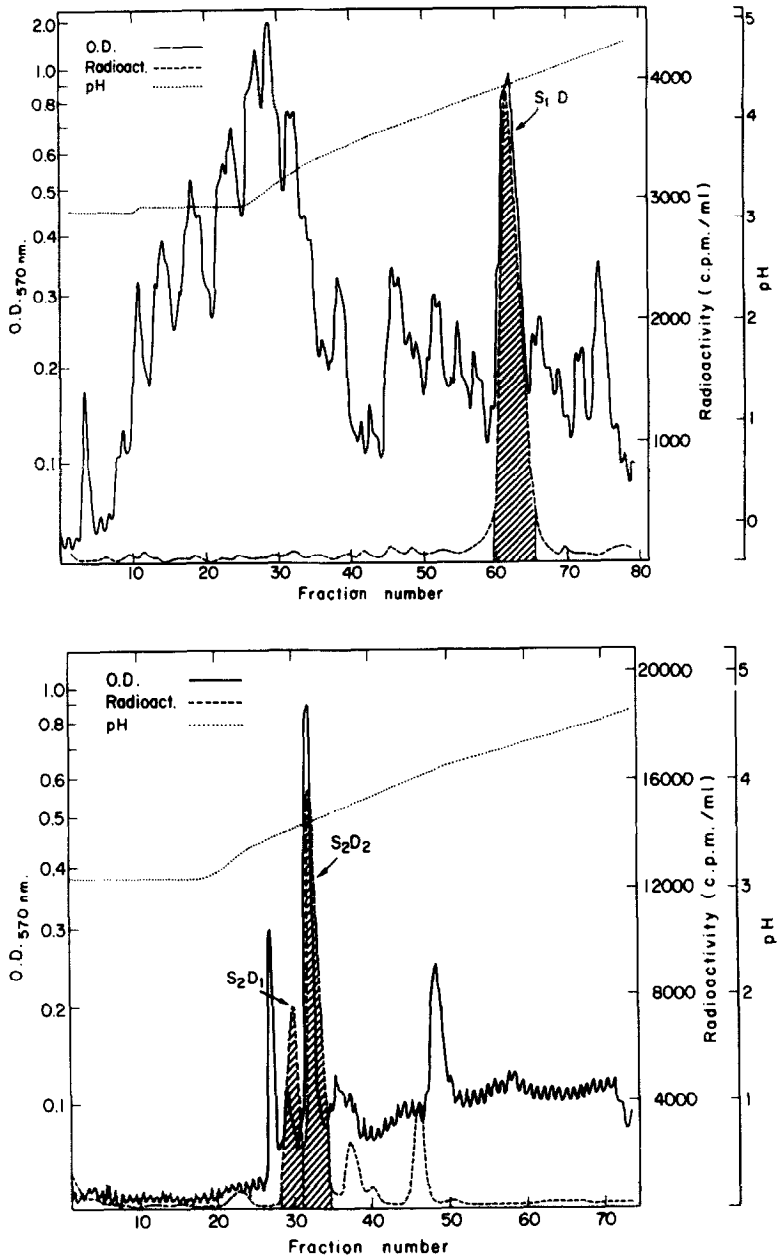

Fig. 2: Fractionation of S_1 (above) and S_2 (below) on Dowex 50-x8. The column (0.9 x 35 cm) was water jacketed and eluted at 50°, first with 0.2 M pyridine acetate, pH 3.1 and then with a linear gradient using 100 ml of this buffer in the mixing chamber and 100 ml of 2 M pyridine acetate (pH 5.1) in the reservoir. The flow rate was 0.5 ml/min; 0.05 ml/min were removed for alkaline hydrolysis and subsequent reaction with ninhydrin. Fractions of 2.5 ml were collected and their ninhydrin color ($O.D_{570\,nm}$) as well as their radioactivity and pH were measured.

of the medium. Under these conditions, 0.95 moles of [^{14}C] DNP groups became covalently attached to each enzyme protomer (M.W. 92500).

