The Binding of Cationic Detergent to Insulin, Zinc-Insulin and Iso-insulin

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Insulin, zinc-insulin and iso-insulin were precipitated by cetyltrimethylammonium bromide when the detergent/protein molar ratio was less than 10:1, whereas no precipitation was observed at ratios greater than 10:1. It is concluded that the detergent binds as a dimer at each binding site at ratios greater than 10:1. U.v. difference spectra indicated that the tyrosine residues were perturbed by detergent binding.

The binding studies of small molecules (such as detergents) to proteins are of great interest, since these interactions are analogous to many biological phenomena. Although a great wealth of data on the binding of anionic detergents to proteins has been reported in the literature (Steinhardt & Zaiser, 1955; Reynolds *et al.*, 1968; Steinhardt & Reynolds, 1969), relatively few studies have been made on the binding of cationic detergents (Few *et al.*, 1955; Foster & Aoki, 1958; Nakaya *et al.*, 1971).

The purpose of the present communication is to report on the binding characteristics of a cationic detergent, cetyltrimethylammonium bromide, to insulin, zinc-insulin and iso-insulin. The insulin molecule is of particular interest, since the primary sequence and the three-dimensional structure are known. The binding equilibria were studied with the help of measurements of u.v. difference spectra. It was also observed that all the insulins were precipitated from their buffer solutions on the addition of cetyltrimethylammonium bromide when the detergent/insulin molar ratio was less than about 10:1 (pH 7.4 and *I* 0.25). These preliminary results are discussed with reference to the number of molecules of cetyltrimethylammonium bromide bound/binding site to the insulin molecule and to the effect of detergent binding on the environment of the tyrosine residues.

Experimental

Materials. All insulin samples were generously provided by Novo Terapeutiske Laboratorium, Copenhagen, Denmark. Insulin (pig) was 5× crystallized. Zinc-insulin was 11× crystallized (Zn content 0.39%). Iso-insulin was a highly purified sample (iso-insulin was prepared by substituting phenyl isocyanate at the *N*-terminal amino groups of phenylalanine and glycine). Cetyltrimethylammonium bromide was used as supplied by Theodor Schuchardt, Munich, Germany (purity approx. 99%). Phosphate buffers were prepared from

reagent-grade Na_2HPO_4 and KH_2PO_4 , final pH 7.4. To all the buffer solutions NaCl (0.15M) was added (to give *I* 0.25).

Precipitation studies. Cetyltrimethylammonium bromide and insulin solutions were prepared in the above-mentioned buffer. For the precipitation studies solutions were prepared where the detergent/insulin molar ratio was varied from 15:1 to 0.01:1. The concentration of insulin was kept constant at 0.05% in all these studies. Each determination was made on at least three samples. The solutions were rotated in a thermostat at 25°C ($\pm 0.1^\circ\text{C}$) for 72h in order to reach equilibrium. After this period the solutions were centrifuged and the clear supernatant was removed for the measurement of the insulin concentration by the u.v. absorption at 277nm (1cm cell). The results obtained with detergent/insulin molar ratios 0.01–1:1 and 1–15:1 are given in Figs. 1(a) and 1(b) respectively. The results obtained with molar ratios in the range below 5:1 were very erratic in all the insulin systems. Even after many attempts no plausible explanation could be found, and this phenomenon is depicted in the plots given in Fig. 1 with error bars correspondingly. On the other hand, the accuracy obtained with molar ratios in the range above 5:1 was very high, as shown in Fig. 1.

Methods. The u.v. spectra were measured on the Cary model 16 spectrophotometer. All measurements were made at 25°C, maintained by circulating water from a thermostat through the cell holder.

Results and discussion

The purpose of the present study was to determine the effect of the cationic detergent cetyltrimethylammonium bromide binding to insulin, zinc-insulin and iso-insulin with respect to the u.v.-spectral changes. Addition of detergent to insulin solutions, however, resulted in the precipitation of these proteins under a certain critical detergent/insulin molar ratio. In order to investigate this

precipitation phenomenon quantitatively, solutions were prepared with various detergent/insulin molar ratios (e.g. from 0.01:1 to 15:1). The amount of insulin in solution (i.e. insulin and detergent-insulin complex) at equilibrium with the precipitate was determined after centrifuging the solutions (Fig. 1). With molar ratios in the range 0.01–1:1 (Fig. 1a) precipitation was observed when the ratio was greater than 0.15:1, with all the insulins. Maximum precipitation is obtained at the molar ratio 0.4:1 with insulin, but when this value is 0.8:1 for zinc-insulin and iso-insulin. These results indicate that when the detergent/insulin molar ratio is less than 0.15:1 any complexes (i.e. detergent-insulin) formed are soluble. Further, the abrupt changes in slope with the increasing detergent concentration provide good evidence that the detergent-insulin complexes are not significantly dissociated at these concentrations. It might imply that at least 2 mol of detergent/mol of hexamer is required for the precipitation.

The plots in Fig. 1(b) are represented by three straight lines, with breaks at molar ratios $5 \pm 1:1$ and 10:1 for insulin and zinc-insulin respectively (and at 12:1 for iso-insulin). It is safe to accept that these values will not be in an error of more than ± 1 unit, even though the results in the range of molar ratios below 5:1 were erratic. It is also worth noticing that all the plots between these two breaks for the respective insulins are rectilinear; the significance of this observation is discussed further below.

