Artificial chaperoning of insulin, human carbonic anhydrase and hen egg lysozyme using linear dextrin chains – a sweet route to the native state of globular proteins

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Abstract Linear dextrins (α-1,4-D-glucopyranoside chains) are known to possess amphiphilic surfaces and solubilize lipophilic compounds. We have assessed the ability of this amphiphilic surface of dextrin to inhibit the self-aggregation and assist the refolding of proteins. Addition of decameric dextrin, or dextrin-10, in the renaturation buffer improves the refolding yield of human carbonic anhydrase from its guanidinium chloride-induced denatured state. It is also seen to inhibit the self-aggregation of insulin. The ability of dextrin-10 to interact with cetyltrimethylammonium bromide and postpone its critical micellar concentration allows the use of dextrin-10 as a 'detergent stripping agent' in a novel artificial chaperoning process described earlier. The aggregation of human carbonic anhydrase and lysozyme upon refolding is prevented by cetyltrimethylammonium bromide due to the formation of a protein-detergent complex; dextrin-10 strips off the detergent from the complex and allow the proteins to fold, thus increasing the renaturation yield. Dextran-4 (the α-1,6-D-glucopyranoside chain), which does not exhibit amphiphilic properties, does not help in such artificial chaperoning.

Key words: Linear dextrin; Dextran; Artificial chaperone; Protein refolding

1. Introduction

Though the information necessary for a polypeptide chain to fold to its native structure resides in its amino acid sequence [1], many proteins do not do so when they are set up to refold from their unfolded state in vitro. Two types of interactions mediated by hydrophobic forces are involved in the folding of globular proteins [2]: intramolecular hydrophobic forces which facilitate the spontaneous folding of the polypeptide chain on the one hand and the competing intermolecular hydrophobic interactions between partially folded species which lead to aggregation and inactivation of proteins on the other. Cells employ molecular chaperones, a class of proteins which bind to the non-native structures of proteins, to prevent aggregation and promote the proper folding of proteins into their native and active conformation [3–5]. Studies on protein folding are important not only to understand the molecular details of this process but also in biotechnology.

Overproduction of many proteins by recombinant DNA technology occasionally has to contend with the problem of the expressed protein forming inclusion bodies, which are aggregates of incompletely folded chains [6]. Inclusion bodies are usually brought into solution by denaturation using guanidinium chloride (GdmCl), and then refolded by dilution with a 'refolding' buffer in order to get active proteins. Aggregation and inactivation are competing processes for productive folding which affect the renaturation yields. Additives such as detergents [7–9] and polyethylene glycols [10,11] have been shown to increase the renaturation yields of proteins by preventing the aggregation. Rozema and Gellman [12–14] have reported a chemical approach for protein renaturation in which the aggregation of proteins is, in the first step, prevented by the addition of detergent molecules, which bind to the protein surface through a weak detergent-protein complex; the detergent molecules are next stripped off the complex by cyclodextrins, a class of amphiphilic hollow drum-like molecules of cyclic oligomeric dextrins, which extract away the protein-bound detergent molecule by accommodating it in their apolar cavity. This allows the detergent-free protein to fold into its native form. This method has been termed 'artificial chaperoning' because it is analogous to the chaperone-assisted protein folding where the stabilized partially folded state of the target proteins complexed with the chaperone is allowed to fold by a slow release of the protein from the complex [12].

We have earlier shown that linear dextrins (α-1,4-D-glucopyranoside chains) possess amphiphilic surfaces while dextrans (α-1,6-D-glucopyranoside chains) and cellulosic chains (β-1,4-D-glucopyranosides) do not have such amphiphilic surfaces. The amphiphilic character of the dextrin chain arises out of stereochemical constraints imposed on it by the quasi-chair conformation of the glucopyranose ring and more importantly by the α-1,4-D-glucopyranoside linkages which dispose the methine hydrogens on one side of the molecule and the hydroxyls on the other [15–17]. We have shown that linear dextrins effectively mimic many of the properties of cyclodextrins (which are cyclic oligomers of α-1,4-D-glucopyranosides). This, plus the fact that linear dextrins are: (a) readily available in a range of chain lengths with longer and more accessible hydrophobic surface than the cyclodextrins, which allows more than monomolecular guest binding and with no cavity size restriction, (b) highly soluble in water, which offers experimental convenience both for their intended use and for later removal, and (c) far less expensive than cyclodextrins, prompted us to use linear dextrins in the 'artificial chaperoning' of the refolding of two globular proteins, namely human carbonic anhydrase B (HCAB) and denatured-reduced hen egg white lysozyme; we find that they successfully assist these proteins to refold into their enzymatically active forms.

