S6 permutein shows that the unusual target topology is not responsible for the absence of rigid tertiary structure in de novo protein albebetin

Ziedulla Kh. Abdullaev[a,b,*], Ramil F. Latypov[c], Azat Ya. Badretdinov[c], Dmitry A. Dolgikh[a], Alexey V. Finkelstein[c], Vladimir N. Uversky[c], Mikhail P. Kirpichnikov[a]

[a]*Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Science, 117871 Moscow, Russia*
[b]*Center 'Bioengineering', Russian Academy of Science, 117312 Moscow, Russia*
[c]*Institute of Protein Research, Russian Academy of Science, 142292 Pushchino, Moscow Region, Russia*

Received 30 July 1997

Abstract Ribosomal protein S6 from *Thermus thermophilus* was modified to form the unusual unique topology designed earlier for a de novo protein albebetin. The S6 gene was cloned, sequenced and circularly permutated by means of genetic engineering methods. The permutated gene was expressed in *Escherichia coli* and the permutein was isolated and investigated by means of circular dichroism, fluorescence spectroscopy and scanning microcalorimetry. The permutated protein revealed a pronounced secondary structure close to that of the wild type S6 protein and a rigid tertiary structure possessing cooperative temperature melting. It means that the unusual new topology of albebetin is compatible with a rigid tertiary structure, it may be realized in natural proteins and it is not responsible for the absence of rigid structure in albebetin.

Key words: De novo protein; Protein design; Ribosomal protein S6; Circular permutation; Permutein

1. Introduction

There is a widespread belief that design and synthesis of man-made amino acid sequences which ensures a desirable 3-D structure is the best way to check whether we understand the principles of protein structure and protein folding. Several successful attempts have been made in this field (see [1–3] for reviews).

It is necessary to emphasize, however, that practically all existing de novo proteins were designed in such a way that they mimic structures of some natural proteins or their domains. The de novo protein albebetin is virtually the only exception. It has been designed to form a predetermined tertiary fold – four-stranded antiparallel β-sheet with one side covered by two α-helixes – with a topology which does not contradict any structural rules, but has never been observed in natural proteins [4–6]. Structural analysis of this protein by high-performance gel filtration, urea-gradient electrophoresis, limited proteolysis, near and far UV CD spectroscopy and microcalorimetry [5–8] has shown that albebetin possesses the properties of the molten globule state [9]. The question arises: Why does albebetin have no rigid tertiary structure? There are at least two possible answers to this question:

1. imperfect design of tight packing of amino acid residues inside the molecule;
2. contradiction of unusual target topology to some unknown rules of protein structure; as a result, the native protein with this topology cannot exist.

It is obvious that finding a natural protein with albebetin-like topology and rigid tertiary structure could help choose between these two possibilities. Unfortunately, so far a small globular protein with this topology has not been found. That is why we decided to perform a circular permutation in natural protein which has a rigid tertiary structure and the secondary structure element packing close to that designed for the de novo protein, but whose topology (connections between the elements of the regular secondary structure) is different from that of albebetin. Such permutation will result in a new protein with albebetin-like topology and rigid tertiary structure.

Previous experience of several research teams in this field shows that permuteins usually have the possibility to form a rigid tertiary structure [10–16] which makes it possible to think that permutation by itself virtually does not ruin a protein structure. To realize this idea, ribosomal protein S6 from *Thermus thermophilus* was selected. It is a small globular protein of 101 amino acid residues; its 3-D structure has been solved with high resolution [17].

With the use of circular dichroism, fluorescence analysis and differential scanning microcalorimetry we showed that the permutated variant of ribosomal protein S6 has a rigid tertiary structure, which can be melted cooperatively. Thus, the albebetin topology does not contradict the requirement of globular protein structure.

2. Materials and methods

2.1. Materials

Restriction endonucleases, T4 DNA ligase, Vent polymerase and other enzymes and chemicals were from New England Biolabs (USA), Pharmacia (Sweden), Sigma (USA), Bio-Rad (USA), and Amersham (UK).

2.2. Genetic engineering

Standard methods [18] were used for genetic engineering procedures such as DNA digestion and analysis with restriction endonucleases, ligation and transformation of *Escherichia coli*, preparation and purification of DNA fragments, etc. *E. coli* strains XL1-Blue [19] and BL21DE3(pLysS) [20] were used for gene cloning and expression. The S6 gene was cloned and permutated using polymerase chain reaction as described elsewhere [21].

