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Structure and Molecular Biology of Steroid Binding Proteins

THE PLASMA SEX STEROID BINDING PROTEIN (SBP OR SHBG). A CRITICAL REVIEW OF RECENT DEVELOPMENTS ON THE STRUCTURE, MOLECULAR BIOLOGY AND FUNCTION

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Summary—Significant developments have taken place within the past five years on the characterization, molecular biology and function of the plasma sex steroid-binding protein, SBP (or sex hormone binding globulin, SHBG). During the span of that time, amino acid sequences of two SBPs have been established, amino acid residues in the steroid-binding site have been identified, the structure of the human SBP gene has been deduced and evidence for the possible existence of a SBP membrane receptor has been presented. This review covers the salient aspects of these and other developments including a critical analysis of the various proposed models and interpretations with regards to the structure, evolution, molecular biology and function of SBP.

The following is a review covering recent developments within the past five years on the characterization, evolution, molecular biology, and function of the plasma sex steroid binding protein, SBP (or sex hormone-binding globulin, SHBG). The most recent comprehensive reviews on the subject were published in 1986 [1, 2] and in 1988 [3]. The second, dealing mostly with molecular aspects, resulted from a lecture presented by the author at the *1st International Congress on Binding Proteins of Steroid Hormones* held in Lyon, France, in 1986. That review was published in an INSERM edition which unfortunately was not readily available outside France. Subsequently, Moore and Bulbrook [3] published an extensive review on the occurrence and fluctuations of SBP in various physiological states and disease as well as the effects of drugs. These subjects will therefore not be covered here. Three other reviews each dealing mostly with glucocorticoid-binding globulin (CBG), have been published [4–6].

INTRODUCTION

The sex steroid-binding protein of human plasma, SBP was discovered in the middle 1960s [7–9]. The human protein specifically binds 5α-dihydrotestosterone (DHT) and testosterone (T) with high affinity, and 17β-estradiol (E_2) with lower affinity [9–13]. Extensive studies on the binding specificity have been published [14–16]. The protein circulates in the plasma of many species [17, 18] including nonhuman primates [18–26], the dog [27], cat [18, 28], rabbit [13, 29–32], goat and ram [20], cow [17, 18, 33, 34], bat [35, 36], hamster [37], badger, fox and hedgehog [38] and marsupials [39, 40]. The protein is absent in mouse [20, 41] and mature rat plasma [17, 18, 42] although T binding activity has been detected in 6-week-old male rats [43] and evidence has been presented suggesting that fetal rat liver synthesizes and secretes SBP [44]. The protein is also absent in the guinea pig [17, 20] where T is specifically-bound to progesterone binding globulin [45], donkey and horse [17, 18, 20], pig [20, 46] and elephant, boar and camel [18]. The protein has been found in nonmammalian vertebrates such as fish [17, 47–50], amphibians [17, 51–55] and reptiles [17, 56–58]. It seems to be absent in birds [17, 18, 59]. All tested primates contain SBPs that bind both T and E_2 whereas SBPs in other species are mainly androgen binding proteins. The SBP levels and steroid-binding properties in these species, including humans, have been reviewed in detail by Westphal [1].

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The various methods used for measuring SBP in plasma and tissue extracts are also discussed in that review, as well as in the INSERM edition covering the *1st International Congress on Binding Proteins of Steroid Hormones* [60].

PURIFICATION

Early studies suggested the existence of two different sex steroid-binding proteins in human plasma, one specific for T [7, 9, 11, 61–63] and the other for E_2 [8, 64, 65]. However, subsequent studies clearly showed that both steroids competed for the same binding site supporting the existence of only one sex steroid-binding protein [66–68]. That realization greatly facilitated the purification work. By the middle 1970s, a number of laboratories had successfully obtained partially-purified preparations of the protein [65, 69–75]. A method for isolating pure human SBP based on affinity chromatography became available in 1975 [76]. To date, many laboratories have published procedures for preparing pure SBP from human [77–85], monkey [86], baboon [87], bovine [34], canine [88], toad [89] and rabbit plasma/serum [13, 32, 90, 91].

These methods can be divided into two main groups, those that incorporate specific affinity chromatography (DHT or T covalently attached to an insoluble matrix) and those that use immunoadsorbants. The protein isolated from the former is steroid-bound. In some applications this may be a disadvantage requiring steroid removal or exchange by exhaustive dialysis. The protein isolated by the latter methods may contain inactive SBP molecules since selection is not made on the basis of steroid-binding activity (it should be noted that hydrophobic and lectin affinity chromatographies are not considered biospecific separations since they do not purify proteins on the basis of their biological activities). In that case, it is advisable to determine the specific steroid-binding activity by radioassay and total protein measurements if the SBP is to be used for structure–function or physiological studies where the specific activity is of prime importance. Also, because SBP is microheterogeneous, inclusion of preparative isoelectricfocusing in the purification protocol may result in the loss of some SBP species.

PHYSICOCHEMICAL CHARACTERIZATION

Availability of pure protein allowed the author to define the physicochemical properties of four SBPs as shown in Table 1. They are dimeric glycoproteins with native M_r ranging from 84 K to 90 K. The first indication that human SBP is a dimer was presented at the *5th International Congress on Hormonal Steroids* in 1978 [92] and, more recently, amino acid sequence determination showed conclusively that the human and rabbit proteins each contain only one polypeptide chain proving that they are homodimers [93, 94]. This was confirmed by other methods [85, 95]. We can therefore conclude that one gene codes for the SBP molecule and that, very likely, other SBPs have homodimeric structures as well.

The SBP dimeric structure is stable and the subunits appear to be tightly-bound to each other since very little monomer, if any, can be detected in the analytical centrifuge [96]. Also, relatively drastic conditions are required to dissociate rabbit SBP (2.5 M guanidine HCl [97]). Once dissociated, the subunits can reassociate after removal of the denaturant indicating that those conditions do not cause irreversible denaturation. The reconstituted homodimers are

Table 1. Physicochemical characteristics of SBP (Petra *et al.* [87])

	Human	Monkey	Baboon	Rabbit
Native M_r (sedim. equil.)	85,600[a] (±3,000)	85,600	84,100 (±1,000)	85,800[a] (±4,000)
Monomeric M_r (SDS-PAGE)	44,000[a]	47,000	47,000	43,000[a]
Carbohydrate content (%)	14	12	12	9
Dimer polypeptide M_r	73,616	75,328	74,008	78,078
Partial specif. vol. (ml/g)	0.720	0.721	0.723	0.727
Molar extinct. coeff. (10^5 cm[su−]1 M^{-1})	1.14	1.10	1.29	1.27
Steroid-binding site per dimer	0.93	0.64	1.19	0.93
K_d—DHT (nM, 9–12°C)	0.30	0.90	1.19	0.89

