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Effect of protein-bound uraemic toxins on the thermodynamic characteristics of human albumin

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Abstract

The ability of albumin to bind drugs and other lipophilic organic acids is decreased in chronic renal failure by the accumulation of albumin-bound uraemic toxins such as hippuric acid, indoxyl sulphate and 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF). This furan acid is the most highly bound and is not removed by haemodialysis. The inhibitory effects of these three uraemic toxins on the interaction of three marker ligands sodium octanoate (for medium chain fatty acids), salicylic acid and phenol red (bilirubin site/site I) with albumin have been investigated by differential scanning microcalorimetry and flow microcalorimetry. CMPF was the most potent inhibitor and its binding site coincided with that of bilirubin (site I). Indoxyl sulphate binds to the site for medium-chain fatty acids and tryptophan (site II) and hippuric acid, the weakest inhibitor, inhibited binding to the salicylic acid site.

Keywords: Albumin; Uraemic toxin; Hippuric acid; Indoxyl sulphate; 3-Carboxy-4-methyl-5-propyl-2-furanpropanoic acid; Binding thermodynamics

1. Introduction

Chronic renal failure decreases the ability of albumin to bind drugs and other lipophilic organic acids [1]. This binding defect is caused largely by the inhibitory effects of the organic acids that accumulate in renal failure [2]. Identification of the key binding inhibitors has been a slow process, however, and some may still remain undiscovered. A number of the organic acids present in uraemic plasma contribute to the inhibitory effect including, for example, hippuric acid [3] and several acidic indoles such as indoxyl sulphate [4,5]. Another and more potent inhibitor is the furan acid, CMPF, which has an association constant around 10^6–10^7 M^{-1} for human albumin [6–8]. The origins of CMPF are not completely clear, a proportion may originate from the diet since precursors are found in food such as fish [9] and fruit [10] and from the breakdown of long-chain fatty acids [11]. CMPF can reach concentrations approaching 400 μM where its molar ratio with albumin starts to approach unity, particularly in a patient with hypoalbuminaemia. Several related furan acids also accumulate and although the 5-pentyl analogue of CMPF, for example, inhibits binding [8] it does not appear to reach a high enough concentration to be a major inhibitor.

We first demonstrated the inhibitory effect of CMPF on binding to albumin with *o*-methyl red [12,13] and subsequently similar effects on a wide variety of both exogenous substances and endogenous ligands (e.g. L-tryptophan, bilirubin and thyroxine) have been observed, notably by several research groups in Japan [14–18]. The evidence from competition experiments and fluorescent probes suggests that CMPF binds mainly to site I (warfarin) but that it can also affect binding to site II [7,8].

CMPF is not removed by haemodialysis because it is ≥99% bound and it disappears relatively slowly after a

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Abbreviations: CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid; IS, indoxyl-3-sulphate; HA, hippuric acid.

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kidney transplant [19]. CMPF is associated with several problems in CRF patients including anaemia, neurological symptoms, low plasma thyroxine and it may also inhibit the active tubular secretion of other organic acids [20,21]. CMPF is actively taken up by rat kidney slices and is a potent inhibitor of the uptake of *p*-aminohippuric acid [21], a marker for active tubular secretion. There is evidence from *in vivo* experiments in the anaesthetised rat that CMPF may be actively secreted [22]. CMPF may, therefore, inhibit the secretion of other organic acids. Neurological symptoms correlate with the plasma concentration of CMPF and these effects may result from interference with organic acid transport across the blood–brain barrier [23].

As regards the significance of the high affinity of CMPF for albumin and its inhibitory effects on other ligands, the situation is akin to that with drug–drug interactions that involve albumin. The interaction at the level of albumin itself may not be of great clinical significance (e.g. phenylbutazone–warfarin), but the interaction may be an indicator of other more critical interactions at the level of metabolism and/or excretion. The affinity of CMPF for albumin and its association with several adverse effects of chronic renal failure suggest that inhibition of organic acid transport and binding is not confined solely to albumin. CMPF is an interesting uraemic protein-bound toxin with important effects and removal of such toxins from the bloodstream of CRF patients is, therefore, an important therapeutic goal. The thermodynamics of the interaction of CMPF, indoxyl sulphate and hippuric acid with human albumin have, therefore, been investigated by differential scanning microcalorimetry and flow microcalorimetry. This approach offers a combined qualitative and quantitative approach to the problem that may be applicable to the investigation of patients and the development of new treatments in the future [24].