2.3. Spectral measurements

Circular dichroism studies were carried out on Jasco-600 and Jasco-41A spectropolarimeters (Japan Spectroscopic) using 0.148 mm and 10 mm quartz cells for the far and near UV CD measurements, respectively. Both instruments were equipped with a temperature-con-

*Corresponding author. Fax: (7) (95) 335-5033.
E-mail: zied@nmr.ru

PII S0014-5793(97)01042-9

trolled holder. Protein concentration was 0.99 mg/ml. The far UV spectra were analyzed in the 200–250-nm region.

Urea-induced unfolding of permutated protein was studied by the characteristic red shift of the maximum of the tryptophan fluorescence spectrum [22] and the unfolding curve was analyzed as described in [23,24]. Protein concentration was 0.05 mg/ml. The value of Δv (the difference in the number of denaturant molecules 'bound' to one protein molecule in its two states) was determined by the following equation [25]:

$$\Delta v^{eff} = 4a_t\left(\frac{\partial\Theta}{\partial a}\right)_{a=a_t} \quad (1)$$

where a_t is the denaturant activity in the transition midpoint.

2.4. Microcalorimetry

Calorimetric measurements were carried out on a new precise differential adiabatic scanning microcalorimeter SCAL-1 (Scal, Ltd., Pushchino, Russia). This microcalorimeter is equipped with a newly designed calorimetric unit with cylindrical glass cells (0.34 ml) providing high chemical resistance and excellent dynamic characteristics. Protein concentration was 2.1 mg/ml; scanning rate was 0.9 K/min.

3. Results and discussion

3.1. S6 permutein engineering

As has already been mentioned, the mutual arrangement of secondary structure elements in ribosomal protein S6 from *Th. thermophilus* is close to that of de novo protein albebetin, but these two proteins differ from each other by their topologies. To visualize this conclusion, Fig. 1 compares the spatial structures of albebetin and S6 protein. One can see that albebetin-like topology can be introduced in S6 protein by the insertion of a loop between its N- and C-termini and cutting the loop between the first α-helix and β-strand.

Circular permutation in S6 protein was designed using the molecular graphic program 'What If' [26]. It was decided to make the following changes [21] (see Fig. 1):

1. remove four amino acid residues at the C-terminus of the protein;
2. remove Met and Arg at its N-terminus;
3. cut the loop between the first α-helix and β-strand (between Asn-13 and Leu-14);
4. link modified N- and C-termini using a loop Ala-Ser-Thr-Thr-Pro-Gly;
5. put Met at the new N-terminus of the chain.

In accordance with these suggestions the S6 gene was modified using a two step polymerase chain reaction. The common direct primer was complementary to the nucleotide sequence coding the first α-helix; two reverse primers consequently generated a sequence corresponding to a β-strand at the C-terminus of the new protein. The product of amplification was a gene, coding a new protein, starting from the α-helix and bearing the β-strand at the C-terminus. The detailed mutagenic procedure is described in [21] as well as expression of the permutated gene in *E. coli*.

3.2. Structural properties of the permutein

Fig. 2A represents the circular dichroism (CD) spectrum of the permutated S6 protein in the near UV region. It is known that the rigid chiral environment of aromatic amino acid residues in native proteins gives rise to specific aromatic CD signals. This means that the presence of pronounced signals in the near UV CD spectrum of permuatted S6 protein is consistent with the conclusion that this protein has a unique tertiary structure. This conclusion is in good agreement with the ^{1}H-NMR spectrum of the permutein, which, being typical for a native globular protein, contains many resonance lines through the whole spectral region with chemical shifts which are not observed for unfolded proteins (data not shown, for details see [21]).

The far UV CD spectrum of the permutated S6 protein is shown in Fig. 2B. It is known that analysis of the shape and intensity of the far UV CD spectrum makes it possible to estimate a secondary structure content in a protein [27]. Quantitative analysis of the far UV CD spectrum of the permutated S6 protein showed that it contains 25% α- and 43% β-structure. These values are close to those calculated from X-ray data for the wild type S6 protein (26% α- and 39% β-structure), indicating that permutein possesses a native-like secondary structure.