[a]These values compare well with subunit polypeptide M_r of 40,386 and 39,796 for human and rabbit SBPs, respectively, as determined from their amino acid sequences [84, 94]. Assuming the carbohydrate contents stated above, the M_r for the native dimeric proteins are 93,400 and 87,404 for human and rabbit SBP, respectively.

active if reassociation is allowed to take place in the presence of T or E_2. In the absence of steroid, homodimers can still reform but a large fraction appears to be inactive. This indicates that a confirmational template, consisting only of steroids that specifically bind to SBP, appears to direct the formation of active dimers. Once formed, T or E_2 can readily dissociate and associate, as they do in plasma, without affecting the dimeric state of the molecule. This is shown by the fact that the pure protein is active after removal of bound DHT while still maintaining a dimeric structure [98]. Since SBP is largely steroid-free in plasma, these findings strongly suggest that the protein exists as a dimer *in vivo* since dissociated monomers would have difficulty associating under nonsaturating T or E_2 concentrations, a condition prevalent in plasma. Furthermore, the data suggest that the presence of T or E_2 might be important during biosynthesis of SBP to allow proper folding and dimerization.

THE AMINO ACID SEQUENCE

The primary structures of human and rabbit SBP [84, 94] are shown in Fig. 1. The human monomer consists of a polypeptide of 373 amino acid residues containing 3 oligosaccharide side-chains and 2 disulfide bonds. Two oligosaccharide side-chains are *N*-linked to Asn^{351} and Asn^{367}, and the other is *O*-linked to Thr^{6}. The rabbit monomer is shorter by 6 residues, has the *N*-linked side-chains at Asn^{345} and Asn^{361}, but lacks the *O*-linked side-chain. Both monomers contain only one tyrosine residue. Location of the two disulfide bonds in human SBP was established by peptide analysis

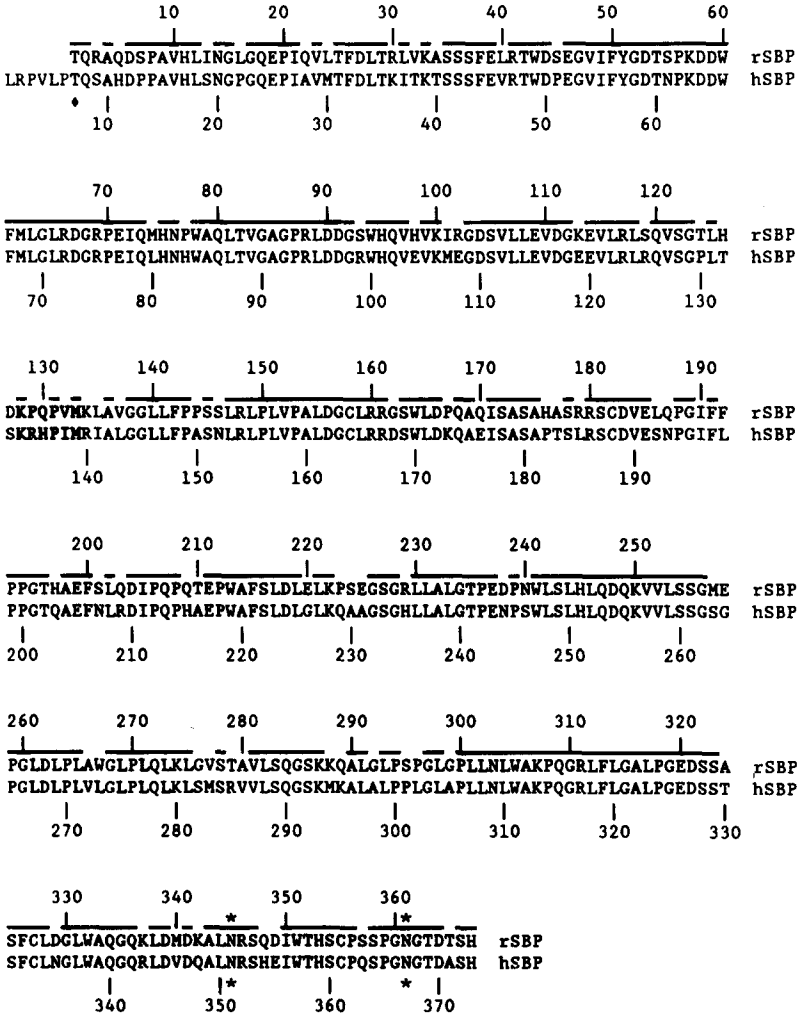

Fig. 1. Amino acid sequences of human and rabbit SBPs [84, 94]. Solid line represents identical sequences. (*) indicates *N*-glycosylation sites and (◆) *O*-glycosylation sites. The segment representing part of the steroid-binding site is shaded, and the alternating leucine segment is indicated (reproduced with permission from the authors).

of unreduced SBP. The data showed that Cys^{164} is connected to Cys^{188} and Cys^{333} to Cys^{361}. The two disulfide bonds of rabbit SBP, connecting Cys^{158} to Cys^{183} and Cys^{327} to Cys^{355}, were placed by mass spectrometry [84]. An interesting sequence can be found in both monomers beginning at Leu^{248} and ending at Gly^{291} (Fig. 1, human numbering). This segment is relatively hydrophobic and contains an internal repeat which is more pronounced in the human protein, and an unusual alternating leucine sequence which is essentially identical in both proteins (residues 267 to 281, human numbering). It was postulated that this segment could represent a portion of the steroid-binding site [99], although recent data indicate another area of the molecule to be involved (see below, [98]), the proposal cannot yet be ruled out. The alternating leucine segments of each subunit could also interact together to form a β-pleated sheet contributing to the stabilization of the dimer. The amino acid sequence of human SBP was subsequently confirmed by cDNA and gene cloning [100–104].*

HOMOLOGY

Comparison of the amino acid sequences of human and rabbit SBPs is shown in Fig. 1. Seventy-nine percent of the residues are identical. Alignment scores using the ALIGN program with the mutational data matrix [105] demonstrate that the two proteins are homologous and have arisen from a common ancestral gene [84]. Since human SBP is homologous to the androgen binding protein (ABP) of rat epididymis [106], the three proteins belong to the same gene family. In fact, cloning experiments indicate that human ABP and human SBP are coded by the same gene [103, 104]. The proteins seem to differ only in their carbohydrate content.

