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Calorimetric Determination of the Enthalpies of Binding of Ions to Deionized Bovine Serum Albumin*

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ABSTRACT: The enthalpy of binding of iodide ions to deionized bovine serum albumin was determined by calorimetry, primary attention being given to binding at the first site. Three sets of experiments were performed, the data being analyzed by a different procedure in each set. The value reported by Scatchard *et al.* (Scatchard, G., Coleman, J. S., and Shen, A. L. (1957), *J. Amer. Chem. Soc. 79*, 12) for the association constant for the first I^- binding site was used to calculate the amount of I^- bound. The mean value for the enthalpy of binding at this site is -17.4 ± 1.0 kcal mole^{-1} at 25°. Combination of this value with the standard free energy of binding gives -42 cal deg^{-1} mole^{-1} for the standard entropy of binding, a surprisingly large unfavorable entropy change. The second set of sites, approximately 8 in number according to Scatchard *et al.*, is characterized by a much smaller binding enthalpy, approximately -3 kcal (mole of sites)$^{-1}$, and the remaining sites, up to 18 reported, have a still smaller enthalpy, -2 kcal mole^{-1}. Less extensive measurements on the binding of Cl^- and the dodecyl sulfate ion were also carried out.

The enthalpies of binding of anions to serum albumin have been estimated in several laboratories by application of the van't Hoff equation to binding equilibria determined at two or more temperatures. A tabulation of representative data for organic anions was published by Kauzmann (1959), and since then additional data have appeared. For example, Scatchard and Yap (1964) investigated inorganic anion binding to HSA,[1] and Reynolds *et al.* (1967) studied the binding of several detergent anions to BSA.

The van't Hoff method for obtaining enthalpies is in general less reliable than the direct calorimetric procedure when the latter can be applied. This situation is not only a result of the inherent inaccuracy of evaluating the slopes of experimentally established curves. The method has been frequently applied in cases where data at only two temperatures are available, and it has therefore been necessary to make the assumption that there is no change in heat capacity accompanying the process. This assumption is apt to be especially poor in the case of processes involving proteins.

In much of the earlier work on the binding of anions to serum albumin the experiments were carried out in the presence of buffers the ions of which could also bind to the protein, or if buffers were not used, the protein had been incompletely deionized.

For these reasons we have determined calorimetrically the enthalpies of binding of several anions to isoionic BSA. A detailed study of the binding of I^- over a wide concentration range has been made, and less detailed studies of the binding of Cl^- and DS^-. Since pH changes take place when anions are bound to BSA, the enthalpy of binding protons to isoionic BSA was also determined.

Materials and Methods

Most of the calorimetric measurements were made with crystalline BSA obtained from Armour and Co. The protein was deionized on Dintzis columns and stored as previously described (Lovrien, 1963). A few calorimetric observations of halide binding were performed with BSA defatted by the method of Chen (1967) and then deionized, and a few with BSA having approximately one molecule of azobenzene bound per molecule of protein. The latter material was prepared by gently stirring a solution of BSA with crystals of azobenzene. Since the experiments with these materials led to results showing no significant differences from those obtained with the

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[1] Abbreviations used are: BMA, bovine mercaptalbumin; BSA, bovine serum albumin; HSA, human serum albumin; DS^-, dodecyl sulfate anion.