Isolation of the Radioactive Peptides. The labeled protein (185 mg) was digested with 10 mg pepsin during 22 hours at 30°, then freeze dried, dissolved in about 3 ml of 2% acetic acid and applied on a Sephadex column (Fig. 1). The fractions from the two radio active peaks (S_1 and S_2) were pooled separately. They contained 90% of the total counts applied on the column, of which 32.5% were in the S_1 pool and 57.5% in S_2. Each of these fractions was subjected to further purification on Dowex 50x8 (Fig. 2). The pool S_1 yielded only one radioactive peak (S_1D) on Dowex which was further purified by preparative paper electrophoresis and chromatography (Fig. 3 A and B). The pool of fractions from the S_2 peak yielded two major radioactive peptides when applied on the

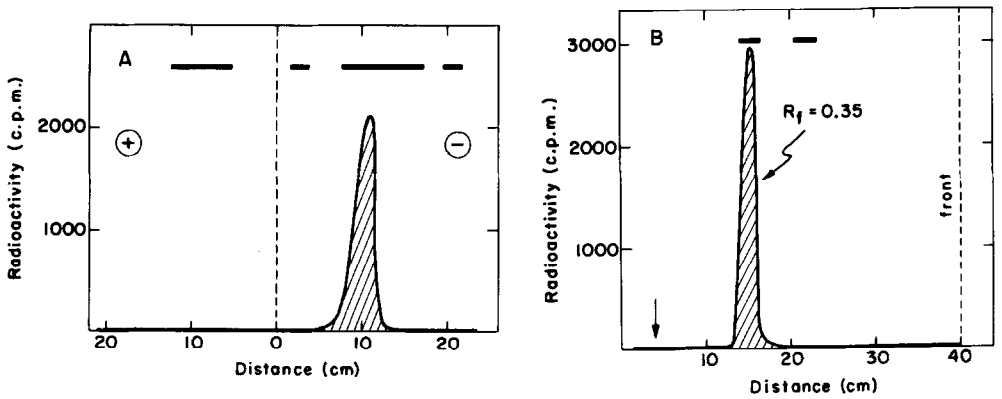

Fig. 3: Purification of S_1D by paper electrophoresis (A) followed by chromatography (B). The electrophoresis was run for 2 hours at 3000 V and pH 6.5 (pyridine acetate). Chromatography was run for 16 hours in 1-butanol: acetic acid: water (4:1:4, upper layer). Both separations were performed on Whatman No. 3 paper. The radioactive peptide was located in each case by cutting a strip of paper (0.5 cm wide) along the chromatogram, dividing it into pieces (1 cm long) and measuring their radioactivity. Horizontal bars indicate ninhydrin-positive materials.

Dowex column. Each of these peptides (S_2D_1 and S_2D_2) was further purified by preparative paper chromatography (Fig. 4).

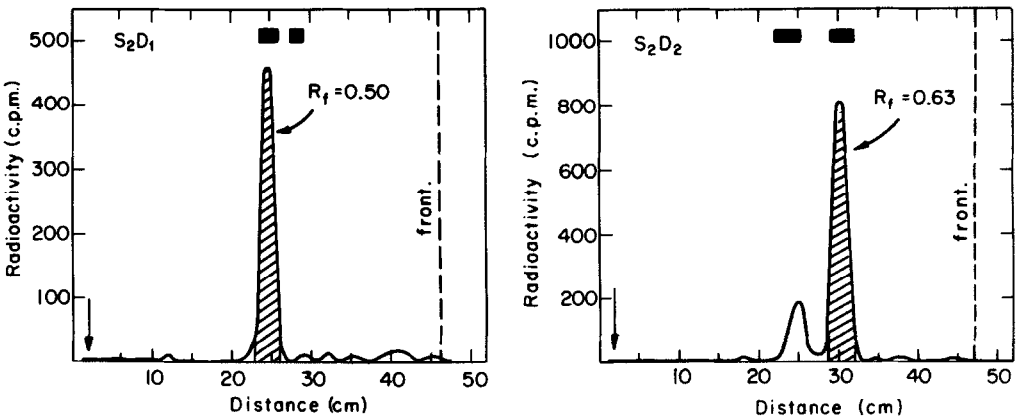

Fig. 4: Purification of S_2D_1 and S_2D_2 by paper chromatography as described in the legend to figure 3.

