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BINDING STUDIES WITH RECOMBINANT HUMAN SERUM ALBUMIN OBTAINED BY EXPRESSION OF A SYNTHETIC GENE IN YEAST

STEREOSELECTIVE BINDING AND ALLOSTERIC INTERACTION WITH BENZODIAZEPINE AND COUMARIN LIGANDS

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Abstract—The specific ligand binding ability of recombinant human serum albumin produced in yeast using the synthetic gene was studied by affinity chromatographic method. It was found that synthetic protein possesses those stereoselective binding and binding interactions for several chiral benzodiazepine and coumarin compounds which are characteristic of the natural human serum albumin, suggesting identical tertiary structures.

Advances in the methodology of oligonucleotide synthesis and alternative gene assembly methods (see Refs 1 and 2 for reviews) have led to the construction of long DNA molecules representing, for example, a totally synthetic plasmid [3] and an artificial gene coding for human serum albumin (HSA) [2]. The latter has also been expressed in yeast to provide recombinant HSA which was shown to be authentic by immunological and physicochemical analyses [2, 4].

Ligand binding can be considered as a functional test which gives information even about the tertiary structure of the protein. Natural mutants were found [5] to have different binding constants for some specific ligands of HSA. In this work we studied whether the recombinant HSA maintained the stereoselectivity of the two main binding sites of the native protein accommodating benzodiazepine [6], as well as coumarin ligands [7], including their allosteric interaction [8]. These characteristics have strict conformational requirements. The protein was immobilized on Sepharose gel, and the chromatographic retentions of chiral 3-substituted 1,4-benzodiazepines and coumarin compounds were measured. Results were compared with those obtained using native HSA [8–11].

MATERIALS AND METHODS

Chemicals

Benzodiazepine and coumarin compounds were obtained as described previously [8–11]. Recombinant HSA produced by the expression of the artificial genes optimized for yeast expression [4] was supplied by Skandigen AB (Stockholm, Sweden) in the form of culture supernatant. Its protein content was analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis, Coomassie staining and western blot, and the immunoreactive HSA content was found to be 1.86 mg/mL. Natural HSA (fatty acid free) was purchased from Miles Labs (Elkhart, IN, U.S.A.).

Binding studies

Ultrafiltrations. These were carried out in Amicon MPS-1 system, using YMT membranes. Free fractions were determined in duplicates by liquid scintillation counting.

Affinity chromatography. We were looking for an efficient binding method which requires a small amount of protein. HSA immobilized covalently on CNBr-activated Sepharose gel was found to maintain the original binding ability of the protein [8, 12]. Chromatographic retentions of ligands on Sepharose–HSA column indicate the binding affinities. Stereoselective binding manifests itself in the resolution of racemic samples and this affinity chromatographic method can also be used to study binding interactions during simultaneous binding of different ligands.

About 30 mL of culture supernatant were washed by Ringer buffer in an ultrafiltration cell using PM-10 membrane, the buffer was changed to the coupling buffer (0.1 M $NaHCO_3$ pH 8.0), the protein solution was concentrated to 8 mL and passed through a 0.2 μ filter to remove the high molecular mass impurities. It was coupled to about a 5-mL swollen CNBr-activated Sepharose 4B gel (Pharmacia, Uppsala, Sweden) and the remaining active CNBr groups were blocked by glycine. A chromatographic column was filled with the HSA–Sepharose gel, washed with the regular eluent (Ringer buffer, pH 7.4, with 0.01% sodium azide). Ligand samples (10 μL of 1 mg/2 mL stock solution) were applied manually, eluting buffer was driven by peristaltic pump (50 mL/hr), detection was made by continuous UV at 250

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Table 1. Comparison of the specific ligand binding of native and recombinant HSA by ultrafiltration

Protein	Ligand	Free fraction
15 μM Native HSA	15 μM	0.63
14 μM Recombinant HSA*	[^{14}C]Diazepam	0.64
15 μM Native HSA	15 μM	0.45
14 μM Recombinant HSA*	*rac*-[^{14}C]Warfarin	0.47

* Culture supernatant was diluted by Ringer buffer (1:1).

or 310 nm and elution volumes were measured. In case of radioactive ligands fractions were collected and subjected to liquid scintillation counting. Elution volumes were measured from a minimum of two chromatographic runs.

RESULTS

The culture supernatant was tested for the binding of diazepam and warfarin ligands which are markers of the two main binding sites on HSA [13]. A natural HSA solution of similar concentration to the culture supernatant (2 mg/mL Ringer buffer) was prepared. Binding affinities for [^{14}C]diazepam and *rac*-[^{14}C]-warfarin were measured by ultrafiltration and the corresponding free fractions of the two HSA-ligand solutions were compared. Results seen in Table 1 show very good agreement.

