Can grafting of an octapeptide improve the structure of a de novo protein?

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Abstract Structural properties and conformational stability of de novo proteins – albebetin and albeferon (albebetin with a grafted interferon fragment) – were studied by means of CD spectroscopy, gel filtration and urea-induced unfolding. The results allow us to conclude that albebetin possesses the properties of the molten globule state. Grafting of the octapeptide to the N-terminus of this de novo protein affects its structure. We show here that albeferon maintains a secondary structure content of albebetin; it becomes more compact and much more stable toward urea-induced unfolding as compared to albebetin and even possesses some weak tertiary structure (at least around Tyr[7]). This means that the structure of the artificial protein albebetin can be improved by a simple procedure of octapeptide grafting to its N-terminus.

Key words: De novo protein; Molten globule; Protein expression; Protein design; Interferon

1. Introduction

The de novo design and production of proteins which ensures a desirable three-dimensional structure and function is one of the most intriguing challenges of modern protein engineering, and the best way to test our understanding of the principles of protein structure and folding. The first successful engineering of a de novo protein – a four-helix bundle – was reported 10 years ago [1]. During the last few years a number of attempts have been made in this direction [2,3]. Most of the designed structures mimicked the structures of natural proteins.

In 1991 the de novo protein albebetin was constructed [4–6]. This protein was designed to form a tertiary fold – a four-stranded antiparallel β-sheet with one side screened by two α-helices – which does not contradict any structural rules, but has never been observed in natural proteins. Analysis of its structure by gel filtration, urea gradient electrophoresis and limited proteolysis showed compactness and relative stability of this protein [5,6].

The next attractive step in the field of de novo protein engineering is the construction of a protein with pre-designed structure and some kind of biological activity. We have grafted an eight amino acid fragment (131–138) of human interferon α_2 (carrying the blast transforming activity of this protein) to albebetin [7,8]. The gene of a chimerical protein was expressed in a wheat cell-free system and the synthesized protein (albeferon) was tested using the approach elaborated for the fast testing of structural properties of de novo proteins at the nanogram level [5,6]. We have shown that albebetin with the grafted octapeptide is practically as compact as natural proteins of corresponding molecular weight and possesses high stability toward urea-induced unfolding [7,8]. Additionally, it was shown that albeferon binds murine thymocyte receptor with high affinity and efficiently activates the thymocyte blast transformation [7,8]. It is important to emphasize here that the previous studies of albebetin and albeferon were performed on radiolabeled proteins obtained in a cell-free system. We believe that this approach – first to test protein obtained in a cell-free system and then to express it in *Escherichia coli* in preparative amounts for detailed analysis – can be a general one for the investigation of de novo and mutant proteins.

In the present paper we describe the expression of genes encoding two de novo proteins (albebetin and albeferon) in *E. coli* and investigations of these proteins. We show that albebetin has a pronounced secondary structure and a high degree of compactness. At the same time grafting of the interferon octapeptide to this protein changes its structural properties. Our data are consistent with the suggestion that the secondary structure content of albeferon is close to that of albebetin, but the modified protein is more compact and substantially more stable toward urea-induced unfolding. It allows us to conclude that grafting the interferon fragment to albebetin can improve the structure of this de novo protein.

2. Materials and methods

2.1. Materials

T4 DNA ligase, $Vent_R$ polymerase, factor Xa protease and other enzymes and chemicals were from New England Biolabs (USA), Pharmacia (Sweden), Sigma (USA), Serva (USA), Bio-Rad (USA), and Amersham (UK).

2.2. Cloning and expression of albeferon

Standard methods were used for genetic engineering procedures such as DNA digestion and analysis with restriction endonucleases, ligation and transformation of *E. coli*, preparation and purification of DNA fragments, etc.