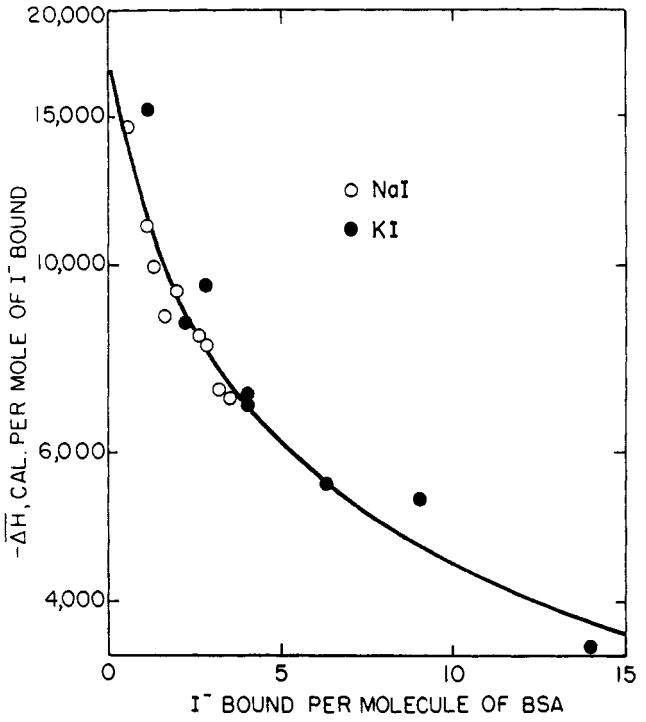

FIGURE 1: The average enthalpy of binding of I^- to deionized BSA at 25° as determined by direct calorimetry. The BSA concentration after mixing was 1.83×10^{-4} M. The curve was calculated using the binding constants, numbers of I^- bound at each of three types of sites and the electrostatic work terms given by Scatchard *et al.* (1957), and the enthalpy values given in Table I.

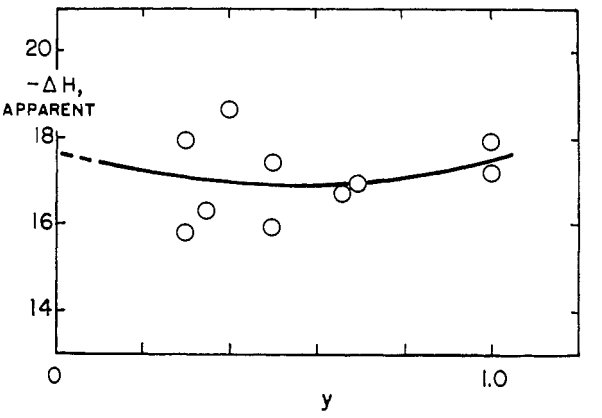

FIGURE 2: The enthalpy of binding of I^- to deionized BSA at 25°. The apparent enthalpy change, equal to the observed heat effect divided by the concentration, (PI), of I^- bound to the first site, is plotted as a function of $y = (I)_0/(P)_0$. The value of (PI) is calculated according to eq 1. The solid curve is a least-squares-fitted quadratic in y.

deionized crystalline protein, all the results reported here refer to the latter type of preparation.

Dodecyl sulfate from Mann Research Laboratories was used in dialysis equilibrium measurements of the binding of DS^-. An apparatus similar to that described by Karush (1956) was employed in these measurements.

Three different calorimeters were employed. Some of the results on the binding of I^- given in Figure 1 were obtained with the apparatus and procedure described by Buzzell and Sturtevant (1951), and some with a Beckman Model 190 microcalorimeter. The remainder of the measurements were obtained with an instrument based on the design of Wadsö (1968) (Lovrien and Anderson, 1969).

There are two principal papers which give association constants for I^- binding to this protein: Scatchard *et al.* (1957) and Saifer *et al.* (1964). A choice between these is not a strong one, but is somewhat in favor of the values given by Scatchard *et al.* For example, if the values of Saifer *et al.* for K_1 for I^- binding to BSA are used instead of Scatchard *et al.*'s value, *i.e.*, 4900 *vs.* 9240, the calculated plot in Figure 1 is much lower than shown, unless we take $n_1 > 1$ or else $\Delta H_1 > 18$ kcal. The values used in this paper are, however, rather close to the Saifer *et al.* values for BSM.

