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The Solubilities of Denatured Proteins in Different Organic Solvents

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The solubilities of heat-denatured and reduced, *S*-carboxymethylated proteins have been investigated in various organic solvents. Polar, protic solvents (formic acid, trifluoroacetic acid, 3-mercaptopropionic acid) were found to be good solvents for the denatured proteins (20–40 mg ml^{-1}), and the solubilities of the reduced, *S*-carboxymethylated proteins were generally higher than those of the heat-denatured forms. Most other organic solvents were less effective in solubilising the denatured proteins. Apolar solvents did not solubilise denatured proteins, but low solubilising powers were observed for polar, aprotic solvents.

Heat-denaturation was observed to result in the formation of large intermolecular aggregates, which, for ovalbumin and lysozyme, were formed by intermolecular S–S bonds, but for bovine serum albumin involved intermolecular isopeptide bonds.

Water is the natural solvent for most biological macromolecules. In water proteins have a sequence-dependent, three-dimensional structure composed of α-helices and β-sheets, which combine with each other to form a compact structure with a hydrophobic core and a more hydrophilic surface.[1–3]

In vivo, the folding of a polypeptide is a highly controlled process supervised by folding catalysts and chaperones.[4] *In vitro*, folding is energetically favoured but may not be kinetically favoured, and insoluble structures may be formed by incorrect folding and aggregation.[3,5] Heat-denaturation also leads to the formation of water-insoluble aggregates[6,7] although water-soluble high molecular weight polymers may be formed by proteins under some conditions.[8–11]

The structure of denatured water-insoluble proteins has not been well defined, but can generally be assumed to have a higher degree of exposure of hydrophobic side chains compared with the native structure. For this reason, the solubility of denatured proteins in organic solvents might *a priori* be expected to differ from the solubility of native proteins in these solvents.

Here we report the solubilities of reduced, *S*-carboxymethylated proteins and heat-denatured proteins in various organic solvents, and compare the results with the solubilities of native proteins in organic solvents.[12]

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Materials and methods

Chemicals. Ethanol was from Danisco (Copenhagen, Denmark). Dimethyl sulfoxide (DMSO), *N,N*-dimethylformamide (DMF), and methanol were from Merck (Darmstadt, Germany). Ethanolamine, trifluoroethanol (TFE), glycerol, 3-mercaptopropionic acid (3-MPA), dithiothreitol (DTT), tris(hydroxymethylamino)methane (Tris), Coomassie Brilliant Blue R-250, urea, pyronin G, bovine serum albumin (BSA, A 7638), ovalbumin (OVA, grade V), and lysozyme (LYZ, grade VI) were from Sigma (St. Louis, USA). Formic acid and 2-propanol were from Sigma–Aldrich (Steinheim, Germany). Gelcode Blue stain reagent was from Pierce (Rockford, USA). Trifluoroacetic acid (TFA), guanidine hydrochloride and 2-mercaptoethanol were from Fluka (Buchs, Germany). Acetic acid and butanol were from Bie & Berntsen (Rødovre, Denmark). Precast 4–12%, 8–12% and 4–20% Tris-glycine gels for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Tris-glycine electrophoresis buffer for SDS-PAGE were from Novex (San Diego, USA). Prestained low molecular weight standard proteins were from Bio-Rad (Hercules, USA).

Reduction and S-*carboxymethylation.* BSA, OVA, or lysozyme (1.0 g) was dissolved in 14.7 ml 6 M guanidine, 0.1 M Tris, pH 8, and 310 μl 2-mercaptoethanol were added. The solution was incubated for 2 h at 37 °C, 1.0 g

Table 1. Solubilities (mg ml^{-1}) of *S*-carboxymethylated (*S*cm) proteins and heat-denatured (hd) proteins in various organic solvents and water.[a]