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2. Materials and methods

2.1. Materials

All biochemicals and reagents were commercially obtained and were of analytical grade. The saccharides dextrin-10, which is a decameric dextrin (MW ca. 1620 Da), and dextran-4, which is a more polydisperse higher oligomer of MW 4000–6000 Da, both obtained from Serva, were used as received. Lysozyme was further purified using a Bio-Rex-70 column, as described by Saxena and Wetlaufer [18].

2.2. Aggregation of insulin

The interchain disulfide bonds of insulin were reduced with 20 mM dithiothreitol (DTT) by adding 20 μl of 1 M DTT to 0.98 ml of 0.225 mg/ml solution of insulin in 10 mM sodium phosphate (pH 7.4) containing 150 Mm NaCl, and the extent of aggregation of insulin was monitored with time by measuring the scattering of 465 nm light in a Hitachi F-4000 fluorescence spectrophotometer. The excitation and emission wavelengths were set at 465 nm with band passes of 1.5 nm. The sample was constantly stirred. To find the effect of additives such as dextrin-10 and dextran-4, the required concentrations of these additives were included in the same buffer.

2.3. Refolding of HCAB

HCAB (5 mg/ml) was denatured by incubation for 2 h at 25°C in 50 mM Tris acetate buffer (pH 7.4) containing 6 M GdmCl; 4 μl of this solution was added to 0.196 ml of the refolding buffer (50 mM Tris acetate, pH 7.4) containing the required concentrations of additives such as dextrin-10 or dextran-4, vortexed for 10 s, incubated at 25°C for about 3 h and the activity was measured.

In another experiment, HCAB was refolded as described above in the refolding buffer, containing 0.6 mM of the detergent cetyltrimethylammonium bromide (CTAB). After incubation at 25°C for 15 min 75 μl of this solution was added to 25 μl of the buffer containing required amounts of additives, incubated at 25°C for about 3 h and the activity of the enzyme measured. HCAB concentration was estimated using the value of absorbance at 280 nm as 17.7, for a 1% solution [19].

2.4. Assay for HCAB

The enzyme activity was measured by the rate of hydrolysis of *p*-nitrophenyl acetate by monitoring the increase in the absorbance at 400 nm with time [20]. The activity recovered on refolding was calculated with respect to that of the native enzyme.

2.5. Heat inactivation of HCAB and its renaturation

HCAB at 100 μg/ml in 50 mM Tris acetate (pH 7.5) containing 0.6 mM CTAB was incubated at 70°C for 7 min and cooled to 25°C. 75 μl of this sample was diluted to 100 μl with the same buffer containing required amounts of dextrin-10, β-cyclodextrin or dextran-4, and the samples were incubated at room temperature for at least 3 h before assaying for enzyme activity.

Circular dichroism (CD) spectra were recorded using a JASCO-715 spectropolarimeter at room temperature.

2.6. Refolding of denatured-reduced lysozyme

Denatured-reduced lysozyme was prepared by dissolving 15 mg of lysozyme in 1 ml of 50 mM Tris acetate buffer (pH 8.1) containing 5 M GdmCl and 50 mM DTT, and incubating at 25°C for 16 h. Refolding of the enzyme at final concentrations of 0.15 mg/ml and 0.3 mg/ml was performed by adding 10 μl and 20 μl, respectively, of the denatured-reduced lysozyme solution to 1 ml of 100 mM Tris acetate buffer (pH 8.1) containing 2 mM DL-cystine hydrochloride and 0.6 mM CTAB, and incubated at room temperature for 10 min. This sample was aliquoted into different tubes, and weighed amounts of solid dextrin-10 were added in order to get the required final concentrations of dextrin-10, and incubated at 25°C for 4 h in order to get the required final concentrations of dextrin-10, and incubated at 25°C for 4 h to facilitate refolding.

