Spectroscopic and Microcalorimetric Study of the Interaction of *n*-Alkyl Sulfates with Insulin in Aqueous Solution

M. Reza Housaindokht,† Malcolm N. Jones* and Joanne F. Newall
Department of Biochemistry and Molecular Biology, University of Manchester, Manchester, UK M13 9PT
Gerardo Prieto and Felix Sarmiento
Department of Applied Physics, University of Santiago de Compostela, Spain

A surfactant-induced conformational transition of bovine insulin that leads to difference spectra assigned to changes in the environment of the tyrosine residues has been studied at pH 10, 25 °C. The transition induced by a homologous series of C_8–C_{12} *n*-alkyl sulfates, below their critical micelle concentrations was studied by difference spectroscopy and the absorbance changes at 295 nm were analysed to obtain values for the Gibbs energies of the transition in water (ΔG^{o}_{w}) and in a hydrophobic environment (ΔG^{o}_{hc}) pertaining to saturated protein–surfactant complexes. The average value of ΔG^{o}_{w}, which was found to be independent of *n*-alkyl chain length, was 14.6 kJ mol^{-1}. The values of ΔG^{o}_{hc} were in the range *ca.* −40 to −100 kJ mol^{-1} for chain lengths from C_8 to C_{12}. The enthalpies of interaction of the *n*-alkyl sulfates were measured over the surfactant concentration ranges of the transition and were used to estimate an enthalpy change for the transition of 97 ± 10 kJ mol^{-1}.

Monomeric insulins are small proteins (*e.g.* bovine insulin has a molecular mass of 5734[1]) and hence are structurally simpler than many larger proteins, however, very little work has been reported on their denaturation and associated conformational changes.[2–4] This is most probably a consequence of the association and low solubility of insulin at neutral pH, although *in vitro* the biological effects are attributed to the insulin monomer which circulates in the blood at concentrations of the order of 10^{-8}–10^{-11} mol dm^{-3}.[5] At neutral pH at higher concentrations insulin self-associates to form first dimers and then hexamers. The self-association of insulins under various solution conditions has been studied[6–8] and there have been studies on the interaction of phenolic[9,10] and surfactant ligands with insulins[11–16]

In our previous study[15] on the interaction of *n*-alkyl sulfates with bovine insulin it was shown that in both acid (pH 3.2) and alkaline (pH 10) solutions insulin binds high levels of surfactants (2.5–3.5 g per g of insulin). We now report a study in which conformational changes of insulin in the presence of a homologous series of *n*-alkyl sulfates (C_8 to C_{12}) have been followed by difference spectroscopy and used to obtain Gibbs energies of unfolding together with further microcalorimetric data carried out under the same conditions of pH and ionic strength as the spectroscopic measurements.

Experimental

Materials

Crystalline insulin from bovine pancreas (No. I 5500 24 I.U. per mg) was obtained from Sigma Chemical Co. The surfactants were obtained from the following sources: sodium *n*-octyl sulfate (No. 5786 Lancaster MTM Research Chemicals Ltd), sodium *n*-nonyl sulfate (No. 6418 Lancaster MTM Research Chemicals Ltd), sodium *n*-decyl sulfate (No. 56027 Cambrian Chemicals), sodium *n*-undecyl sulfate (No. 6429 Lancaster MTM Research Chemicals Ltd) and sodium *n*-dodecyl sulfate (specially pure) (No. 44244 BDH Chemicals Ltd). Two buffers were used; glycine (50 mmol dm^{-3})–sodium hydroxide pH 10.0, ionic strength 0.0312 and phosphate (50 mmol dm^{-3}) pH 4.0, ionic strength 0.0500. The ionic strengths of the buffers were calculated from the glycine second dissociation constant pK_a (2) = 9.7796[17] and the phosphate second dissociation constant pK_a (2) = 7.21.[17] At an ionic strength of 0.03 (temperature 21–25 °C) the critical micelle concentrations (c.m.c.s) of the *n*-alkyl sulfates are as follows[18]: C_8, 118 mmol dm^{-3}; C_9, 52.8 mmol dm^{-3}; C_{10}, 21.1 mmol dm^{-3}; C_{11}, 9.48 mmol dm^{-3}; C_{12}, 3.08 mmol dm^{-3}.

Apparatus and Methods

Difference spectra were measured with a Cary 219 UV-VIS spectrophotometer operating in the double-beam mode with a full scale expansion of 0.2 absorbance units and thermostatted at 25 °C. All the measurements were made using insulin solutions of concentration 0.05% w/v (0.5 × 10^{-3} kg dm^{-3}) in each of a pair of carefully matched quartz cuvettes (1 cm^3 capacity) in the wavelength range 280–320 nm. One cell contained surfactant in the required concentration range. Measurements were made after insulin and surfactant had been incubated for over 30 min after which time the difference spectra did not change.

Enthalpy measurements were made at 25 °C with an LKB 10700 batch microcalorimeter which utilizes the twin-vessel principle, each vessel being divided into two compartments.[19] The microcalorimeter was frequently calibrated electrically during the course of the study. On the most sensitive range used for the measurements (30 μV), the mean sensitivity of the detectors in the heat sinks of the two vessels was 14.66 ± 0.32 μW μV^{-1} (*i.e.* ±2.2%). The two detector sensitivities differed by only 0.37%, which is less than the standard deviation of the sensitivity measurements of the two detectors *i.e.* the vessels were matched.

