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BINDING CAPACITIES OF HUMAN SERUM ALBUMIN MONOMER AND DIMER BY CONTINUOUS FRONTAL AFFINITY CHROMATOGRAPHY

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SUMMARY

Human serum albumin monomer and dimer obtained by fractionation of a commercial preparation were immobilized on CH-Sepharose 4B by covalent coupling. For salicylic acid, warfarin, phenylbutazone, mefenamic acid, sulphamethizole and sulphonylureas, the binding capacities of the monomer and dimer were compared by continuous frontal affinity chromatography. The salicylate-binding capacities of both monomer and dimer were essentially retained upon immobilization. For these drugs, the dimer showed only about 10–30% less capacity per monomeric unit than that of the monomer, the reduction being associated for most drugs with the intrinsic binding constant rather than with the number of binding sites.

INTRODUCTION

In our laboratories we have been studying the use of albumin covalently coupled to insoluble carriers for the simple and precise *in vitro* evaluation of the relative affinity of drugs to serum albumin in conjunction with frontal affinity chromatography. The latter technique was first demonstrated for the quantitative evaluation of various trypsin-inhibitor complexes using glycylglycyl-L-arginine immobilized on agarose beads[1]. Utilizing bovine serum albumin (BSA) we have previously shown[2,3] that, when the albumin is covalently attached to agarose gel matrix through a six-carbon-atom spacer, the binding properties of albumin are essentially retained. This technique was further extended to the simultaneous binding of two drugs to human serum albumin (HSA) whereby mutual displacement phenomena can be directly observed[4].

This paper demonstrates another application of this technique to proteins that are usually not readily available, using the dimer of HSA as a model, whereby comparison of the binding of many ligands by HSA monomer and dimer is expected to be readily possible. We have already reported that the fractionation of an HSA preparation on Sephadex G-200 columns using 0.1 *M* sodium chloride solution as the eluting medium and shown by equilibrium dialysis that the salicylate-binding capacity of the dimer per monomeric unit was about 80% of the monomer[5]. In order to investigate the generality of this relationship for a variety of drugs that show strong affinity, we

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have immobilized the monomer and dimer under the same conditions and the binding of several drugs to these affinity columns was quantitatively investigated by continuous frontal analysis, which is a modification of the previously employed technique[2].

EXPERIMENTAL

Materials

Human serum albumin (HSA), fraction V (essentially fatty acid free, lot no. 76C-7480), was purchased from Sigma (St. Louis, MO, U.S.A.) and fractionated on Sephadex G-200 columns as described previously[5] using 0.1 *M* sodium chloride solution as the eluting medium. The monomeric fraction contained over 95% monomer, whereas the dimeric fraction contained about 90% dimer. Activated CH-Sepharose 4B was purchased from Pharmacia (Uppsala, Sweden). Chlorpropamide was obtained from Sankyo (Tokyo, Japan). Mefenamic acid and tolbutamide were products of Upjohn (Kalamazoo, MI, U.S.A.) and Ono Pharmaceutical (Osaka, Japan), respectively. Other drugs were as described previously[3]. All other chemicals were of analytical-reagent grade and water was deionized and doubly distilled, the second distillation being performed in all-glass apparatus.

Immobilization of HSA monomer and dimer on CH-Sepharose 4B

To about 75 ml of either the monomer or dimer fraction containing about 1% HSA in 0.1 *M* sodium chloride solution, appropriate amounts of sodium hydrogen carbonate and sodium chloride were added so that the solution became 0.1 *M* and 0.5 *M* with respect to sodium hydrogen carbonate and sodium chloride, respectively. These HSA solutions were mixed with swollen activated CH-Sepharose 4B (15 g) and reacted at room temperature for 1 h in screw-capped test-tubes which were rotated end-over-end.