A real surprise came when no homology was found between human SBP and the cDNA-derived sequences of several steroid receptors [94, 107]. This held true when the androgen receptor was subsequently reported [84]. It is clear then that there are at least three different gene families of steroid-binding proteins, the third being represented by CBG which is not homologous to SBP [108]. Since human and rabbit SBPs have steroid-binding specificities very similar to those of the androgen receptor, one would have expected some structural similarity. It should be noted, however, that alignment scoring is obtained by comparing linear sequences and not three-dimensional structures. Since deletion studies suggest that the steroid-binding site of receptors arises from folding of the entire steroid-binding domain [109, 110], a number of residues located at distant positions from each other in the primary structure could be involved in forming the site; if such were the case, any possible homology between SBP and steroid receptors would not have been detected by aligning the sequences. The steroid-binding sites of the two gene families could still be related through a process called *convergent molecular evolution* which has been observed in proteins [111] (see Steroid-Binding Site section below for further discussion). Therefore, even if the steroid-binding sites turn out to be homologous with regards to a few critical amino acid residues, the steroid receptors and SBP are not likely to have similar three-dimensional structures. This reasoning is based on the fact that the amino acid sequence of the SBP monomer is 30% longer than the consensus E domain of steroid receptors which fold into the steroid-binding site [112], and it requires two monomers to form the SBP steroid-binding site (Fig. 2) whereas only one E domain is sufficient to create the steroid-binding sites of receptors. It is therefore tempting to think of the SBP molecule as being large enough to contain other biological information besides that of binding steroids. This point will become apparent when the issue of membrane-binding is discussed (see Physiological Role section below).

Shortly after the discovery of homology between rat ABP and human SBP, Baker *et al.* [113] found that rat ABP was also homologous to bovine protein S, a vitamin K-dependent protein [114] which had just been sequenced [115]. When the sequence of human protein S was reported [116, 117], human SBP was found to be homologous to the carboxy-terminus of both bovine [100] and human protein S [100, 102], as expected. Protein S is thought to function in the blood clotting "cascade" as co-factor for activated protein C, a protease that degrades factor Va and VIIIa [118, 119]. It also appears to serve as a regulating factor of the complement system [120]. Unlike the other

*Sites of post-translation modifications such as disulfide bonds and oligosaccharide side-chains cannot be assigned from deduced cDNA sequences. This is particularly important for oligosaccharide side-chains because consensus sequences are not always used by glycosylating enzymes.

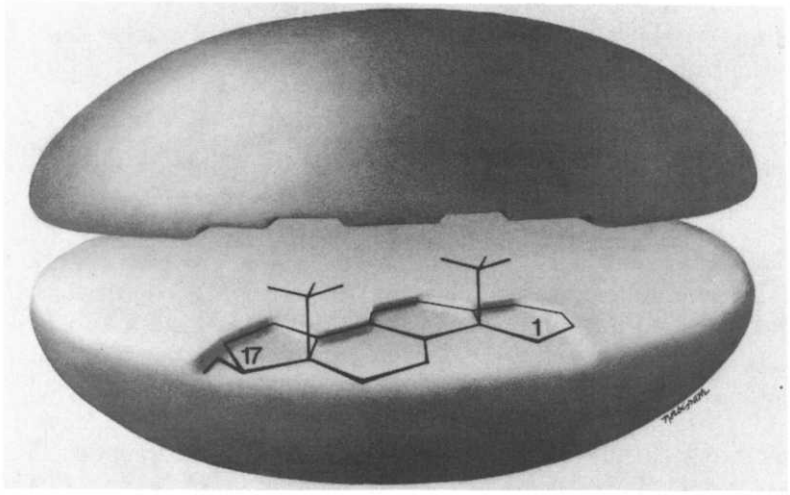

Fig. 2. Hypothetical representation of the SBP molecule.

vitamin K-dependent clotting factors which contain a serine protease sequence at the carboxy-terminus, that sequence in protein S is replaced by one that is homologous to SBP. Interestingly, the polypeptide segment of human SBP thought to be part of the steroid-binding site, Pro^{130}–Leu^{143} (see below), is not homologous with the corresponding sequence in protein S [99], and recent data show that purified human protein S does not bind DHT or cross-react with antihuman SBP (B. Que, G. L. Long and P. H. Petra, unpublished data). Therefore, the biological significance of the homology remains unknown.

THE MICROHETEROGENEITY PROBLEM

The proposed homodimeric structure of SBP should predict the existence of only one band in SDS-PAGE. Yet, many laboratories have reported that the pure protein displayed microheterogeneity. A heated debate was then precipitated in an attempt to explain the observed microheterogeneity. Since most glycoproteins are microheterogeneous much effort, particularly from the laboratory of Strel'chyonok and Avvakumov [6], has been directed at establishing the carbohydrate structure of SBP. They showed that the human protein contains two biantennary *N*-linked and one *O*-linked sugar chain. These were subsequently placed in the primary structure by protein sequencing methods [94] (Fig. 1). Although the microheterogeneity could be explained by variability in sugar content, determination of the amino acid sequence revealed two other factors which appeared to contribute: variability in the amino acid sequence at the amino-terminus and chemical modification arising from base-catalyzed rearrangement of Asn-Gly bonds [94].

The microheterogeneity then arises from the following: presence of a significant number of SBP monomers migrating as three SDS-PAGE bands, one major at about 44 K, and two minor ones containing monomers with either slightly higher molecular weights or slightly lower [87, 121]. There are minor variations in the reported molecular weights but the SDS-PAGE patterns are the same. The higher molecular weight third band is not always observed [81, 83, 85, 98, 121], it is present in about 20% of the population as determined on 121 individuals of both sexes [121]. The presence of this SDS band therefore depends on the plasma source used to purify the protein and likely represents genetic variants. It is important to realize that each band is composed of a mixture of monomers and not a pure monomer as shown by two-dimensional electrophoretic techniques [85]. Therefore, the terms "heavy" and "light" protomer adopted by some authors for both SBP and ABP correspond to "heavy" protomers and "light" protomers. This has been a source of confusion in the past because the heavy–light protomer terminology implied that SBP was composed of two nonidentical subunits, coded by two different genes [122]. Clearly, this is not the case [94]. A recent experiment on cell-free translation of human liver mRNA using a wheat germ system shows SBP migrating as a single band in SDS-PAGE at 42,000 [123], a value similar to 40,449 obtained from the polypeptide sequence of the SBP monomer [94]. This observation is consistent

with carbohydrate variability as a major contributor to the microheterogeneity. Dimerization of microheterogeneous monomers should lead to the formation of many SBP molecules having different mobilities in native PAGE and isoelectricfocussing. From 5 to 12 different species of human and rabbit SBPs have been detected by the latter method [32, 85, 96, 121].

In conclusion, there are three oligosaccharide attachment sites in human SBP and two in rabbit SBP (Fig. 1). The microheterogeneity arises from variability of the monosaccharide content of these oligosaccharides since the structural data obtained by protein sequencing methods do not support the existence of other oligosaccharide attachment sites. Because the protein is purified from pooled plasma/serum, the monosaccharide content will vary from preparation to preparation as reflected from SDS-PAGE and focussing patterns. Furthermore, it is likely that sugar residues are removed during collection, storage and processing of plasma/serum prior to purification adding to the complexity of the final product. In any case, the microheterogeneity problem of SBP is now well-understood and all existing data are still consistent with the proposition of a homodimeric model.