The reaction vessel was charged with (2 ± 0.1) × 10^{-3} kg of insulin solution (concentration 0.1% w/v (1 × 10^{-3} kg dm^{-3}) and (2 ± 0.1) × 10^{-3} kg of surfactant solution of the required concentration. The reference vessel was charged with (2 ± 0.1) × 10^{-3} kg of buffer solution and (2 ± 0.1) × 10^{-3} kg of surfactant solution identical with that in the reaction vessel. On mixing the surfactant solutions the enthalpies of dilution cancel and the enthalpy of dilution of insulin is negligible. The final insulin concentration (0.5 × 10^{-3} kg dm^{-3}) was the same as that used in the spectroscopic experiments.

† Current address: Department of Chemistry Faculty of Science, Shiraz University, Iran.

Results

Fig. 1 shows the difference spectra in the wavelength range 280–320 nm at pH 10.0, between insulin and insulin plus the C_{12} sulfate as a function of the surfactant concentration. Addition of surfactant results in the development of an absorption band at 295 nm, negative relative to native insulin and characteristic of the phenolic side-chain of tyrosine.[20] The difference spectrum absorbance for the C_{12}, surfactant (the strongest denaturant) was checked for reversibility. Within experimental error (*ca.* 2%) the difference spectral absorbance for a solution diluted from a high surfactant concentration (*ca.* 5 mmol dm^{-3}) to a lower surfactant concentration (*ca.* 2.5 mmol dm^{-3}) was the same as that of insulin that had not been exposed to a high surfactant concentration, so that the conformational changes giving rise to the 295 nm difference spectral peak are reversible within experimental error. Similar spectra to those of Fig. 1 were obtained for all the other alkyl sulfates (C_8 to C_{11}). It should be noted that all the experiments were carried out with the insulin disulfide linkages intact. Addition of the disulfide reducing agent mercaptoethanol at pH 10 gave rise to very much larger spectral changes. Under these conditions the A and B chains are separated, resulting in gross conformational changes.

Attempts were made to obtain a corresponding set of spectra in acid solution pH 4.0. This was not possible however because of the insolubility of the insulin–surfactant complexes in acid solution. Limited data was obtained at high surfactant concentrations *i.e.* for C_8 (>100 mmol dm^{-3}), C_9 (>45 mmol dm^{-3}), C_{10} (>20 mmol dm^{-3}), C_{11} (>10 mmol dm^{-3}). The complexes formed with the C_{12} surfactant were insoluble over the entire range of composition. The limited data at pH 4 showed that the absorbance of insulin plus surfactant was positive relative to native insulin, also the absorbance maxima showed a shift to shorter wavelengths (*ca.* 290 nm). Since the phenolic hydroxy group of tyrosine will be fully protonated at pH 4 the data suggest that transfer of the protonated form to the more hydrophobic environment of the complexes causes an increase in absorbance in contrast to protonation at pH 10 which causes a decrease in absorbance.

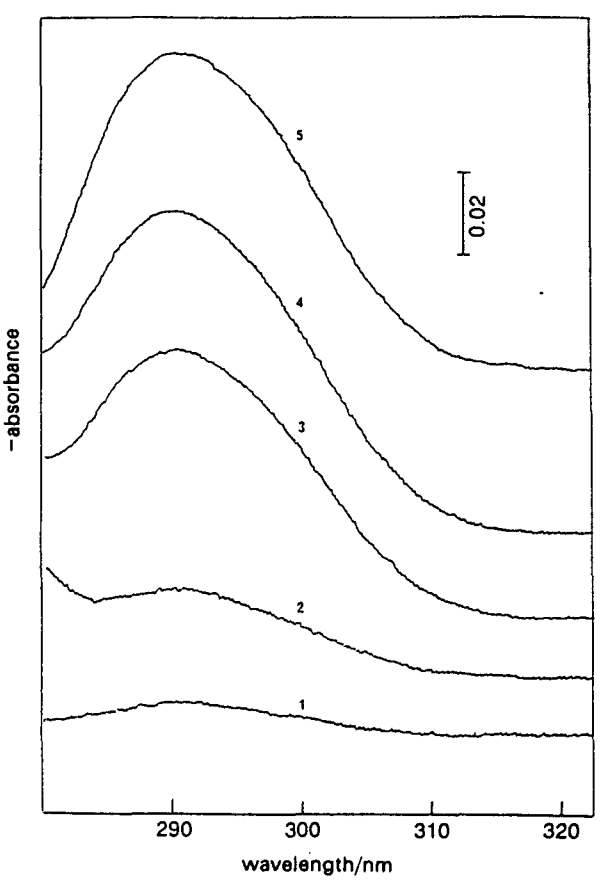

Fig. 1 Difference spectra of bovine insulin in the presence of sodium *n*-dodecyl sulfate relative to native insulin at pH 10, 25 °C. The insulin concentration was 0.5 × 10^{-3} kg dm^{-3} and the sodium *n*-dodecyl sulfate concentrations were: 1, 1.25; 2, 2.0; 3, 3.0; 4, 5.0 and 5, 8.0 mmol dm^{-3}. The spectra were recorded continuously throughout the wavelength range.