2. Materials and methods

2.1. Materials

The following analytically pure reagents were used: polyethylene glycol 400 (Merck), QAE-Sephadex A-50, bromophenol blue (BPB; sodium salt), phenol red (sodium salt), salicylic acid, sodium octanoate, crystalline human albumin (fraction V, $M_r = 66{,}500$ essentially fatty acid free), hippuric acid, 3-indoxyl-sulphate, trifluoroacetic acid (potassium salt) (Sigma Chemical Co.). CMPF was a generous gift from Prof. G. Spiteller (University of Bayreuth, F.R.G.) and had been synthesised as described by him [25] or in our laboratory by a slight modification of his method [26]. All HPLC solvents were ''HPLC'' grade and were obtained from FSA Laboratory Supplies. Water used for HPLC was double distilled and deionized. All other reagents were of ''Analar'' grade and used without additional purification.

2.2. Plasma samples from patients

Blood samples were obtained from 18 patients with end-stage renal failure (age range 30–71 years, average age 55 years). All patients included in the study were on maintenance haemodialysis treatment (4–5 hr treatment time, three times per week) for at least 1 year. The haemodialysis regimen included bicarbonate dialysate, cellulose membranes (surface: 1.8 m^2), 200–250 mL/min blood flow, 500 mL/min dialysate flow and heparin for anticoagulation. Patients with active infections, diabetes mellitus, and/or hyperlipidaemia were excluded from the study. Blood samples were obtained on an empty stomach before haemodialysis treatment. The plasma was promptly frozen and stored for not more than 1 week. Control investigations were performed on the serum/plasma of healthy donors (18 male volunteers, average age 54 years) sampled on an empty stomach. To obtain sufficient HSA, equal volumes of plasma from pairs of patients of similar age were pooled. Plasma from healthy donors was pooled as well as for uraemic patients. Figs. 1 and 2 demonstrate the results obtained for paired plasma pools. HSA samples were isolated from the plasma of healthy donors and uraemic patients by the method of Vasileva *et al*. [27], dialysed for 48 hr at 4° against distilled water and freeze-dried.

Defatted human albumin (75 μM) was titrated with each of the three ligands over a range of concentrations (CMPF: 30–833 μM; indoxyl sulphate: 90–591 μM; hippuric acid: 20–440 μM) for differential scanning and flow microcalorimetry experiments. Model complexes of defatted human albumin with uraemic toxins were prepared by supplementing albumin solution (606 μM in isotonic sodium phosphate–chloride buffer, pH = 7.2–7.4) with indoxyl sulphate up to a final concentration of 400 μM, hippuric acid up to 600 μM and CMPF up to 250 μM.

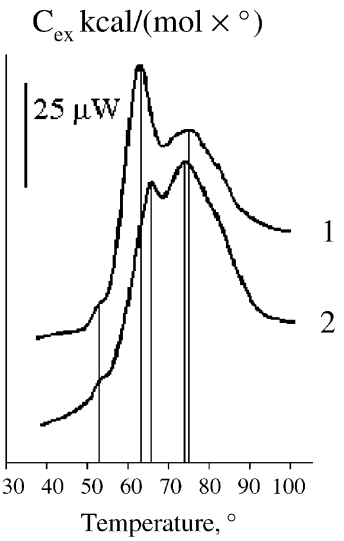

Fig. 1. Melting thermograms of plasma from healthy donors (1) and patients with end-stage renal disease (2).

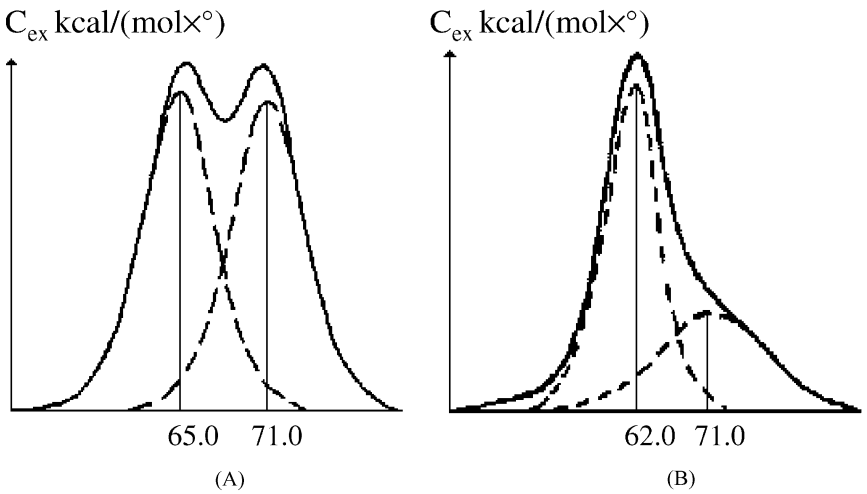

Fig. 2. Melting thermograms of albumin isolated from the plasma of patients with end-stage renal disease (A) and healthy donors (B).

The plasma from healthy donors, with an albumin concentration of 530 μM determined by a dye binding method [28], was supplemented with hippuric acid, indoxyl sulphate and CMPF at the same concentrations. Albumin was isolated as described elsewhere [27]. The samples of albumin were dialysed for 48 hr at 4° against distilled water and freeze-dried. The aqueous solubility of hippuric acid, indoxyl sulphate and CMPF is limited and so solutions of these uraemic toxins were prepared in methanol, then freeze-dried and dissolved in a solution of buffered human albumin.

