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# Identification of Lys[190] as the primary binding site for pyridoxal 5′-phosphate in human serum albumin

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The covalent binding of pyridoxal 5′-phosphate (PLP) to human serum albumin (HSA) is important in the regulation of PLP metabolism. In plasma, PLP is bound to HSA at a single high-affinity and at two or more nonspecific sites. To characterize the primary PLP binding site, HSA was incubated with [$^3$H]PLP, and the Schiff base linkage was reduced with potassium borohydride. Tryptic peptides were purified, and the major labeled peptide was sequenced. Amino acid analysis confirmed a homogeneous peptide Leu-Asp-Glu-Leu-Arg-Asp-Glu-Gly-Xaa-Ala-Ser-Ser-Ala-Lys which corresponds to residues 182–195 of HSA. The data indicate that Lys[190] is the primary PLP binding site. This Lys residue is distinct from other sites of covalent adduct formation; namely, the primary sites for nonenzymatic glycosylation (Lys[525]) and acetylation by aspirin (Lys[199]).

Pyridoxal 5′-phosphate; Vitamin B6; Binding site; Albumin (human)

## 1. INTRODUCTION

Pyridoxal phosphate (PLP), the major cofactor form of vitamin B6, is required in many of the reactions of amino acid metabolism. In plasma, more than 95% of the circulating PLP is bound to human serum albumin (HSA) [1]. Since PLP bound to HSA is protected from hydrolysis by phosphatases [1,2], HSA serves as a reservoir and mediates PLP transport to tissues. Although PLP binds as a Schiff base to both BSA and HSA, recent studies suggest that PLP binds differently to HSA than it does to BSA [3]. Equilibrium binding data indicate a single high affinity and two or more nonspecific sites for PLP in HSA [3]. In previous studies, PLP was shown to be localized to a region of HSA containing residues 162–197, but sequencing was not performed [4].

In contrast, BSA contains two high affinity PLP binding sites, one of which has been identified as Lys[223] or Lys[220], a site for which there is no homologous Lys in HSA [5]. The specificity of high affinity covalent binding of PLP to BSA appears to be dependent on interactions of the anionic phosphate ester with neighboring cationic lysine or arginine residues in the protein [6]. The role of the phosphate ester in binding at the primary site also appears to be different in HSA than in BSA since phosphorylated compounds do not inhibit the binding of PLP to HSA, but do inhibit PLP binding to BSA [3].

In addition to PLP, HSA also forms covalent adducts with cysteine, aspirin and glucose [5]. Cysteine forms a disulfide bond with Cys[34], aspirin acetylates Lys[199] and glucose forms a ketoamine derivative at Lys[525] [5,7]. The present study was undertaken to identify the PLP binding site on HSA and determine its relationship to covalent adduct formation at Lys[199] (aspirin) and Lys[525] (glucose). Tryptic peptides of radiolabeled HSA were purified by high performance liquid chromatography (HPLC) and characterized by amino acid analysis and sequencing of pyridoxylated peptides.

*Abbreviations:* PLP, pyridoxal 5′-phosphate; HSA, human serum albumin; BSA, bovine serum albumin; NEG, nonenzymatic glycosylation; PBS, phosphate-buffered saline.

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## 2. MATERIALS AND METHODS

### 2.1. *Materials*

Human serum albumin (Fraction V) was purchased from the American Red Cross and further purified by the method of Feldhoff and Ledden [8]. PLP was obtained from Sigma (St. Louis, MO). [$^3$H]Sodium borohydride (348 mCi/mmol) was purchased from NEN (Boston, MA). All experiments involving PLP and PLP-modified HSA were performed in a room illuminated only with gold fluorescent lights (Sylvania F15T8-GO). Phosphate-buffered saline (PBS) contained sodium chloride, 8.0 g/l; potassium chloride, 0.2 g/l; sodium phosphate dibasic, 1.15 g/l; potassium phosphate monobasic, 0.2 g/l; and sodium azide, 0.2 g/l.