2.7. Lysozyme assay

Lysozyme activity was measured at 25°C as per Fischer et al. [21]. The rate of enzymatic lysis of *Micrococcus lysodeikticus* cells, suspended in 100 mM sodium phosphate buffer (pH 6.3), was obtained by measuring the decrease in turbidity of the cell suspension at 450 nm with time. The percentage recovery of activity upon refolding was calculated with respect to that of the native enzyme.

3. Results and discussion

Polysaccharide chains are usually considered to be hydrophilic, since they contain no obvious apolar moieties but a large number of polar hydroxyl groups. Yet, the cyclic oligosaccharides called cyclodextrins [22] exhibit amphiphilic properties: they solubilize lipophilic compounds by accommodating them in their inner hydrophobic cavities. Our earlier studies on linear polysaccharides show that linear dextrins are amphiphilic in nature. The twisted chair conformation of the D-glucose monomers and the α-1,4-glycosidic linkage impart stereochemical constraints on the chain in a manner that places all the methine hydrogens of the sugar on one side of the chain, while the other face of the ribbon has the hydroxyls (see Fig. 1). Polymeric α-1,4-linked dextrin, i.e. α-amylose, is a 7_2 helix with the interior of the helix being a relatively apolar tube. This enables the linear dextrins to enhance the solubility of lipophilic compounds such as tyrosine, pyrene, estradiol and progesterone in water severalfold; on the other hand, the α-1,6-linked dextran or the β-1,4-linked cellulose do not exhibit such an amphiphilic property [15–17]. In the present study we have investigated whether the hydrophobic surfaces of linear dextrin can facilitate the refolding of proteins. Disulfide bonds are broken using the reducing agent DTT. This aggregation is effectively prevented upon the addition of the eye lens protein α-crystallin, which is recognised to be a natural chaperone-like molecule [23,24], known to prevent such self-aggregation by providing appropriately placed host hydrophobic surfaces [24]. α-Crystallin thus offers us a benchmark in studying the effect of dextrin-10 and dextran-4 on the aggregation of insulin. As evident from Fig. 2, dextrin-10 reduces the aggregation though to a lesser extent than α-crystallin [23,24]. These results suggest that the interaction

Table 1
Renaturation of GdmCl-denatured HCAB

A: By dilution[a]		B: By artificial chaperoning[b]	
Additives	Recovery of activity (%)[c]	Additives	Recovery of activity (%)
Buffer	11.0	0.6 mM CTAB/buffer alone	10.0
25 mM dextrin-10	34.5	10 mM dextrin-10	77.0
50 mM dextrin-10	54.1	7.5 mM dextran-4	2.0
7.5 mM dextran-4	0.0	3.75 mM β-cyclodextrin	77.0

[a]The refolding was done by dilution of a 5 mg/ml solution of HCAB by a factor of 50, using 50 mM Tris acetate (pH 7.4).
[b]HCAB was refolded at 100 μg/ml from 6 M GdmCl in 50 mM Tris acetate (pH 7.4) buffer containing 0.6 mM CTAB. The enzyme was subsequently renatured at 75 μg/ml by adding varous additives (see Section 2 for details).
[c]The percentage recovery of activity is with respect to that of the native enzyme.