Chromatographic results

Binding of benzodiazepines. It is known that HSA stereoselectively binds the 3-substituted 1,4-benzodiazepines in favour of the (*S*)-enantiomers [6]; the measure of stereoselectivity depends on substitution [8, 9]. Table 2 summarizes the elution volumes obtained on the recombinant HSA–Sepharose column for a series of chiral benzodiazepines measured just after the immobilization, after some washing and after the regeneration of the column. Data obtained previously on a native HSA–Sepharose column [8] are also given. It can be observed that the immobilized recombinant HSA did resolve the racemic samples, i.e. stereoselectively binds these compounds. The $R < S$ elution order was established with oxazepam hemisuccinate enantiomers. We assume that analogous to native HSA [6, 8], the first eluted enantiomer with V_1 elution volume belongs to the (*R*)-, while V_2 to the (*S*)-enantiomer, in all cases in Table 2. It is to be noted that at the beginning of the chromatographic study the results showed some discrepancies compared to those obtained on regular HSA: N(1)-Me substitution was expected to increase the binding of the second eluted enantiomer [8] and (*S*)-oxazepam hemisuccinate was also supposed to have a higher binding affinity [9]. These irregularities seemed to vanish by extensive washing by buffer and after regeneration of the column the normal substituent effect was observed. Figure 1 shows the highly stereoselective binding of temazepam. The regeneration [12] was made after the interaction studies (see later) and it involved a washing procedure by 1% HSA solution and removal of the native protein by 1 M NaCl and Ringer buffer. It is probable that the culture supernatant contained some substance which bound to HSA influencing its binding affinity.

Binding of coumarins. Table 3 shows the elution volumes obtained for the enantiomers of warfarin, acenocoumarol and phenprocoumon. The elution volumes are higher than expected, but the relations between the enantiomers ($K^S > K^R$ for warfarin and phenprocoumon while $K^R > K^S$ for acenocoumarol) are in accordance with the previous findings on native HSA [10, 11].

Binding interactions. It was found previously [8, 11] that there exists an allosteric interaction between the two main binding sites on HSA, which manifests itself by either mutually increased or decreased binding depending on the structure of the benzodiazepine and on the chiral configuration of both ligands. The most remarkable enhancements could be observed in the simultaneous binding of (*S*)-lorazepam acetate and (*S*)-warfarin.

Table 4 summarizes the alteration of elution volumes of a series of 3-substituted 1,4-benzodiazepines on a recombinant HSA–Sepharose column when the eluent contained (*R*)- or (*S*)-warfarin. It is assumed that V_1 and V_2 belong to the (*R*)- and (*S*)-benzodiazepine enantiomers, respectively. Data obtained previously on a native HSA–Sepharose column [8] are also given. The following observations suggest good agreement for the two proteins. (1) (*R*)-Warfarin enhanced the binding of (*S*)-lorazepam acetate only. (2) (*S*)-Warfarin enhanced the binding of (*S*)-oxazepam acetate and (*S*)-lorazepam acetate to very high extent. The latter practically stuck to the column and could be eluted by albumin solution. (3) The presence of N(1)-Me substituent on the benzodiazepine prevented the enhancement.

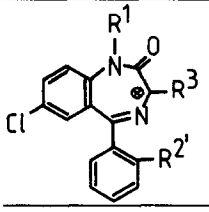
Figure 2 shows the chromatograms of lorazepam acetate which produces the most pronounced induced stereoselectivity.

The regeneration of the column was performed after the interaction studies to remove warfarin.

DISCUSSION

The ligand binding behaviour of recombinant HSA produced in yeast using the synthetic gene was found to be very similar to that of the native protein. The chromatographic technique allowed us to follow the relative differences between the stereoisomers,

Table 2. Elution volumes (V_1, V_2 in mL) of racemic benzodiazepines obtained on a recombinant HSA–Sepharose column (V_o = 5.5 mL)



R^1	R^3	$R^{2'}$	
H	OH	H	Oxazepam (Ox)
CH_3	OH	H	Temazepam (Tem)
H	OH	Cl	Lorazepam (Lor)
CH_3	OH	Cl	Lormetazepam (Lmet)

Compound	After immobilization V_1	V_2	After washing V_1	V_2	After regeneration V_1	V_2
Oxazepam	12	20	12	21	11 (11)	30 (35)
Temazepam	9	20	9	27	9 (10)	45 (48)
Lorazepam	16*				14 (13)*	18
Lormetazepam	12*				13 (18)	25
Ox acetate	12	32	12	23	12 (12)	25 (31)
Tem acetate	8	12	8	22	9 (9)	55 (41)
Lor acetate	14	26	14	19	15 (13)	20 (20)
Lmet acetate	11	14	10	16	11 (11)	23 (28)
Ox hemisuccinate	12	21	12	26	12	40

Data in parentheses were obtained on a native HSA–Sepharose column (V_o = 6.4 mL), taken from Ref. 8, eluent: Ringer buffer.
* Not resolved.

