Covalent Linkage of Phospholipid to Myelin Basic Protein: Identification of Serine-54 as the Site of Attachment†

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ABSTRACT: The peptide portion of the lipopeptide isolated from bovine myelin basic protein contained glycine, lysine, and serine in a 2:1:1 molar ratio as determined by amino acid analysis. The N-terminus of the peptide was determined to be glycine. The tetrapeptide Gly_{53}-Ser-Gly-Lys_{56} was the only segment of myelin basic protein that matched the above two characteristics. This tetrapeptide is highly conserved among the myelin basic proteins sequenced so far. After the selective degradation of the lipopeptide, phosphoserine was identified in the acid hydrolysate, thus indicating that Ser-54 of myelin basic protein in bovine brain is the site of attachment of polyphosphoinositide. Interestingly, serine-54 of myelin basic protein can be phosphorylated by the endogenous protein kinase myelin. However, myelin basic protein phosphorylated by the catalytic subunit of an exogenous soluble protein kinase failed to produce radioactively labeled lipopeptide. Hence the endogenous enzymes of myelin are thought to be involved in the formation of the covalent linkage between polyphosphoinositide and myelin basic protein. The conservation in sequence suggests a possible important structural role for the "phospholipidation" of myelin basic protein.

Myelin purified from the central nervous system (CNS) has been shown to undergo phosphorylation. Miyamoto et al. (1974) reported that a soluble protein kinase of brain, which is stimulated by adenosine 3′,5′-cyclic monophosphate (cAMP), phosphorylated myelin basic protein. Besides being a substrate for this soluble protein kinase, the myelin basic protein was also found to be phosphorylated by an endogenous protein kinase of myelin (Carnegie, 1974; Steck & Appel, 1974). This endogenous protein kinase was shown to phosphorylate the basic protein (Carnegie, 1974; Miyamoto, 1976) but not the proteolipid nor the Wolfgram protein. The endogenous kinase was thought to be magnesium-dependent and calcium-stimulated (Petrali et al., 1980 a,b; Sulakhe et al., 1980a; Deshmukh et al., 1984). However, it is still not well characterized.

It has been determined that several amino acid residues in myelin basic protein are capable of being phosphorylated. Threonine-33, serine-54, and serine-109 were all shown to be phosphorylated (Carnegie, 1974), by specific kinases. The endogenous kinase of myelin phosphorylates serine-54 preferentially, whereas the water-soluble protein kinase of brain phosphorylates serine-109 (Carnegie, 1974).

Myelin contains both phosphatidylinositol kinase and phosphatidylinositol phosphate kinase activities (Eichberg & Dawson, 1965; Jacobelli, 1969; Schacht, 1976). Their products, phosphatidylinositol 4-phosphate (PhIP)[1] and phosphatidylinositol 4,5-bisphosphate ($PhIP_2$), were shown to be much more abundant in brain than in any other tissue. Neither PhIP nor $PhIP_2$ can be extracted into chloroform–methanol (2:1 v/v) unless concentrated HCl is added. This suggests some interaction between the lipid and the myelin (Gonzalez-Sastre, 1970). In the preceding paper in this issue (Yang et al., 1986), we have demonstrated that when myelin basic protein is phosphorylated by endogenous protein kinase and then digested with trypsin or carboxypeptidase Y, or both proteases sequentially, a lipophilic fragment is released. This fragment was shown to contain PhIP and $PhIP_2$ covalently attached. Thus a covalent interaction between the PPI and myelin basic protein has been proposed. This finding supports the observation that myelin basic protein, although a very basic and highly polar protein with only 31% of its amino acid residues either neutral or hydrophobic, can be extracted readily into a chloroform–methanol (2:1 v/v) mixture.

The nature of the peptide moiety and the covalent linkage between peptide and lipid in the lipopeptide was still unknown and is the subject of this study.

Materials and Methods

Materials. Dansyl chloride, trypsin type IX from frog pancreas, carboxypeptidase Y, and phospholipase C type IV from *Clostridium perfringens* (*Clostridium welchii*) were from Sigma. [γ-^{32}P]ATP was from ICN. Myelin was prepared from bovine brain white matter as described by Norton and Poduslo (1973).