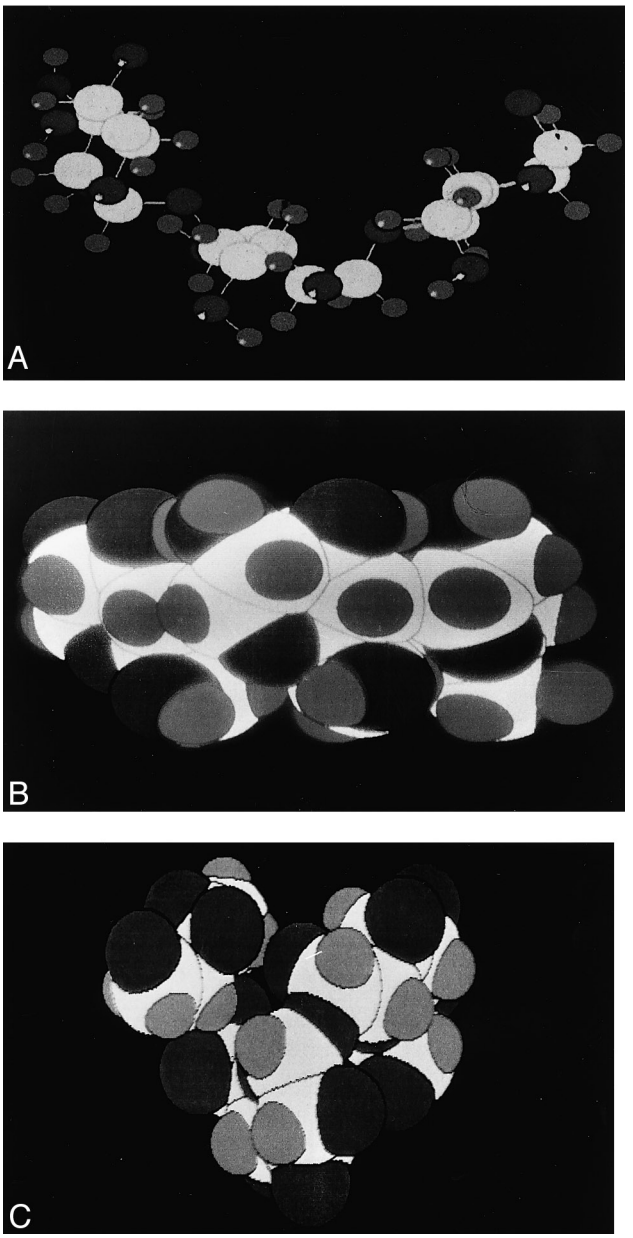

Fig. 1. The energy minimized conformations of dextrin and dextran trimers. A and B: Dextrin trimer. C: Dextran trimer. (Adapted from [15].)

of dextrin-10 with insulin is weaker but it does reduce the extent of aggregation, possibly through its apolar surfaces. Such an interaction is not exhibited by dextran-4 which, on the other hand, is seen to promote the self-aggregation of the protein.

Intermolecular hydrophobic aggregation often competes with the proper refolding of globular proteins and reduces their renaturation yield. Table 1 shows the renaturation yields of HCAB (measured as described in Sections 2.3 and 2.4), upon refolding from its unfolded state (in 6 M GdmCl) in the presence of various additives in the refolding buffer. The renaturation yield in buffer alone is 10%. dextrin-10 (50 mM) increases the renaturation yield to approximately 54%. Here too, dextran-4 is ineffective.

As mentioned earlier, Rozema and Gellman [12–14] have taken an approach for protein renaturation in which the aggregation of proteins is prevented by detergents at premicellar concentrations by forming a detergent-protein complex. The detergent molecules are then slowly stripped off the protein surface by cyclodextrins, allowing the protein to fold. This approach requires an efficient 'detergent stripping agent'. In an effort to assess whether dextrin-10 can act as such a 'detergent stripping agent', we measured the critical micelle concentration (CMC) of CTAB in the absence and presence of dextrin-10 using the fluorescence probe diphenylhexatriene

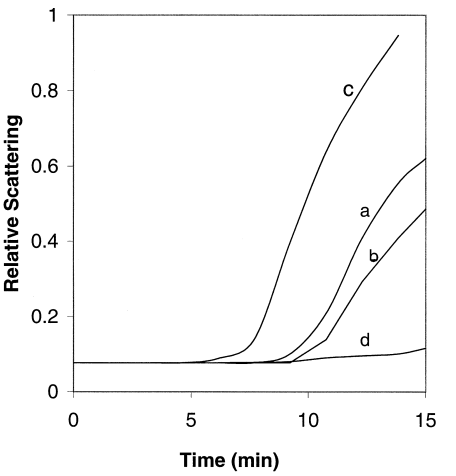

Fig. 2. Aggregation of insulin (0.225 mg/ml) upon breaking the interchain disulfide bonds with 20 mM DTT in buffer alone (curve a), buffer containing 40 mM dextrin-10 (curve b), 12.7 mM dextran-4 (curve c) and 1.6 mg/ml of α-crystallin (curve d) Buffer = 10 mM sodium phosphate (pH 7.4) containing 150 mM NaCl.