### 2.2. *Preparation and purification of [$^3$H]PLP*

[$^3$H]PLP was synthesized by reduction of PLP with [$^3$H]sodium borohydride followed by oxidation with manganese dioxide [9]. The [$^3$H]PLP was purified by chromatography on Dowex AG-1X2, 200–400 mesh (Bio-Rad Labs, Richmond, CA) [10]. The purity of the radiolabeled PLP was determined by HPLC using a Spectra Physics 8700XR system with a Vydac 401TP cation-exchange column [11]. The effluent was reacted with sodium bisulfite, and the fluorescence was monitored at 400 nm following excitation at 332 nm. Radioac-


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tivity was determined by liquid scintillation counting. At least 91% of the total fluorescence and 92% of the total radioactivity eluted from the HPLC column at the time corresponding to a PLP standard. The specific radioactivity was 15.6 mCi/mmol.

2.3. *Labeling of PLP binding sites on HSA*

HSA (50 mg) was reacted with an equivalent molar amount of PLP (0.75 $\mu$mol containing 4.2 $\mu$Ci [$^3$H]PLP) in PBS, pH 7.4, for 1 h at room temperature. The sample was then reacted for 15 min with 7.5 $\mu$mol potassium borohydride to reduce the Schiff base formed between HSA and PLP. The sample was dialyzed exhaustively against 25 mM ammonium bicarbonate, pH 8.0, and lyophilized. The protein specific activity (≈ 180,000 dpm/mg) corresponded to a 0.9:1 PLP/HSA ratio.

2.4. *Denaturation, disulfide cleavage, carboxymethylation, and tryptic digestion*

The pyridoxylated HSA was dissolved in 2.5 ml of 1 M Tris-HCl, pH 8.0. After the addition of 0.5 ml of 10 mM EDTA in 0.6 N NaOH, 0.5 ml of 40 mg/ml dithiothreitol, and 2.86 g of guanidine-HCl, the mixture was incubated for 1 h at 37°C. Iodoacetic acid (65 mg) was added, and the sample was incubated in the dark, under $N_2$, for 1 h at 37°C. The carboxymethylated sample was dialyzed exhaustively against 25 mM ammonium bicarbonate, pH 8.0. Protein concentration was estimated by absorbance at 280 nm using a specific absorbance of 0.53 $mg^{-1} \cdot ml^{-1} \cdot cm^{-1}$.

The PLP-HSA (30 mg at 2 mg/ml) was combined with 2.25 ml of 50 mM octanesulfonic acid, 2.25 ml of 0,25 M ammonium bicarbonate, pH 8.0, and 3.0 ml of TPCK (tosylphenylalanine-chloromethyl ketone)-treated trypsin (0.1 mg/ml). The protein was digested for 18 h at 37°C. No turbidity was observed.

2.5 *Separation and purification of radiolabeled peptides*

Radiolabeled peptides were purified on a Waters HPLC system with detection at 225 nm. The tryptic peptides were separated first on a 0.46 × 25 cm Synchropak AX-300 anion-exchange column (SynChrom, Lafayette, IN) using a 30-min linear gradient of 0.02 M to 0.5 M ammonium bicarbonate, pH 8.0, and a flow rate of 1.5 ml/min. Aliquots of the collected fractions were assayed for radioactivity, pooled and lyophilized. The lyophilized sample was dissolved in 0.5 ml of 25 mM ammonium bicarbonate and incubated for 1h at 37°C with 6 $\mu$g of alkaline phosphatase (20 U/mg) to remove the phosphate group. The dephosphorylatedpeptides were resolved again by anion-eschange chromatography. The primary radioactive fractions from the second chromatographic run were purified by reversed-phase HPLC on a 0.46 × 15 cm Zorbax ODS column (Dupont, Wilmington, DE) using a 20-min linear gradient of 10% to 50% acetonitrile (0.1% to 0.0075% trifluoroacetic acid) at a flow rate of 1.0 ml/min.