Fig. 3 presents data on urea-induced unfolding of permu-

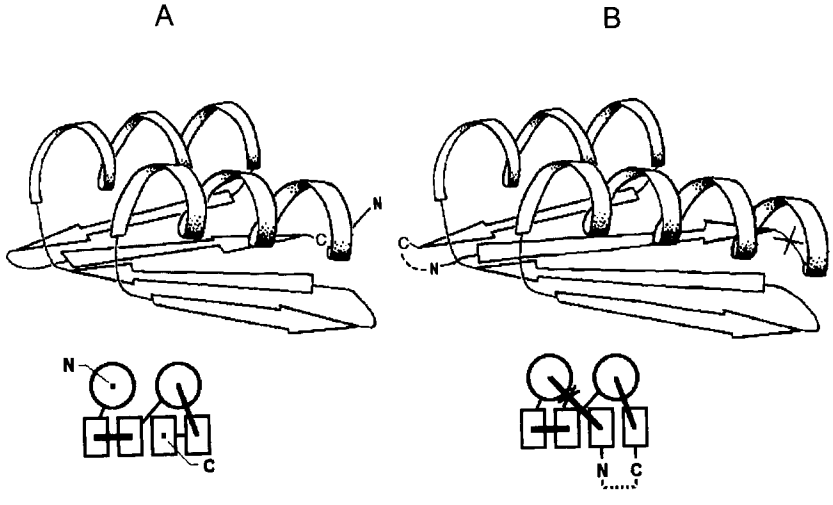

Fig. 1. A: Pre-designed 3-D structure of albebetin [2–4]. B: 3-D structure of ribosomal protein S6 [16]. Topology of both proteins is shown below. To transform the S6 protein molecule into albebetin topology, one has to introduce the loop between two β-strands (dashed line) and cut the loop between the first β-strand and β-helix (crossed).

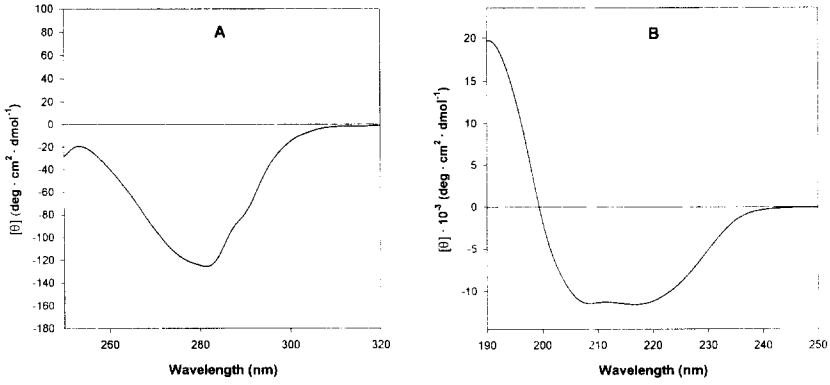

Fig. 2. Near (A) and far (B) UV CD spectra of the permuted S6 protein in 20 mM potassium phosphate buffer pH 7.37 at 20°C.

tein followed by the parameter I_{370}/I_{330} (I_{370} and I_{330} represent intensities of Trp fluorescence at 370 and 330 nm, respectively). It is known that changes in this parameter upon protein unfolding can be used to monitor the characteristic red shift of the tryptophan fluorescence spectrum maximum.

Recently it has been shown [23,24] that the steepness of the urea- or guanidinium chloride-induced unfolding curve depends strongly on whether a given protein has a unique tertiary structure (i.e. it is native) or it is already denatured and exists as a molten globule. This means that additional information about the rigidity of the tertiary structure can, in principle, be obtained by the analysis of the denaturant-induced unfolding curve. To this end the corresponding value of Δv^{eff} (see Section 2) should be determined. Then this value should be compared with $\Delta v^{eff}_{N\rightarrow U}$ and $\Delta v^{eff}_{MG\rightarrow U}$ values corresponding to the native-coil and molten globule-coil transition, respectively. These two parameters can be estimated from [24]

$$\log \Delta v^{eff}_{N\rightarrow U} = 0.97 \log M - 0.07 \quad (2)$$

$$\log \Delta v^{eff}_{MG\rightarrow U} = 0.89 \log M - 0.4 \quad (3)$$

where M is the protein molecular weight in kDa. The value of standard deviation (± 2.0) in these cases was determined from the corresponding figures in [24].

Thus, $\Delta v^{eff}_{N\rightarrow U} = 8.9 \pm 2.0$ and $\Delta v^{eff}_{MG\rightarrow U} = 3.4 \pm 2.0$, respectively, for native-coil and molten globule-coil transitions in natural proteins of 11 kDa (molecular weight of the permutein). The corresponding value for the permutated protein obtained from the data of Fig. 3 using Eq. 1 is $\Delta v^{eff} = 9.6 \pm 0.5$, therefore the cooperativity of urea-induced unfolding of permutated S6 protein is typical for native globular protein of this molecular weight.