Solvent	*S*cmOVA	*S*cmBSA	*S*cmLYZ	hdOVA	hdBSA	hdLYZ
Water	0.6	0.2	1.6	0.6	4.8	0.6
Glycerol	ns	ns	ns	ns	ns	ns
Methanol	ns	ns	0.1	ns	ns	0.2
Ethanol	ns	ns	0.1	ns	ns	ns
2-Propanol	ns	ns	ns	ns	ns	ns
Butanol	ns	ns	ns	ns	ns	ns
2-Mercaptoethanol	ns	ns	0.1	ns	0.1	2.1
Trifluoroethanol	1.7	3.6	0.5	0.4	0.4	0.7
Ethanolamine	ns	0.2	0.8	1.6	0.1	2.2
DMF	ns	ns	ns	0.1	ns	0.6
DMSO	0.5	0.4	22.7	0.4	0.4	6.8
Acetic acid	0.1	0.8	ns	0.1	0.1	1.2
3-MPA	6.8	18.2	3.2	5.6	3.8	1.4
Formic acid	24.0	41.8	28.5	12.3	4.2	2.5
TFA	31.6	38.2	31.2	12.4	1.2	1.8

[a]ns: not significant ($\leqslant 0.05$ mg ml^{-1}).

iodoacetamide was added, and the mixture was incubated for 30 min in the dark at room temperature. The solution was dialysed five times against 2 l of water. After centrifugation at 2600*g* for 30 min the pellet was lyophilised.

Heat-denaturation. BSA, OVA or lysozyme (1.0 g) was dissolved in 100 ml phosphate-buffered saline (50 mM sodium phosphate, pH 7.2, 0.15 M NaCl), autoclaved at 105 °C for 1 h and lyophilised.

Determination of solubility. 10 mg of either reduced, *S*-carboxymethylated-BSA, -OVA, or -lysozyme, or heat-denatured-BSA, -OVA or -lysozyme were added to 0.2 ml solvent, and the mixtures were incubated overnight at room temperature on a shaking table. The solutions were centrifuged (15 000*g*, 15 min), and 50 µl aliquots were withdrawn into hydrolysis vials, dried *in vacuo*, and subjected to hydrolysis and quantitative amino acid analysis.

Alternatively, for experiments with glycerol, which could not be removed *in vacuo*, and for experiments with ethanolamine, which gave rise to a large interfering peak on the amino acid analyser, 10 µl were transferred to 1 ml 8 M urea and the absorbance read at 280 nm. The amount of protein was then calculated using a standard curve for proteins dissolved in 8 M urea.

Amino acid analysis. Amino acid analyses were performed according to Barkholt and Jensen.[13] Samples were hydrolysed in 6 M HCl–0.05% phenol–0.05% dithiodipropionic acid for 24 h at 110 °C, dried *in vacuo*, redissolved, and analysed by ion-exchange chromatography with post-column orthophthalaldehyde derivatisation.

Electrophoresis. SDS-PAGE was performed according to Laemmli,[14] using 4–12%, 8–12% or 4–20% precast gels (Novex). Samples were mixed with an equal amount of sample buffer (280 mM SDS–250 mM Tris–35% glycerol–10% pyronin G, pH 6.8) and heated at 100 °C for 1 min. DTT (260 mM) or 8 M urea was included in the sample buffer as indicated. Gels were run at 100–150 V for approximately 90 min and then stained with 0.1% Coomassie Brilliant Blue–40% methanol–10% acetic acid. Destaining was carried out in 40% methanol–10% acetic acid. Alternatively, gels were stained with Gelcode Blue reagent for 1 h according to the manufacturers instructions.

Results

Table 1 shows the solubilities in fourteen different organic solvents and water of the three heat-denatured and reduced, *S*-carboxymethylated proteins.

The solubilities of both forms of the three proteins were insignificant ($\leqslant 0.05$ mg ml^{-1}) in glycerol, 2-propanol and butanol. In ethanol the solubilities were also insignificant except for a low solubility of reduced, *S*-carboxymethylated lysozyme. A similar result was obtained for methanol, but heat-denatured lysozyme also had a low solubility in this solvent. 2-Mercaptoethanol was a sligthly better solvent than methanol since reduced, *S*-carboxymethylated lysozyme and heat-denatured BSA had a small solubility in this solvent, and heat-denatured lysozyme had a solubility of 2.1 mg ml^{-1}. Ethanolamine was a sligthly better solvent that 2-mercaptoethanol, and trifluoroethanol was an even better solvent, since all the proteins had low solubilities ranging from 0.4 to 3.6 mg ml^{-1} in this solvent. Therefore, within the alcohols the following order of solubilising power was found: glycerol = 2-propanol = butanol < ethanol < methanol < 2-mercaptoethanol < ethanolamine < trifluoroethanol.