Determination of the amount of monomer or dimer immobilized on the gel matrix (CH-Sepharose 4B)

Direct spectrophotometric method. HSA-coupled gel (0.5 ml) in water was measured and water was added so that the total volume became 5 ml. This gel suspension (1 ml) was transferred into a 1-cm cell and 2 ml of 75% glycerin were added. The turbidity due to the gel matrix of the sample cell was balanced with that of the reference cell with either Sepharose 4B or CH-Sepharose 4B in 50% glycerine. The difference spectrum was then recorded in the second cell compartment of a Shimadzu Model UV-300 double-beam spectrophotometer (in which the cells were positioned directly in front of the photomultiplier for turbid samples). A molecular weight of 66,250[6] was used for the monometric unit of HSA. Absorbance values ($A^{1\%}_{1\,cm}$ at 278 nm) of 5.30 and 5.47 were used for the monomer and dimer, respectively[5].

Lowry's method[7]. HSA-coupled gel (1 ml) was measured and water was added to make the total volume 100 ml. To 1 ml of this suspension 0.5 ml of 1 *M* sodium hydroxide solution was added and the mixture was boiled for about 10 min. Alkaline copper reagent (5 ml) was added and the mixture was stirred occasionally for 11 min. A 1-ml volume of Folin–Ciocalteu reagent was then added and the mixture was left for about 30 min with occasional stirring. The absorbance of the supernatant solution

at 750 nm was measured. The amounts of the monomer and dimer coupled to the gel were determined from calibration graphs obtained with the monomer and dimer of HSA used for coupling.

The amounts of monomer immobilized as measured by the direct spectrophotometric method and Lowry's method were in fairly good agreement. On average, about 11 mg of HSA were coupled per millilitre of gel. For some batches, particularly those of the dimer, we experienced difficulty in balancing the turbidity due to the gel matrix in the direct method and no satisfactory difference spectrum was obtained. In such instances, the values obtained by Lowry's method were employed in the calculation of the binding parameters.

Determination of drugs bound by continuous frontal affinity chromatography

The HSA-coupled gel was packed into either a Pharmacia column (K16/20) or a Whatman column (MS-PC 1020; Whatman Biochemicals, Maidstone, Great Britain) with flow adaptors. Upward elution was carried out by means of a Pharmacia Model P-3 peristaltic pump at a rate of 10 ml/h. The temperature of the column was maintained at 4°C with water circulated through the jacket of the column by a Haake Model FK 10 constant-temperature circulator. All binding experiments were carried out in 0.05 *M* Tris buffer containing 0.1 *M* sodium chloride (pH 7.87) at 4°C. When the columns were not in use they were constantly eluted with buffer containing 0.01 % sodium azide. Prior to use they were pre-equilibrated with the buffer for about 16 h. The void volume of the column (V_0), the sum of the volume of the gel bed available for eluting media and that of the tubing, was determined by elution of 0.1 % EDTA (disodium salt), which has less affinity to the HSA-coupled gel than the sodium azide employed previously for this purpose[2–4].

Basically the previously described frontal analysis technique was followed except that after application of a known concentration of drug solution, which is the concentration of the free (or unbound) drug in this method, to the equilibrium state, a second solution with a different concentration of drug was directly applied to the column without regeneration of the column with the buffer as had been done previously[2,3]. The column eluate was collected by a fraction collector in pre-weighed test-tubes and exact volume determined by weighing. All of the drugs studied showed negligible affinity towards the gel matrix itself, with the exception of mefenamic acid, for which corrections were made by determining the adsorption isotherm of the drug to the gel by frontal analysis.

RESULTS AND DISCUSSION

Continuous frontal analysis

Fig. 1 shows that two different concentrations of drug solutions can be applied continuously without regeneration of the column with the buffer after application of each drug solution. This is demonstrated for the determination of the amounts of salicylic acid bound to an 8.2-ml gel column of immobilized HSA monomer ($1.34 \cdot 10^{-6}$ mole) corresponding to two different concentrations of free (or unbound) salicylic acid, i.e., $2.25 \cdot 10^{-5}$ and $3.64 \cdot 10^{-5}$ *M*. The amount of salicylic acid bound corresponding to the lower concentration can be calculated from area B to be $1.34 \cdot 10^{-6}$ mole and that corresponding to the higher concentration from the sum of