Whether the carbohydrate side-chains play a role in steroid-binding is still unclear. Most of the results suggest that they do not. However, this conclusion is based on data obtained with neuraminidase and other glycosidases that do not remove entire carbohydrate side-chains. More recently, *N*-glycanase and *O*-glycanase (Genzyme Corp.), enzymes that cleave oligosaccharides at asparagine and serine/threonine, have been used with some success [85]. These enzymes deglycosylate most efficiently under *denaturing* conditions, and there lies the difficulty since SBP must remain active in order to assess the role of sugar on steroid-binding activity. So far, it has not been possible to remove the entire carbohydrate side-chains of rabbit or human SBP without destroying their native structures and even under these conditions sugar still remains [85]. Resistance to enzyme attack suggests that the side-chains may be partially buried in the interior of the protein. Thus, unless mild enzymatic procedures are found which completely deglycosylate SBP, a clear understanding of the role of the oligosaccharide chains in either the steroid-binding process or any other function will have to wait until a full-length SBP–cDNA clone can be expressed in bacteria.

THE PROPOSED MOLECULAR STRUCTURE

All the structural and most of the steroid-binding data obtained within the past ten years led to the proposal of a working model for the structure of SBP [96] which is depicted in Fig. 2. The model consists of a homodimer of noncovalently-bound subunits containing one steroid-binding site. Although, the subunits are believed to be held tightly through protein–protein interaction, the steroid appears to contribute to the energy of dimerization [97]. The main evidence for the binding stoichiometry of 1 mol steroid/mol SBP dimer comes from Scatchard analysis [87]. The fact that the dimer is made up of identical subunits binding 1 mol of steroid provides an argument for placing the steroid at the interface between the subunits [96]. Inserting the steroid in the binding site with C-17 and C-1 facing to the outside of the molecule is suggested by SBP binding to agarose derivatives (used in purification) containing DHT attached at either C-17 [77] or C-1 [78] meaning that that surface of the steroid must be exposed when bound in the site.

Another model was also proposed in which the steroid binds to one subunit, inducing a conformation change in the other, and preventing the binding of a second mole of steroid [99]. Evidence for this negative co-operativity model came from steady-state steroid-binding analyses in polyacrylamide gels [124]. SBP preparations which had been kept frozen for extended periods appeared to bind more than 1 mol steroid per dimer. This suggested an irreversible change in the conformation abolishing negative co-operativity and allowing 2 mol of steroid to bind. The binding data using an improved filter assay [98], however, did not support that interpretation. All pure SBP preparations tested did not yield values higher than 1 mol steroid-bound per dimer. Since it is difficult to rationalize a biological role for negative co-operativity of sex steroid-binding in plasma, it is doubtful that this model is an adequate representation of the native molecule.

THE STEROID-BINDING SITE

As indicated in the Introduction, extensive studies have been published defining structural features in the steroid nucleus that are required

for efficient binding to human SBP. The salient features are: a planar steroid nucleus, a 17β-OH group, and a ketone or hydroxyl-group at C-3 (α or β). Changes in any of these leads to a dramatic decrease of binding activity. Also, presence of a group at C-11 eliminates binding. DHT is the best natural ligand, it binds twice as strongly as T and about ten times better than E_2. Among all steroid derivatives tested, there are some that are particularly interesting because their ability to bind in relation to their structure reveals specific information on the topology of the steroid-binding site. They can be divided into two groups: those modified in the A-ring and those in the D-ring. The first group includes 2-methoxyestradiol [125], 2-iodoestradiol [126], 1α-carboxy-methyl-DHT [78] and danazol [127]. These are modified at either C-1 or C-2 and yet exhibit binding as tight as DHT, except for danazol which contains a fused ring at C-1 and C-2 and binds only ten times less than estradiol. 2-Hydroxyestradiol, however, does not bind [125]. The second group can accommodate a substitution at C-17α without affecting the binding affinity, they include 17α-methyl-DHT and 17α-methyl-T [128], and 17α-hexanoic-DHT [77]. The conclusion reached from these data is that the steroid-binding site is "open" at both ends and exhibits a certain structural flexibility in those regions. The consequence is that affinity labels containing reactive groups in rings A and D are likely to be effective probes for studying the structure of the binding site (see below). Moreover, those are the characteristics that make it possible to attach linker "arms" at C-17α or C-1 for synthesizing steroid–agarose derivatives useful in affinity chromatography [77, 78, 80, 81, 83].

Although much has been learned about the steroid requirements, very little is known about the protein determinants involved in the binding process. Because all steroid hormones have a common structure, it seems reasonable to expect that all steroid-binding sites should contain common structural elements. This is the main reason for presenting the argument of convergency in the evolution of the three families of steroid-binding proteins, as discussed above. In addition, unique features should exist within these sites to explain the molecular basis of steroid-binding specificity. One of the first experiments designed to answer such questions was carried out with the fluorescent estrogen, d-1,3,5(10),6,8-estrapentaene-3,17β-diol (dihydroequilenin) [129]. The results indicate that the SBP steroid-binding site is a low-dielectric cavity containing one or more proton acceptors that interact with the C-3 hydroxyl group of equilenin [130].

Physical methods are useful for learning about protein structure, but other approaches such as affinity labeling are needed for assigning functional roles to amino acid side-chains. Since the amino acid sequences of human and rabbit SBP are known, this approach becomes especially powerful because functional amino acid residues can be placed within the primary structure. It then becomes feasible to "map" the site using different labels. A number of affinity labels have been used with steroid-binding proteins [131]. One is the alkylating reagent, 17β-[(bromoacetyl)oxy]-5α-androstan-3-one (DHTBr), which was shown to specifically label yeast 3α,17β-hydroxysteroid dehydrogenase [132, 133], rat liver 3α-hydroxysteroid dehydrogenase [134] and the androgen receptor [135, 136]. Another is Δ^6-T, a photoaffinity label which specifically inhibits rat ABP [137], rabbit SBP [32, 138] and human SBP [99, 139]. In the case of human SBP this label appears to bind covalently to Met^{139} [140], suggesting that this residue is in close proximity to the C-3 of the steroid. The Met^{139} assignment, however, needs to be confirmed since it was made from a peptide recovered in a 1% yield, and other data suggested a different location [99, 101].

Affinity labeling of human SBP with DHTBr led to the identification of Lys^{134} in the steroid-binding site [98].* The proposed mechanism of the labeling reaction is shown in Fig. 3. Only one of the two Lys^{134} residues was found labeled suggesting that the steroid-binding site is constructed through an association of the two subunits in an AB to BA "sandwich" configuration. This interpretation lends support to the hypothesis placing the steroid at the interface between the subunits [96]. This molecular arrangement would explain how the SBP homodimeric structure is able to recognize the asymmetric ligand since the AB/BA configuration would present a difference surface to each

*A different result claiming that DHTBr alkylates His^{235} instead of Lys^{134} in human SBP has recently been published [180]. The data in that report, however, do not support that conclusion. Edman degradation of their radioactive peptide yields PTH-histidine in relatively good yield (30%) at position 235, indicating that His^{235} is not alkylated by DHTBr. Also, alkylation at lysine cannot be ruled out in that work because a carboxymethyllysine standard was not included in their analyses.