Fig. 2 and 3 show the absorbance changes for the 295 nm difference spectral band as a function of surfactant concentration for the homologous series of surfactants at pH 10. The data show that for all the surfactants there is a transition region over which the absorbance changes steeply with concentration. As the surfactant chain length increases the transition regions becomes sharper and the total change in absorbance becomes greater for the range C_8 to C_{10}. The shift in the transition region to lower concentrations follows the decrease in the c.m.c.s of the surfactants.

The enthalpy of interactions between insulin and the *n*-alkyl sulfates is shown in Fig. 4 and 5 as a function of the surfactant concentration after mixing. The enthalpy curves for the series C_8 to C_{11} all show endothermic maxima which decrease and occur at lower surfactant concentrations with

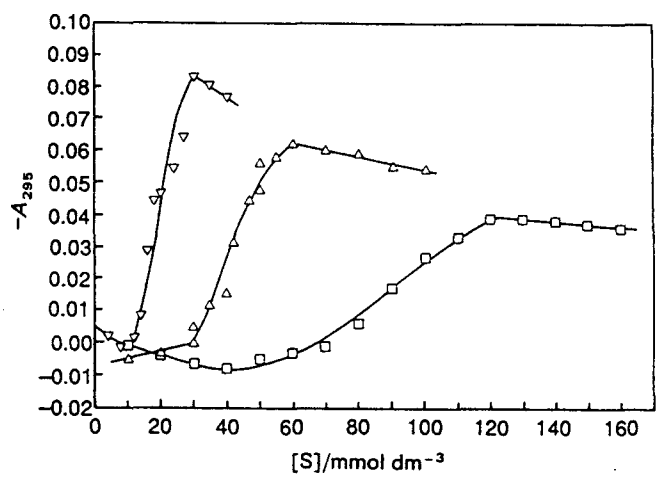

Fig. 2 Absorbance of bovine insulin at 295 nm in the presence of surfactants relative to native insulin at pH 10, 25 °C. (□) sodium *n*-octyl sulfate, (△) sodium *n*-nonyl sulfate, (◇) sodium *n*-decyl sulfate. The insulin concentration was 0.5 × 10^{-3} kg dm^{-3}.

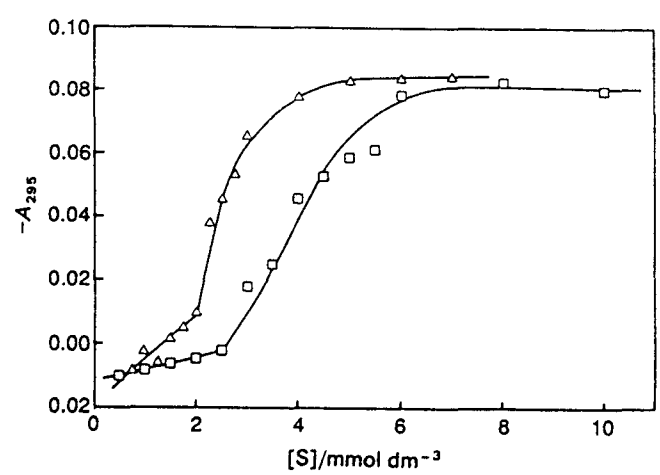

Fig. 3 Absorbance of bovine insulin at 295 nm in the presence of surfactants relative to native insulin at pH 10, 25 °C. (□) sodium *n*-undecyl sulfate, (△) sodium *n*-dodecyl sulfate. The insulin concentration was 0.5 × 10^{-3} kg dm^{-3}.

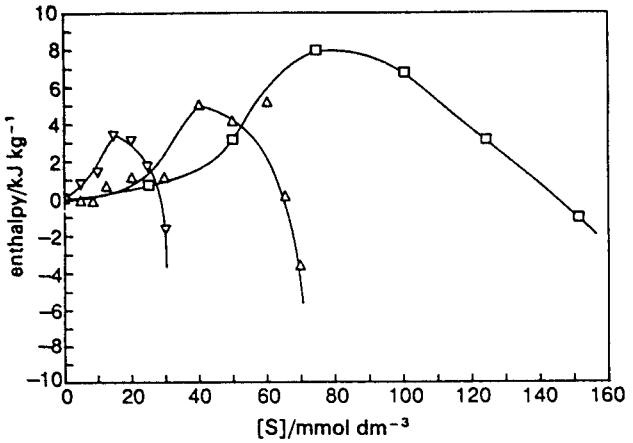

Fig. 4 Enthalpy change in kJ (kg insulin)$^{-1}$ at 25 °C on interaction of surfactants with bovine insulin at pH 10, 25 °C. (□) sodium *n*-octyl sulfate, (△) sodium *n*-nonyl sulfate, (▽) sodium *n*-decyl sulfate. The insulin concentration was 0.5×10^{-3} kg dm^{-3}.