Phosphorylation of myelin basic protein and isolation of lipopeptides were the same as outlined in the preceding paper (Yang et al., 1986).

Total Acid Hydrolysis of Lipopeptide. Total acid hydrolysis of lipopeptide was carried out with 4 mL of 6 N HCl in a sealed ampule heated at 110 °C for 24 h. The hydrolysate was then extracted with ether twice and dried by rotary evaporation. The HCl was removed with several additions of methanol to the dried hydrolysate and dried by rotary evaporation.

Amino Acid Analysis. Amino acid analysis was carried out on a Beckman 120C amino acid analyzer.

Identification of N-Terminal Amino Acid by Dansylation. The process was modified from the procedure by Hartley (1970). The lipopeptide was first treated with 0.5 N KOH

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[1] Abbreviations: HPLC, high-performance liquid chromatography; PPI, polyphosphoinositide; PhIP, phosphatidylinositol 4-phosphate; $PhIP_2$, phosphatidylinositol 4,5-bisphosphate; DNS (dansyl), 5-(dimethylamino)naphthalene-1-sulfonyl; TLC, thin-layer chromatography; PEI, poly(ethylenimine); MBP, myelin basic protein.

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for 30 min at room temperature to remove the acyl group. The solution was then extracted with ether twice, and the aqueous phase was neutralized with Dowex 50-4X 200-mesh hydrogen form. The Dowex 50 was then removed by gravity filtration. The filtrate was dried by rotary evaporation and redissolved in 0.5 mL of 0.2 M sodium bicarbonate in a small test tube and lyophilized. Deionized water (0.5 mL) and dansyl chloride (0.5 mL; 2.5 mg/mL in acetone) were then added to the dried test tube to give a 1-mL final solution containing 5 mM dansyl chloride and 50% (v/v) acetone, pH 9.8. The test tube was sealed with Parafilm and incubated for 1 h at 37 °C. At the end of incubation, the solution was evaporated and redissolved in 2 mL of 6 N HCl. The solution was transferred, sealed in an ampule, and heated at 110 °C for 18 h. The hydrolysate was dried by rotary evaporation. The HCl was removed with repeated addition of methanol and then dried by rotary evaporation.

Two-dimensional TLC was carried out on a 10 cm × 10 cm polyamide TLC plate (Schleicher & Schuell). The dansylated hydrolysate was spotted on side 1, and the dansylated amino acid standards DNS-Lys, DNS-Gly, DNS-Ser, DNS-Asp, DNS-amide, and $(DNS)_2$-Lys were spotted on the opposite side of the plate. TLC was carried out in the first phase with 1.5% (v/v) formic acid. After the TLC plate was dried completely in an oven at 110 °C for 10 min, the second phase was performed in benzene–acetic acid (9:1 v/v). The dansylated amino acid was then visualized under UV light in the dark room, and a photograph was taken by using Polaroid 3000 film. A third-phase TLC was also run in solvent containing ethyl acetate–methanol–acetic acid (20:1:1 v/v/v) in the same direction as that of the second phase, and the photograph was taken of the dansylated amino acid under UV light.

β-Elimination of Lipopeptide. The removal of PPI from lipopeptide via β-elimination was modified from the method outlined by Toyama et al. (1983) (Figure 4):

(*A*) *Phospholipase D Treatment.* The lipopeptide was suspended in 2 mL containing 15 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES), pH 5.6. Then 1.5 units of phospholipase D was added, and the solution was incubated at 37 °C for 1 h and extracted twice with an equal volume of ether.

(*B*) *Phosphatase Treatment.* The aqueous layer from the previous step was dried by rotary evaporation and redissolved either in 2 mL of 10 mM sodium acetate, pH 4.0, or in 2 mL of 10 mM sodium borate, pH 10.0. Acid phosphatase or alkaline phosphatase (1.5 units) was added to the respective solution, and incubation was carried out at 37 °C for 30 min.