Results

Binding of Iodide Ions. The results of the first two sets of experiments are summarized in Figure 1. It should be noted that these two sets were performed with different calorimeters and with different preparations of BSA, at times a few years apart. As expected, KI gives essentially the same results as NaI. The extent of binding, $\bar{\nu}$ = the average number of I^- bound per molecule of protein, was calculated from the total protein and I^- concentrations, following the method and using the binding constants given for BMA by Scatchard *et al.* (1957). These authors concluded that their binding data were consistent with the existence of 3 classes of I^- binding sites on BMA, accommodating 1, 8, and 18 ions, respectively, and characterized by three different intrinsic binding constants. Binding studies by Saifer *et al.* (1964) also indicate a single especially tightly binding site for I^-. The calculation of $\bar{\nu}$ includes the effect of electrostatic interactions between bound and unbound I^- ions, with the electrostatic work terms calculated as outlined by Scatchard *et al.* (1957). Their eq 11, weighted with enthalpy values for the various classes of sites, ΔH_i, yields a total enthalpy for binding several iodides, *i.e.* $\Delta H_{\text{total}} = \Sigma_i \bar{\nu}_i \Delta H_i$. The average heat per bound iodide, $\Delta H = \Sigma_i \bar{\nu}_i \Delta H_i / \Sigma_i \bar{\nu}_i$, is plotted as the ordinate of Figure 1. It was calculated using for the binding enthalpies at the three types of sites the values $\Delta H_1 = -18.0$ kcal mole^{-1}, $\Delta H_2 = -3.2$ kcal mole^{-1}, and $\Delta H_3 = -2.0$ kcal mole^{-1}.

A third set of measurements is summarized in Figure 2, in which the apparent value of the enthalpy of binding to the first site is plotted against the ratio $y = (I)_0/(P)_0$, where $(I)_0$ and $(P)_0$ are, respectively, the total concentrations of I^- and BSA. The apparent enthalpy is calculated as $\Delta Q/(PI)$, where ΔQ is the heat effect observed per liter of solution and (PI) is the concentration of I^- bound at the first site, the latter quantity being evaluated from the approximate relation for the binding constant for the first class of sites

$$K_1 \approx \frac{(PI)}{[(P)_0 - (PI)][(I)_0 - (PI)]} \qquad (1)$$

This expression becomes exact as y approaches zero, and is actually not very inaccurate at $y = 1$ since K_2, the binding constant for the second class of sites, is small compared to K_1. In using eq 1, concentrations expressed in molarities were employed although K_1 is in units of (molality)$^{-1}$ (Scatchard *et al.*, 1957). The intercept of the plot in Figure 2 gives $\Delta H_1 = -17.5 \pm 1.0$ kcal mole.

Two additional sets of measurements were performed at two different protein concentrations. In these experiments the results were expressed as $\Delta Q/(I)_0$; this quantity is plotted

TABLE I: Enthalpies of Binding of Anions to Isoionic Bovine Serum Albumin at 25°.

Data in Figure	No. of Sites (i)	No. of Ions Bound, n	$-\Delta H_i$ (kcal mole^{-1})	$-\Delta G_i°$[a] (kcal mole^{-1})	$\Delta S_i°$ (cal deg^{-1} mole^{-1})
			Binding of Iodide Ions		
1	1	1	18.0	5.41	−42
	2	8	3.2	3.53	+1.1
	3	18	2.0	1.51	−1.6
2	1	1	17.5 ± 1.0		
3	1	1	16.8 ± 1.0		
		Mean	17.4 ± 1.0		
			Binding of Chloride Ions		
	1	1	8.3 ± 3.0	4.62	−12.3
	2	8	3.1 ± 1.0	2.73	−1.2
			Binding of Dodecyl Sulfate Ions		
	1	1	18 ± 4		

[a] Values reported for BMA by Scatchard *et al.* (1957).