The albebetin and albeferon genes were expressed in *E. coli* in a fusion expression system with maltose binding protein purchased from New England Biolabs (NEB). The genes were cloned into plasmid pMALc following the N-terminal part of the maltose binding protein gene. Expression, purification, cleavage of the fusion proteins with factor Xa protease as well as the final affinity chromatography purification of de novo proteins on amylose binding resin were performed according to the NEB manual as described elsewhere [9]. The proteins were monitored by SDS-polyacrylamide gel electrophoresis at 10% gel according to Laemmli [10] as well as at 12.5% gel in Tris-tricine buffer system [11,12].

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2.3. Spectral measurements

Circular dichroism (CD) studies were carried out with Jasco-600 and Jasco-41A spectropolarimeters (Japan Spectroscopic Co., Ltd, Tokyo, Japan). Both instruments were equipped with a temperature-controlled holder. Protein concentration was 1.7 and 0.88 mg/ml for near and far UV CD measurements, respectively. Cell pathlength was 0.148 and 10 mm for far and near UV CD measurements, respectively. To investigate the temperature dependence of the spectra, a scanning rate of 0.9°C/min was used.

3. Results

3.1. Tertiary structure (near UV CD spectra)

Fig. 1 represents near UV CD spectra of two de novo proteins, albebetin (solid line) and albeferon (dashed line). One can see there are no specific signals in the near UV CD spectrum of albebetin which is quite natural since this protein has no tryptophan and tyrosine residues. As for albeferon, this protein gets a tyrosine residue in position 7 (Tyr^7) from the grafted interferon fragment. The chirality of the Tyr^7 environment may be responsible for the appearance of specific bands in the near UV CD spectrum of this protein (e.g. minima at 265 and 276 nm, see curve 2 in Fig. 1). On the other hand, Fig. 1 shows that the overall intensity of the near UV CD spectrum of this protein is relatively low. This observation allows us to conclude that albeferon has some tertiary structure (at least, in the environment of Tyr^7).

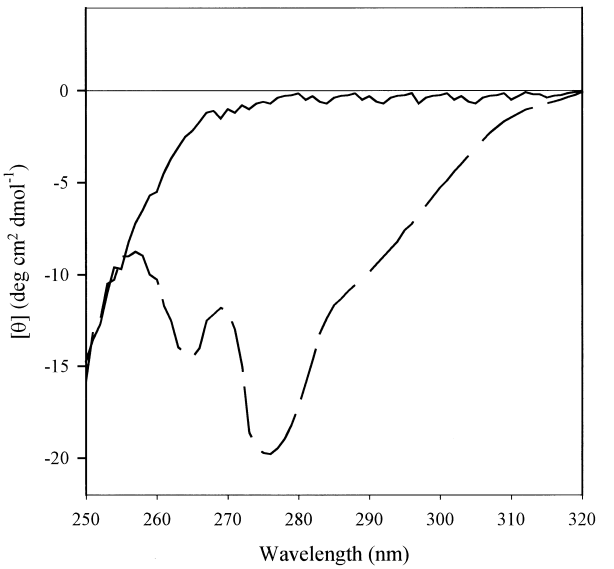

Fig. 1. Circular dichroism spectra of albebetin (solid line) and albeferon (dotted line) in the near UV region. Measurements were carried out in 100 mM potassium phosphate buffer, pH 8.0 at 25°C. Protein concentration was 1.7 mg/ml. Cell pathlength was 10 mm.

3.2. Secondary structure (far UV CD spectra)

Far UV CD spectra of albebetin and albeferon are shown in Fig. 2 as solid and dashed lines, respectively. As follows from Fig. 2, both de novo proteins have similar far UV CD spectra at the conditions studied (100 mM potassium phosphate buffer pH 8.0, 25°C). The quantitative analysis of these spectra according to Provencher and Glöckner [13] showed that albebetin contains 29% α- and 40% β-structure, while albeferon has 27% α- and 35% β-structure. These values are close to the pre-designed ones (30% α- and 36% β-structure), suggesting that the secondary structure design of the proteins was successful.