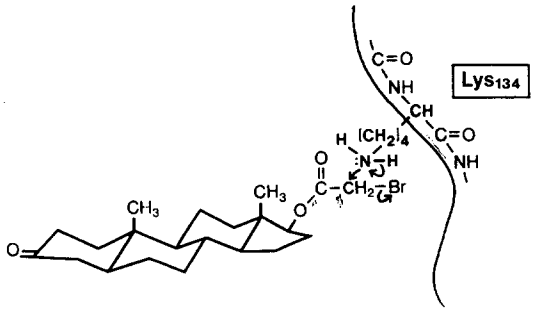

Fig. 3. Mechanism of affinity labeling of the steroid-binding site of human SBP by DHTBr according to Namkung *et al.* [98].

of two faces of the steroid. The data support the location of one Lys[134] near the D-ring, while the other Lys[134] of the other subunit could be either at the opposite end of the site or somewhere else in the protein.

Although the nature of the results does not allow us to define a specific role for Lys[134], its proximity to the 17β-OH of the steroid nucleus nevertheless suggests ways in which it could participate in the binding process, one of which is schematized in Fig. 4. First, the ϵ-amino group of Lys[134] could form a hydrogen-bond with the 17β-OH group providing energy of binding for stabilizing the steroid in the binding site. The hydrogen of the 17β-OH of DHT or T is required for high affinity, derivatives that contain a ketone or an α-H at C-17 do not bind to SBP. This mechanism would require that Lys[134] be mostly unionized at pH 7 and thus have an unusually low pK for a primary amino group. Since the environment of the steroid-binding site constitutes a medium of low dielectric constant [130], the proper condition exists for maintaining such a low pK. A different

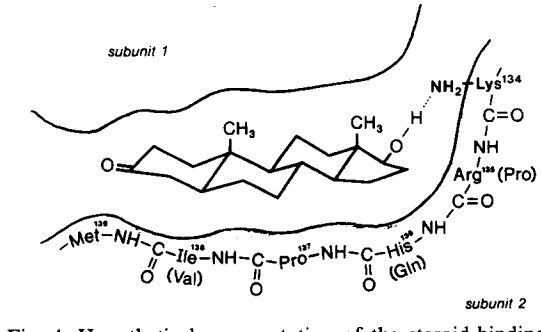

Fig. 4. Hypothetical representation of the steroid-binding site of human SBP. Proposed formation of a hydrogen-bond between DHT and Lys[134]. Another possible mechanism involving Lys[134] as an ion-pair is not shown. The corresponding sequence in rabbit SBP is identical except for the residues in parentheses.

mechanism would involve Lys[134] in a salt-bridge (with a nearby negatively-charged group) which would be required for maintaining the active conformation of the steroid-binding site. Alkylation of Lys[134] would then disrupt the ion-pair and result in loss of activity.

In summary, the data suggest that the polypeptide segment encompassing the sequence—PLTSKRHPIMRIAL—is likely to form part of the steroid-binding site. Preliminary results indicate that rabbit SBP is also inhibited by DHTBr with similar kinetics and stoichiometry (P. C. Namkung and P. H. Petra, to be published). Although the corresponding polypeptide fragment in rabbit SBP has a slightly different sequence (Fig. 4), the lysine and methionine residues occupy the same positions. Interestingly, the binding sites of steroid receptors are thought to contain a methionine residue where the A-ring of the steroid binds [141] and a lysine/arginine residue near the D-ring [142] lending support to the mechanism of *convergent molecular evolution* for the SBP and steroid receptor gene families, as presented in this review (see Homology section above). It should be noted that a number of other sequences in the SBP molecule are apt to be involved in the formation of the site, including a contribution from the other subunit. A consensus sequence first noted in *P*450 enzymes has been proposed to exist in all steroid-binding sites and judged necessary for steroid-binding [143]. The sequence is located between Met[107] and Pro[130] in human SBP (Fig. 1). Although that sequence is well-conserved in both SBPs as well as rat ABP, many other sequences located throughout the molecules are also conserved. Interestingly, the sequence is located next to the segment Pro[130]–Leu[143] now thought to be in the steroid-binding site [98]. Further experiments, however, have indicated that the consensus sequence is not required for steroid-binding [144]. Short of having an X-ray diffraction model, it is clear that more data are needed before we can fully understand what a steroid-binding site looks like and what structural features are responsible for directing steroid-binding specificity.

MOLECULAR BIOLOGY AND GENE STRUCTURE

Exploration into the structure and physiological role of SBP will require the techniques of molecular biology. The possibility of preparing mutant SBP molecules will greatly expand the repertoire of experiments, some of which are

impossible to perform on the pure protein. Within the past several years significant progress has taken place in this area resulting in the cloning and sequencing of a number of SBP-cDNAs and in the determination of the structures of the human SBP and rat ABP genes.

Gershagen *et al.* [100] isolated a λgt11 clone from a human liver cDNA library by screening with affinity-purified antibodies to human SBP. It contained a 1.1 kb insert with an open reading frame coding for 356 amino acids. Except for the first nine residues at the amino-terminus, the deduced sequence is identical to human SBP from Ile^{29} to His^{273} (Fig. 1). The anomalous nine residues were found to originate from an open reading frame in the minus-strand of the 5′-flanking region of exon I (complementary to nucleotides 323 to 351 of the plus strand [103]). They were placed in the coding region as a result of an artifact produced during construction of the library. Two other cDNAs were sequenced in human liver libraries, one containing the entire coding region plus eight residues of the signal peptide [101], and the other a partial cDNA coding for the SBP sequence starting at Gly^{92} [102]. Restriction maps have been defined [100, 101]. Two other partial cDNAs were isolated and sequenced from an Hep G2 cDNA library [B. Que, F. Hagen and P. Petra, unpublished results] one coding for SBP starting at Glu^{115} and the other at Asp^{117}. All these cDNAs contain a potential polyadenylation signal ATTAAA as part of the His^{273} codon and the termination codon TAA, followed by 18 to 30 untranslated nucleotides and a poly(A) tail. These cDNA probes were used to identify SBP-mRNAs of 1.6 [103, 104 [and 2.5 kb [104] in both human liver and testis, and 1.6 kb in Hep G2/H5A [145], a human liver cancer cell line. No message was found in other human cancer cell lines from the breast (MCF-7), endometrium (RL95-2) and prostate (LNCaP) [145]. Recently, a full-length human SBP-cDNA was cloned (F. Hagen, C. Arguelles, P. Seidel, L. M. Sui and P. Petra, manuscript in preparation). It was constructed from three partial cDNAs, two amplified by PCR using Hep G2 poly A selected RNA as a template, and the other a Hep G2 cDNA clone (previously isolated as mentioned above). The 5′ end cDNA fragment was amplified using a sense PCR primer based on the 5′ flanking region of the SBP gene (we thank S. Gershagen, A. Lundwall and P. Fernlund for making the SBP gene sequence available to us prior to publication, see Ref. [103]). After assembling the full-length cDNA, it was moved to a eucaryotic expression vector. Expression in mammalian cells is in progress.