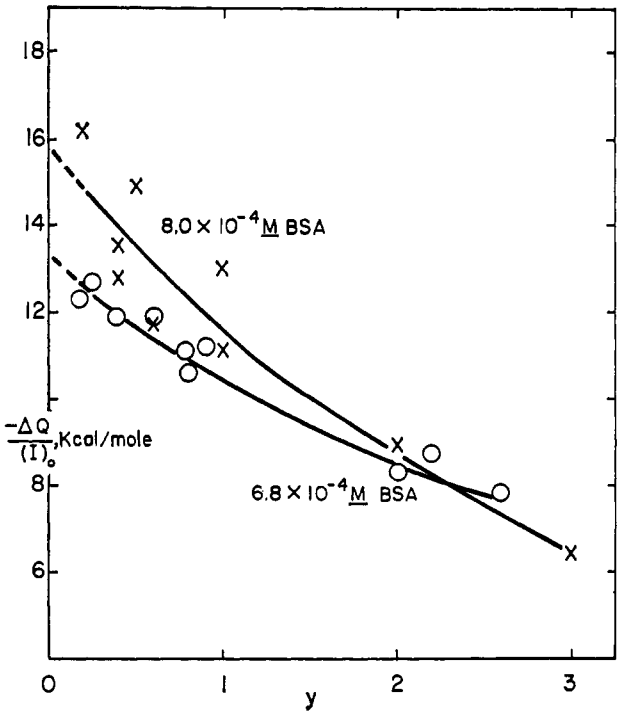

FIGURE 3: The enthalpy of binding of I^- to deionized BSA at 25°. The observed heat effect divided by the total I^- concentration is plotted as a function of $y = (I)_0/(P)_0$ for two different protein concentrations. The solid curves are least-squares-fitted quadratics in y.

against y in Figure 3. It is easily shown that for single-site binding

$$\frac{(PI)}{(I)_0} = \frac{K(P)}{1 + K(P)} \equiv j \tag{2}$$

where K is the binding constant. From this equation it follows that

$$\frac{j}{1 - j} = (1 - \bar{\nu})K(P)_0 \tag{3}$$

As y approaches zero, $\bar{\nu} \ll 1$, and j can be calculated from the value of K (Scatchard *et al.*, 1957) and the total protein concentration. Thus the binding enthalpy for the first site is given by

$$\frac{1}{j} \lim_{y \to 0} \frac{\Delta Q}{(I)_0}$$

The curves in Figure 3 were obtained by least squaring the data points to quadratic expressions in y; from the intercepts and the calculated limiting values of j, ΔH_1 is calculated to be -18.0 ± 1.9 and -15.5 ± 1.2 kcal mole^{-1} for the two sets of data in Figure 3. The negative slopes apparent in the figure are to be expected in view of the lower binding enthalpies characterizing the less tightly binding sites.

The results for the binding of I^- to BSA are summarized in Table I. The various sets of experiments are listed according to the figures in which they are recorded. We conclude that the enthalpy of binding of I^- at the single tightest binding site is $\Delta H_1 = -17.4 \pm 1.0$ kcal mole^{-1} at 25°.

Saifer and Steigman (1961) studied the binding of I^- to deionized BMA, using a tracer equilibrium dialysis procedure, obtaining results in good agreement with those of Scatchard *et al.* (1957). Saifer *et al.* (1964) applied the same procedure to the binding of I^- to BSA with results significantly different from those for BMA. For unknown reasons, our calorimetric data are more accurately fitted using the binding data for BMA than those for BSA. Fortunately, the value for the limiting enthalpy of binding at very low values of $\bar{\nu}$, ΔH_1, is relatively insensitive to the value of K_1 used in the calculations.

The standard entropies listed in Table I include an uninteresting contribution reflecting the loss of translational entropy when two molecules react to form a single molecule. Gurney (1953) has suggested adoption of the standard state of unit activity at unit mole fraction as an approximate means for elimination of this contribution. The resulting so-called unitary entropies are obtained in the present case by adding $R \ln 55.5 = 8.0$ cal deg^{-1} mole^{-1} to the values given in Table I.

Binding of Chloride and Dodecyl Sulfate Ions. The binding of Cl^- to BMA has been studied by Scatchard *et al.* (1957). On the assumption that their binding constants apply to BSA (again neglecting differences between molalities and molarities), we can calculate the extents of occupancy of the various types of sites in calorimetric experiments, and estimate binding enthalpies, as was done in the case of I^-. Eight calorimetric experiments gave the results of $\Delta H_1 = -8.3 \pm 3.0$ kcal mole^{-1} (one site) and $\Delta H_2 = -3.1 \pm 1.0$ kcal mole^{-1} (8 sites).

In the case of DS^-, the determination, by equilibrium dialysis, of binding at low concentrations is very difficult, and no value for the binding constant for the first molecule bound is available at present. In interpreting our calorimetric data, we have assumed that at low values of y only one site is occupied, and that j is practically unity. This latter assumption is equivalent to assuming a binding constant of at least 10^6 M^{-1}.