Fig. 4 shows the data of a microcalorimetric study of the permutein. Scanning microcalorimetry is a powerful tool to investigate thermal denaturation of globular proteins [28,29]. The heat absorption peak(s) in a calorimetric curve presents evidence for cooperative unfolding of a protein molecule [29], and predominantly of tertiary structure disruption [9]. Microcalorimetry study of the permutein (Fig. 4) revealed a heat absorption peak at 69°C upon the temperature melting. Thus, it is also evidence indicating that the permutated protein possesses a rigid tertiary structure.

Finally it should be emphasized that the above results taken together leave no doubt that the permutated version of ribosomal protein S6 has a native-like secondary structure and

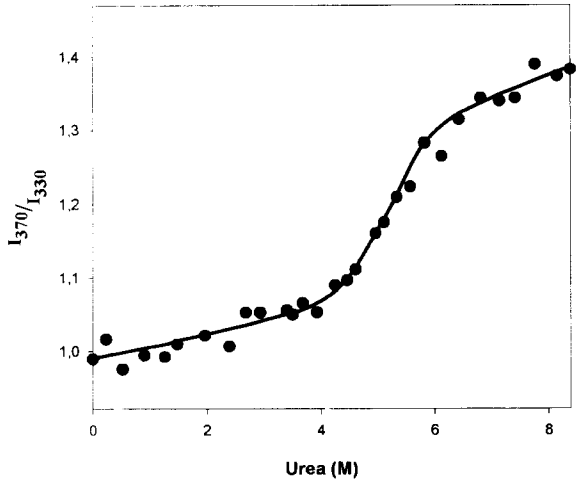

Fig. 3. Urea-induced unfolding of the permutated S6 protein monitored by the characteristic red shift of the maximum of the tryptophan fluorescence spectrum. I_{370} and I_{330} are intensities of Trp fluorescence at 370 and 330 nm, respectively. Measurements were carried out in 20 mM potassium phosphate buffer pH 7.37 at 23°C.

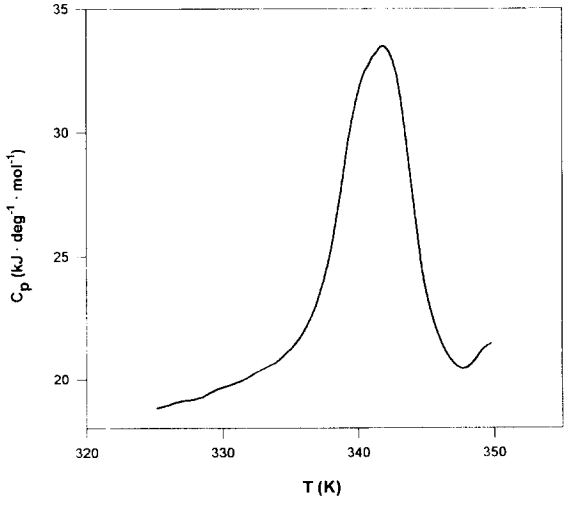

Fig. 4. Temperature dependence of partial heat capacity C_p of the permutated S6 protein in 20 mM potassium phosphate buffer at pH 7.37.

stable rigid tertiary structure. This allows us to presume that the circular permutation in S6 protein (as well as some small modifications of its amino acid sequence) does not prevent the permutein from forming a rigid tertiary structure. Unfortunately, we have no direct evidence on the topology of permutated S6 protein. However, all the permuteins studied before either had a 3-D structure very similar to that of initial protein, or had no stable 3-D structure at all [10–16]. Thus, from our point of view, the suggested design and the obtained results allow us to think that permutein actually possesses the albebetin-like topology. Consequently, a protein with unusual albebetin-like topology can have a unique rigid 3-D structure. This means that this topology is not responsible for the absence of a rigid tertiary structure in the de novo protein albebetin.

Acknowledgements: We thank M.B. Garber, O.B. Pitsyn, and A.S. Arseniev for valuable discussions. We are grateful to D.V. Shcherbakov, K.S. Vassilenko and T.N. Melnik for providing a part of experiments and E.V. Bocharov for his help in NMR spectra measurements and fruitful discussions. This work has been supported in part by an International Research Award from the Howard Hughes Medical Institute to A.V.F.