(*C*) *Sodium Periodate Oxidation.* To the inositol peptide solution, an equal volume of 1 mM sodium periodate was added. After incubation for 60 min in the dark at room temperature, a 5-fold molar excess of ethylene glycol relative to periodate was added to destroy unreacted periodate.

(*D*) *β-Elimination.* The oxidized peptide solution was either (1) adjusted to pH 11 with 1 N NaOH and incubated at 50 °C for 60 min or (2) treated with 0.3 M aniline (pH 5.3) for 3 h at room temperature for β-elimination.

(*E*) *Hydrolysis.* To the NaOH-treated solution was added an equal volume of 6 N NaOH. The alkaline solution was sealed in an ampule and heated at 110 °C for 30 min (Toyama, 1983). The solution treated with aniline was adjusted to 2 N HCl and heated in a sealed ampule at 110 °C for 1 h. The phosphohydroxy amino acids were stable under this condition (Congote, 1982; Rothberg et al., 1978). The hydrolysate was neutralized either by removing the HCl with several additions of methanol and then drying with rotary evaporation or by the addition of Dowex 50-4X 200-mesh hydrogen form.

Table I: Summary of Amino Acid Analysis of Lipopeptide II[a]

amino acid	relative area to Lys	ratio
Ser	0.785	1
Gly	1.745	2
Ala	0.209	
Ile	0.179	
Leu	0.248	
Lys	1.000	1
NH_3	58.942	

[a] Amino acid analysis of lipopeptide II was performed on a Beckman 120C amino acid analyzer. The integrated area under the peak of lysine was used as a reference. The table contained amino acids with their relative area (to that of lysine) larger than 0.178. The "ratio" of each amino acid was the relative area that had been rounded off to unit value. Serine eluted at 32.5 min, glycine at 54.7 min, and lysine at 151.6 min.

HPLC Analysis of Phosphoamino Acids. P–O type phosphoamino acids were separated by HPLC using an anion-exchange Partisil-10 SAX column as described previously (see preceding paper in this issue). P–N type phosphoamino acids were analyzed by HPLC using a Chromex anion-exchange column eluted with 15 mM potassium phosphate, pH 7.5. Fractions of 1 min were collected, and the radioactivity was measured.

TLC System for P–O Type Phosphoamino Acids. Thin-layer chromatography was performed on cellulose NM-300 plates (Macherey-Nagel) developed in 1-butanol–2-propanol–formic acid–water (3:1:1:1 v/v/v/v) (Ushiro & Cohen, 1980).

Results

Amino Acid Analysis of Lipopeptide II. Three preparations of lipopeptide II were isolated as outlined in the preceding paper (Yang et al., 1986) and hydrolyzed in 6 N HCl. The resulting hydrolysate was analyzed for its amino acid content by employing a Beckman-120C amino acid analyzer. Glycine, serine, and lysine were the three major amino acids contained in the lipopeptide, and their molar ratio is Gly:Ser:Lys = 2:1:1 (Table I). The only tetrapeptide in myelin basic protein containing the correct ratio of Gly:Ser:Lys is the sequence Gly-Ser-Gly-Lys (residues 53–56) in the bovine myelin basic protein. Amino acid analysis of lipopeptide I gave a similar result (data not shown), and we believe it differs from lipopeptide II possibly by its fatty acid content. The additional alanine, leucine, and isoleucine that were seen in the analysis may indicate a small contamination of another lipopeptide or incomplete digestion to the tetrapeptide.

