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Note

Isotachophoretic investigation of the binding of 8-anilino-1-naphthalenesulphonic acid to human serum albumin*

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Serum albumin has the ability to bind adsorptively a vast range of ligands, and the transport function of this protein is based on its interaction with small hydrophobic compounds. A listing of the substances whose binding to serum albumin has been investigated is presented by Chignell[1]. Visible and fluorescent dyes in particular have been employed for the experimental characterization of ligand binding to albumin. One such fluorescent dye is 8-anilino-1-naphthalenesulphonic acid (ANS), and a great deal of effort has been invested in the elucidation of the number and types of binding site for this ligand on the albumin molecule.

Typical analytical methods for this problem are fluorescence spectroscopy[2,3] and circular dichroism[4], from which it has been determined that bovine serum albumin possesses at least five binding sites for ANS, with differing affinities[4], and possibly also cooperativities[5,6]. An analysis of equilibrium constants and binding capacities using a modified Scatchard method from fluorescence measurements[3] yielded linear relationships both for human and bovine serum albumins, with $n = 3$ for ANS.

In conventional analytical techniques for binding studies, either the free or bound ligand is determined. As an alternative, analytical capillary isotachophoresis offers the opportunity to analyse simultaneously the protein–ligand complex and the free ligand. Thus, this technique has been applied to the analysis of free and bound indomethacin (1-*p*-chlorobenzoyl-5-methoxy-2-methylindole-3-acetic acid) and sodium dodecyl sulphate[7,8]. The appearance of free ligand at particular stoichiometric ratios was taken to indicate the limit of binding. In the present work, we show that this is not necessarily the case, and that further information on the nature of the binding can be obtained from the isotachopherograms.

MATERIALS AND METHODS

8-Anilino-1-naphthalenesulphonic acid was obtained from E. Merck (Darmstadt, G.F.R.) and human serum albumin (HSA) from Behring (Marburg, G.F.R.). The reagents for the leading and terminating electrolytes, hydrochloric acid, 6-aminohexanoic acid and 3-amino-1,2-propanediol were purchased from E. Merck, and

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hydroxypropylmethyl-cellulose (HPMC) from Ega-Chemie (Steinheim, G.F.R.). All reagents were of the highest available purity, and were used as supplied.

The incubations were carried out in unbuffered neutral solution, since high concentrations of buffer ions lead to longer analysis times in the isotachophoretic analyser. A series of mixtures containing 0.1 mmol/l HSA and ANS in the range 0.1–0.8 mmol/l were employed. A volume of 5 μl of the incubation mixtures was sufficient for qualitative and quantitative analysis by capillary isotachophoresis.

Isotachophoretic analyses were carried out on the LKB 2127 Tachophor (LKB, Bromma, Sweden), fitted with a PTFE capillary (23 cm × 0.5 mm I.D.). The temperature of the capillary block was maintained at 20°C during the analyses. Detection of the zones was both by thermal and UV-signals at 280 nm.

The zones were evaluated as follows. The UV signal (percent transmisson) of the LKB-Tachophor was converted into extinction units using a desk computer. The lengths of the UV-absorbing zones were measured in seconds at half maximal height, with a constant current of 50 μA for the zones passing the detectors. The zones were integrated in terms of (extinction units × seconds). The thermo-signals were used for qualitative analysis of the protein zones. Reciprocal reference unit (RRU) values were calculated from the step heights of the leading electrolyte, h_L, terminating electrolyte, h_T, and sample zone, h_X, by the following expression:

$$\mathrm{RRU} = \frac{(h_T - h_L)}{(h_X - h_L)} \cdot 100$$

The RRU value increases with increasing mobility. A stable plateau of at least 30 sec duration was taken as the minimal criterion for evaluation of this parameter, owing to the slow response and relatively poor resolving power of the thermodetector.

RESULTS

Fig. 1 shows isotachopherograms derived from various stoichiometric ratios of ANS and HSA. From the UV-signals at 280 nm, it is apparent that higher extinctions are obtained with increasing amounts of bound ANS, due to the inherent UV-absorption of the dye itself. Up to a ratio of 3:1, very little free dye, which migrates behind the protein zone, is seen. Thus, it appears that at least three molecules of ANS are bound very tightly to the protein. At ratios greater than 3:1, a further increase in UV-absorption occurs, but very much smaller than the initial increase up to a ratio of 3:1. However, this does indicate that further molecules of dye are bound. With increasing degree of ANS binding, the protein zone loses its characteristic rectangular form, indicating non-homogeneity of the zone. This behaviour, again, is particularly marked at ANS:HSA ratios greater than 3:1. Use of a longer capillary did not influence this pattern, so that the effect is not purely an artefact of the short capillary.