Sequence of the Labeled Peptides. Table I summarizes the sequence of the three radioactive peptides as well as their yield.

Table I: Sequence of the Labeled Peptides from Apophosphorylase b

Peptide	R_f [a]	Sequence [b]	Yield [c]
S_1D	0.35	DNP Asx-Glx-Lys-Cys-Gly-Gly	18.6
S_2D_1	0.50	DNP Asx-Ala-Cys-Asp	22.0
S_2D_2	0.63	DNP Ala-Cys	39.0

[a] In 1-butanol: acetic acid: water (4:1:4, upper layer).

[b] The amino acid composition (——) was determined by quantitative amino acid analysis except for S-DNP-cysteine which was determined from the radioactivity of the sample. The symbol —► indicates a determination by the Dansyl-Edman procedure and ⇒ represents an N-terminal determination by the FDNB method. Carboxypeptidase A was used for C-terminal analysis (◄—).

[c] Represents the percentage of radioactivity in S_1D, S_2D_1 and S_2D_2 out of the total radioactivity found in the fractions obtained from the two Dowex columns (Fig. 2).

Assigning the Peptides Ala-Cys and Asx-Ala-Cys-Asp to the PLP Binding Site.

When apophosphorylase b is reacted with 1.5 moles of FDNB per mole of enzyme protomer, there is a 90% loss of activity (as measured after reconstitution with PLP). However, when the reaction is performed with only 1 mole FDNB per mole of protomer, the loss of activity is 50-55% although 0.9-1.0 moles FDNB become covalently attached to each enzyme protomer. This already indicates that some of the label may be attached to a site where it does not inhibit the potential enzymatic activity of the apoenzyme. Therefore, one is faced with the problem of deciding which of the DNP-labeled peptides originate from the PLP binding site.

Several pieces of evidence seem to indicate that two out of the three labeled peptides isolated are derived from the PLP binding site. These two peptides are Ala-Cys and Asx-Ala-Cys-Asp. Since both of them contain the sequence Ala-Cys, it is possible that the dipeptide is a fraction of the tetrapeptide and that both contain the same cysteine residue in the peptide chain. It should be mentioned, however, that Zarkadas et al. (1968), who determined the sequence around all 9 cysteine residues in phosphorylase found two different peptides containing the sequence Ala-Cys and therefore it is possible that some or all of the Ala-Cys peptide originates not from the tetrapeptide Asx-Ala-Cys-Asp but from the other sequence Ala-Cys-Ala. Nevertheless, the assumption that the two peptides in S_2 contain the same cysteine residue in the peptide chain could explain several observations summarized below, so that it seems to be at the moment a useful working hypothesis.

The yield of the two peptides together (the S_2 fraction) is 57.5%. Therefore, the loss of enzymatic activity (50-55%) could be accounted for by the extent of labeling of these peptides.

By partial thiolysis of the dinitrophenylated apoenzyme we were able to obtain an apoenzyme preparation which regained 60-70% of its catalytic activity upon reconstitution with PLP. This apoenzyme preparation had 65% of its PLP sites vacant and accessible for reconstitution with the cofactor as determined by following the PLP fluorescence of the enzyme*. Moreover, this apoenzyme preparation had 0.3 moles of [^{14}C]DNP groups covalently bound to each mole of enzyme protomer and all of the label was found in the S_2 fraction of the Sephadex column. We have thus three independent parameters (catalytic activity, fluorescence and radioactive labeling) which seem to correlate quantitatively with each other and support the suggestion that the two peptides Ala-Cys and Asx-Ala-Cys-Asp originate from the PLP site.

*PLP, when bound to its native site in phosphorylase, has a characteristic green fluorescence (Shaltiel and Fischer, 1967).

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