Determination of N-Terminus of Lipopeptide II. In order to confirm the lipopeptide sequence to be the tetrapeptide above, the peptide was first reacted with dansyl chloride and then acid hydrolyzed. The hydrolysate was then analyzed by two-dimensional TLC (Figure 1) as outlined under Materials and Methods. The standards DNS-Asp, DNS-Gly, ε-DNS-Lys, DNS-Ser, DNS-amide, and $(DNS)_2$-Lys were spotted on the opposite side of the TLC plate. The dansylated lipopeptide hydrolysate gave rise to several spots under UV light. The one labeled with "X" comigrated with DNS-Gly. The spot labeled with "Y" is hydrolyzed dansyl chloride. The two faint spots near the solvent front of the second-phase TLC did not comigrate with any standard and were believed to be DNS-NH_2. The N-terminal amino acid residue was thus identified as glycine. This supported the finding that the tetrapeptide Gly-Ser-Gly-Lys was the site of the covalent attachment of the lipid. It is not clear why ε-DNS-Lys did

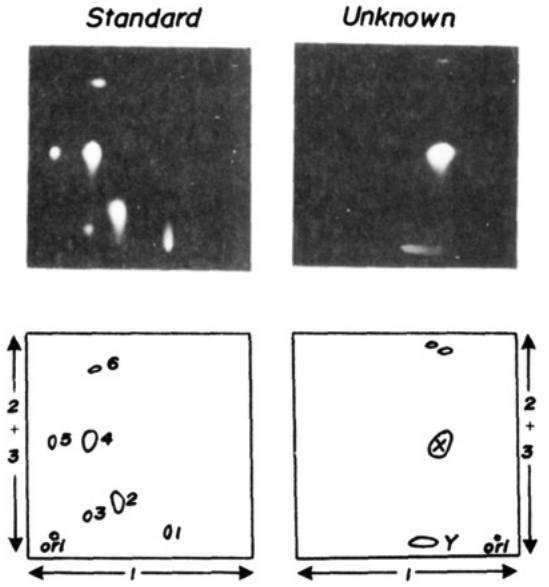

FIGURE 1: Dansylation analysis of the N-terminal amino acid of the peptide moiety in lipopeptide II. Lipopeptide II (unknown) was reacted with dansyl chloride as outlined under Materials and Methods. The sample was then analyzed by two-dimensional TLC using a polyamide TLC plate. TLC was carried out in the first phase with 1.5% formic acid, then in the second phase with benzene–acetic acid (9:1 v/v), and finally in the third phase with ethyl acetate–methanol–acetic acid (20:1:1 v/v/v) in the direction of second-phase TLC as described under Materials and Methods. Standard contained (1) DNS-Lys, (2) DNS-Ser, (3) DNS-Asp, (4) DNS-Gly, (5) DNS_2-Lys, and (6) DNS-amide.

not show up clearly, although it is possible that it was masked by DNS-NH_2 and DNS-OH.

Identification of the Site of Attachment of PPI in Myelin Basic Protein. Both lysine and serine in the tetrapeptide are possible sites for the covalent attachment of PPI with myelin basic protein. However, phosphoryl bonds to lysine or to serine will have very different stabilities to acid or base treatments. The P–O type phosphodiester bond is relatively base labile. On the other hand, a phosphoramide linkage to lysine would be very acid labile and generally base stable. Figure 2 illustrates the strategy of investigating the site of the attachment. Neutral or acidic conditions were maintained during the search for phosphoserine, and neutral or basic conditions were maintained during the search for phospholysine.

The lipopeptide was treated with phospholipase D and then extracted with ether, and any monoester phosphate was removed by treating with either acid phosphatase or alkaline phosphatase. Sodium periodate oxidation of the putative inositol–peptide was followed by β-elimination with either KOH (basic condition) or aniline (neutral condition). After acid hydrolysis, the hydrolysate was analyzed either by PEI TLC using a cellulose NM-300 plate or by HPLC using an SAX anion-exchange column. [^{32}P]Phosphoserine was identified by both SAX HPLC (Figure 3) and TLC (Figure 4). In the search for phospholysine under neutral or basic conditions, neither HPLC nor paper electrophoresis showed the presence of phospholysine. These results indicate that PPI is attached to serine-54 of the myelin basic protein from bovine brain through a phosphodiester bond (Figure 5).