2.3. Differential scanning microcalorimetry and flow microcalorimetry

The albumin samples were dissolved at a concentration of 5 mg/mL in isotonic sodium phosphate–chloride buffer, pH = 7.4, for differential scanning microcalorimetry and flow microcalorimetry. Albumin concentration was checked spectrophotometrically at 279 nm using $A_{1\%} = 5.31$. The melting thermograms of albumin samples from healthy donors and uraemic patients were recorded on a DASM-4 microcalorimeter (Biopribor) at a scanning rate of 1°/min. The temperature maxima (T_1 and T_2) of the components were obtained by mathematical deconvolution [29] of the melting curve. The calorimetric enthalpies (ΔH_1 and ΔH_2) were measured as the area under the melting curve.

The temperature effects of albumin complexing with different marker ligands (sodium octanoate, salicylic acid and phenol red) in equimolar concentrations with albumin were measured with a flow microcalorimeter 2277 thermal activity monitor (LKB) at ambient temperature. The enthalpies of complexing were calculated [30].

Preliminary experiments with 5-fold repeated measurements of albumin thermal denaturation and complexing with marker ligands demonstrated that a deviation of the temperature maxima of more than 0.5° and a melting enthalpy of more than 7% coincided with a significance level of $P < 0.01$. A deviation of complexing heats of more than 10% coincided with a significance level of $P < 0.05$.

2.4. Equilibrium dialysis

Binding experiments were carried out with 1 mL dialysis cells in a Dianorm apparatus (Dianorm GMBH) in a waterbath at 37°. Semipermeable cellulose membranes (Visking tubing, Medicell International Ltd.) with a molecular weight cut-off of approximately 12,000 were used and binding was expressed as the percentage unbound as described previously [31]. Results are expressed as the mean ± SD and the non-paired Student's *t*-test was used for statistical comparison.

2.5. Measurement of CMPF and indoxyl sulphate by HPLC

2.5.1. Extraction of CMPF from plasma

The plasma sample (0.4 mL) was added to 100 μL of formic acid and 6 mL of dichloromethane, shaken, centrifuged (4,000 *g*) for 5 min. The organic layer was dried, reconstituted in 100 μL of mobile phase and then analysed for CMPF by HPLC.

2.5.2. Extraction of indoxyl sulphate from plasma

The plasma sample (0.4 mL) was added to 0.6 mL of acetonitrile in a screw-topped microcentrifuge tube (1.5 mL). The tubes were then vigorously vortexed and stored at −20° overnight. On reaching room temperature the tubes were again vortexed and then centrifuged (11,500 *g*) for 30 min in a microcentrifuge. The supernatant was dried and reconstituted in 100 μL of mobile phase and then analysed for indoxyl sulphate by HPLC.

The methods for measurement of CMPF and indoxyl sulphate were based on those published by Mabuchi and Nakahashi [32], Takeda *et al.* [33] and Vanholder *et al.* [34]. A Spectra Physics analytical HPLC system (Thermoseparation Products) was used along with a Ultratech 5 ODS column (25×0.46 cm i.d.) obtained from Phase Separations. The mobile phase was acetonitrile:water:trifluoroacetic acid (40:60:0.05 by vol., pH = 2.4) and the elution was isocratic at a flow rate of 0.5 mL/min at room temperature. Samples of 50 μL were injected onto the column and the CMPF in the column effluent was monitored by UV absorbance at 265 nm (spectra system UV 1000). Quantitative calculations were based on peak height measurements. Standard curves for CMPF and indoxyl sulphate were made as described earlier [31]. The unknown concentrations of CMPF and indoxyl sulphate were calculated with the linear regression program Linefit. The correlation coefficients of all standard curves, also calculated with Linefit, were greater than 0.997.

3. Results

3.1. Melting thermograms

The melting thermograms for plasma from healthy donors and from patients with end-stage renal disease are illustrated in Fig. 1. The melting curve of uraemic plasma shows a different shape with a reversal in amplitude of the two peaks and a shift to the right at higher temperature. These changes are attributed to an excessive load of hydrophobic uraemic toxins bound to the albumin molecule [35]. Fig. 2 illustrates the process of thermodenaturation of human albumin isolated from the plasma of healthy donors and uraemic patients. The melting thermogram of uraemic albumin displayed two maxima: at 65 ± 0.5 and $71 \pm 1.0^{\circ}$. The normal albumin was characterised by a main peak at $62 \pm 0.5^{\circ}$ and with a less pronounced shoulder that formed a second maximum ($71 \pm 1.0^{\circ}$).

