A NEW APPROACH TO THE STUDY OF THE CONTRIBUTION OF PEPTIDE CARRIERS TO ANTITUMOUR ACTIVITY; BINDING OF THE PEPTIDE MOIETY TO HUMAN SERUM ALBUMIN

M. SZEKERKE, M. HORVÁTH and J. ÉRCHEGYI
Institute of Organic Chemistry, L. Eötvös University, Budapest 1088, VIII. Múzeum krt 4/b, Hungary

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1. Introduction

Many attempts have been made to improve the specificity of action of antitumour drugs so that their cytotoxicity should be concentrated in malignant cells. As a result of encouraging clinical trials [1,2] interest increased recently in the use of larger molecular weight materials as carriers of cytotoxic drugs. It was postulated that the resulting conjugates might be taken up selectively by tumour endocytosis [1]. Daunorubicin was administered as its intercalated complex with DNA [1], antimetabolites have been covalently bound to globulin [3], while alkylating agents have been found to fibrinogen, albumin, globulin and their polypeptidyl derivatives [4,5]. One interesting finding was that the nitrogen mustards merely absorbed to protein, i.e., as complexes could also be more selective than the mustard alone [4,6]. A detailed investigation seemed necessary to elucidate the factors influencing the establishment of favourable transport forms. The comparison of the biological properties of a series of complexes [6] suggested, that the specificity of protein binding hinges mainly on the carrier part of the alkylating agents.

In the search for new approaches to study the mechanism of carrier function we have started experiments to analyse the interaction of peptide-type carriers with biopolymers, e.g. with DNA [7] and with proteins. The subject of the present paper is the investigation of the binding of L-phenylalanine and its methyl ester (the carrier part of Melphalan) to human serum albumin (HSA). Furthermore a series of dipeptides and dipeptide esters containing aromatic amino acids were prepared and their binding to HSA measured.

HSA seemed a suitable choice since it was one of the carriers previously applied [4–6] and at the same time it is a widely used standard substance in protein investigations because many drugs are bound to plasma albumin [8].

The measurements have been made by the classical equilibrium dialysis method [8,9].

2. Experimental

The ultraviolet absorption spectra were obtained in a Beckman DU (G2400) spectrophotometer, or with Unicam SP 700 and Specord UV VIS recording spectrophotometers. All solutions were filtered on Sartorius filters SM 11306 (0.45 μm).

Details of the experimental procedure were described elsewhere [10]. Negligible amounts of the small molecules were found to be bound to the membrane. The protein concentration used in these experiments was 1.43×10^{-4} M (1% solution). All experiments were carried out at 4°C.

HSA was obtained from 'Human' Serum and Vaccine Institute, Budapest and was further purified by dialysis and freeze-drying. The molecular weight of HSA was taken (69 000) [11] and the $\epsilon_{280}^{1\%}$ as 5.3 [12]. The albumin was homogeneous by electrophoresis on acetylated cellulose at pH 8.6.

Amino acids were purchased from Reanal (Budapest). Amino acid and peptide derivatives were prepared in our laboratory, partly according to

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Table 1

Compound[a,b]	Molar ext. coeff.	Wave-length (nm)	Conc. range investigated (M)	Time req. for equil. (hr)	pH[d]
Phe (Reanal)	195.1	257.5	$3-9 \times 10^{-3}$	9	9
	193	257.5	$3-9 \times 10^{-3}$	9	7.35
DL-C$^{\beta}$-Phe–Ser (Reanal)	201.2	258	$2-5 \times 10^{-3}$	24	7.35
PheOMe.HCl [13]	187.5	257.5	$2-7 \times 10^{-3}$	24	6
Glu–Phe [14]	217.8	257.5	$2-8 \times 10^{-3}$	16	7.35
Glu(OMe)–PheOMe.HCl[c]	180.5	257.5	$2-7 \times 10^{-3}$	24	6
Glu(PheOMe)–OH.HCl[c]	183.1	257.5	$2-7 \times 10^{-3}$	24	6
Pro–PheOMe.HCl [15]	205.5	257.5	$2-7 \times 10^{-3}$	24	6
Glu(OMe)–TyrOMe.HCl[c]	1102.5	274	$2 \times 10^{-4}-1 \times 10^{-3}$	24	6
Glu(OH)–TyrOMe.HCl[c]	1201	275	$4.5 \times 10^{-4}-2 \times 10^{-3}$	24	6
Ser–TyrOMe.HCl [16]	1295.6	275	$3 \times 10^{-4}-2 \times 10^{-3}$	24	6
Lys–TyrOMe.2 HCl [7]	1265.2	275	$4 \times 10^{-4}-2 \times 10^{-3}$	24	6
Ser–TrpOMe.HCl [16]	5161.6	279	$6 \times 10^{-5}-1 \times 10^{-3}$	24	6
Lys–TrpOMe.2HCl [7]	4931.4	279	$7 \times 10^{-5}-8 \times 10^{-4}$	24	6