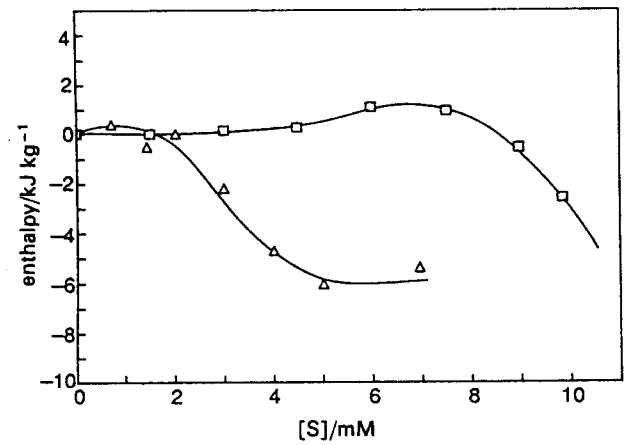

Fig. 5 Enthalpy change in kJ (kg insulin)$^{-1}$ at 25 °C on interaction of surfactants with bovine insulin at pH 10, 25 °C. (□) sodium *n*-undecyl sulfate, (△), sodium *n*-dodecyl sulfate. The insulin concentration was 0.5×10^{-3} kg dm^{-3}.

increasing alkyl chain length. Previous enthalpy measurements for the C_8, C_9 and C_{10} *n*-alkyl sulfates made at pH 10 but at considerably higher ionic strength (0.125) also gave endothermic maxima.[15] The shift of the curves to lower surfactant concentrations reflects the decrease of the c.m.c.s as for the absorbance curves. It was also observed that for the series C_8 to C_{10} above surfactant concentrations of 50 mmol dm^{-3} (C_8), 30 mmol dm^{-3} (C_9) and 20 mmol dm^{-3} (C_{10}) the thermograms observed on mixing surfactant with insulin showed that two process were occurring: an initial endothermic process followed by an exothermic process. Some examples of the thermograms are shown in Fig. 6. The biphasic thermograms for the C_8 to C_{10} surfactants all show a sharp initial endothermic peak consistent with an endothermic effect which is rapidly compensated by an exothermic process. Although the rate of heat flow from the mixed solutions is governed by the heat conductivity of the sinks of the microcalorimeter the occurrence of thermograms showing endo- and exo-portions indicates that at least two processes are taking place with opposite enthalpy signs. For higher alkyl chain lengths (C_{11} and C_{12}) the thermograms did not show two effects which suggests that the two processes are occurring concomitantly so that only the net exothermic effect is seen. It should also be noted that in these cases the thermograms are broader, displaying a time course characteristic of a single almost instantaneous process. The exothermic enthalpy found for the C_{12} sulfate is consistent with that previously observed at higher ionic strength at pH 10.[15]

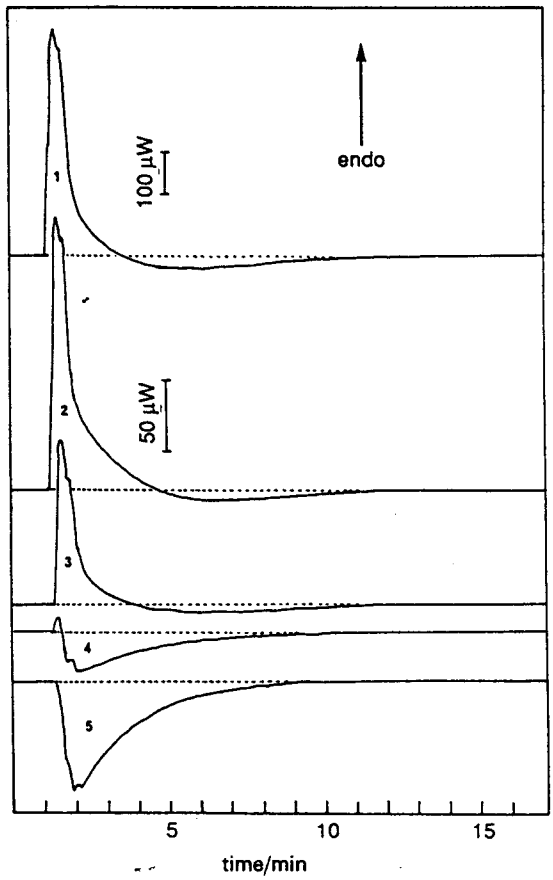

Fig. 6 Thermograms produced on mixing nominally equal masses of insulin solutions (1×10^{-3} kg dm^{-3}) with surfactant solutions at pH 10, 25 °C. 1, sodium *n*-octyl sulfate (199.9 mmol dm^{-3}); 2, sodium *n*-nonyl sulfate (99.96 mmol dm^{-3}); 3, sodium *n*-decyl sulfate (50.04 mmol dm^{-3}); 4, sodium *n*-undecyl sulfate (20 mmol dm^{-3}); 4, sodium *n*-dodecyl sulfate (14 mmol dm^{-3}). The final concentrations of insulin and surfactants were, nominally, half the above values. The upper scale bar applies to curve 1 and the lower scale bar to curves 2–5.

