BUOYANT DENSITIES OF SUBTILISIN CARLSBERG IN DISSOLVED AND CRYSTALLINE STATES

by

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The buoyant densities of subtilisin Carlsberg in dissolved and crystalline states, including glutaraldehyde cross-linked crystals, have been determined by equilibrium centrifugation at neutral pH in the analytical ultracentrifuge in concentrated solutions of CsCl. Due to the negligible diffusion of suspended crystals they form a sharp band at the position in the gradient corresponding to their density. Different crystal preparations may be separated in a single gradient when their densities differ by more than $0.005 \text{ g}\cdot\text{ml}^{-1}$ and the results are reproducible to $\pm 0.001 \text{ g}\cdot\text{ml}^{-1}$. Glutaraldehyde stabilized crystals were found to have higher buoyant densities than crystals of unmodified subtilisin. The increase in density due to the glutaraldehyde cross-linking can be explained if a polymer composed of approximately 5 glutaraldehyde residues is bound per pair of blocked lysine residues. Unmodified subtilisin Carlsberg shows identical densities in the crystalline and soluble states in CsCl solutions. The observation is consistent with identical molar volumes of subtilisin Carlsberg in solution and crystals and no charged groups being involved in intermolecular binding in the crystals.

1. INTRODUCTION

In addition to application in nucleic acid research, equilibrium centrifugation in density gradients has been established as a useful technique for the characterization of proteins

and polypeptides (3, 7, 9). Unfortunately, in the present technique, based on density gradients produced by cesium salts, diffusion makes it difficult to measure densities of proteins with molecular weights below 25,000 and

high precision is only obtained with considerably larger molecules. However, during determinations of buoyant densities of proteins it was occasionally observed, e.g., at extreme pH-values, that proteins aggregated due to denaturation and then could be observed as sharp bands in the ultracentrifuge. The densities measured from these bands could be determined with high precision (5, 9), but were higher than the densities of the dissolved proteins. In crystalline states, proteins would be expected to band with similar precision as denatured proteins, and since a variety of physico-chemical investigations have indicated that protein conformations are very similar in dissolved and crystalline states, they might also be expected to have identical buoyant densities.

In the present report this has been tested by measuring the density of dissolved and crystalline subtilisin Carlsberg. Some measurements were performed with crystals stabilized to different degrees of substitution with glutaraldehyde. Other measurements could be made with unmodified crystals since they only dissolve very slowly in the cesium chloride solution used to form the gradients. The results are discussed in relation to a recently formulated model for protein hydration, which assumes all of the bound water to be associated with the charged residues and their counterions (9).

2. MATERIALS AND METHODS

Subtilisin type Carlsberg, lot. no. 60420, was a gift from Novo Industry A/S, Copenhagen. Glutaraldehyde was a 25% (w/v) aqueous solution obtained from Fluka, Switzerland. The CsCl, product number 1115-470, was from Pierce Chemical Co., Illionis, and stated to be 99.9% pure. All buffer salts were reagent quality. Distilled water was used throughout.

The lyophilized enzyme preparation was purified by amorphous precipitation with sodium sulphate and two recrystallizations by slow additions of saturated sodium sulphate at pH 5.5 and room temperature. The crystals from sodium sulphate were cross-linked by treatment with 1% glutaraldehyde at pH 5.5 as previously described (15). Before use in the ul-

tracentrifuge the crystals of the stock suspensions were washed in small centrifuge tubes with solutions of CsCl of lower density than the solutions used to form the gradient.

The protein content of the crystals and the extent of lysyl residue modification, which is supposed to reflect the degree of cross-linking, were determined by amino acid analysis (15). Samples of crystals containing about 1 mg of protein were hydrolyzed with 1 ml 6M-HCl in evacuated, sealed tubes for 24 hours at 110°C and analyzed in a Durrum model 500 amino acid analyzer.

The densities of the gradient solutions were determined by way of their refractive indices, n_D^{25} (6), measured on a Hilger refractometer, thermostated to $25.0 \pm 0.1^\circ\text{C}$. Refractive indices were measured before and after every run, and were corrected for the contribution from the protein.

Measurements of pH were made with a Radiometer pH meter, model PHM 63 equipped with a combination electrode. Standardization of the instrument was made according to the National Bureau of Standards scale with buffers obtained from Radiometer. The crystal suspensions were kept at pH 7.0 in phosphate buffers of ionic strength 0.01.

Ultracentrifugation measurements were performed in a Spinco Model E ultracentrifuge using the schlieren optics. An An-D rotor was used in conjunction with a standard 12 mm Kel-F centerpiece. The temperature was kept at $25.0 \pm 0.1^\circ\text{C}$ and the speed was 52640 rpm. The CsCl solutions containing the different crystals were prepared following standard procedures (4). Equilibrium was found to be reached in 16 hours. After this time exposures were taken and a photographic method (9) was used in measuring the relevant positions in the cell.

Buoyant densities were calculated using an iterative computer program written in FORTRAN for a Honeywell Bull computer (9).