3.2. Effect of uraemic solutes on the melting temperature of albumin

The relationships between the concentration of hippuric acid, indoxyl sulphate and CMPF and the melting temperatures of the complex between defatted albumin and the uraemic toxin are illustrated in Fig. 3. CMPF induced the highest elevation of the temperature of denaturation (T_{max}) curve, followed by indoxyl sulphate and the weakest effect was with hippuric acid. There was thus a direct relationship between the affinity of these three uraemic toxins for the albumin molecule and their thermostabilizing abilities. The addition of CMPF also induced a sharp elevation of the melting curve over the base line, which was reflected by the value of melting enthalpy represented as the area under

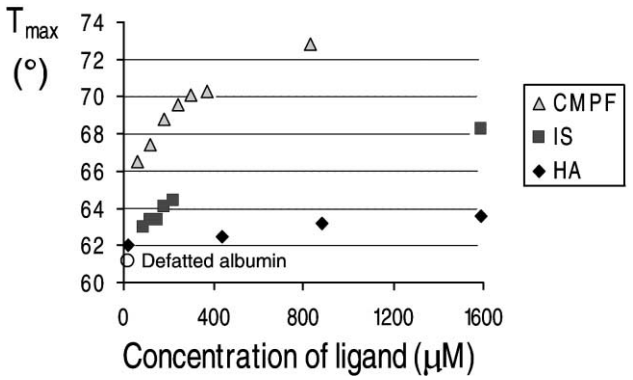

Fig. 3. Effect of CMPF (△), indoxyl sulphate (■) and hippuric acid (◆) on the melting point (T_{max}; °) of human albumin. The melting point of defatted human albumin in the absence of any added uraemic solute (binding inhibitor) is shown on the *y*-axis by an open circle (○).

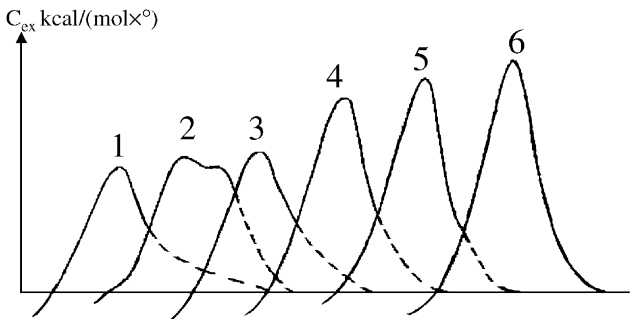

Fig. 4. Melting thermograms of defatted human albumin (75 μM) before (1) and after its supplementation by CMPF (2, 3, 4, 5, 6) at concentrations of 30, 60, 120, 300 and 833 μM, respectively.

the thermogram (Fig. 4). The addition of CMPF to a solution of defatted albumin at a concentration of 833 μM nearly doubled the value of enthalpy for defatted albumin (Table 1).

Table 2 shows that the hippuric acid was completely removed during the isolation of the albumin fraction from the plasma of healthy donors that had been supplemented with hippuric acid, indoxyl sulphate or CMPF and likewise from the plasma of uraemic patients. Indoxyl sulphate and CMPF, however, induced a conformational change in the

Table 1
The effect of supplementation with various concentrations of CMPF on the enthalpy of thermodenaturation of defatted human albumin

CMPF concentration (μM)	ΔH (kcal/mol)	CMPF/albumin ratio
60	328.6	0.8
120	390.3	1.6
180	348.1	2.4
240	354.4	3.2
340	359.6	4.5
370	370.4	4.9
833	430.8	11.1
Defatted albumin	257.5	–

Table 2
The enthalpy of complexing ($-\Delta H_c$, kJ/mol) of human albumin preparations with protein-bound uraemic toxins in comparison with the enthalpies of HSA isolated initially from the plasma of healthy donors

Sample	Ligand		
	Hippuric acid	Indoxyl sulphate	CMPF
Control: albumin from healthy donors	2.85 ± 0.33	15.17 ± 0.84	21.70 ± 0.60
Albumin isolated from healthy donor plasma preloaded with HA	2.72 ± 0.25	15.10 ± 1.03	21.40 ± 0.60
Albumin isolated from healthy donor plasma preloaded with IS	2.76 ± 0.23	13.41 ± 0.98	19.60 ± 0.50
Albumin isolated from healthy donor plasma preloaded with CMPF	2.79 ± 0.30	13.61 ± 0.30	16.40 ± 0.30

A deviation in the heat of complex formation of more than 10% coincided with a significance level of $P < 0.05$.