[a] Amino acids are of L configuration unless otherwise stated.
[b] Compounds were checked for purity by microanalyses and TLC.
[c] Prepared by the mixed anhydride method: the dipeptide esters isolated as hydrochlorides are amorphous, hygroscopic solids.
[d] The following buffers were used: pH 9, 0.05 M Tris–HCl; pH 7.35 and pH 6, 0.1 M phosphate.

literature methods, partly by using the mixed anhydride method for coupling *N*-benzyloxycarbonyl-protected amino acids with the appropriate amino acid esters, followed by the removal of the protecting groups by catalytic hydrogenation.

In table 1 are listed the compounds studied, with references to the method of preparation, to values used for the spectrophotometric determination of the concentration of the compounds in the ultraviolet region and to the experimental conditions used in the equilibrium dialysis experiments.

The UV absorption spectra of the compounds did not change significantly in the presence of varying amounts of HSA. The presence of Cl^- ions in the range 0.005 M–0.0001 M did not influence the binding of dipeptide esters. This is in agreement with the results of McMenamy [17] who reported that only with negatively charged indole derivatives, like tryptophan were salt effects pronounced, while with uncharged analogues they were small, except at high salt concentrations.

Binding data were treated according the equation developed by Scatchard [18] correlating $\bar{n}$, the mean number of moles of compound bound per mole of protein; A, the molar concentration of unbound compound; N, the theoretical number of binding sites and k, the association constant.

3. Results and discussion

Although insufficient data were obtained to characterize fully the binding curves for each compound, some conclusions can be drawn about their relative ability to be bound to HSA.

For Phe the percentage bound at various Phe concentrations was determined at pH 7.35 and 9 (fig. 1). A considerable increase of binding was observed at pH 9. A similar behaviour has been reported for Trp [19]. In studies of competitive binding Phe was found to inhibit Trp binding to HSA to a slight extent at pH 9, having a much smaller association constant than does Trp. This is in good agreement with our present results. The Scatchard plot of data obtained at pH 9 indicates that there are about 22 binding sites on HSA for Phe, with equal, but rather low affinities. (fig. 1b).

Binding studies with PheOMe and with dipeptide

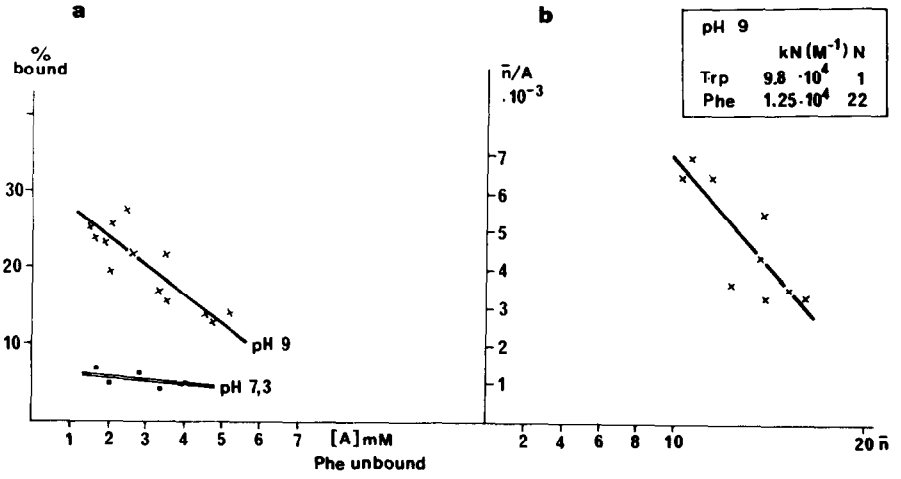

Fig. 1: a) Comparison of per cent Phe bound to HSA at various free amino acid concentrations at pH 7.35 and pH 9; data obtained by equilibrium dialysis, 4°C; b) Scatchard plot for the binding of Phe to HSA (1% solution) at pH 9, 4°C. Insert: binding parameters for Trp [19] and Phe at pH 9.