It is of interest that in eight equilibrium dialysis experiments with DS^- at 2°, with $\bar{\nu} = 6$–12, in phosphate buffer, we obtained data in good agreement with those of Reynolds *et al.* (1967). Since the detergent–protein interaction is sensitive to impurities, this agreement suggests that our BSA preparation was free of significant contamination.

Binding of Protons. Since the pH of a solution of isoionic BSA increases when anions are bound by the protein, it is evident that the degree of protonation of one or more of the acidic groups in the protein must increase. Consideration of the relative numbers of carboxyl and imidazole groups, and their states of ionization in the isoionic region (Tanford *et al.*, 1955), makes it probable that the protons taken up by the protein go to carboxylate groups. The van't Hoff estimate for the heat of protonation of these groups is -1.6 kcal mole^{-1} (Tanford *et al.*, 1955). The calorimetric heats of protonation of several aliphatic carboxylate ions given by Christensen *et al.* (1967) lie for the most part in the range 0 to -1.5 kcal mole.

We have measured calorimetrically the average heat of protonation of about 10 carboxylate groups around the isoionic region. Experiments in the presence of 0.02 and 0.15 M NaCl gave indistinguishable results, and there was no statistically significant variation in the heat effect per proton bound with the net charge on the protein. The average value was $\Delta H = -1.4 \pm 0.3$ kcal/mole of proton added.

It appears that protonation heats cannot make any very significant contribution to the anion binding enthalpies reported here. Other linked effects, such as changes in hydration, could make very substantial contributions, but no methods are available for detecting the presence or absence of such effects, or for estimating their magnitudes.

Discussion

Scatchard and Yap (1964) determined the binding of I^- to HSA at 0 and 25°, and from these data estimated for the three types of binding sites $\Delta H_1 = -7.03$ kcal mole^{-1}, $\Delta H_2 = -3.26$ kcal mole^{-1}, and $\Delta H_3 = 0$ kcal mole^{-1}. Agreement with our calorimetric results for BSA is reasonably good with respect to all except the first site, their value being only 40% of our value. This difference seems too large to be attributable to the difference in proteins, especially since there is very little difference in $\Delta G_1°$ between HSA and BMA (Scatchard *et al.*, 1957). In view of the recent observation of very large changes in apparent heat capacity in certain protein reactions (Velick *et al.*, 1971; Hinz *et al.*, 1971), it is of interest to see whether the binding constants for I^- with HSA at 0 and 25° (Scatchard and Yap, 1964) and our binding enthalpy for I^- with BSA at 25° can be reconciled on the basis of a reasonable value for ΔC_p. If we set

$$\Delta H = \Delta H_0 + T\Delta C_p \tag{4}$$

and use the resulting integrated form of the van't Hoff equation

$$R \ln \frac{K_1}{K_2} = -\Delta H_0\left(\frac{1}{T_1} - \frac{1}{T_2}\right) + \Delta C_p \ln \frac{T_1}{T_2} \tag{5}$$

we have two equations to solve for ΔH_0 and ΔC_p. Inserting the appropriate numbers and solving gives $\Delta C_p = -870$ cal deg^{-1} mole^{-1} for binding at the first site, a value which we consider to be of unreasonably large magnitude. It thus appears either that the enthalpy and entropy contributions to $\Delta G_1°$ in HSA are very different from those in BSA, or the van't Hoff enthalpy of Scatchard and Yap (1964) is in error.

It is evident that the binding of the first I^- and Cl^-, and in all probability also the first DS^-, to BSA at 25° is energy rather than entropy driven. This is overwhelmingly the case for the first site for I^- where the entropy of binding is highly unfavorable.

It is not at all easy to suggest convincing sources for an enthalpy of -18 kcal mole^{-1} in the binding of I^- to a protein. Presumably anions bind at protein sites having one or more positive charges in their vicinities, so that the possibility exists of enthalpic contributions from Coulombic interactions. The simplest formulation (Kauzmann, 1959) of the formation of an ion pair in a continuous medium of dielectric constant D gives for the relations between free energy, enthalpy, and entropy the expressions

$$\Delta H = \frac{\mathrm{d} \ln D}{\mathrm{d} \ln T}\Delta G \qquad -T\Delta S = \left(1 - \frac{\mathrm{d} \ln D}{\mathrm{d} \ln T}\right)\Delta G \tag{6}$$

In water at 25°, $\mathrm{d} \ln D/\mathrm{d} \ln T = -1.34$, so that the attraction of two charges of opposite sign is presumably entropy driven. It is impossible to say what the temperature dependence of the effective dielectric constant would be in the situation of interest here.