Table 3
The enthalpy of complexing ($-\Delta H_c$, kJ/mol) of albumin, isolated from the plasma of healthy donors and uraemic patients, with different marker ligands

Sample	Ligand		
	Sodium octanoate	Salicylic acid	Phenol red
Healthy donor albumin	19.00 ± 0.70	27.60 ± 0.90	6.50 ± 0.50
Uraemic albumin	10.40 ± 0.50	21.70 ± 0.50	3.70 ± 0.20

A deviation in the heat of complex formation of more than 10% coincided with a significance level of $P < 0.05$.

albumin molecule and inhibited its binding ability (Table 3). The greatest inhibitory effect (Table 3) was observed for the binding site of medium-chain fatty acids (marker ligand: sodium octanoate) and for the bilirubin site (marker ligand: phenol red) [36].

3.3. Effect of uraemic solutes on the binding of marker ligands

Fig. 5 and Table 4 confirm that isolation of albumin from whole uraemic plasma and from the plasma of a healthy donor supplemented with indoxyl sulphate did not affect the interactions of albumin with indoxyl sulphate and CMPF. In this experiment indoxyl sulphate and CMPF were present at concentrations of 186 ± 17 and 19 ± 5 μM, respectively.

Table 5 and Fig. 6 summarise the evidence that the binding site of indoxyl sulphate coincides with that for medium-chain fatty acids (drug binding site II), that CMPF and bilirubin have the same binding region (site I) and that the hippuric acid binding site is in the region where salicylate binds to human albumin.

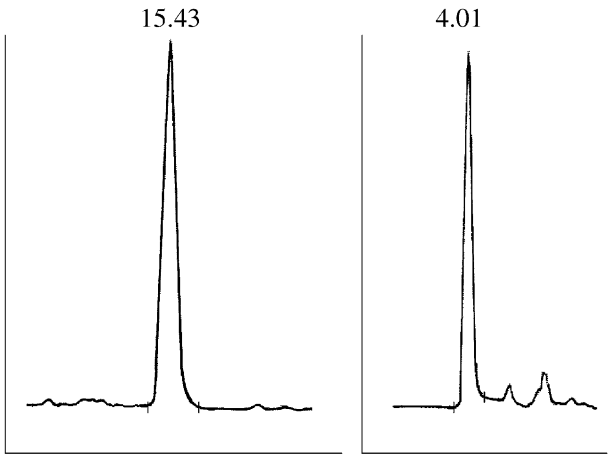

Fig. 5. Representative chromatograms for separation and quantification of CMPF and indoxyl sulphate in an extract of uraemic albumin: (A) the CMPF peak at 15.43 min yielded 65,800 area units equivalent to 24.4 μmol/L in the original sample; (B) the indoxyl sulphate peak at 4.01 min yielded 198,900 area units equivalent to 202 μmol/L in the original sample.

Table 4
The enthalpy of complexing ($-\Delta H_c$, kJ/mol) of marker ligands and human albumin isolated from the plasma of healthy donors preloaded with hippuric acid and indoxyl sulphate

Sample	Ligand		
	Sodium octanoate	Salicylic acid	Phenol red
Control: healthy donor albumin	19.06 ± 0.70	27.60 ± 0.90	6.50 ± 0.30
Albumin isolated from healthy donor plasma preloaded with HA	18.20 ± 0.60	30.50 ± 0.70	6.50 ± 0.60
Albumin isolated from healthy donor plasma preloaded with IS	15.80 ± 0.20	29.90 ± 0.40	5.40 ± 0.30

Table 5
The enthalpy of complexing ($-\Delta H_c$, kJ/mol) of different marker ligands with defatted human albumin supplemented by hippuric acid, indoxyl sulphate and CMPF

Sample	Ligand		
	Sodium octanoate ($K_1 = 5.5 \times 10^5\ M^{-1}$, $n_1 = 1$)	Salicylic acid ($K_1 = 1.9 \times 10^5\ M^{-1}$, $n_1 = 1$)	Phenol red ($K_1 = 0.28 \times 10^5\ M^{-1}$, $n_1 = 1$)
Control: defatted albumin	25.50 ± 0.50	31.50 ± 0.50	11.20 ± 0.30
Defatted albumin + HA (HA $K_1 = 0.5 \times 10^5\ M^{-1}$, $n_1 = 1$)	23.20 ± 0.60	26.80 ± 1.40	6.50 ± 0.40
Defatted albumin + IS (IS $K_1 = 16.0 \times 10^5\ M^{-1}$, $n_1 = 1$)	0.06 ± 0.03	20.30 ± 0.60	3.40 ± 0.10
Defatted albumin + CMPF (CMPF $K_1 = 4.8 \times 10^5\ M^{-1}$, $n_1 = 1$)	25.40 ± 0.80	32.50 ± 1.10	12.20 ± 0.50