The binding of an ionic detergent to a protein will be dependent on the electrostatic (long-range) and the hydrophobic (short-range) interactions. In general (Steinhardt & Reynolds, 1969) the affinity of a detergent ligand increases with increasing length of the hydrocarbon chain. In solution at the pH studied, the insulin monomer has six negatively

charged carboxyl groups (Blundell *et al.*, 1971). It is reasonable to conclude that the precipitation by the cationic detergent molecule cetyltrimethylammonium bromide is mainly due to the neutralization of the negative charges of the insulin molecule.

It is obvious that the binding constant needs to be known before any quantitative description can be given. However, from the results in Fig 1(a) it can be concluded that approx. 0.15 mol of detergent/mol of insulin is present unbound, which indicates that the affinity for insulin is high, and thus the concentration of the unbound detergent in solution can be expected to be rather low. The number of mol of detergent bound/mol of protein (ν) can be approximately determined from the total detergent concentration (below a molar ratio of 10:1). Similar arguments have been found to be acceptable in binding studies whenever the binding constants were expected to be of high values (Steinhardt & Reynolds, 1969). It is reasonable to conclude that the first break in the plots in Fig. 1(b) corresponds to $\nu \sim 5$, whereas the next break corresponds to $\nu \sim 10$. Further, since the respective plots with molar ratios in the range 5–10:1 are linear, the detergent-insulin complex that is soluble when the molar ratio is greater than 5:1 consists of a constant composition. Since the value of ν at the first break is about half of that at the second break, this suggests that a dimer of detergent is bound at each binding site in the insulin molecule at $\nu \sim 10$. I believe that these binding equilibria are of a general character, as preliminary studies carried out with another cationic detergent, cetylpyridinium chloride, show similar precipitation behaviour (K. S. Birdi, unpublished work). Similar observations of a dimer detergent binding have been reported by other investigators for other protein-detergent systems (Putnam & Neurath, 1944; Steinhardt & Reynolds, 1969). It can be argued that, owing to the hydrophobic

Fig. 1. Precipitation of insulin by cetyltrimethylammonium bromide

Amount of protein in solution (E_{277}^{1cm}) is plotted versus the detergent/protein molar ratio (a, 0.01–1:1; b, 1–15:1). ▲, Insulin; □, zinc-insulin; ●, iso-insulin. The molecular weight of insulin used is that of the subunit.

Fig. 2. Difference spectra for protein (0.05%) solution in phosphate buffer, pH 7.4 and 0.25, plus cetyltrimethylammonium bromide (detergent/protein molar ratio 16.5:1)

▲, Insulin; □, zinc-insulin; ●, iso-insulin.

interactions between the apolar side chains of the proteins and the apolar part of the detergent molecule, a dimer formation of detergent is energetically favourable.

The slopes of the plots given in Fig. 1(b) with molar ratios in the range 5–10:1 were found to be equal to $5(\pm 0.5)$ mol of detergent/mol of insulin for insulin and zinc-insulin. For iso-insulin this value was $7(\pm 0.5)$ mol/mol of protein. These results indicate that after an initial binding corresponding to the first break, i.e. $\nu \sim 5$, each 1 mol of protein dissolved requires approx. 5 mol of detergent. The detergent-binding equilibria consistent with the above observations can be described as follows. Initially 5 ± 1 mol of detergent binds at the negative carboxyl groups, and because of the charge neutralization the detergent-insulin complex of $\nu \sim 5$ exhibits very low solubility. On further binding of 5 mol of detergent/mol of protein the detergent-insulin complex of $\nu \sim 10$ is formed, and this is soluble. The complex of $\nu \sim 10$ can only be soluble in water if the last 5 mol of detergent/mol binds such that their positively charged hydrophilic groups are pointing towards the aqueous phase.

Difference spectra were recorded between 260 and 300 nm. The insulin solution (0.05%) was used as the reference and the sample cell contained insulin + detergent (detergent/insulin molar ratio 16.5:1). The ratio of 16.5:1 was used since at this value no precipitation is observed (Fig. 1). The results given in Fig. 2 show that, within the experimental accuracy, the difference spectra are similar for the various insulins. The u.v. difference spectra give information about the interaction between the ligand and the chromophoric side chains of the protein. The changes in the spectra measured between 260 and 300 nm are consistent with the perturbation of the tyrosine residues (Rupley *et al.*, 1967; Steinhardt & Reynolds, 1969; Steiner & Weinryb, 1971) arising from the detergent binding. The difference spectra given in

Fig. 2 suggest that the environment of the tyrosine changes to a more apolar character after detergent binding. It has been reported that in solution, when the insulin molecule is in the associated state (dimer or tetramer), the chromophoric side chains interact (Rupley *et al.*, 1967). It has also been shown that in the solid state an aromatic cage consisting of tyrosine and phenylalanine residues is present in the insulin dimer (Blundell *et al.*, 1971). It would thus be reasonable to expect that the association equilibria of the insulin and zinc-insulin will be affected as a result of the detergent binding.

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