An interesting and pertinent system was studied calorimetrically by Papenmeier and Campagnoli (1969). They found that mixing the anionic detergent sodium dodecyl sulfate with the cationic detergent cetylpyridinium chloride in equimolar amounts at 37° resulted in an enthalpy change of -18 kcal mole^{-1}, and an entropy change of approximately -30 cal deg^{-1} mole^{-1}. Here, as in the case of interest to us, it is impossible to state the source of such a large enthalpy change. However, an electrostatic contribution in the enthalpy of mixing oppositely charged detergents is suggested by comparison with the enthalpy of micellization of a single kind of detergent, *e.g.*, decyl sulfate. The latter is quite small, or positive (Goddard *et al.*, 1957). Association of long paraffin chains in water is probably roughly equivalent from system to system, in enthalpy. Therefore differences in micellization enthalpy between single detergents and mixed ones appears to originate in the behavior of the ionic groups upon close packing.

A source of enthalpy change might lie in changes of solvation of anions upon binding, with possible release of solvent molecules to the bulk solvent, if the bound ion is stabilized by interaction with other charges or polarizable groups in the protein instead of with water molecules. Particularly interesting in connection with the problem of solvation of anions is the work of Arshadi *et al.* (1970). They determined the thermodynamics of gas-phase reactions

$$X^-(H_2O)_{n-1}(g) + H_2O(g) \rightleftarrows X^-(H_2O)_n(g) \tag{7}$$

by mass spectrometry of ions equilibrated with water vapor at various pressures and temperatures. The enthalpy change accompanying hydration of I^- was found to range from -10.2 kcal mole^{-1} for the first H_2O molecule to -9.4 kcal mole^{-1} for the third one. If the heat of vaporization of water, 10.5 kcal mole^{-1}, is taken into account, it appears that the process

$$I^-(g) + H_2O(l) \rightleftarrows I^-(H_2O)_n(g) \tag{8}$$

is accompanied by very little net enthalpy change. These considerations suggest that even if the bound I^- is approximately anhydrous, the change in the hydration state of the ion may not make a large contribution to the binding enthalpy.

There is evidence that conformational changes in the pro-

tein may be induced by ligand binding. Bigelow and Sonenberg (1962) and Polet and Steinhardt (1968) obtained aromatic difference spectra in the dodecyl sulfate–serum albumin complex at moderately low ($\bar{\nu} \sim 8$) levels at neutral pH which are consistent with conformation control exerted by the detergent. Specific viscosities reflecting an unusual amount of conformational tightening were found by Reynolds *et al.* (1967) in the same system. Markus *et al.* (1967) showed that methyl orange binding to human serum albumin, which appears to have $n_1 = 2$ strong sites, causes marked tightening of the protein as judged by its resistance to enzyme-catalyzed proteolysis. Benson and Hallaway (1970) observed major effects on the tritium exchange of bovine serum albumin by small amounts of DS^-, again consistent with marked tightening of the protein conformation. Foster (1968), reviewing a series of papers from his laboratory, and also by other authors, notes that detergent and halide ions can control the refolding reaction of the serum albumin molecule in microheterogenity studies. The conformational transition taking place in the region of pH 4 (the N–F transition in Foster's terminology) also comes under anion binding control.

From these and other studies, there is little doubt that serum albumin is considerably more compact and less conformationally motile when anions at low binding levels are bound than in the absence of such ligands. In Karush's older (1950) but descriptive terminology, the isoionic protein is configurationally adaptable. Therefore, the general tightening of the protein upon anion binding may also be a source of the enthalpy, especially in the sensitive (low) ranges of ligand concentration levels. The tightening phenomenon may produce heat in either the folding of the protein itself, or from water leaving the domain of the protein upon conformational tightening, or both. The negative entropies observed in the binding of various anions at the first binding site are consistent with these concepts.

Acknowledgments

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