Discussion

The data in Fig. 1–3 suggest that on interaction with surfactant at pH 10 insulin undergoes a significant change in conformation. The absorption band at 295 nm (Fig. 1) is characteristic of phenolic groups[20] associated with the four tyrosine residues in insulin (two in both the A and B chains). The pK_a of the phenolic hydroxyl group in free tyrosine at 25 °C is 10.05.[20] In insulin the pK_a may be changed to a small extent but it is reasonable to suppose that the tyrosine residues will be *ca.* 50% ionised. The absorbance becomes more negative relative to native insulin with increasing surfactant concentrations and since the molar absorption coefficient of tyrosine increases with ionisation[20] it follows that surfactant binding causes an increase in the proportion of protonated residues consistent with transfer to a more hydrophobic environment *i.e.* transfer to the environment characteristic of *n*-alkyl chains. The steepness and position of the curves in Fig. 2 and 3 in relation to the surfactant c.m.c. suggests that it is associated with cooperative binding of surfactant and that the transition is also highly cooperative.

Studies[6–8] on the self-association of insulin in the pH range 2–10 assumed that insulin adopted a native conformation in this pH range and there appears to be no evidence that this is

not so. Furthermore at pH 10 at the concentration used here (0.5×10^{-3} kg dm^{-3}) the extent of dimerisation will be of the order of 0.22.[21] For a first approximation, interaction between native insulin (N) and surfactant (S) may be expressed by the equilibrium.

$$N + \bar{\nu}S \rightleftharpoons DS_{\bar{\nu}} \quad (1)$$

where $\bar{\nu}$ is the average number of surfactant molecules bound to the denatured complex ($DS_{\bar{\nu}}$). In writing eqn. (1) it is assumed that the binding of the surfactant and the conformation changes induced by binding are reversible. The reversibility of the difference spectral absorbance at 295 nm on dilution of the surfactant from high to lower concentrations suggests that this is a reasonable assumption. The equilibrium constant (K) for reaction (1) can thus be written

$$K = \frac{[DS_{\bar{\nu}}]}{[N][S]^{\bar{\nu}}} = \frac{K_S}{[S]^{\bar{\nu}}} \quad (2)$$

where K_S is the ratio of denatured complex and native molecules respectively and [S] is the equilibrium concentration of free surfactant. Because the insulin molarity was very low (87 mmol dm^{-3}) in the experiments it is assumed that [S] is negligibly different from the total surfactant concentration in the system. Values of K_S as a function of [S] in the transition region were calculated from the absorbance curves (A_{295}) in Fig. 2 and 3 from the extent of denaturation (α) taken as

$$\alpha = \frac{A_{295} - A^N_{295}}{A^D_{295} - A^N_{295}} \quad (3)$$

where A^N_{295} and A^D_{295} are the absorbance for the native and denatured states, respectively, and $K_S = \alpha/(1 - \alpha)$. Two methods were used, (1) in which A^N_{295} and A^D_{295} were taken from the start and finish of the transition region and (2) in which the pre- and post-transition curves, where possible were fitted by least-squares linear plots and the transition region was fitted by a polynomial; α and K_S were then calculated by the method described by Pace.[22] Fig. 7 and 8 show plots of ln K_S as a function of surfactant concentration as calculated by method (1); method (2) also gave very similar plots. The linearity of ln K_S as a function of [S] is consistent with the relations

$$\Delta G^0 = \Delta G^0_w - m[S] \quad (4)$$

$$\ln K_S = \ln K_w + \frac{m}{RT}[S] \quad (5)$$

where ΔG^0_w is the value of ΔG^0 for the transition in the absence of surfactant and m is a measure of the dependence of ΔG^0 on surfactant concentration. Although there is some curvature in the plot of ln K_S *vs.* [S] for the C_9 sulfate and possibly for the C_{12} sulfate at high [S], of these plots and those of ln K_S *vs.* ln[S] (Fig. 9) curvature was not a consistent feature and analysis based on linearity was considered more appropriate than higher-order regression.

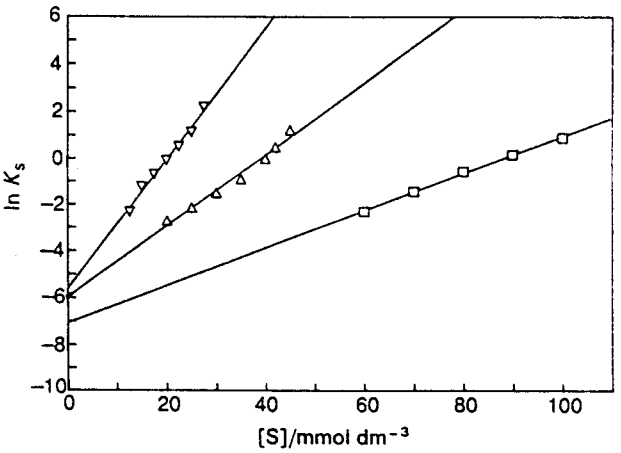

Fig. 7 Relationship between ln K_S and surfactant concentration for bovine insulin at pH 10, 25 °C. (□) sodium *n*-octylsulfate, (△) sodium *n*-nonylsulfate, (▽) sodium *n*-decylsulfate.