A plot of integrated area of the protein zone against ANS:HSA ratio is depicted in Fig. 2. A linear increase is observed up to a stoichiometry of 3:1, followed by a less steep increase at higher ratios. This is indicative of two types of binding, the first three molecules of ANS being bound more tightly than those at higher ratios. Fig. 3 represents a control plot for that in Fig. 2. Here, the total surface areas of the protein

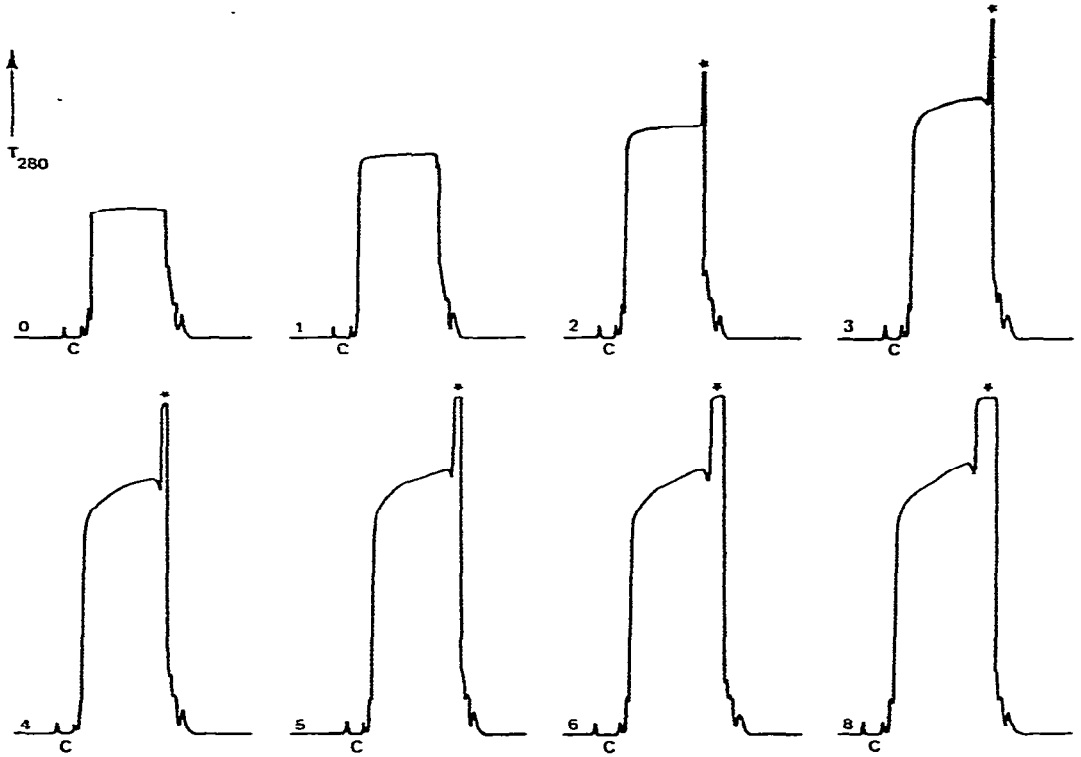


Fig. 1. Isotachopherograms derived from incubations of ANS with HSA at various stoichiometric ratios in the range 0 – 8, as indicated at the left-hand side of each trace. The UV signals at 280 nm are shown. The carbonate zone, always present to a small extent at high pH, is indicated by C, and the free dye zone by *.

and free dye zones at the various ANS:HSA ratios have been added, and, as expected, the plot is linear over the whole range.

Not only the UV signals, but also the thermo signals altered following dye binding. Fig. 4 shows this effect: with increasing degree of dye binding, the thermal step height of the protein zone is lowered, representing a shift to higher mobility.

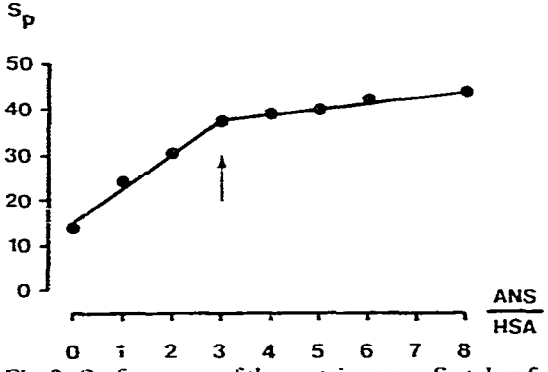
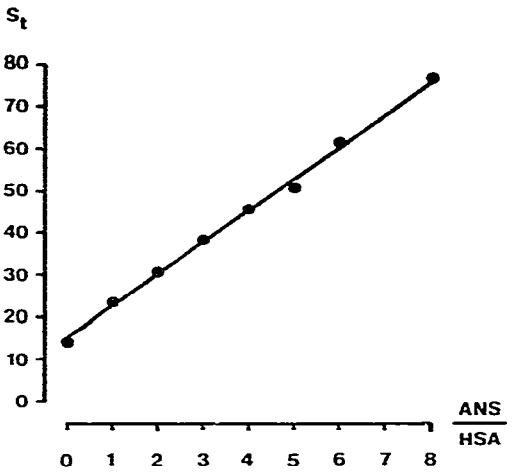


Fig. 2. Surface areas of the protein zones, S_p, taken from the isotachopherograms in Fig. 1, plotted against the respective ANS:HSA ratios.