The structure of the human SBP gene was determined independently by Gershagen *et al.* [103] and Hammond *et al.* [104]. The sequence was obtained from 4 [103] and 6.1 kb [104] restriction fragments isolated from human cosmid genomic clones. The gene is organized into eight exons separated by seven small introns (Fig. 5). The first exon codes for a 29 amino

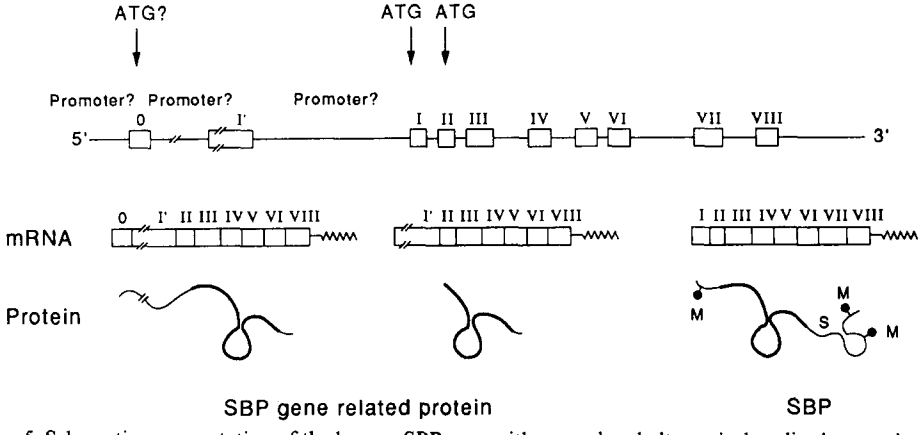

Fig. 5. Schematic representation of the human SBP gene with normal and alternatively spliced transcripts, and translated products, according to Gershagen *et al.* [103]. The "□" represent exons with their roman numerals. Native SBP is shown at the right of the figure containing its oligosaccharide side-chains marked (M) and the alternating leucine sequence marked (S). The other two SBP gene related protein sequences were deduced from alternatively spliced transcripts that lack translated portion of exons I, part of II and VII. The solid line in the proteins represent common sequences to all three, the thin lines reflect differences. I′ and O represent alternate exons postulated to exist upstream of exon I (reproduced and adapted with permission from the authors).

acid signal peptide (MESRGPLATSRLLLLL-LLLLLRHTRQGWA) and continues up to Gln^{8}. For the rest of the amino acid sequence, exon II ends at Thr^{38}, III at Gln^{102}, IV at Pro^{156}, V at Arg^{209}, VI at Gln^{255} and VII at Pro^{324}. Exon IV is believed to represent part of the steroid-binding domain [98], while exon VII may code for a protein-stabilizing structural domain, or perhaps contribute to the binding site. There are sequences in intron 6 and in the 5′-flanking region of the gene which are related to the repetitious *Alu*-type oligonucleotides sequences [103, 104] mainly found in introns of eucharyotic mRNAs and thought to be involved in gene recombination [146]. The 6.1 kb genomic clone contains an additional open reading frame located upstream from exon I and an *Alu*-type sequence [104], the nature of which will be discussed below. Presence of these two sequences were confirmed by primer extension [103]. Southern blot analyses indicate that only one SBP gene exists in the human genome [103, 104]. A distinct SBP gene promoter could not be identified, no TATA or CCAAT boxes were found in the 5′-flanking region of the coding sequence [103, 104]; however, a sequence related to a liver-specific enhancer was identified 115 bp upstream from the proposed Met start site in the 6.1 kb clone [104]. No distinct promoter could be identified in the rat ABP gene as well, although Joseph *et al.* [147] suggest the presence of a somatostatin cAMP regulation site in the 5′-flanking region of the gene. Absence of the liver-specific enhancer in the rat ABP gene perhaps explains why it is not expressed in the liver [104].

Homology between rat ABP and human SBP prompted the screening of human testis cDNA libraries with human SBP-cDNAs to investigate the relationship between the two proteins in the same species. Northern blots indicated that testis mRNAs hybridized with SBP-cDNA probes (see above) setting the stage for determining the sequence of the testis message. Gershagen *et al.* [103] isolated a partial cDNA clone (1.1 kb) and found the sequence to be identical to a previously-isolated liver SBP-cDNA [100] except for a shorter 5′-end (starting at Thr^{31} in exon II) and a shorter untranslated 3′-segment between the stop codon and the poly(A) tail. Hammond *et al.* [104] also found a clone having the same sequence as their previously-isolated human liver SBP-cDNA [101] but missing 116 bp at the 5′ end. Although different ABP and SBP transcripts may be produced, the data indicate that the coding sequences are identical, therefore protein differences must result from post-translational modification. The rat ABP gene also contains 8 exons [147], and comparison with the human SBP gene reveals similar exon/intron boundaries at identical locations and a sequence 5′ of the start codon coding for a 30 amino acid signal peptide. The main differences are in the leader sequence upstream from the start codon and in the size and sequence of introns.

Screening the human testis cDNA library with human SBP-cDNA revealed the presence of other clones markedly different from SBP/ABP-cDNA. Gershagen *et al.* [103] described one having exons I and VII missing (Fig. 5). In addition, a completely different 96 nucleotide open reading frame is spliced to exon II, and splicing of exon VI to VIII causes a shift in the translation reading frame giving rise to a stop codon and a shorter clone. The clone does not have a poly(A) tail but the polyadenylation signal is at the normal position. Sequencing a 0.6 kb genomic restriction fragment placed the 5′-end open reading frame at about 1.5 kb upstream of the normal start codon of exon I. A similar clone was also found in liver libraries. Hammond *et al.* [104] identified and sequenced two clones identical to those described above plus a third one containing a sequence inversion at the 3′ end.