Discussion

Characterization of lipopeptide II demonstrated the presence of glycine, lysine, and serine in a ratio of 2:1:1. Glycine was further identified as occupying the N-terminus of the lipopeptide by the dansylation method. These results suggest that a unique tetrapeptide in myelin basic protein, Gly-Ser-Gly-Lys,

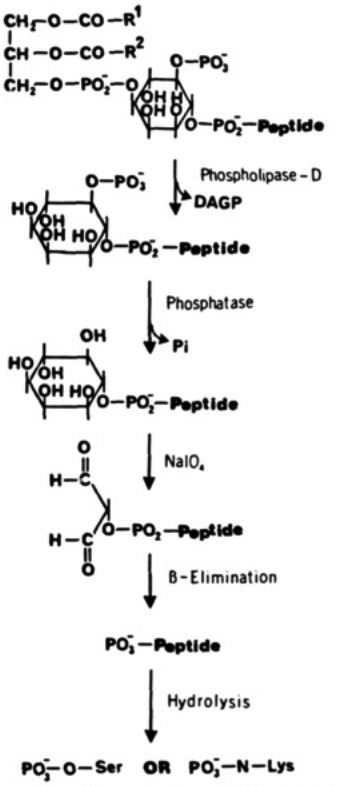

FIGURE 2: Strategy for determination of the attachment site of PPI in myelin basic protein. The strategy to determine the site in the myelin basic protein to which PPI was covalently attached was outlined. For the identification of phosphoserine, the reactions were performed under neutral or acidic conditions where acid phosphatase was used to remove the monoester phosphate and aniline was used for β-elimination. For the identification of phospholysine, the reactions were performed under neutral or basic conditions where alkaline phosphatase was used to remove the monoester phosphate and aniline was used for β-elimination.

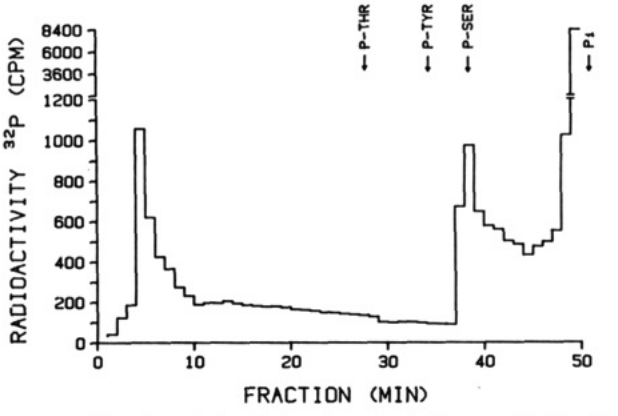

FIGURE 3: HPLC analysis of phosphoserine in lipopeptide II. Lipopeptide II was first treated with phospholipase D and acid phosphatase, then oxidized by sodium periodate, then subjected to β-elimination with aniline, and finally subjected to total acid hydrolysis as described under Materials and Methods. The hydrolysate was analyzed by HPLC using an SAX anion-exchange column. The column was eluted with 10 mM sodium phosphate, pH 3.0, and the flow rate was set at 1 mL/min. Fractions of 1 min were collected and were counted on a Beckman LS-100 liquid scintillation counter with 5 mL of Aquasol added.

was released by proteolysis and contained PPI covalently attached. A similar amino acid sequence was found in the lipopeptide I fraction as well. We believe that lipopeptides I and II differ in their lipid components.

A phosphodiester linkage between phosphatidylinositol phosphate and serine-54 would be expected to be labile to dilute acid and dilute base (D. M. Brown, personal commu-