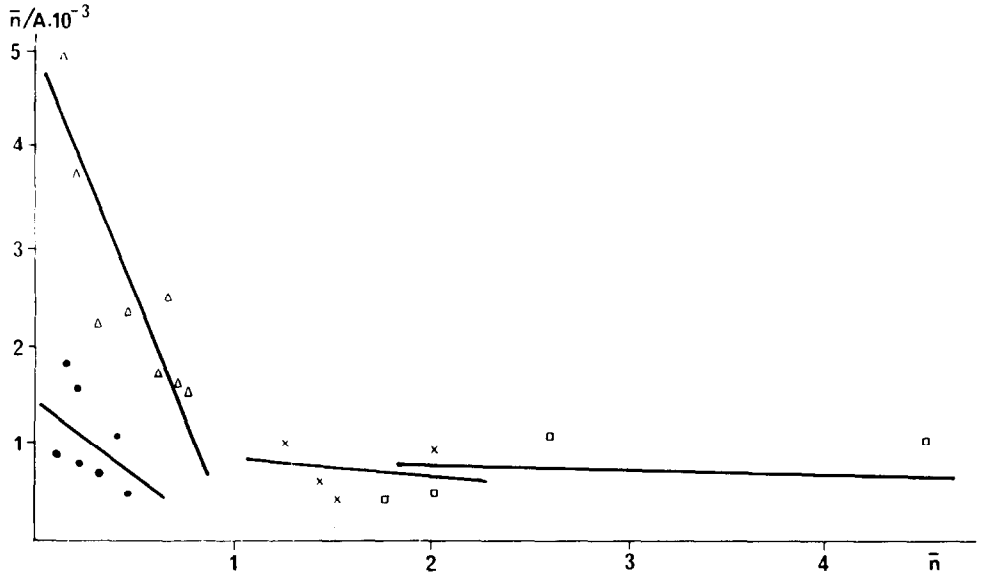

Fig. 2. Binding of PheOMe (–□–), Pro–PheOMe (–×–), Lys–TrpOMe (–△–), and Ser–TrpOMe (–●–) to HSA (1% solution). Scatchard plots for data obtained by equilibrium dialysis at pH 6, 4°C.

esters were carried out at pH 6, the pH of incubation used in preparing protein–cytotoxic drug complexes [6]. HSA seems to bind PheOMe also at several, but equally weak sites (fig. 1). Phenylalanine peptides shows very little affinity for HSA, as illustrated by Pro–PheOMe (fig. 2). No binding could be measured with Glu(OMe)PheOMe, Glu(PheOMe)OH, and DL-C^{β}-Phe–Ser.

The plots of $\bar{n}/A$ vs $\bar{n}$ obtained for Tyr peptides indicate that the number of binding sites is not as high

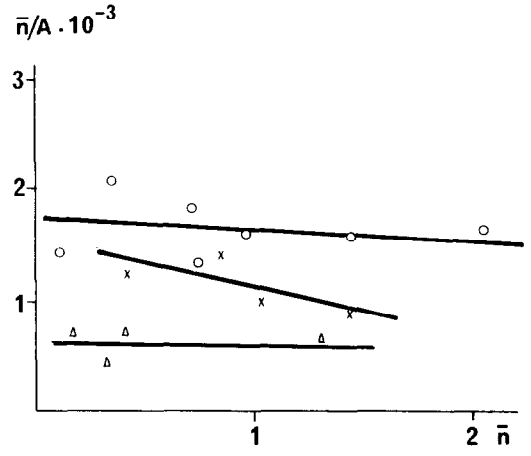

Fig. 3. Binding of Ser–TyrOMe (–o–), Lys–TyrOMe (–x–), and Glu–TyrOMe (–△–) to HSA (1% solution). Scatchard plots for data obtained by equilibrium dialysis at pH 6, 4°C.

as for Phe derivatives and the association constants also have rather low values (fig. 3). The strongest binding properties were observed with Trp peptides (fig. 2). These results are in agreement with findings reported in the literature [20,21] that the indole compounds investigated so far, including glycine peptides of Trp, all bind to HSA at one specific site, and in some cases at a few more very weak sites.

On the basis of binding parameters found for Phe an explanation can be offered for the results of previous animal tests, which indicated, that no improvement of antitumour activity could be achieved by complexing Melphalan or its ethyl ester with protein carriers [6,22]. It is likely, that albumin can also bind a considerable amount of Melphalan in appropriate conditions, but the low value which is likely for the association constant would not favour transport in vivo. Our experimental result suggest, that the use of Melphalan peptides containing Trp would be more suitable for the formation of protein complexes with higher stability. Synthestic work has been started to prove our hypothesis by preparing the proposed peptide–protein complexes and to study their antitumour activity.

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