Existence of such mRNAs means that regulation of the SBP/ABP gene may be complex giving rise to a number of different transcripts through alternative splicing. Gershagen *et al.* [103] have suggested that since a distinct promoter could not be identified, untranslated exons may exist upstream where the putative promoter is located. Alternatively, the gene may be regulated by a different mechanism. To be noted is that the translation products coded by these transcripts have not been reported in various preparations of the human protein by either immunochemical or sequencing methods. These authors further suggest that absence of exon VII, implicated in the steroid-binding domain [94] (although now questionable) may give rise to inactive translation products. These could have been lost in affinity chromatography. However, since it is thought that dimerization of monomers is required for the formation of the steroid-binding site (Fig. 2), the entire coding sequence may be needed to generate an active steroid-binding protein. Thus, a likely

explanation for the absence of gene products arising from the translation of altered transcripts is that truncated polypeptides unable to fold properly are degraded by intracellular proteases.

The regulation of SBP biosynthesis is still not well understood. Measurements of SBP levels in various physiological conditions including pregnancy as well as in hormonal therapies and in disease states indicate that E_2 increases the levels of the protein in plasma whereas T does the opposite. However, the possibility exists that these SBP fluctuations may reflect pharmacological rather than physiological effects [148]. Moreover, in Hep G2 cells, the liver cancer cell line found to synthesize SBP [149], both steroids induce its secretion [150, 151, 152], although a negative response had been reported for both [153]. Insulin and prolactin have been shown to inhibit the production of SBP by Hep G2 cells [152] and recently somatomedin-C and other growth factors were proposed as regulators of SBP [148]. The clearest effect so far shown is that of thyroxin since the level of SBP is high in hyperthyroidism [154] and the hormone induces secretion of SBP in Hep G2 cells [152, 153, 155]. It seems then that other hormones besides steroids must be considered as candidates for regulating SBP biosynthesis and secretion.

In summary, molecular biology studies have revealed exciting information and have opened new avenues of investigation on SBP research. Although the molecular basis of SBP/ABP gene expression and regulation is still unknown, the ground work making it possible to obtain the answer has been achieved. Availability of expressible SBP-cDNAs will allow us to design experiments that should clarify our understanding of regulatory mechanisms.

PHYSIOLOGICAL ROLE

A great deal of effort has been spent in trying to understand the role of SBP in sex steroid hormone action. The early ideas of Tait and Burstein [156] suggesting that unbound steroids in plasma incorporate into tissues came just one year before the first report on the discovery of SBP [7]. It was proposed that the newly-discovered protein would function in maintaining the steady-state concentration of unbound sex steroid thereby controlling diffusion of the free fraction from plasma into tissues. Physiological studies supported this idea since the metabolic clearance rate (MCR) of sex steroids in the plasma of males and patients with low SBP levels was faster than in those with high levels such as normal and pregnant females [157, 158] indicating that SBP plays a role in the regulation of MCR of sex steroids. The proposal was further substantiated by studies in monkeys where MCR-T and MCR-E_2 were found to decrease after the infusion of pure human or monkey SBP and to increase after infusion of SBP antibodies [159, 160]. *In vitro* studies also showed that addition of pure SBP to endometrial cancer cells maintained in culture appeared to inhibit the incorporation of steroids [161] supporting the premise that the unbound fraction enters into cells and represents the active form of the hormone.

That interpretation, however, raises some questions. The effect of SBP on sex steroid clearance in plasma is not at all surprising since it is a reflection of its specific steroid-binding activity. But this physico-chemical property does not necessarily rule out a role for SBP in intracellular transport, it is conceivable that the protein could serve both functions as suggested previously [162]. As far as the *in vitro* data on SBP quenching of steroid incorporation is concerned, a different situation exists. If there is a specific SBP membrane receptor, it may be limiting such that addition of a certain concentration of SBP–steroid complex could saturate it to the extent that nonspecific diffusion of steroid into the cells would account for most of the steroid incorporated. The specific process would then escape detection and the rate of steroid incorporation would simply be proportional to the concentration unbound steroid. This scenario is difficult to rule out because the extent of specific membrane binding is unknown and could vary from tissue to tissue under changing physiological states. This situation complicates the experimental design in setting the proper steroid concentration for maximizing the chances of detecting the phenomenon, particularly if the active membrane components are present in very low concentrations. In short, the existing *in vivo* and *in vitro* data cannot rule out a direct role of SBP in the transport of sex steroids across membranes.

What about the data supporting a role of SBP as a trans-membrane steroid transporter? The first indication for such a possibility came from the detection of immunofluorescence in MCF-7 cells exposed to SBP [163]. The results are particularly significant since recently these cells were found to lack SBP-mRNA [145] ruling

out *de novo* synthesis of the protein. Immunofluorescence was also found in prostate, testis, epididymis, liver and adrenal tissue cells [155, 163, 164]. Interpretation of these data, however, is difficult for a number of reasons. First, although antibodies against human SBP were shown not to cross-react with steroid receptors [163] they do so with human ABP [165], SBP's homolog. This protein is synthesized in the testis [166, 167] but its message has also been detected in the human prostate [168]. If ABP is present in this tissue, the immunofluorescence would certainly reflect its presence. Second, the antibodies might recognize degraded SBP following internalization as part of a membrane clearing mechanism, in that case SBP internalization may not necessarily represent a specific process. Nevertheless, the immunofluorescence still implies that specific membrane binding must have occurred prior to endocytosis. The data would then support a different mechanism for SBP-mediated steroid transport which is discussed below.

Strel'chyonok and Avvakumov [169] first proposed that the SBP–steroid complex specifically binds to the outside of the plasma membrane without incorporating intracellularly. They also offered a similar mechanism for CBG-mediated cortisol transport [170]. This new hypothesis localizes the SBP–steroid complex on the cell surface and allows diffusion of the free steroid into the cell to occur at a higher rate than in the absence of SBP. In theory, the process is similar to that proposed by Pardridge [171], which he termed "enhanced dissociation", except that in this case the local steroid concentration is increased by specific binding of the complex, rather than nonspecific membrane interaction inducing dissociation of the steroid. In support of this mechanism, specific binding of ^{125}I-SBP to human plasma membrane fractions has been demonstrated in decidual endometrium [169], placental syncytiotrophoblast [172], benign prostatic hyperplasia [173] and a human prostatic cancer cell line (LNCaP) [174]. Treatment of the prostatic membranes with the detergent [(3-cholamido-propyl)dimethylammonio]-1-propane-sulfonic acid (CHAPS) yields a soluble fraction having two classes of ^{125}I-SBP-binding sites with association constants of $6.8 \times 10^8\ M^{-1}$ and $4.7 \times 10^6\ M^{-1}$ [175]. The binding component in the soluble fraction has an apparent M_r of 167 K as measured by gel filtration on Sepharose CL-6B. These encouraging results support the existence of an SBP membrane receptor and lead the way for further biochemical characterization.