References

[1] Hecht, M. (1996) in: P.L. Carey (Ed.), Protein Engineering and Design, Academic Press, London, pp. 1–50.
[2] Sander, C. (1994) Trends Biotechnol. 12, 163–167.
[3] Dolgikh, D.A., Kirpichnikov, M.P., Ptitsyn, O.B. and Chemeris, V.V. (1996) Mol. Biol. (Moscow) 30, 149–156.
[4] Dolgikh, D.A., Fedorov, A.N., Chemeris, V.V., Chernov, B.K., Finkelstein, A.V., Schulga, A.A., Alakhov, Yu.B., Kirpichnikov, M.P. and Ptitsyn, O.B. (1991) Dokl. Akad. Nauk. SSSR (Moscow) 320, 1266–1270.
[5] Fedorov, A.N., Dolgikh, D.A., Chemeris, V.V., Chernov, B.K., Finkelstein, A.V., Shulga, A.A., Alakhov, Yu.B., Kirpichnikov, M.P. and Ptitsyn, O.B. (1992) J. Mol. Biol. 225, 927–931.
[6] Chemeris, V.V., Dolgikh, D.A., Fedorov, A.N., Finkelstein, A.V., Kirpichnikov, M.P., Uversky, V.N. and Ptitsyn, O.B. (1994) Protein Eng. 7, 1041–1052.
[7] Uversky, V.N., Aphasizheva, I.Yu., Dolgikh, D.A., Abdullaev, Z.Kh., Kirpichnikov, M.P. and Ptitsyn, O.B. (1997) Protein Eng. (in press).
[8] Aphasizheva, I.Yu., Dolgikh, D.A., Abdullaev, Z.Kh., Latypov R.F., Tiktopulo, E.I., Uversky, V.N., Ptitsyn, O.B. and Kirpichnikov, M.P. (1997) Biofizika (Moscow) (in press).
[9] Ptitsyn, O.B. (1995) Adv. Protein Chem. 47, 83–229.
[10] Goldenberg, D.P. and Creighton, T.E. (1983) J. Mol. Biol. 165, 407–413.
[11] Luger, K., Hommel, U., Herold, M., Hofsteenge, J. and Kirschner, K. (1989) Science 243, 206–213.
[12] Buchwalder, A., Szadkowsky, H. and Kirschner, K. (1992) Biochemistry 31, 1621–1630.
[13] Horlick, R.A., Cooke, G.M., Tritch, R.J., Newton, R.C., Dwivedi, A., Lischwe, M., Salemme, F.R. and Horuk, R. (1992) Protein Eng. 5, 427–431.
[14] Zhang, T., Bertelsen, E., Benvegnu, D. and Albert, T. (1993) Biochemistry 32, 12311–12318.
[15] Protasova, N.Yu., Kireeva, M.I., Murzina, N.V., Murzin, A.G., Uversky, V.N., Gryaznova, O.I. and Gudkov, A.T. (1994) Protein Eng. 7, 1373–1377.
[16] Uversky, V.N., Kutyshenko, V.P., Protasova, N.Yu., Rogov, V.V., Vassilenko, K.S. and Gudkov, A.T. (1996) Protein Sci. 5, 1844–1851.
[17] Lindahl, M., Svensson, L.A., Liljas, A., Sedelnikova, S.E., Eliseikina, I.A., Fomenkova, N.P., Nevskaya, N., Nikonov, S.V., Garber, M.B., Muranova, T.A., Rykunova, A.I. and Amons, R. (1994) EMBO J. 13, 1249–1254.
[18] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning. A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[19] Bullock, W.O., Fernande, J.M. and Sort, J.M. (1987) Biotechniques 5, 376–378.
[20] Studier, F.W. and Moffatt, B.A. (1986) J. Mol. Biol. 189, 113.
[21] Latypov, R.F., Abdullaev, Z.Kh., Badretdinov, A.Ya., Bocharov, E.V., Melnik, T.N., Arseniev, A.S., Dolgikh, D.A., Uversky, V.N. and Kirpichnikov, M.P. (1997) Mol. Biol. (Moscow) (in press).
[22] Stryer, L. (1968) Science 162, 526–540.
[23] Ptitsyn, O.B. and Uversky, V.N. (1994) FEBS Lett. 341, 15–18.
[24] Uversky, V.N. and Ptitsyn, O.B. (1996) Folding Design 1, 117–122.
[25] Aune, K.C. and Tanford, C. (1969) Biochemistry 8, 4586–4590.
[26] Vriend, G. (1990) J. Mol. Graphics 8, 52–56.
[27] Provencher, S.W. and Gloeckner, J. (1981) Biochemistry 20, 33–37.
[28] Privalov, P.L. (1979) Adv. Protein Chem. 33, 167–241.
[29] Privalov, P.L. and Potekhin, S.A. (1986) Methods Enzymol. 131, 4–51.