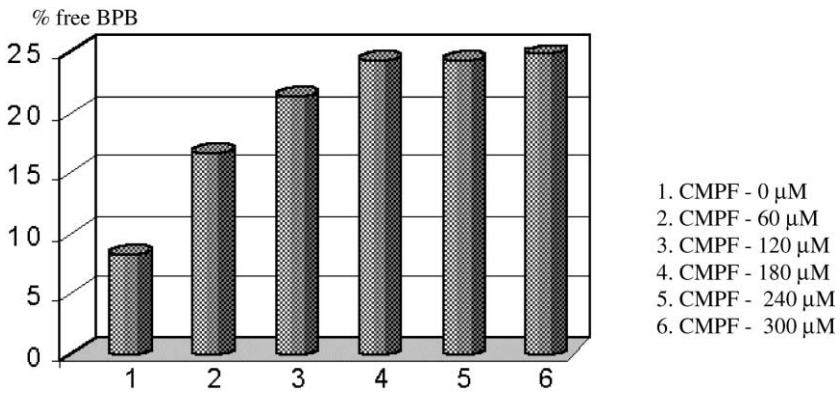

Fig. 6. The binding of BPB by defatted human albumin before (1) and after the addition of CMPF (2, 3, 4, 5) at concentrations of 60, 120, 180, 240 and 300 μM, respectively.

4. Discussion

The melting thermograms for pooled plasma from healthy donors and patients with end-stage renal disease are shown in Fig. 1. Both thermograms have a complicated character with three temperature maxima. Recently we have demonstrated that a maximum melting temperature around 63 ± 0.5° corresponds to the albumin component, the two other high-temperature maxima are caused by the thermodenaturation of the immunoglobulins [37]. The melting endotherm of uraemic plasma has a different shape with a shift to the right at higher temperatures, and the melting endotherm of albumin and immunoglobulins fractions overlap. In addition to the increases in the maximum values of the melting temperatures for human albumin isolated from the plasma of healthy donors and uraemic patients, the character of denaturation process of uraemic plasma exhibited an increase in the height above the base line. This was attributed to a higher thermoresistence of the albumin component, most probably because of the high concentration of protein-bound uraemic toxins.

The melting endotherms for albumin fractions (Fig. 2) are in general similar to the central part of the total thermograms of whole plasma. The main peak of the curve for albumin from the healthy donors is around 62 ± 0.2°. The melting curve for uraemic albumin is represented by two maxima, the first one at 65 ± 0.5° and the second (main maximum) at 71 ± 1.0°. An increase in the number of intramolecular bonds, because of the binding of uraemic toxins to albumin, increases the thermal stability of the protein molecule. The degree of effect of each uraemic toxin depends on its affinity for the albumin molecule.

Fig. 3 demonstrates the relationship between the melting temperature of defatted albumin and appropriate concentrations of hippuric acid, indoxyl-3-sulphate and CMPF. The addition of CMPF to human albumin induced the highest elevation of the denaturation temperature curve, followed by indoxyl sulphate and the weakest effect was with hippuric acid. This rank order is identical to the relative affinities of each ligand for albumin, CMPF having the highest and hippuric acid the lowest. The possible formation of additional intramolecular bonds between amino acid residues and the carboxylic groups of CMPF may also increase the stability of albumin.

The highest concentrations of CMPF that have been reported in uraemic plasma are around 370–400 μM [38] and an ''average'' range may be around 75–85 μM. Both a low concentration and also a molar ratio of CMPF:albumin equal to unity can induce an increase (up to 6°) in the denaturation temperature of defatted albumin. This contrasts with hippuric acid, for instance, where a pathological concentration (880 μM) increased the melting temperature (T_m) by only 2°. A higher concentration (nearly 1600 μM) elevated the denaturation temperature by only 0.6°. The

maximum concentration of indoxyl sulphate that was added to defatted albumin stabilised the protein molecule by only 7° in comparison with 61° for the initial human albumin without added ligand. At the same time, CMPF at a concentration of 370 μM shifted the endotherm to the region of higher temperatures by nearly 10°. Thus there is a direct relationship between the affinity of these three uraemic toxins for the albumin molecule and their thermostabilizing abilities.

The concentration of the added ligand also influences the thermal stabilisation of the albumin molecule. Fig. 4 shows that the effects on T_m and the amplitude of the melting curve became apparent at an initial concentration of 60 μM CMPF and a molar ratio CMPF:HSA equal to 0.8. There were further concentration-related increases in the values of T_m and total enthalpy of thermodenaturation (Fig. 5). The effect of indoxyl sulphate became apparent at an initial concentration of 90 μM followed by a slow rise of T_m. Addition of hippuric acid (440 μM) increased the T_{max} by only 1°, and further increase in HA concentrations had very little effect (Fig. 3).

The effect of the increase in the thermoresistance of HSA was also reflected in the value of the melting enthalpy that was represented as the total area under the melting curve. Fig. 4 demonstrates the melting endotherms for defatted albumin and the samples of human albumin containing CMPF at a concentrations of 30, 60, 120, 300 and 833 μM, respectively. CMPF induced a gradual and concentration-related increase in the amplitude of the second high-temperature component of the endotherm above the base line. At the same time the second peak became narrower at its base (Fig. 4). This change is probably connected with an alteration in the native conformation of the albumin, with an increase in the number of intramolecular bonds caused by CMPF. Therefore, the binding of this ligand increases the cooperativity of the melting process over this narrow temperature interval.