3.3. Compactness (gel filtration)

Compactness of the de novo proteins at various experimental conditions was studied by means of high performance liquid chromatography. This approach makes it possible to estimate the hydrodynamic dimensions of protein conformers [14–18] with high accuracy at low concentrations. A summary of the obtained results [6–8] is shown in Table 1. The table presents the hydrodynamic dimensions of albebetin and albeferon in comparison with those of native and unfolded globular proteins of corresponding molecular weights estimated according to empirical equations [16] based on the intrinsic viscosity data [19]:

$$\log R_S^N = 0.369 \log(M) - 0.254 \quad (1)$$

and

$$\log R_S^U = 0.533 \log(M) - 0.682 \quad (2)$$

where R_S^N and R_S^U are Stokes radii of the native (N) and unfolded (U) protein, respectively, while M is the protein molecular weight (in Da). Table 1 shows that in the absence of denaturant the R_S value of albeferon is somewhat smaller than that of albebetin. This difference is consistent with the situation when the grafted octapeptide is attracted to albebetin since in the opposite case one can expect some increase in R_S value rather than a decrease. On the other hand, the R_S value for albeferon in the absence of denaturant exactly coincides with that of natural native protein of the same molecular weight. Thus Table 1 suggests that albeferon is as compact as native natural globular protein of the same molecular weight, whereas R_S of albebetin exceeds the corresponding values by a factor 1.13, which is the usual case for molten globules [16,17,20].

Table 1
Hydrodynamic dimensions of albebetin and albeferon in different conformational states

Protein	Conditions	R_S (Å)	R_S/R_S^N
Albebetin	0 M urea	17.2 ± 0.5	1.13
	9 M urea	24.1 ± 0.8	1.59
Natural protein of the same M (7760 Da)[a]	0 M urea	15.2	1.00
	8 M urea	24.6	1.62
Albeferon	0 M urea	16.0 ± 0.5	1.01
	9 M urea	25.3 ± 0.8	1.59
Natural protein of the same M (8640)[a]	0 M urea	15.9	1.00
	8 M urea	25.9	1.63

[a]Stokes radius values in the native and completely unfolded states were calculated in accordance with Eqs. 1 and 2 (see text).

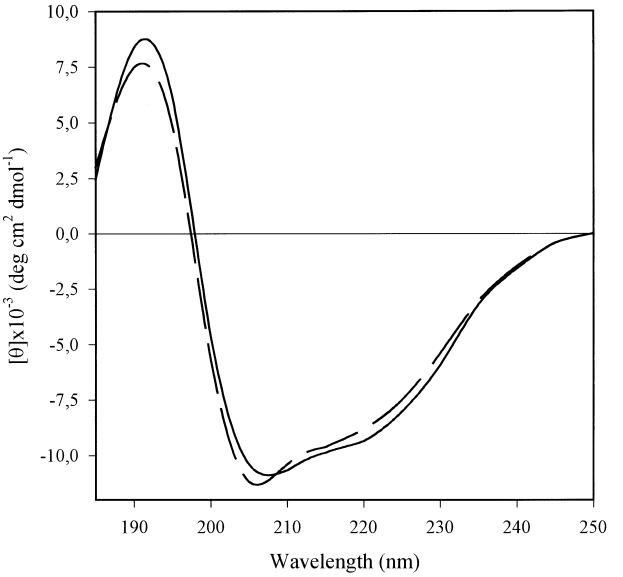

Fig. 2. Circular dichroism spectra of albebetin (solid line) and albeferon (dotted line) in the far UV region. Measurements were carried out in 100 mM potassium phosphate buffer, pH 8.0 at 25°C. Protein concentration was 0.88 mg/ml. Cell pathlength was 0.148 mm.