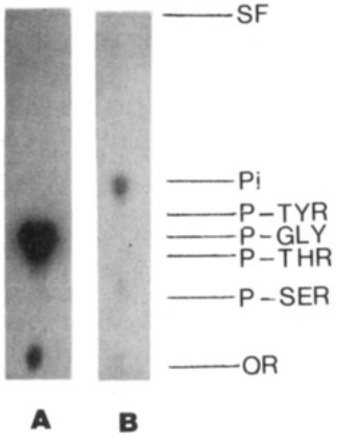

FIGURE 4: TLC analysis of phosphoserine in lipopeptide II. Lipopeptide II was first treated with phospholipase D and acid phosphatase, then oxidized by sodium periodate, then treated with aniline, and finally subjected to total acid hydrolysis as described under Materials and Methods. The hydrolysate was analyzed by TLC using a cellulose NM-300 plate as outlined under Materials and Methods. The autoradiography was taken using a Du Pont Cronex 4 X-ray film with an intensifier. P-GLY was glycerol phosphate obtained from a glycerol kinase reaction employing [γ-^{32}P]ATP. (Lane A) ^{32}P-labeled glycerol phosphate; (lane B) hydrolyzed ^{32}P-labeled lipopeptide.

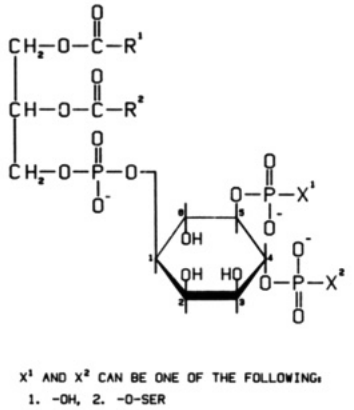

FIGURE 5: Models for attachment of $PhIP_2$. The proposed models for phospholipid attachment to myelin basic protein involve the covalent link of $PhIP_2$ to serine-54 of the MBP.

nication) just as is the phosphodiester bond between glycerol phosphate and methanol (Brown & Todd, 1955). Thus dilute acid treatment would easily remove PPI from myelin basic protein and would change its solubility properties in $CHCl_3$ and methanol, as is observed. Nonetheless, treatment of such a phosphodiester with strong acid such as is used in the hydrolysis of peptides during the search for phosphohydroxy amino acids would quickly cause cleavage randomly on either side of the phosphorus atom, yielding phosphoserine as an apparent product at least 50% of the time.

The phosphoserine of the lipopeptide corresponds to serine-54 of bovine myelin basic protein and is reported to be the major amino acid residue phosphorylated by endogenous protein kinase, but not by soluble protein kinase (Carnegie, 1974). Thus the covalent attachment is probably mediated in part by the action of the endogenous protein kinase. This was confirmed by the fact that phosphorylation of myelin basic protein by the catalytic subunit of the soluble protein kinase did not yield lipopeptide after proteolysis. The factors that regulate the activity of the endogenous protein kinase and the molecular details of the attachment of phosphatidylinositol phosphate are still unknown.

The tetrapeptide sequence Gly-Ser-Gly-Lys was found to be conserved among the myelin basic proteins of human, rat, bovine, sheep, rabbit, chimpanzee, monkey, and guinea pig (Dunkley et al., 1974; Westall et al., 1975; Shapira et al., 1971; Carnegie et al., 1984). This conservation in sequence indicates a possible important functional role in myelin basic protein. In the previous paper (Yang et al., 1986), we demonstrated that lipopeptides of similar properties can be generated from both rat and bovine myelin basic proteins. It is thus expected that such a covalent linkage between myelin basic protein and PPI may be a general phenomenon among animal species possessing myelinated nervous systems.

According to present evidence (Braun, 1984) myelin basic protein is considered to be an extrinsic protein having one or more domains in limited contact with the hydrophobic interior of the membrane bilayer. Further, it is located exclusively at the cytoplasmic appositions. If one analyzes the sequence of either human or bovine myelin basic protein for membrane binding tendency by the methods of Eisenberg et al. (1984), wherein a moving window algorithm is utilized to detect transmembrane helices in conjunction with a hydrophobic moment plot on which the hydrophobic moment of each helix is plotted as a function of its overall hydrophobicity, one finds no membrane binding tendency of myelin basic protein whatsoever. Thus the covalent attachment of phosphatidylinositol to serine-54 could provide the anchor that would permit membrane association of this polar protein.