The effect of T, DHT, and E_2 on ^{125}I-SBP-binding to membranes are both interesting and puzzling. Most of the data indicate that steroid-bound SBP binds very tightly to membranes with K_d orders of magnitude lower than steroid-free SBP. Also, binding is steroid specific. For instance, membranes prepared from decidual endometrium, an estrogen-responsive tissue, bind the E_2–^{125}I-SBP complex but not T–^{125}I-SBP [176]. The K_d is about 10^{-12} M (4°C) which is 100-fold lower than the SBP and E_2 concentrations in plasma [169] implying that binding of the complex is dependent only on the SBP "receptor" concentration. A dose response curve shows that membrane-binding increases with E_2 concentration indicating that SBP must be E_2-bound before it can interact [169]. The authors further suggest that E_2 may induce a conformation change in SBP allowing the complex to bind to membranes. This is certainly possible, although recent results indicate that fairly rigorous conditions are required to detect conformational changes in SBP as measured by fluorescence [97], however, subtile conformation changes could be difficult to detect. Similar results were obtained for premenopausal endometrium where the SBP–E_2 complex binds a 100-fold better than SBP alone (from about 10^{-9} M to 10^{-11} M) while T inhibits binding [N. Fortunah; F. Fissore, A. Fazzar; L. Berta, M. Giudili and R. Fhairia: *Steroids* **56** (1991) In press]. Steroid specificity was clearly shown in the case of placental syncytiotrophoblast where the T–^{125}I-SBP and DHT–^{125}I-SBP complexes bind with K_d of about 10^{-12} M [172]. All these results are consistent with the proposed hypothesis.

Rosner *et al.* [174, 177], on the other hand, report that unliganded SBP is the active species that binds to membranes of human prostatic tissue and LNCaP cells, and that membrane-binding is actually inhibited by DHT, T and E_2 in the order of their relative binding affinities to SBP. They propose that the steroid induces a reversible allosteric isomerization of the SBP molecule that prevents it from binding to membranes. In order to explain the conflicting results, they construe that since E_2 does not bind as tightly to SBP as do T and DHT, the allosteric transition is not fully expressed allowing the complex to bind to the membranes. However, this argument falls short in the case of the syncytiotrophoblast data where T–^{125}I-SBP

and DHT–^{125}I-SBP were shown to bind to the membranes with very high affinity [172].

Although the model and interpretations proposed by Rosner *et al.* are interesting, there are a number of problems with them. First, the dissociation constant for unliganded SBP to membranes is orders of magnitude higher than SBP complexed with steroids reported by others. It seems strange that weaker binding should have more physiological meaning than specific high affinity. Second, their model describes a reversible allosteric transition in the SBP molecule induced by steroid which eliminates the binding site recognizing the membrane receptor. Addition of steroid to the membrane-bound unliganded SBP should then promote dissociation of SBP from the membrane. In fact, the opposite is found. Third, the CHAPS-extracted "receptor" binds unliganded SBP with a K_d orders of magnitude lower than the membrane preparations, indicating that detergent treatment may have affected the activity or may have removed a vital component of the "receptor". Fourth, it is extremely difficult to make sure that SBP is steroid-free in the presence of membranes. The tissues from which they were obtained contain varying amounts of endogenous T or E_2 which may bind to the lipid portion of the membranes during their preparation. When steroid-free ^{125}I-SBP is added to the membrane fraction, some steroid–^{125}I-SBP complexes may form and membrane-binding will occur. The same argument applies to whole cells which may contain sex steroids within their intact membranes.

In conclusion, in view of these theoretical and experimental problems, it is more reasonable at this time to interpret the available data according to Avvakumov *et al.* [6, 169] who propose that the incorporation of sex steroids into cells requires the prior formation of the SBP–steroid complex. The model makes biological sense because binding affinity and specificity is directly regulated by the hormone. Part of the "shuttle" mechanism proposed by Avvakumov *et al.* in which steroid receptors are involved in membrane steroid transport can be omitted since steroid receptors have now been localized in cell nuclei [178]. One further point, the issue of whether a membrane steroid transport mechanism exists in those species that do not possess SBP, such as the rat, is still not resolved and the question of specificity of steroid transport in those species still needs to be addressed. Finally, the endocytotic mechanism proposed earlier [163] involving incorporation of the SBP–steroid complex into cells probably reflects a membrane clearing process and it is doubtful that it represents a specific mechanism for incorporating steroids. Recent data showing destruction of specific membrane-binding by pronase treatment support this conclusion [174]. Whether membrane-binding brings about physiological consequences other than steroid transport is still unknown, although there are indications that the intracellular concentration of cAMP seems to increase in LNCaP cells when T–SBP binds [174]. More data are needed to clarify the meaning of that observation.

A few words should be mentioned with regards to the so-called free hormone hypothesis as it applies to the role of SBP in the transport of sex steroids into tissues. As recently reviewed [179], the hypothesis states that the concentration of intracellular sex steroids is affected by the unbound rather than the protein-bound fraction of hormone in plasma. The author further states the possibility that SBP serves no function in plasma and that it binds sex steroids by accident.* The evolution of a single gene for expressing a 95 K protein that binds DHT by accident with a K_d of 0.1 nM and a stoichiometry of 1:1 is inconceivable. Furthermore, discovery of specific binding of SBP to membranes seriously casts doubt on the validity of the free hormone hypothesis as it applies to sex steroid hormone transport in primates and in other species that have SBP. Complicated kinetic models (containing assumptions difficult to prove) have seldom played important roles for elucidating complex biological phenomena. In the end, it is the isolation and characterization of biologically important molecules that is crucial towards understanding physiological processes.

CONCLUDING REMARKS

In the light of the extensive amount of data accumulated within the past 22 years since the discovery of SBP, a certain amount of pessimism was expressed at the close of the *3rd International Congress on Steroid Binding Proteins* (held in conjunction with the *VIIIth International Congress on Hormonal Steroids*, The Hague, 1990) with regards to our continuing

*Quoting from p. 264 of Ref. [179]: "... plasma hormone-binding proteins ... have no function ... they bind their hormones by "accident" like other lower affinity nonspecific binding component in plasma".

inability to define a physiological role. This author does not share that view. Since the 1st meeting held in Lyon in 1986 [60], a great deal of information has been published in a relatively short time as described in this review. Although the information has not yet gelled into a clear definition of SBP function, the nature of the information should significantly help to speed up our understanding.

Much progress on the biochemistry of SBP has taken place within the past four years. The amino acid sequences of two SBPs have been established which allows us to interpret future experiments in terms of structure. Characterization of the steroid-binding site is well under way; short of X-ray diffraction data, the information will provide a basis for "mapping" the site, and will establish general rules on how steroids are recognized by proteins. Knowledge of the human SBP/ABP gene structure opens the way for understanding regulation of the gene in normal and disease organisms. The powerful DNA recombinant techniques can now be used not only to expand our knowledge on the structure of SBP but also to explore its biological role in ways not previously possible. The important discovery of specific membrane interaction will allow us to search for an SBP receptor which, if shown to exist, will have a major impact towards solving the physiological role. The future looks bright.

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