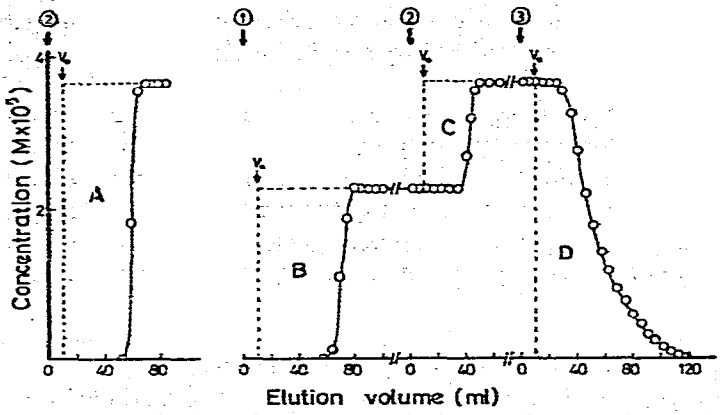

Fig. 1. Determination of the amount of salicylic acid bound by continuous frontal affinity chromatography. Column: 8.2 ml of HSA monomer ($1.34 \cdot 10^{-6}$ mole) immobilized CH-Sepharose 4B gel equilibrated at 4°C with 0.05 *M* Tris buffer containing 0.1 *M* NaCl (pH 7.87), V_0 = 10.5 ml. The arrows indicate the points of application of the following salicylic acid solutions and the buffer: (1) 2.25×10^{-5} *M*; (2) 3.64×10^{-5} *M*; and (3) the buffer. See text for areas A–D.

areas B and C to be $1.80 \cdot 10^{-6}$ mole. Agreement between the sum of areas B and C and either area A for the direct application of the solution of higher concentration ($1.77 \cdot 10^{-6}$ mole) or area D ($1.77 \cdot 10^{-6}$ mole) corresponding to the amount released following elution with the buffer justifies the present continuous application of drug solutions.

Fig. 2 shows a typical continuous frontal analysis elution pattern for four concentrations of free salicylic acid applied to a 15.2-ml HSA dimer-immobilized column. This procedure about halves the time required for the determination of binding parameters compared with the previous procedure[2].

Drug-binding characteristics of the immobilized HSA monomer and dimer

Salicylate-binding characteristics of the immobilized HSA monomer and dimer

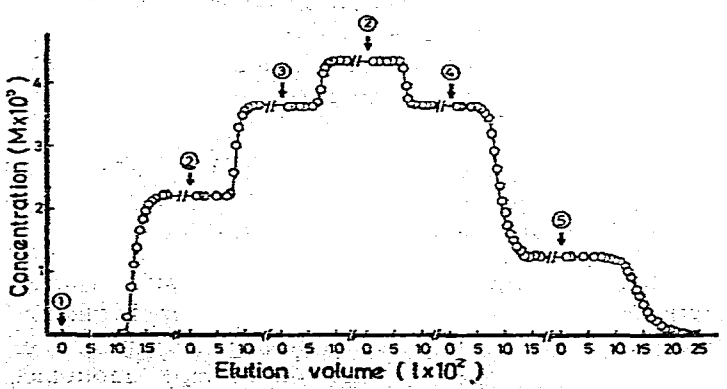

Fig. 2. Elution profile of a consecutive application at 4°C of four solutions of different concentrations of salicylic acid in 0.05 *M* Tris buffer containing 0.1 *M* NaCl (pH 7.87) and the buffer to 15.2 ml of HSA dimer ($2.66 \cdot 10^{-6}$ mole) immobilized column, V_0 = 19.1 ml. The arrows indicate the points of application of salicylic acid solutions and the buffer: (1) $2.22 \cdot 10^{-5}$ *M*; (2) $3.64 \cdot 10^{-5}$ *M*; (3) $4.38 \cdot 10^{-5}$ *M*; (4) $1.29 \cdot 10^{-5}$ *M*; and (5) the buffer.

are compared in Fig. 3, in the form of Scatchard plots, with those of soluble species determined previously[5] by equilibrium dialysis under the same experimental conditions, where r is the number of moles of salicylic acid bound per mole of a monomeric unit of HSA and D_f is the concentration of free salicylic acid. The Scatchard plots show reasonable linearity; the data were treated as previously on the basis of the presence of only one class of n binding sites per monomeric unit, which possess an equal intrinsic binding constant K.