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Steroid-Binding Site of Human and Rabbit Sex Steroid Binding Protein of Plasma: Fluorescence Characterization with Equilenin†

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ABSTRACT: The interaction of the estrogen *d*-3-hydroxy-1,3,5(10),6,8-estrapentaen-17-one (equilenin) with the human and rabbit sex steroid binding proteins (hSBP and rSBP, respectively) has been investigated by using fluorescence and absorption spectroscopy. Equilenin competes for the binding of 5α-dihydrotestosterone. The calculated binding constant of equilenin for rSBP is 1.9×10^7 M^{-1} at 4 °C, which can be compared with the binding constant of 5.7×10^7 M^{-1} reported for hSBP [Ross, J. B. A., Torres, R., & Petra, P. H. (1982) *FEBS Lett. 149*, 240]. The results of fluorescence quenching experiments with the collisional quenchers KI and acrylamide indicate that the bound steroid has limited accessibility to the bulk solvent and that there are no anionic surface groups near the steroid-binding site. The fluorescence excitation spectra of SBP–equilenin complexes are similar to the absorption spectra of equilenin in low-dielectric solvents. The fluorescence emission of the SBP–equilenin complexes, however, exhibits wavelength shifts (red shifts) opposite to those of the steroid in low-dielectric solvents or complexed with β-cyclodextrin (blue shifts) but similar to the red shift produced by addition of the proton acceptor triethylamine to equilenin in cyclohexane. These data indicate that the steroid-binding site of hSBP and rSBP is a nonpolar cavity containing a proton acceptor that participates in a specific interaction, possibly a hydrogen bond, with the 3′-hydroxyl group of the bound steroid.

The sex steroid binding protein (SBP)[1] of vertebrate serum binds sex steroids with high affinity, especially 5α-dihydrotestosterone (DHT) and testosterone, with binding constants on the order of 10^9 M^{-1} at 4 °C (Petra, 1979). Functional and structural properties of SBPs from human, macaque, baboon, and rabbit have been reviewed and compared with each other in several recent papers (Petra et al., 1983, 1986a,b; Turner et al., 1984). There is suggestive evidence that SBP may be involved in the transport of sex steroids into target cells (Bordin & Petra, 1980). In addition, pure human or macaque SBPs have been shown to directly affect the metabolic clearance rate of testosterone in the macaque (Petra et al., 1985). The human (hSBP) and rabbit (rSBP) proteins also bind estrogens such as 17β-estradiol, estrone, and equilenin, but with less affinity than androgens (Mickelson & Petra, 1978; Lata et al., 1980; Ross et al., 1982). Compared with hSBP, rSBP generally exhibits a weaker affinity for estrogens, suggesting evolutionary differences in the steroid-binding site (Petra et al., 1983).

Equilibrium sedimentation studies show hSBP and rSBP to be dimers of about 85 000 and 86 000 daltons, respectively (Petra et al., 1986a). According to sequence analysis of the half-cystine peptides, hSBP is a homodimer (Petra et al., 1986b). In addition, metal-binding studies using the lanthanide terbium indicate that rSBP and hSBP each have four metal-binding sites (Ross et al., 1985). Steroid-binding studies indicate that 1 mol of steroid is bound per SBP dimer (Petra, 1979; Turner et al., 1984; Petra et al., 1986a). On the basis of steroid–SBP stoichiometry and preliminary sequence data, Petra et al. (1983) suggested the interface of the two identical SBP subunits as a candidate for the steroid-binding site.

The present investigation was first to determine if the steroid binds at the protein surface or is buried within the polypeptide structure and second to characterize the specific chemical interactions involved in the steroid binding. Relatively little is known about the detailed structure of the steroid-binding site of different SBPs or about the interactions that account for the specificity and high affinity of steroid binding. Previous

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[1] Abbreviations: SBP, sex steroid binding protein; hSBP, human sex steroid binding protein; rSBP, rabbit sex steroid binding protein; equilenin, *d*-3-hydroxy-1,3,5(10),6,8-estrapentaen-17-one; DHT, 5α-dihydrotestosterone; β-CD, β-cyclodextrin; TEA, triethylamine; PPO, 2,5-diphenyloxazole; HPLC, high-pressure liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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