DMF was a poor solvent for most of the proteins except that low solubilities were observed for heat-denatured OVA and lysozyme. DMSO was able to solubilise small amounts of the two forms of OVA and

BSA but was a very good solvent for lysozyme, and especially for the reduced, *S*-carboxymethylated form.

Among the organic acids, acetic acid was a poor solvent, 3-mercaptopropionic acid was a good solvent, and formic acid and trifluoroacetic acid were very good solvents.

Among the good solvents, the solubilities of the heat-denatured forms of the proteins were generally lower than those of the *S*-carboxymethylated forms, and was highest for heat-denatured OVA, for which it was approximately half of the solubility of *S*-carboxymethylated OVA. It was hypothesised that the lower solubilities of the heat-denatured forms may be caused by disulfide-mediated polymerisation.

In order to determine the importance of disulfide bond status for solubility, the solubility of the heat-denatured proteins in TFA, DMF and trifluoroethanol was investigated in the presence of 1% 2-mercaptoethanol, and this showed essentially no difference (not shown). Furthermore, SDS-PAGE analysis showed that the electrophoretic mobilities of the *S*-carboxymethylated forms were essentially unchanged by inclusion of DTT in the sample buffer (Fig. 1). These results indicated that the *S*-carboxymethylated forms existed as monomers, and this conclusion was supported by the amino acid analyses, which showed that all cysteines were converted into *S*-carboxymethylcysteine.

Under non-reducing conditions all three heat-denatured proteins were insoluble, and formed large aggregates, which could not be analysed by SDS-PAGE (Fig. 1A). Upon inclusion of DTT in the sample preparation buffer, OVA and lysozyme were solubilised, and showed the same electrophoretic mobilities as the reduced, *S*-carboxymethylated forms (Fig. 1B). The same results were obtained for OVA and lysozyme upon prolonged (20 min) boiling in non-reducing sample buffer. However, heat-denatured BSA was seen as high molecular weight polymers under all these sample preparation conditions.

Amino acid analyses of the heat-denatured proteins showed that cysteines and cystines were intact in all the three proteins investigated (not shown). In order to investigate further the behaviour of BSA upon heat-denaturation we subjected BSA to heat-denaturation in various buffers followed by analysis by SDS-PAGE (Fig. 2). This showed that the formation of high molecular weight aggregates was most pronounced in phosphate buffer, and less pronounced in Tris buffer and in water. Heat-denaturation under acid or basic conditions totally abolished aggregate formation and led to hydrolysis of the polypeptide chain. From these results we inferred that BSA had a tendency to form high molecular weight aggregates by intermolecular, isopeptide bond-mediated, cross-linking as a result of heat-denaturation. Further evidence for this was that the formation of intermolecular bonds in BSA was less pronounced at higher dilutions, where intermolecular encounters are less frequent, although it was still very substantial (not shown).