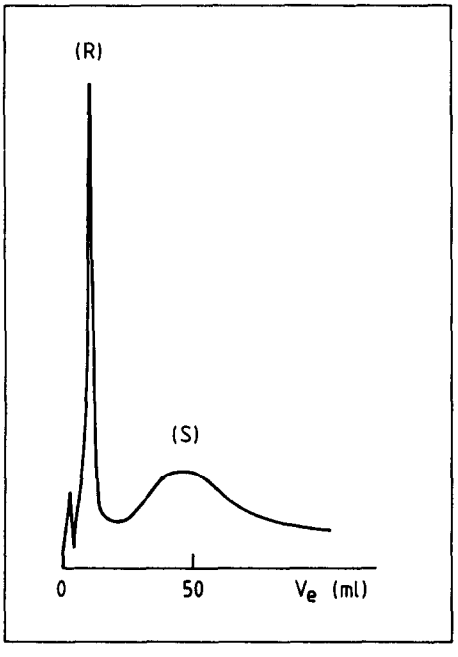


Fig. 1. Chromatographic resolution of temazepam on a recombinant HSA–Sepharose column (V_o = 5.5 mL).

Table 3. Elution volumes (V) of coumarin compounds obtained on a recombinant HSA–Sepharose column (V_o = 5.5 mL), as well as on a native HSA–Sepharose column (V_o = 3.5 mL) taken from Ref. 11, eluent: Ringer buffer

Compound	V (mL) Recombinant HSA	Native HSA
(*R*)-Warfarin	70	34
(*S*)-Warfarin	80	43
(*R*)-Phenprocoumon	70	46
(*S*)-Phenprocoumon	80	80
(*R*)-Acenocoumarol	70	39
(*S*)-Acenocoumarol	53	24

the changes during simultaneous binding of two ligands and the effect of substitution in a series of ligands. Since the binding stereoselectivity and the allosteric binding interactions must have strict conformational requirements realised in the tertiary structure of the protein, these results can be considered as further evidence for the authenticity of recombinant HSA.

Up to now much work has been done to locate the specific binding sites or binding regions on HSA, approaching the problem from the ligand and the protein side [5, 13–18]. In 1981 Fehske *et al.* [18]

Table 4. Effect of warfarin enantiomers (10^{-4} M) on the elution volumes (V_1 and V_2 in mL) of racemic benzodiazepines on a recombinant HSA–Sepharose column (V_0 = 5.5 mL)

Compound	Eluent: Ringer V_1	Ringer V_2	(*R*)-Warfarin V_1	(*R*)-Warfarin V_2	(*S*)-Warfarin V_1	(*S*)-Warfarin V_2
[^{14}C]Ox acetate	12	22	10	18	10	40
	(12)	(31)	(12)	(28)	(12)	(67)
[^{14}C]Tem acetate	9	22	9	18	9	18
	(9)	(41)	(9)	(34)	(9)	(35)
[^{14}C]Lor acetate	14	18	12	50	12	>100
	(13)	(20)	(12)	(45)	(13)	(>130)
[^{14}C]Lmet acetate	11	16	10	16	10	16
	(11)	(28)	(11)	(26)	(11)	(24)

Data in parentheses were obtained on a native HSA–Sepharose column (V_0 = 6.4 mL), taken from Ref. 8.

Abbreviations: Ox, oxazepam; Tem, temazepam; Lor, lorazepam; Lmet, lormetazepam.

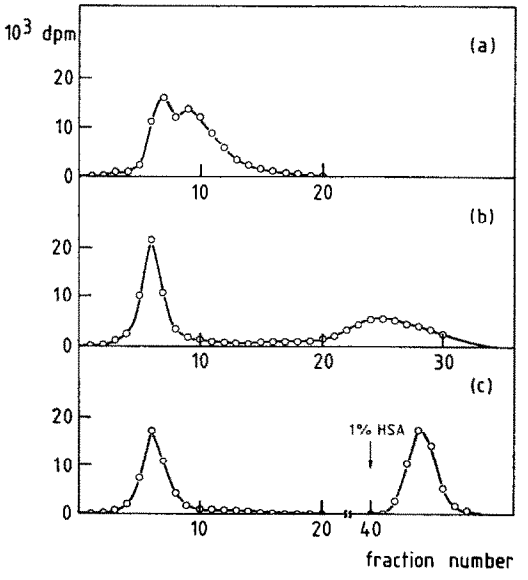


Fig. 2. Radiochromatogram of *rac*-[^{14}C]lorazepam acetate on a recombinant HSA–Sepharose column (V_0 = 5.5 mL, $V_{fraction}$ = 2 mL). Elution was by (a) buffer, (b) 10^{-4} M (*R*)-warfarin and (c) 10^{-4} M (*S*)-warfarin followed by 1% HSA solution.

suggested that the most advanced approach might be to rebuild this site synthetically.

Our results show that the ligand binding behaviour of the recombinant HSA is very similar to that of the natural HSA. It seems feasible, therefore, that the localization of the binding sites on the HSA molecule could be effectively approached by site-directed mutagenesis and heterologous (gene) expression of the mutant proteins.

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