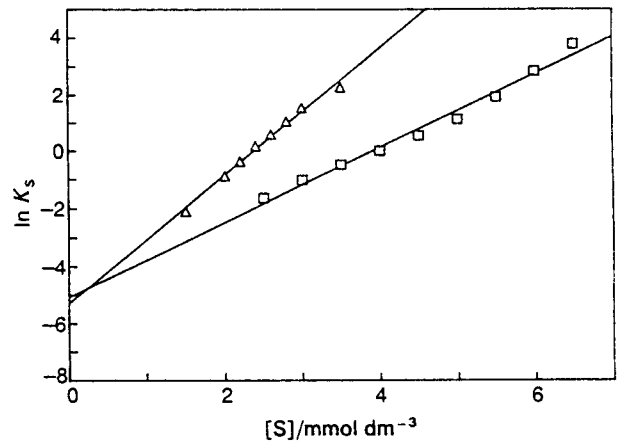

Fig. 8 Relationship between ln K_S and surfactant concentration for bovine insulin at pH 10, 25 °C. (□) sodium *n*-undecyl sulfate, (△) sodium *n*-dodecyl sulfate.

An equation of the form of eqn. (4) is found for protein denaturation by urea.[22,23] In principle the value of ΔG^0_w should be independent of the surfactant used to induce the transition.

Table 1 lists the parameters ΔG^0_w and m calculated from eqn. (5). While m increases steeply with *n*-alkyl chain length there is no systematic variation of ΔG^0_w with surfactant and both methods of data analysis give similar average values of ΔG^0_w.

From eqn. (2) it follows that

$$\ln K = \ln K_S - \bar{\nu} \ln[S] \quad (6)$$

At a surfactant concentration of 1 mol dm^{-3}, ln K = ln K_S and the equilibrium constant K might then be considered to

Table 1 Parameters characterising the surfactant-induced conformation changes in bovine insulin, pH 10.0, 25 °C

n-alkyl chain length	$[S]_{1/2}$ /mmol dm^{-3}	$m^a/10^3$ J mol^{-1} (mol dm^{-3})$^{-1}$	$m^b/10^3$ J mol^{-1} (mol dm^{-3})$^{-1}$	$\Delta G^0_w{}^a$ /kJ mol^{-1}	$\Delta G^0_w{}^b$ /kJ mol^{-1}	$\bar{\nu}^a$	$\bar{\nu}^b$	$\Delta G^0_{hc}{}^a$ /kJ mol^{-1}	$\Delta G^0_{hc}{}^b$ /kJ mol^{-1}	$\Delta(\Delta_{tr}G^0)$ /kJ mol^{-1}
C_8	88.0	119.4 ± 5.0	176.5 ± 10.1	17.56 ± 0.41	14.65 ± 0.83	6.31 ± 0.14	5.33 ± 0.58	−38.16 ± 0.87	−40.75 ± 3.68	−56 ± 3
C_9	40.0	352.5 ± 21.1	478.6 ± 28.7	14.10 ± 0.69	20.70 ± 1.24	6.64 ± 1.58	7.87 ± 0.78	−52.70 ± 12.60	−61.67 ± 6.16	−75 ± 8
C_{10}	20.3	691.7 ± 33.4	507.4 ± 44.1	14.01 ± 0.69	10.26 ± 0.99	5.30 ± 0.36	4.31 ± 0.47	−51.69 ± 3.50	−41.96 ± 4.48	−59 ± 7
C_{11}	3.90	3232 ± 157	2666 ± 211	12.60 ± 0.73	11.41 ± 0.98	5.39 ± 0.52	4.65 ± 0.43	−74.75 ± 7.06	−63.15 ± 5.80	−81 ± 8
C_{12}	2.37	5546 ± 223	6684 ± 175	13.17 ± 0.57	17.20 ± 0.52	5.30 ± 0.18	7.73 ± 0.23	−79.75 ± 2.67	−108.2 ± 3.38	−109 ± 20
mean				14.3 ± 1.9	14.8 ± 4.3	5.79 ± 0.64	5.98 ± 1.70			

[a] Calculated assuming $A_D - A_N$ is constant (see text). [b] Calculated by the method of Pace.[22]

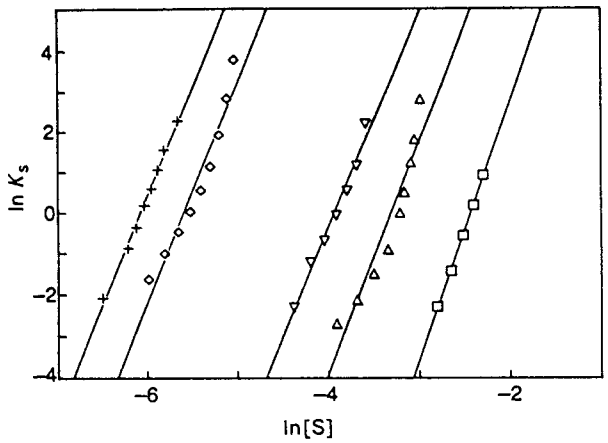

Fig. 9 Relationship between ln K_S and ln[surfactant] for bovine insulin at pH 10, 25 °C. (□), sodium *n*-octyl sulfate, (△) sodum *n*-nonyl sulfate, (◇) sodium *n*-decyl sulfate, (▽) sodium *n*-undecyl sulfate, (×) sodium *n*-dodecyl sulfate.