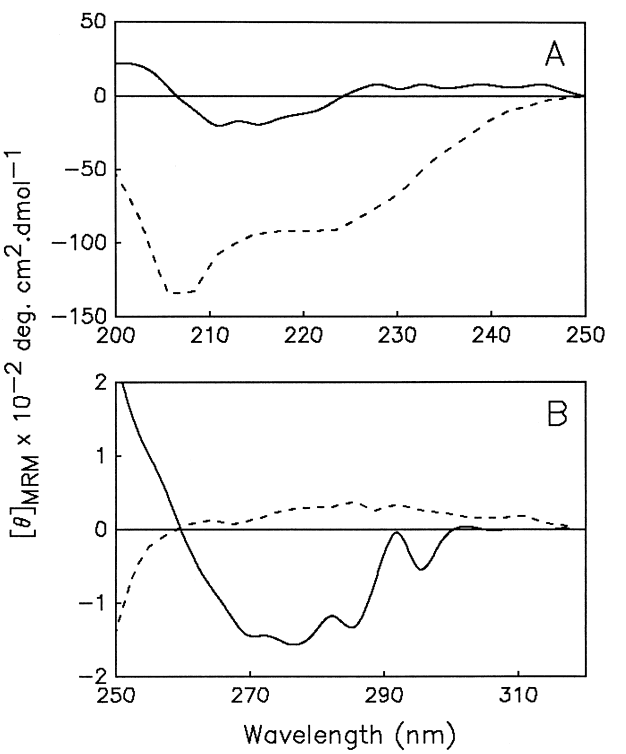

Fig. 3. Far (A) and near (B) UV-CD spectra of native HCAB (solid line) and heat-inactivated HCAB in the presence of 0.6 mM CTAB (dotted line).

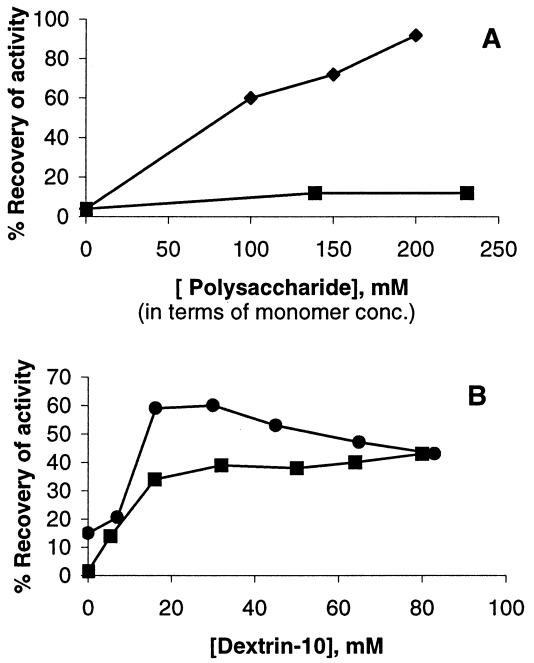

Fig. 4. A: Renaturation of heat inactivated HCAB by 'artificial chaperoning'. The protein in 50 mM Tris acetate buffer (pH 7.4) containing 0.6 mM CTAB was denatured by incubating it at 70°C for 7 min. The percentage recovery of the enzyme activity (with respect to that of the native enzyme) upon subsequent addition of dextrin-10 (♦) or dextran-4 (■) is shown. The concentration on the *x*-axis is in terms of the concentration of monomer units. HCAB was heat-inactivated at a concentration of 100 μg/ml and renatured in the presence of various additives at 75 μg/ml. B: Artificial chaperoning of the refolding of lysozyme from its denatured-reduced state at 0.15 mg/ml (•) and at 0.3 mg/ml (■). Lysozyme was refolded at the indicated final concentrations in 100 mM Tris acetate (pH 8.1), containing 2 mM DL-cystine and 0.6 mM CTAB and the sample was incubated at room temperature for 10 min. Weighed amounts of dextrin-10 were then added to the sample to give the indicated final concentrations and the samples incubated for 4 h for refolding.