Table 1 illustrates how the values of the melting enthalpy increases with the ligand concentration and the increase in the molar ratio of CMPF:albumin. The addition of CMPF at 833 μM increased the enthalpy of denaturation for defatted albumin nearly 2-fold whereas hippuric acid at 1180 μM had little effect on ΔH_m (kcal/mol). The increase in the thermodenaturation enthalpy was not very pronounced, i.e., $\Delta H^{ha}_{117.9} = 306$ kcal/mol. The maximum concentration of indoxyl sulphate in our experiments (1591 μM) elevated this parameter to about 1.4 times. At the same time, neither hippuric acid nor indoxyl sulphate at their usual concentrations in uraemic plasma affected the character/shape of melting curve. Thus, in relation to Fig. 2 it is likely that CMPF as a uraemic toxin with a high affinity for albumin is one of the main causes of the big change in the nature of the melting process for defatted albumin. It is also possible, however, that several albumin-bound uraemic toxins, some of which may not yet have been identified, may collectively contribute in the case of whole plasma and albumin isolated from patients with end-stage renal disease.

The data in Table 2 provide information about which of these uraemic toxins were retained in the albumin samples that had been isolated by the gentle procedure of polyethylene glycol precipitation, ion exchange chromatography and dialysis against distilled water. Hippuric acid gave similar results for all the samples isolated from the plasma of healthy donors preloaded with hippuric acid, indoxyl sulphate or CMPF. In relation to the binding site for HA on the albumin molecule, it should be noted that the presence of hippuric acid itself in the albumin preparation decreased the value of the enthalpy of complexing ($-\Delta H_c$, kJ/mol) during the titration by another molecule of hippuric acid. In our experiments, we did not observe any difference between the complex forming abilities of albumin isolated from the initial healthy donors' plasma and the same plasma preloaded with hippuric acid. Therefore, hippuric acid due to its relatively low affinity for albumin [7] has been lost during the isolation of albumin from whole plasma.

There was a small drop in the enthalpy of complexing with albumin from healthy volunteers that had been preloaded with indoxyl sulphate (Table 2). There are at least two explanations for this phenomenon. Firstly, indoxyl sulphate was not completely removed during isolation of the albumin. The primary binding site is thus still occupied and so the added indoxyl sulphate molecule occupies the secondary binding site on albumin. There is, therefore, a less pronounced heat effect of the interaction. Another possibility is that during isolation from plasma, the indoxyl sulphate–albumin complex has changed its native conformation. In this situation, the heat effect observed represents the reaction of the free indoxyl sulphate molecule with a secondary binding site that possesses a lower affinity. The addition of indoxyl sulphate to the CMPF–albumin complex caused some decrease in the $-\Delta H_c$ (kJ/mol) value, probably because of a conformational change in the albumin molecule with the additional ligand. Thus, the primary site for indoxyl sulphate molecule may overlap and so consequently in the presence of CMPF it binds to human albumin with a lower affinity.

The addition of further CMPF to the CMPF–albumin complex demonstrated a quite pronounced difference in heat effects between healthy donor albumin isolated from the initial plasma ($-\Delta H_c = 21.7 \pm 0.6$ kJ/mol; Table 2) and whole plasma preloaded with CMPF ($-\Delta H_c = 16.4 \pm 0.3$ kJ/mol; Table 2). Therefore, it was concluded that during the isolation of albumin from both the original uraemic plasma and from that of healthy donors, which had been preloaded with hippuric acid, indoxyl sulphate or CMPF, the hippuric acid was completely removed because of its low affinity for albumin. Thus in relation to Figs. 1 and 2 it is likely that indoxyl sulphate, CMPF and possibly other unknown uraemic toxins of a hydrophobic nature and high affinity for

albumin affect the melting thermogram of whole plasma from end-stage renal failure patients.

Table 3 demonstrates that these uraemic toxins inhibit the complex-forming function of albumin, one of its main functions in the bloodstream. The main inhibitory effect was on the binding site for medium-chain fatty acids and tryptophan (marker ligand: sodium octanoate), drug binding site II and that for bilirubin (marker ligand: phenol red) drug binding site I [39].

Table 4 shows that isolation of human albumin from the plasma of end-stage renal disease patients did not damage the albumin–indoxyl sulphate complex and the interaction occurs in the area of drug binding site II. The HPLC data for indoxyl sulphate and CMPF (Fig. 5) revealed that preparations of human albumin isolated from the plasma of uraemic patients contained indoxyl sulphate and CMPF at concentrations of 186 ± 17 and 19 ± 5 μM, respectively.