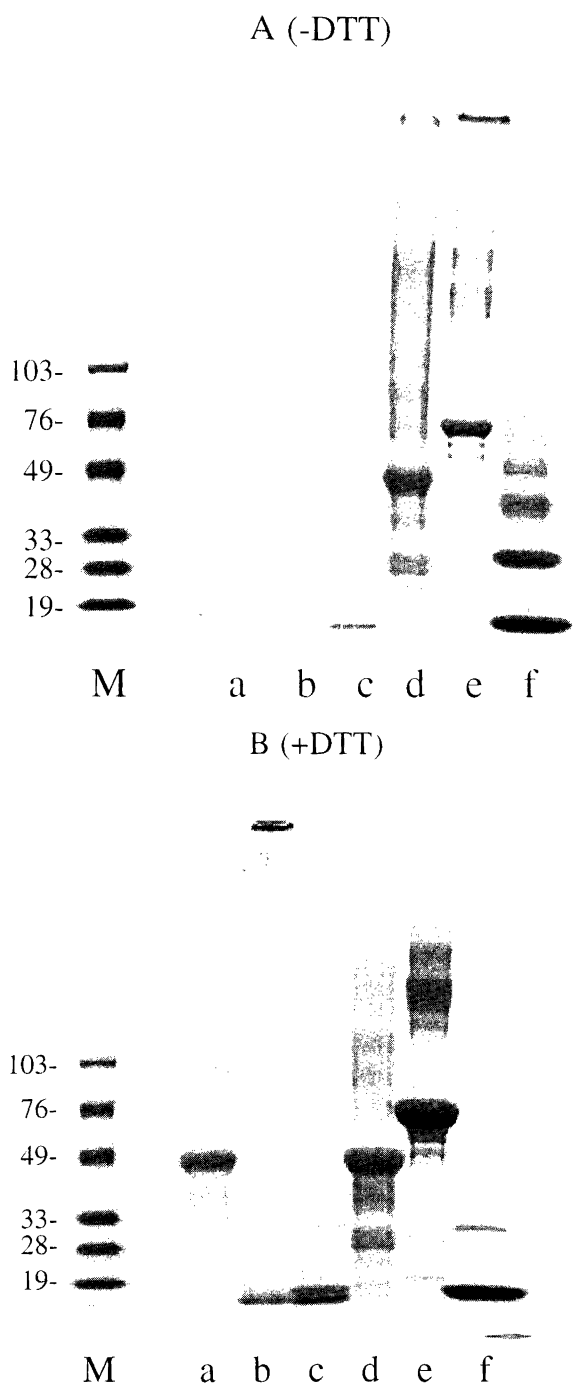

Fig. 1. SDS-PAGE analysis of the heat-denatured and reduced, *S*-carboxymethylated proteins: **A**, non-reducing conditions (−DTT); **B**, reducing conditions (+DTT); **M**, relative molecular mass marker proteins; **a**, heat-denatured OVA; **b**, heat-denatured BSA; **c**, heat-denatured lysozyme; **d**, reduced, *S*-carboxymethylated OVA; **e**, reduced, *S*-carboxymethylated BSA; **f**, reduced, *S*-carboxymethylated lysozyme.

The solubilities of the various protein preparations in water were very low (less than 1 mg ml^{-1}) except for

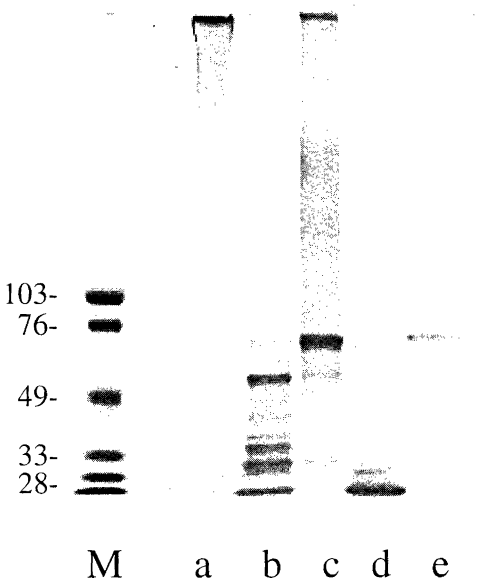

Fig. 2. SDS-PAGE under reducing conditions (+DTT) of BSA denatured in various solvents. **M**, relative molecular mass marker proteins; **a**, 50 mM sodium phosphate, pH 7.3; **b**, 50 mM acetic acid, pH 3.0; **c**, 10 mM Tris, pH 7.5; **d**, 50 mM sodium carbonate, pH 10.9; **e**, water, pH 7.0.

heat-denatured BSA, which had a solubility of approximately 5 mg ml^{-1} in water. SDS-PAGE analysis of the water-soluble proportions of reduced, *S*-carboxymethylated or heat-denatured proteins showed that these proportions appeared to be similar to the denatured preparations with regard to relative molecular weight distribution, except that the average molecular weight of the water-soluble proportion of heat-denatured BSA was somewhat lower (not shown).