3. RESULTS

In equilibrium ultracentrifugation experiments it was found that crystals stabilized with glutaraldehyde rapidly formed sharp and easily measurable bands in the cesium chloride

Figure 1. Sedimentation equilibrium experiments in cesium chloride gradients.

a. Glutaraldehyde cross-linked crystals of subtilisin Carlsberg, $60 \mu\text{g}\cdot\text{ml}^{-1}$.

b. Unmodified crystals, $700 \mu\text{g}\cdot\text{ml}^{-1}$.

c. Unmodified and cross-linked crystals together.

d. Dissolved enzyme, $1400 \mu\text{g}\cdot\text{ml}^{-1}$.

gradients as seen from Figure 1a. The unperturbed baseline confirms that no protein has leaked out from the crystals during the experiment. Crystal preparations with either 5.6 or 3.0 modified lysyl residues per mole banded equally well in the gradients, although at positions corresponding to different densities (Table I). Since crystals of unmodified subtilisin dissolved only very slowly in concentrated solutions of cesium chloride, it was possible to determine the buoyant density of such crystals directly once equilibrium of the salt gradient had been reached after approximately 16 hours (Figure 1b and Table I). The magnitudes of the symmetrical perturbation of the base-line on both sides of the crystal band indicate that

approximately one third of the crystals had dissolved before equilibrium was reached. In a mixture of cross-linked and unmodified crystals two well-separated bands were observed (Figure 1c) as was expected from their difference of $0.007 \text{ g}\cdot\text{ml}^{-1}$ in buoyant density.

The buoyant density of soluble subtilisin Carlsberg could only be determined with less precision due to the pronounced diffusion effect (Figure 1d). However, within the precision of the method, the buoyant density was identical with the value determined for the unmodified crystals (Table I).

Furthermore, Figure 2 indicates that the buoyant density increases linearly with the degree of cross-linking. Thus, in cases where

Table I

Buoyant densities of crystals, cross-linked and unmodified, and of soluble subtilisin Carlsberg at 52640 rpm, 25°C, in CsCl, pH 7.0.

Run no.	State	Modified lysines	ρ_0 , buoyant densities observed	mean
1176	Cryst.	5.6	1.3293	1.3293
1183	–	–	1.3294	
1178	Cryst.	3.0	1.3265	1.3264
1186	–	–	1.3263	
1180	Cryst.	0	1.3227	1.3224
1183	–	–	1.3222	
1180	Dissolved	0	1.321	
1182*)	–	–	1.319	1.321
1184*)	–	–	1.322	

*) In 1182 and 1184 the enzyme was pretreated with phenylmethyl sulfonylfluoride to decrease the autolysis. PMSF is known to react only with the active serine residue no. 221.

Figure 2. Buoyant densities, ρ_0 , of crystals of subtilisin Carlsberg in CsCl as a function of the number of lysine side chains modified, m .

non-cross-linked crystals dissolve too rapidly to permit direct measurement in equilibrium ultracentrifugation, it should be possible to determine the correct density by a linear extrapolation of densities measured with crystals of different cross-linking.

4. DISCUSSION

Buoyant density reflects, in addition to the amino acid composition, the amount of water and salt being bound to a protein molecule. As demonstrated in a previous paper from this laboratory (9), the individual contributions arising from the binding of hydrated anions and cations of salts, respectively, may be estimated by varying the pH of the experiment and the nature of the gradient forming salt. Using ovalbumin as a model practically all of the preferentially bound water could be accounted for as water being bound to hydrated charge-ion complexes, i.e., the hydration of the charged amino acid side chains of the protein plus the hydration of their oppositely charged counter-ions. Only negligi-

ble amounts of water appeared to be preferentially bound to the uncharged protein surface.

To test whether a similar distribution of preferentially bound water exists in subtilisin Carlsberg, buoyant density values (Table I) were used together with equation [1] derived for the buoyant density of ovalbumin.

$$\rho = \frac{M_p + n_c W_c + n_a W_a}{M_p \bar{v}_p + n_c V_c + n_a V_a} \quad [1]$$

For subtilisin the molecular weight, M_p , is 27287 and at pH 7 it is estimated from the amino acid composition (13) that the protein has 15 negative and 17 positive charges. Thus, n_c , the number of bound cations should be 15 cesium ions and, n_a , the number of bound anions should be 17 chloride ions.

As outlined by PEDERSEN and IFFT (9) the hydration of charge-ion complexes on the protein surface is a function of the water activity in the salt solution. A cesium chloride solution of density 1.3226 g ml^{-1} corresponds to a water activity of 0.911 (10). Using this number in the graphs Figure 3 of ref. (9) the charge-cesium ion complex is seen to bind 15 water molecules and from Figure 4 we find a molecular weight, W_c , of 399 g and a molar volume, V_c , of 286 ml for the hydrated complex. The corresponding numbers of the hydrated charge-chloride-ion complex at a water activity of 0.911 are 12 moles of bound water, a molecular weight, W_a , of 259 g and a molar volume, V_a , of 248 ml. Inserting these numbers into equation [1] a value of 0.7322 ml g^{-1} is found for the partial specific volume, $\bar{v}_p$. This value is slightly higher than the value of 0.730 ml g^{-1} calculated by the method of COHN and EDSALL (1) but it may be adjusted to 0.730 by inclusion of only 15 extra water molecules in addition to the 433 water molecules presumably bound to subtilisin in the form of hydrated charge-ion complexes. This small discrepancy is hardly significant.