(DPH) [25] and found that the CMC of CTAB is postponed from 0.9 mM in water to about 6 mM in 25 mM dextrin-10, indicating that dextrin-10 can serve as a good 'detergent stripping agent'; dextran, as expected, does not.

Table 1 also shows the renaturation yield of HCAB upon refolding of the enzyme in the refolding buffer containing 0.6 mM CTAB with subsequent stripping of the detergent molecules from the protein surface using dextrin-10. With 10 mM dextrin-10, 77% of the activity of the enzyme is recovered, an efficiency of linear dextrins that is seen to be about the same as that of cyclodextrin [12]; thus it is an effective and less expensive substitute for the latter. It is important to note that the renaturation yield obtained by this process (Table 1B) is higher than that obtained when the enzyme is refolded in the presence of dextrin alone (Table 1A).

We next studied the renaturation of HCAB that has been inactivated by heat. When the protein is incubated at 70°C for about 7 min, it aggregates and loses its activity. In the presence of 0.6 mM CTAB, such aggregation is completely prevented. CD spectra reveal that almost all the tertiary structure of the protein is lost (near-UV CD spectrum, Fig. 3B), while the chiral backbone structure is increased (Fig. 3A). This result shows that the detergent stabilizes the enzyme in its partially unfolded state which exhibits CD spectra similar to those of the molten globule state of the protein [26,27]. Fig. 4A shows the renaturation yield of the enzyme after stripping off the protein-bound detergent molecule by dextrin-10 at different concentrations. The renaturation yield increases up to 90% as the concentration of dextrin-10 increases. Dextran-4, on the other hand, does not lead to recovery of the enzyme activity.

We have also studied the refolding of denatured-reduced hen egg white lysozyme by this 'artificial chaperoning process'. Rather than return to is native and enzymatically active form upon refolding from its denatured-reduced state, this protein tends to aggregate and remain inactive [2,18,28]. An earlier study from our laboratory has shown the presence of an aggregation-prone refolding intermediate state, which exhibits a substantial amount of secondary structure, highly solvent exposed hydrophobic surfaces and no tertiary structure, on the refolding pathway of denatured-reduced lysozyme [28]. This aggregation is seen to be prevented by CTAB but the resulting solution does not exhibit significant activity (Fig. 4B). This shows that CTAB molecules bind to the intermediate of the enzyme and inhibits its oxidative refolding. Fig. 4B shows that the percentage recovery of lysozyme activity upon stripping of the protein-bound detergent by dextrin-10 at various concentrations. At the final concentration of 0.15 mg/ml of the refolded enzyme, the percentage recovery of activity increases up to 60% until a concentration of 30 mM dextrin-10 is reached; the gradual decrease with further increase in dextrin-10 concentration is most likely due to the binding of the sugar to the enzyme. Likewise when the protein concentration is doubled, the percentage of recovery of activity tapers off to 40%. Thus an optimal choice of protein, detergent and dextrin concentrations would lead to significant recovery in the oxidative refolding of enzymes.

We conclude that linear dextrins possess amphiphilic surfaces which can interact with the folding protein, thus preventing self-aggregation and enhancing its renaturation yield. Linear dextrins interact with detergents and can be used as efficient detergent-stripping agents in the artificial chaperoning of proteins. Unlike the α-1,4-linked dextrin chain, the α-1,6-linked dextran is ineffective. The stereochemical constraints imposed on the polysaccharide chain, depending on the nature of the glycosidic linkage, can generate amphiphilic surfaces on polysaccharides. These amphiphilic compounds may prove useful in biotechnological applications such as renaturation of proteins from their inclusion bodies.

Finally, in a lighter vein, we found that while β-cyclodextrin and dextrin-10 are very mildly sweet, dextran, cellulose (and even α-crystallin) are not. We may thus have an inexpensive artificial chaperone with a sweet touch in corn starch!

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