The question arises which binding sites on the albumin molecule are usually affected by indoxyl sulphate and CMPF? Data for the binding ability of defatted albumin before and after its loading with one of the uraemic toxins with a marker ligand for the medium-chain fatty acids site, bilirubin site and binding region for salicylate are given in Table 5. The working concentration of albumin was 606 μM and the concentrations of indoxyl sulphate, hippuric acid and CMPF were 600, 400 and 250 μM, respectively. These concentrations are related to those in the plasma of uraemic patients. The value of $-\Delta H_c$ (kJ/mol) for the interaction of defatted human albumin and sodium octanoate were largely unaffected by hippuric acid. There are three possible interpretations. Firstly, sodium octanoate and hippuric acid have different binding sites. Secondly, since the association constant of octanoate is $K_1 = 5.5 \times 10^5\ M^{-1}$, $n_1 = 1.0$ [39] and that for hippuric acid is $K_1 = 1.0 \times 10^4\ M^{-1}$, $n_1 = 1.0$ [7] the octanoate molecule can displace hippuric acid from its binding site on albumin. Thirdly, a very small decrease in the $-\Delta H_c$ (kJ/mol) value is probably because of conformational changes caused by the binding of hippuric acid to albumin.

The association constant for salicylic acid ($K_1 = 1.9 \times 10^5\ M^{-1}$, $n_1 = 1.0$) [39] is higher than that for hippuric acid and lower than that for sodium octanoate. Salicylic acid is, therefore, only likely to be a weak inhibitor of the binding of hippuric acid, and indeed only a weak thermal effect was observed with salicylic acid. Another possibility is that salicylic acid was bound to its secondary site because of a change in the native conformation of albumin that affected the value of $-\Delta H_c$ (kJ/mol).

The binding of phenol red ($K_1 = 0.28 \times 10^5\ M^{-1}$) [39] showed a 2-fold inhibition of the enthalpy of complexing with albumin. There are several possible reasons. Firstly, bilirubin and hippuric acid may share a common binding site, and in this case we could not detect the thermal effect of phenol red because the binding site was occupied. A second possibility is that the decrease of ΔH_c was due to a change in the conformation of albumin arising from some hippuric acid still present. Hippuric acid and bilirubin have different discrete binding sites, but owing to a conformational change the phenol red site became inaccessible.

In another experiment, the defatted human albumin was supplemented by indoxyl sulphate. This investigation of the medium-chain fatty acid binding site demonstrated a zero effect on the enthalpy of the interaction of defatted albumin with sodium octanoate (Table 5). Indoxyl sulphate and octanoate, therefore, have the same binding site on the albumin molecule. The inhibition of the thermal effects of the interaction of phenol red with the indoxyl sulphate–albumin complex suggests that it is very close to the binding site of middle-chain fatty acids and tryptophan. The binding of salicylic acid and phenol red probably took place at their secondary binding sites. In addition, it is important to take into account the molar ratio between albumin and the uraemic toxin. The consistent values of the enthalpies of binding of marker ligands with defatted human albumin and the defatted human albumin–CMPF complex were observed only because the albumin:CMPF ratio equalled 2.5 and they were not due to the discrete binding sites for CMPF and sodium octanoate or phenol red. All the marker ligands bound to the second unloaded molecule of albumin.

Earlier work in our laboratory [40] and more recent work [41] has shown that CMPF inhibits the binding of bilirubin to albumin and there is competition between these two dicarboxylated endogenous substances for a common binding site. We have used BPB as a marker ligand for the bilirubin site and it has an association constant ($K_1 = 1.5 \times 10^6\ M^{-1}$, $n_1 = 3.0$) [39] comparable with that of CMPF ($K_1 = 10^6$–$10^7\ M^{-1}$; [6–8]). Because the value of n_1 is about three for BPB, a working molar ratio of albumin:BPB of 1:3 was used. Fig. 6 shows that CMPF produced a concentration-related increase in the unbound fraction of BPB. The increasing free fraction of dye was observed over a range of molar ratios of albumin:CMPF from 2.5–3.0 to 0.83. Further decreases in the ratio did not effect the unbound concentration of BPB. Flow microcalorimetry data for titration of the albumin–CMPF complex at a molar ratio equal to one demonstrated a sharp inhibition of phenol red binding (data not presented).

5. Conclusion

The three uraemic toxins hippuric acid, indoxyl sulphate and CMPF in the plasma of patients with end-stage renal disease contribute to inhibitory effect on binding to several sites on albumin. The binding site of indoxyl sulphate coincides with that for medium-chain fatty acids and tryptophan (site II). The CMPF binding site coincides with

the site for bilirubin (site I) and the hippuric acid site is in the region of salicylate. Conventional haemodialysis treatments do not remove albumin-bound uraemic toxins [42] and new methods such as the use of highly adsorbent carbons [24] should be investigated.

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