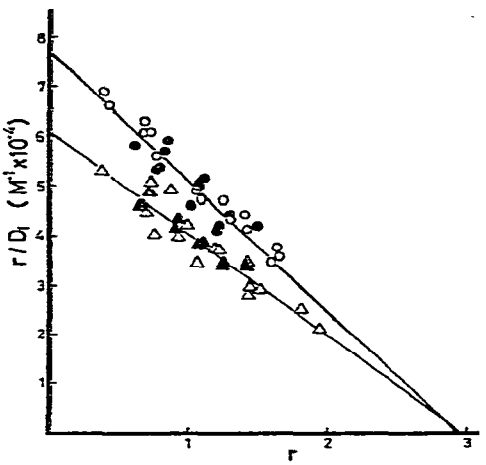

Fig. 3. Scatchard plots for the interaction of HSA monomer and dimer with salicylic acid at 4°C in 0.05 M Tris buffer containing 0.1 M NaCl (pH 7.87). By continuous frontal analysis: HSA monomer (●) and HSA dimer (▲). By equilibrium dialysis: HSA monomer (○) and HSA dimer (△). The straight lines represent the regression lines for the continuous frontal affinity chromatographic data.

These column data were obtained from four monomer and three dimer columns, each consisting of 8–20 ml of albumin-immobilized gel of different batches. Salicylate-binding parameters determined from each column by continuous frontal analysis of three solutions with different concentrations of salicylic acid are presented in Table I.

The monomer column data in Fig. 3 show considerable scatter. The scatter can be seen in Table I to exist between columns, *i.e.*, for each column the data give almost parallel lines, so that K values show much better agreement than n values. It should be pointed out that n depends upon the amount of total albumin present in the column, whereas K does not. The amount of total albumin present in the column is subject to some experimental errors originating, for instance, in the determination of albumin immobilized per millilitre of gel, in the measurement of gel volume before packing and in the leakage of covalently coupled albumin. Less scatter of the dimer data probably means that we could control at least some of these factors as we became used to handling these gels.

The salicylate-binding parameters of soluble HSA calculated previously[5] from the equilibrium dialysis data presented in Fig. 3 are $K = 2.58 \cdot 10^4\ M^{-1}$, $n = 3.03$ for the monomer, and $K = 2.05 \cdot 10^4\ M^{-1}$, $n = 2.96$ per monomeric unit of the dimer. Good agreement between these values and corresponding values for the immobilized

TABLE I

DRUG-BINDING PARAMETERS OF HSA MONOMER AND DIMER

Studied at 4°C in 0.05 *M* Tris buffer containing 0.1 *M* NaCl (pH 7.87) by continuous frontal affinity chromatography. *nK*, *K* and *n* were calculated by the method of least squares from the Scatchard plot of three sets of data points. D_f ranged from $0.8 \cdot 10^{-5}$ to $4.5 \cdot 10^{-5}$ *M*.

Drug	*HSA*	$nK \cdot 10^{-4}$ (M^{-1})	$K \cdot 10^{-4}$ (M^{-1})	*n*	$-\varrho^*$
Salicylic acid	Monomer	8.13	2.64	3.08	0.997
		7.31	2.63	2.78	0.999
		7.87 } 7.69 ± 0.38**	2.65 } 2.64 ± 0.01**	2.97 } 2.91 ± 0.14**	0.999
		7.45	2.65	2.81	0.999
	Dimer	5.94	2.00	2.97	0.998
		6.30 } 6.07 ± 0.20**	2.10 } 2.05 ± 0.05**	3.00 } 2.97 ± 0.04**	0.991
		5.98	2.04	2.93	0.999
Warfarin	Monomer	18.1	8.69	2.08	0.993
	Dimer	16.7	8.17	2.05	0.993
Phenylbutazone	Monomer	32.9	20.8	1.58	0.920
	Dimer	26.5	17.2	1.54	0.999
Sulphamethizole	Monomer	3.73	1.37	2.72	0.998
	Dimer	2.63	0.97	2.71	0.999
Mefenamic acid	Monomer	49.0	5.86	8.36	0.997
	Dimer	42.9	5.07	8.46	0.998
Chlorpropamide	Monomer	3.78	1.57	2.41	0.999
	Dimer	3.00	1.41	2.13	0.999
Tolbutamide	Monomer	8.39	3.90	2.15	0.999
	Dimer	6.79	3.43	1.98	0.998