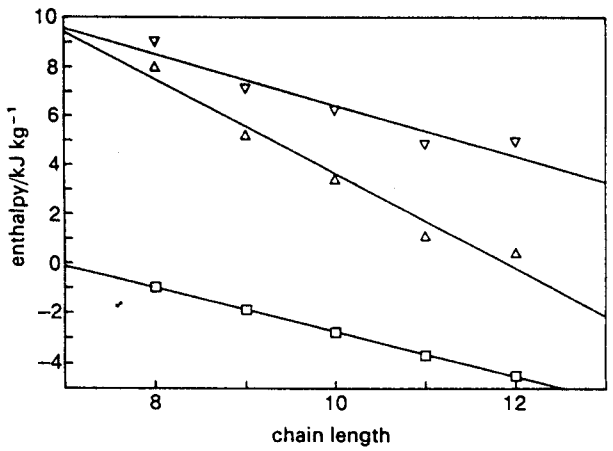

Fig. 10 Enthalpy contributions in kJ (kg insulin)$^{-1}$ to the enthalpy of interaction of *n*-alkyl sulfates with bovine insulin at pH, 25 °C as a function of *n*-alkyl chain length. (□) ΔH_{cal}, (△) maximum endothermic effect observed (ΔH_{obs}^{max}), (▽) $\Delta_u H + \Delta H_{hc}$.

correspond to the transition in a surfactant-saturated complex approximating to that in a very hydrophobic environment with a corresponding Gibbs energy change, ΔG_{hc}^0. Plots of ln K_S *vs.* ln[S] are shown in Fig. 9. Least-squares analysis of these plots (and a corresponding set using the Pace method of analysis[22]) gave the values of ΔG_{hc}^0 in Table 1. ΔG_{hc}^0 and ΔG_w^0 can be related by the following scheme

$$\begin{array}{ccc} N_w & \xrightarrow{\Delta G_w^\circ} & D_w \\ \Big\uparrow\Big\downarrow \Delta_{tr}G^\circ(N) & & \Big\uparrow\Big\downarrow \Delta_{tr}G^\circ(D) \\ N_{hc} & \xrightarrow{\Delta G_{hc}^\circ} & D_{hc} \end{array}$$

from which it follows that

$$\Delta G_{hc}^0 - \Delta G_w^0 = \Delta_{tr}G^0(D) - \Delta_{tr}G^0(N) \qquad (7)$$

where $\Delta_{tr}G^0(D)$ and $\Delta_{tr}G^0(N)$ are the standard Gibbs energies of transfer of denatured and native insulin from water to a hydrophobic environment. Average values of $\Delta G_{hc}^0 - \Delta G_w^0$, defined as $\Delta(\Delta_{tr}G^0)$, calculated by the two methods of data analysis are shown in Table 1. Although the errors on the values of $\Delta(\Delta_{tr}G^0)$ are large, partly because of the long extrapolations to ln[S] = 0 required to estimate ΔG_{hc} from the plots of ln K_S *vs.* ln[S] across the homologous series, $\Delta(\Delta_{tr}G^0)$ becomes increasingly negative with increase in alkyl chain length.

The binding of *n*-alkyl sulfates to globular proteins is in general exothermic[24] and protein unfolding is endothermic.[25] For the C_8–C_{10} surfactants the thermograms showed an initial endothermic process followed by an exothermic process whereas the C_{11} and C_{12} surfactants, where the binding energy is greater, showed only exothermic thermograms. The data strongly suggest that the enthalpy change corresponding to the conformational transition induced by the surfactants is endothermic (Fig. 4 and 5) but the observed enthalpies of interaction decrease with alkyl chain length as the exothermic contributions of the interaction of the surfactants with cationic sites on insulin increase. While surfactant binding must trigger the conformational change for the C_8 to C_{10} surfactants, the enthalpy of initial binding of surfactant molecules is not sufficiently large to conceal the endothermic effect whereas for the C_{11} and C_{12} surfactants it is. The sharpness of the transition from an endothermic to an exotheric thermogram signal for the C_8, C_9 and C_{10} surfactants (Fig. 6) suggests that even for these surfactants the initial endothermic effect is very rapidly followed by the exothermicity of further binding.

Insulin has four cationic amino acid residues in the B chain (B-5 His, B-10 His, B-22 Arg and B-29 Lys) and two N-terminal primary amines (B-1 Phe and A-1 Gly). At pH 10 the histidyl residues ($pK_a \approx 6.4$–7.0[26]) will be deprotonated and the primary amine groups of lysyl ($pK_a \approx 10.5$) and the N-terminal amino groups will be *ca.* 50% deprotonated. The enthalpies of interaction of C_8 to C_{12} n-alkyl sulfates with the polypeptides poly (L-lysine) and poly (L-arginine) have been measured[27] and were used to estimate the enthalpy contributions arising from binding to cationic sites making the assumption that the N-terminal amino groups behave as lysyl side chains. To a first approximation the observed enthalpy (ΔH_{obs}) of interaction can be expressed in terms of the enthalpies of interaction with cationic sites (ΔH_{cal}), hydrophobic binding of surfactant (ΔH_{hc}) and an enthalpy of unfolding ($\Delta_u H$)