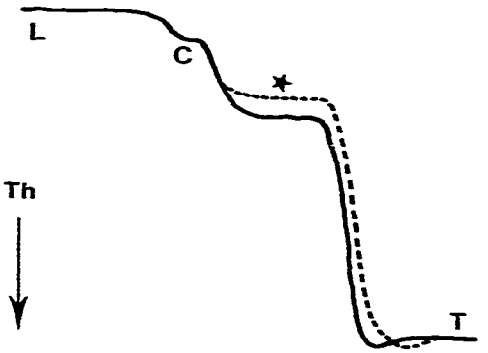


Fig. 3. Sum (S_t) of the surface areas of the protein and free dye zones in the isotachopherograms in Fig. 1, plotted against the respective ANS:HSA ratios.

Fig. 4. Thermo signals obtained for HSA without (continuous line) and with (discontinuous line) bound ANS. The protein zone is indicated by *, the zone preceding it, represented by C, is carbonate. L and T are the signals from the leading and terminating ions, respectively.

Thus, under the analytical conditions employed, *i.e.*, separations as anions at high pH, the albumin acquires additional negative charges following binding of the dye, resulting in an increase of the effective anionic mobility of the protein. This is possibly the first direct evidence that the dye binds as an anion rather than the free acid, neutralizing positive charges on the protein molecule. The RRU values, calculated from the expression given in the Materials and methods section, yield the plot shown in Fig. 5 with respect to ANS:HSA ratio. There is an excellent correlation with the form of the plot of surface area in Fig. 2, two straight lines intersecting at a ratio of 3:1. Thus, the mobility shifts are larger following binding of the first three molecules of dye.

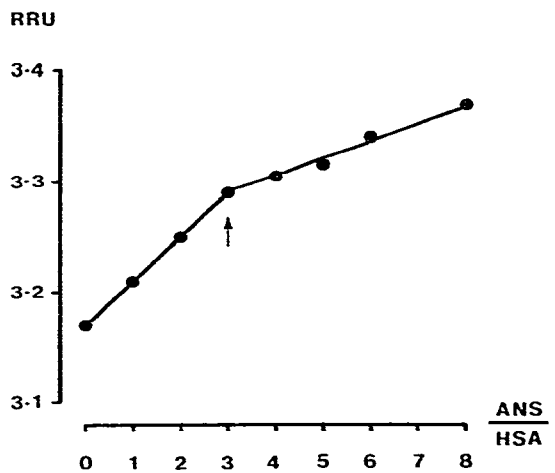


Fig. 5. Plot of RRU values, derived from the step heights of the thermo signals, against ANS:HSA ratios.

In the present work, we have demonstrated that analytical capillary isotachophoresis can be applied to the investigation of ligand–protein interactions. The fluorescent dye ANS has been chosen as a model ligand, since its binding to serum albumin has already been examined in some detail by other methods. Binding of the dye to the albumin leads to an increase in the extinction of the protein zone, due to the intrinsic UV absorption of the dye. Integration of the UV signal gives a measure of the dye binding, whence the binding character can be elucidated from the slope of the relevant plot, and the appearance of free dye. The surface area rather than the extinction itself must be taken as the parameter, since there is a slight alteration of the zone width after dye binding, resulting either from an alteration in the protein conformation (molecular volume) or a mobility shift. The dye binding can also be monitored by use of a detector which responds to differing mobilities, in this case, the thermodetector. Dye binding leads to a shift to higher mobilities, presumably due to an increase in the net negative charge of the protein molecule. Again, a two-phase process is noted, indicating at least two types of binding site.

From work with other techniques[3], the tight binding of three molecules of ANS to a molecule of HSA had been reported. Furthermore, it was shown that ANS:HSA ratios greater than 3:1 can be obtained, since there are lower affinity sites in addition to the three high affinity sites[5,6]. Thus, the results of our isotachophoretic study seem to correlate quite well with these data. The obvious advantage of isotachophoresis over conventional methods is that both the protein–dye complex and the free dye can be analysed separately in a single run.

This technique could find application, for example, in the study of enzyme–substrate interactions, or the identification of a particular binding protein in a complex mixture.

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