In this connection it should be remembered that titration studies with subtilisin Novo suggested 4 histidine residues and 4 carboxylate groups to be present in uncharged form at pH 7 (8). In case a similar masking of groups should exist in the structurally related subtilisin

Carlsberg, equation [1] would result in a value for $\bar{v}_p$ of 0.734 ml g^{-1} and 24 additional water molecules would be required to decrease the value to 0.730. Thus, equation [1] seems also to fit reasonably well to the hydration data of subtilisin Carlsberg and accordingly it will in the following be assumed that practically all preferentially bound water is present as hydration on charge-ion complexes.

The effect of cross-linking can be described by a modified form of equation [1]. This equation, [2], accounts for a possible change of the overall charge due to modified lysines as well as for the mass and volume contributed by the cross-linking agent itself.

$$\rho = \frac{M_p + n_c W_c + (n_a - x) W_a + y W_g}{M_p \bar{v}_p + n_c V_c + (n_a - x) V_a + y V_g} \quad [2]$$

In equation [2], W_g corresponds to the molar weight and V_g to the molar volume of the cross-linking agent. The letter y is used to denote the number of moles of this agent incorporated per mole of protein present in the crystals while x stands for the number of charge-chloride ion complexes lost if the cross-linking reaction involving lysine residues should result in a net loss of positive charge.

In 1968 RICHARDS and KNOWLES (12) suggested the following structure for the smallest and presumably most common type of cross-link formed by glutaraldehyde in proteins:

The two secondary amines linked by the glutaraldehyde polymer have a pK_a which renders them positively charged at pH 7 where our experiments were performed, and thus, they would still be involved in hydrated charge-chloride ion complexes. The density of this polymer was calculated according to a method by TRAUBE (14) and found to be less than the density of the unmodified crystals. Consequently, cross-linking the crystals would be expected to result in a decrease of their buoyant density. Since Figure 2 shows an increase in buoyant density upon cross-linking, a polymer of this type is unable to explain our data.

More recently a different type of polymer was proposed by MONSAN et al. (11) to explain the reaction between glutaraldehyde and protein.

In this formula, lysines of the protein have reacted to give imino bonds which are stabilized by conjugation. The pK_a 's of such Schiff bases are probably no higher than 5, and thus at pH 7 modified lysines carry no positive charges. To estimate the influence on the buoyant density of modification with such a reagent we will first calculate the influence of two charge-chloride ion complexes (of density 1.04 g ml^{-1} from W_a and V_a given earlier) being lost as a result of cross-linking, by using $x=2$ and disregarding W_g and V_g , viz. $y=0$. The density of the remaining crystals would increase from 1.3226 to 1.3275 g ml^{-1} for two substituted lysine residues, an increase which corresponds to about twice the actually recorded value in Figure 2. Next, we will consider the additional effect of W_g and V_g as calculated for glutaraldehyde polymers of the MONSAN type based on TRAUBE's rules. The density, W_g/V_g , is low, 1.08 - 1.12 g ml^{-1} for $n=1$ - 4 , and the introduction of these polymers into the protein would thus counteract the increase in buoyant density arising from the loss of charge-chloride ion complexes. For the case of a MONSAN-polymer bound to two lysines (assuming $n=3$; $x=2$; $y=1$) the calculated value for ρ_0 (1.3249 g ml^{-1}) is close to the experimentally found value for $x=2$ in Figure 2. Higher values of n would further decrease the buoyant density. Conceivably, some polymers are bound to only a single lysyl residue and thus would not participate in cross-linking. Such species would have an even lower buoyant density, while a polymer bound through 3 or 4 lysines would decrease the buoyant density to a lesser degree. Consequently, our results are consistent both with cross-linking by introducing a single type of MONSAN-polymer (composed of approximately 5 glutaraldehyde residues) or a mixture of polymers of different length and being bound to different numbers of lysine residues.

According to equation [1] buoyant densities are sensitive to variations in the number of charged residues on the protein. For this reason the identical buoyant densities of dissolved and unmodified crystalline subtilisin Carlsberg make it unlikely that the contact areas in the crystal involve the intermolecular neutralization of oppositely charged amino acid residue side chains. Thus, subtilisin Carlsberg seems in this respect to resemble subtilisin BPN' where the x-ray crystallographic studies of DRENTH et al. (2) did not reveal any such charge pairs. Therefore, the identical buoyant densities in the two states are consistent with the same molar volume of dissolved and crystalline subtilisin Carlsberg.

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