3.4. Stability (urea-induced unfolding)

To obtain an idea of the conformational stability of different proteins analysis of their stability toward denaturant-induced unfolding is commonly used. Assuming an all-or-none mechanism of the transition between the initial (native) and final (unfolded) states, it is possible to estimate the free energy of the initial state relative to that of the unfolded one, ΔG_{fold}, under the given conditions as [19]:

$$\Delta G_{\text{fold}} = -RT \ln K_{\text{eq}} \quad (3)$$

where K_{eq} is the equilibrium constant of the transition. It is known that within the transition region, where it can be measured, the value of ΔG_{fold} varies linearly with the denaturant concentration [21]:

$$\Delta G_{\text{fold}} = \Delta G_{\text{fold}}^{\text{H}_2\text{O}} + m\,[\text{denaturant}] \quad (4)$$

The parameter m reflects the dependence of the free energy on the denaturant concentration, [denaturant]; typically the value of m is $\sim$1 kcal/mol for urea-induced unfolding of native natural proteins [22]. The values of ΔG_{fold} are usually extrapolated linearly to determine its value in the absence of denaturant, $\Delta G_{\text{fold}}^{\text{H}_2\text{O}}$; typical values of this parameter for the native natural proteins are between -5 and -10 kcal/mol [22].

A detailed description of the urea-induced unfolding of albebetin and albeferon was presented in our previous publications [6–8]. Although the all-or-none character of the urea-induced unfolding was established only for albeferon [7,8], and although unfolding transitions for these de novo proteins were obtained by different methods (urea-gradient electrophoresis for albebetin [6] and gel-permeation chromatography for albeferon [7,8]), we assume that it is possible to use these data for the analysis suggested. Results of this analysis are summarized in Table 2, which also contains the urea concentrations corresponding to the middles of corresponding unfolding transitions ($[\text{urea}]_{1/2}$ values) and the averaged values of parameters m and $\Delta G_{\text{fold}}^{\text{H}_2\text{O}}$ estimated for urea-induced unfolding of native natural proteins [22]. As follows from Table 2, albebetin and albeferon, being relatively stable toward urea-induced unfolding ($[\text{urea}]_{1/2} > 4.5$ M), show considerable differences in unfolding behavior. Table 2 shows that all parameters of the unfolding of albebetin ($[\text{urea}]_{1/2}$, m, $\Delta G_{\text{fold}}^{\text{H}_2\text{O}}$) are substantially lower than those of albeferon. This means that the grafting of an octapeptide to albebetin results in a considerable stabilization of this de novo protein.

4. Discussion

The most important finding of our work is that grafting the octapeptide changes the structure of albebetin. Indeed, albeferon is a bit more compact than the non-modified protein ($R_S = 16.0 \pm 0.5$ Å, as compared with $R_S = 17.2 \pm 0.5$ Å) and possesses cooperativity toward urea-induced unfolding almost as high as that of natural protein of this molecular weight (see Table 2). It was also shown that this modified protein has a pronounced secondary structure (see Fig. 2). The near UV CD spectrum of albeferon (Fig. 1) suggests that this de novo protein has some tertiary structure. On the other hand, it is necessary to emphasize here that microcalorimetric studies showed that neither albebetin nor albeferon has the rigid cooperatively melted tertiary structure characteristic of native natural proteins [9]. This means that either the tertiary structure of albeferon is too weak to be accompanied by any cooperative heat absorption upon disruption or it is situated only in the near environment of Tyr^7 and does not touch the rest of the molecule. This might be the reason for the relatively low intensity of the near UV CD spectrum of albeferon as compared with native natural proteins.

It seems that our data make it possible to conclude that engineering of specific fragments and grafting them to the N- and/or C-terminus of de novo proteins is a possible way to improve their design. It has already been mentioned that the current state of the art in the engineering of artificial proteins makes it possible to design successfully only more or less ordered molten globules [23–26]. This means that an important piece of knowledge in this area is still missing. The approach which we have used to design the chimerical albebetin (attachment of the peptide to the terminus of de novo protein) can be considered one more step toward the creation of artificial proteins with a native-like unique structure.

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Table 2
Parameters of urea-induced unfolding of albebetin and albeferon

Protein	$\Delta G_{\text{fold}}^{\text{H}_2\text{O}}$ (kcal/mol)	m (kcal/mol	$[\text{urea}]_{1/2}$ (M)
Albebetin	-2.9 ± 0.3	0.56 ± 0.07	4.6
Albeferon	-5.2 ± 0.3	0.99 ± 0.07	5.6
Natural native protein	$-10 \div -5$	~ 1	–

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