$$\Delta H_{obs} = \Delta H_{cal} + \Delta H_{hc} + \Delta_u H \qquad (8)$$

Fig. 10 shows the maximum endothermic enthalpy observed (ΔH_{obs}^{max}), ΔH_{cal} and ($\Delta_u H + \Delta H_{hc}$) plotted as a function of alkyl chain length. Extrapolation of ($\Delta_u H + \Delta H_{hc}$) to zero chain length where $\Delta H_{hc} \rightarrow 0$ gives a value of $\Delta_u H$ of 16.9 ± 1.9 J g^{-1} (97 ± 10 kJ mol^{-1}). This estimate is in the range of unfolding enthalpies (*ca.* 8–20 J g^{-1}) for small globular proteins.[25] Taking ΔG_w^0 as 14.6 kJ mol^{-1} (Table 1) and $\Delta_u H$ as 97 kJ mol^{-1} gives an estimate of the entropy of insulin unfolding of 276 J mol^{-1} K^{-1}. The entropic contribution to ΔG_w^0 arising from the conformational change ($T\Delta_u S$) at 298 K is thus 82 kJ mol^{-1}, so that the increase in entropy on unfolding corresponds to *ca.* 85% of the energy (enthalpy) required to bring about the conformational transition. As for the unfolding of many globular proteins[26] the stability of insulin is determined by a fine balance between the large enthalpic and entropic changes which occur on unfolding.

References

1 W. H. Ward, in *A Laboratory Manual of Analytical Methods of Protein Chemistry including Polypeptides*, ed. P. Alexander and H. P. Lundgren, Pergamon Press, Oxford, 1966, vol. 4, p. 153.
2 A. Hvidt and S. O. Nielson, *Adv. Protein Chem.*, 1966, **21** 287.
3 N-T. Yu, C. S. Liu and D. C. O'Shea, *J. Mol. Biol.*, 1972, **70**, 117.
4 E. J. Dodson, G. G. Dodson, R. E. Hubbard and C. D. Reynolds, *Biopolymers*, 1983, **22**, 281.
5 R. C. Bi, Z. Dauter, E. Dodson, G. Dodson, F. Giordano and C. Reynolds, *Biopolymers*, 1984, **23**, 391.

6 A. E. Mark and P. D. Jeffrey, *Biol. Chem.*, 1990, **371**, 1165.
7 J. F. Hansen, *Biophys. Chem.*, 1991, **39**, 107.
8 P. D. Jeffrey, *Biol. Chem.*, 1986, **367**, 363.
9 S. E. McGraw, D. T. Craik and S. Lindenbaum, *Pharm. Res.*, 1970, **7**, 600.
10 S. E. McGraw and S. Lindenbaum, *Pharm. Res.* 1990, **7**, 600.
11 K. S. Birdi, *Biochem. J.*, 1973, **135**, 253.
12 A. Ushiwata, K. Nakaya and Y. Nakamura, *Biochim. Biophys. Acta*, 1975, **393**, 215.
13 J. J. Garcia Dominquez, P. Erra, M. R. Julia and M. R. Infante, *J. Com. Exp. Deterg*, 11th Meeting, 1980, 487.
14 B. Jirgenson, *Makromol. Chem. Rapid Commun.*, 1981, **2**, 213.
15 F. Sarmiento, G. Prieto and M. N. Jones, *J. Chem. Soc., Faraday Trans.*, 1992, **88**, 1003.
16 M. N. Jones, E. M. Gilhooly and A. R. Nicholas, *J. Chem. Soc., Faraday Trans.*, 1992, **88**, 2733.
17 *Handbook of Chemistry and Physics*, ed. R. C. Weast, CRC Press, Boca Raton, FL., 57th edn., 1976, p. D147.
18 P. Mukerjee and K. J. Mysels, *Critical Micelle Concentrations of Aqueous Surfactant Systems*, Natl. Stand. Data Ser., no. 36, National Bureau of Standards, Washington, DC, 1971.
19 I. Wadso, *Acta Chem. Scand.*, 1968, **22**, 927.
20 C. Tanford and G. L. Roberts, *J. Am. Chem. Soc.*, 1952, **74**, 2509.
21 A. E. Mark, L. W. Nichol and P. D. Jeffrey, *Biophys. Chem.*, 1987, **27**, 103.
22 C. N. Pace, *Tibtech*, 1990, **8**, 93.
23 C. N. Pace and D. V. Laurents, *Biochemistry*, 1989, **28**, 2520.
24 M. N. Jones, in *Biochemical Thermodynamics*, ed. M. N. Jones, Elsevier, Amsterdam 2nd edn, 1979. ch. 5.
25 W. Pfeil, in *Biochemical Thermodynamics*, ed. M. N. Jones, Elsevier, Amsterdam, 2nd edn, 1979. ch. 3.
26 D. E. Metzler, *Biochemistry*, Academic Press, New York, 1977, ch. 2. p. 59.
27 M. I. Paz Andrade, M. N. Jones and H. A. Skinner, *J. Chem. Soc., Faraday Trans. 1*, 1978, **74**, 2923.

Paper 2/06221F; *Received* 23*rd November*, 1992