2.6 *Peptide sequence analysis*

The purified radiolabeled peptide was sequenced using an ABI (Foster City, CA) 470A/120A gas-phase protein sequencer. Approximately 1 nmol of the major radiolabeld peptide was sequenced. The amino acid sequence was confirmed by amino acid analysis using a Waters Pico Tag System (Milford, MA).

## 3. RESULTS

Fig. 1. shows the purification of a peptide containing the major PLP binding site in HSA. The elution profile of radiolabeled tryptic peptides from anion-exchange HPLC appears in panel A. Fraction 11 contained 42% of the total radioactivity, and no other fraction contained more than 10% of the total. The recovery of radioactivity from each chromatographic run was at least 95%. The equivalent fractions from five separate

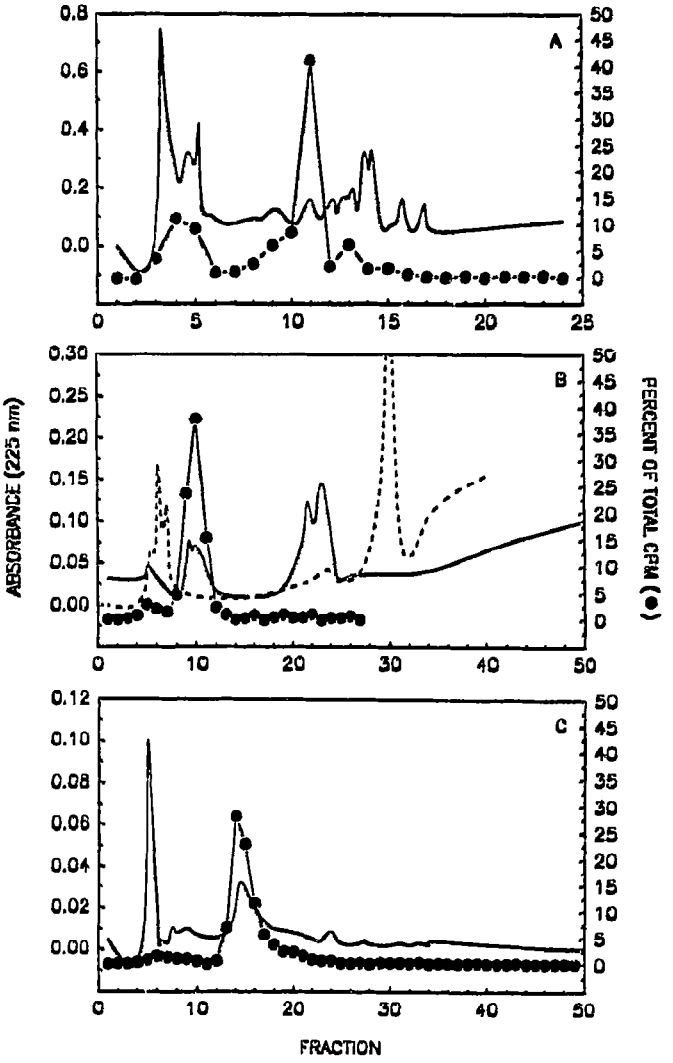


Fig. 1. Purification of [$^3$H]pyridoxylated tryptic peptides of HSA. A: anion-exchange HPLC of total labeled tryptic peptides. Peptides were resolved as described in Section 2, and fractions (1.5 ml) were collected and analyzed for radioactivity. Fraction 11, from 5 separate runs, was pooled, lyophilized, de-phosphorylated and resolved by anion-exchange re-chromatography shown in (B). Dashed line in (B) shows elution of 100 $\mu$g of alkaline phosphatase. Radiolabeled fractions 9–11 (0.5 ml) from re-chromatography were pooled and purified by reversed-phase HPLC in (C). Fractions 14 and 15 (0.5 ml) were collected for sequencing and amino acid analysis.

runs were pooled, lyophilized, and treated wiht phosphatase to remove the phosphate group.