* ϱ = correlation coefficient.
** Average value (± S.D.).

species (Table I) indicates that the binding of salicylic acid to both HSA monomer and dimer is essentially unaffected by immobilization on the insoluble carrier.

The binding characteristics of several drugs to the immobilized HSA monomer and dimer were evaluated similarly employing two columns each for the monomer and the dimer, and the results are presented in Table I. Variations in *r* for a fixed value of D_f between columns were corrected by employing salicylic acid as a standard drug. Thus, continuous analysis with three concentrations of salicylic acid was carried out and, employing the average values of *K* and *n* given in Table I, the total amount of albumin present in the column was calculated. A standard solution of salicylic acid was always applied before proceeding to a different drug and a check on albumin leakage was made. When leakage of albumin was suspected from such a check, salicylic acid was re-analysed by continuous frontal analysis and when the *K* value was unchanged a new value of total albumin present was re-calculated. The binding characteristics of these drugs were similar to those of salicylic acid, and the dimer showed slightly lower capacities (about 10–30% less) per monometric unit than the monomer, which for most drugs appears to reflect the greater reduction in *K* than in *n* values. This trend is not so clear with the sulphonylureas.

Sollenne *et al.*[8], however, recently observed that HSA dimer did not bind L-tryptophan, nor did it have the esterase activity of HSA monomer, suggesting that the tryptophan-binding site of HSA monomer, in which the esterase activity is considered

to reside[9], is lost upon dimerization. The linkage of two monomer units occurs at or near the tryptophan-binding site. In view of their observations, our present data may suggest that the primary binding site(s) of the drugs studied here is (are) different from the tryptophan-binding site.

Characterization of the specific binding sites present in a HSA molecule has been the subject of recent investigations[10–14]. Although many contradictory results have been reported, it is likely that an HSA molecule has at least two or more major independent drug-binding sites, one of which is the indole–benzodiazepine-binding site[10] (tryptophan-binding site, called site II by Sudlow *et al.*[14]) located around the tyrosine 411 residue of HSA, and the other being warfarin-binding site(s) (called site I by Sudlow *et al.*[14]), located around the lone tryptophan residue of HSA. According to this classification none of the drugs studied here is definitely classified as binding HSA only at the tryptophan-binding site. Therefore, these drugs probably bind to HSA primarily at the warfarin-binding site(s), which is (are) relatively unaffected upon dimerization, and the slight reduction in binding may be due to the allosteric effects. However, HSA dimer was reported to bind indole analogues[15] and progesterone[16] to the same extent as the two monomers.

Although a comparison of binding capacities of the monomer and dimer of HSA is an indirect approach, the present technique may be of value in the elucidation of site specificity of drugs. We are now directing our efforts in this direction by examining the drugs that are reported to bind to HSA at site II of Sudlow's classification.

CONCLUSION

Both HSA monomer and dimer essentially retained the salicylic acid-binding capacity upon immobilization on agarose beads by means of activated CH-Sepharose 4B. The activated beads are particularly suitable for covalent coupling of purified HSA monomer and dimer because no dimer or polymers are expected to be formed during the coupling reaction. Further, the reaction is very rapid under very mild conditions.

As with salicylic acid, for all of the drugs studied here the binding capacity of the dimer was only slightly less than that of the two monomers and for most drugs this reduction was associated with a reduction in K values rather than n values. The binding of these drugs to HSA probably takes place at site(s) other than the site of the linkage of two monomer units in dimer formation.

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