Discussion

The solvents used can be grouped according to structure and functional groups, or according to dielectric constant (polar/apolar) and hydrogen-bond-donating efficiency (protic/aprotic).

Among the alcohols, the solubilities were insignificant in the apolar ones (butanol, 2-propanol), and only very low solubilities were observed for the polar alcohols. Water may be grouped together with the alcohols, since it ranges just above or below trifluoroethanol for the proteins investigated. From these results it seems that polarity is a major determinant of denatured protein solubility among the alcohols, although the solubilities were low.

A similar trend was observed for the organic acids. The apolar solvent acetic acid was a poor solvent for denatured proteins, 3-mercaptopropionic acid was a fairly good solvent, and the strongly polar solvents formic acid and trifluoroacetic acid were very good solvents for the heat-denatured proteins, especially heat-denatured OVA.

Among the polar, aprotic solvents, DMF was a poor solvent, and this also applied to DMSO except for lysozyme, which had a high solubility in DMSO in both denatured forms. This may result from interactions of basic groups on lysozyme ($pI=9.1$) with dipoles in this dipolar solvent ($\varepsilon=46.7$).

The solubilities of heat-denatured proteins were generally less than those of the *S*-carboxymethylated forms for the good solvents. A notable exception was the solubility of heat-denatured lysozyme in DMSO which is comparable to the high solubility of *S*-carboxymethylated lysozyme in DMSO.

The relatively high solubility of heat-denatured lysozyme in 2-mercaptoethanol compared with the low solubility in most of the other solvents suggests that the heat-denatured proteins may exist in aggregated and polymerised forms with intermolecular S–S bonds. This was supported by SDS-PAGE analysis of the denatured proteins, which showed that the heat-denatured proteins formed large aggregates which for BSA could not be reduced to monomers. This is in agreement with the results of others, showing that some heat-denatured proteins may form linear disulfide-linked aggregates, but also shows that the behaviour of BSA is different from that of OVA and lysozyme. From the experiments with heat-denaturation of BSA in various buffers and under basic or acidic conditions, it can be concluded that the intermolecular cross-links in heat-denatured BSA were formed by isopeptide bonds. Thus, the behaviour of different proteins upon heat-denaturation depends on the individual properties of the proteins and might possibly depend on the presence of specific amino acid sequences. The low solubility of reduced, *S*-carboxymethylated proteins and heat-denatured proteins in glycerol compared with the high solubility of native proteins in glycerol[12] shows that the solvating properties of this solvent are very similar to those of water. This conclusion is supported by the recent finding that lysozyme will fold correctly in glycerol.[15]

The solubilities of *S*-carboxymethylated proteins in organic solvents generally followed the solubilities of native proteins in these solvents.[12] Thus, strongly polar, protic solvents had great solubilising power, whereas polar, aprotic solvents only had low solubilising power. The solubilities in apolar solvents were essentially zero. This suggests that native proteins are denatured in polar protic solvents, but retain their structure in apolar, aprotic solvents. This conclusion is supported by results of others showing that many enzymes retain activity in apolar, aprotic solvents.[16,17] The structure of native proteins solvated in polar, aprotic solvents presumably depends on the structure of the solvent and the individual properties of the proteins.

In conclusion, it was observed here that aprotic solvents did not solubilise denatured proteins very well except for lysozyme, which interacted favourably with DMSO. Apolar, protic solvents were also poor solvents, and the solubilising power seemed to increase with increasing

dielectric constant. Polar, protic solvents, however, were good solvents.

With regard to the structure of denatured proteins, it may be inferred that the solubility depends on the interactions of the polypeptide backbone and of ionic, hydrophilic, and hydrophobic side-chains with the solvent. The amide backbone is a good acceptor and donator of hydrogen bonds. This should contribute favourably to solubility in protic solvents, and in solvents capable of accepting hydrogen bonds. Conversely, the poor solubilities in solvents of low polarity must be assumed to result from unfavourable interactions with the backbone and ionic and hydrophilic side-chains.

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