Rechromatography of the dephosphorylated sample separated the major labeled peptide from several unlabeled peptides (Fig. 1B). The PL-peptide had a shorter retention time on the anion-exchange columnt than the PLP-peptide. More than 95% of the total radioactivity was recoverd from reversed-phase HPLC within a single broad band (Fig. 1C). The tow peak fractions were pooled for protein sequencing and amino acid analysis. Protein sequencing (Fig. 2) revealed a homogenous peptide with the sequence Leu-Asp-Glu-Leu-Arg-Asp-Glu-


Fig. 2. Protein sequencing analysis of the primary pyridoxylated peptide from HSA. The purified major labeled peptide from Fig. 1C was sequenced as described in Section 2. The number of picomoles of each residue which were recovered during 14 consecutive sequencing steps is indicated. The sequence given in the one letter code corresponds to Leu-Asp-Glu-Leu-Arg-Asp-Glu-Gly-Xaa-Ala-Ser-Ser-Ala-Lys. Residue Xaa corresponds to the radiolabeled, pyridoxylated $Lys^{190}$. No other residue was greater than 10% of the recovered amino acid.

Gly-Xaa-Ala-Ser-Ser-Ala-Lys corresponding to residues 182–195 in the known sequence of HSA [5]. Residue Xaa represents the modified $Lys^{-190}$. An aliquot of the peptide was subjected to amino acid analysis, and the theoretical and experimental amino acid compositions were as follows: Ala (2 residues expected; experimental 1.9), Arg (1; 1.2), Asp (2; 1.7), Glu (2; 2.0), Gly (1; 1.2), Leu (2; 2.0), Lys (2; 1.0), and Ser (2; 2.0). The composition data are consistent with that obtained by peptide sequencing.

## 4. DISCUSSION

We utilized amino acid analysis and protein sequencing of a purified peptide containing [$^3$H]PLP to identify $Lys^{190}$ as the primary PLP binding site in HSA. The isolation of this peptide, which contained more than 40% of the total label, demonstrated resistance to trypsin cleavage at two sites within the peptide: $Arg^{185}$ and the PLP-modified $Lys^{190}$. $Arg^{185}$ may be naturally resistant to trypsin digestion owing to its position in the HSA molecule or, alternatively, the phosphate ester of PLP may interact with $Arg^{185}$ and inhibit cleavage. Incomplete trypsin cleavage at $Arg^{185}$ and nearby sites would result in the production of other radiolabeled peptides containing $Lys^{190}$. This suggests that the 42% PLP labeling we found at $Lys^{190}$ of HSA is a minimum estimate. Several different elution conditions and HPLC columns were used in attempts to resolve the early-eluting, radiolabeled peptides. However, it was not possible to obtain, from these fractions, peptides of a quality suitable for sequencing.

Our investigations failed to identify a PLP binding site in the vicinity of $Lys^{525}$, which is the primary site of nonenzymatic glycosylation (NEG) in HSA. The pK, accessibility, and microenvironment of specific Lys amino groups may determine the sites of Schiff base formation with a particular ligand. A phosphate binding region on HSA has been proposed to play a role in site-specific NEG [12]. Furthermore, it was reported that a high concentration of PLP (10 mM) may block NEG sites of HSA and BSA [13,14]. However, our results indicate that PLP at physiological concentrations would not compete for the primary NEG site of HSA. Regardless, we cannot rule out the presence of a low affinity PLP or phosphate binding site in the region of $Lys^{525}$ since only the major labeled peptide was purified and sequenced.

Equilibrium binding data indicating that HSA has one high affinity and two or more nonspecific sites for PLP [3] are consistent with our identification of one primary site. Sequence differences in the PLP binding sites of HSA ($Lys^{190}$) and BSA ($Lys^{220/223}$) [5] support previous results which demonstrated differences in PLP binding to these albumins [3].

Identification of $Lys^{190}$ as the specific PLP-binding residue on HSA allows for the use of PLP as a site-specific affinity probe of HSA and may permit the design of studies to elucidate the mechanisms which control PLP binding to HSA and regulate human PLP metabolism in normal and disease states.

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