FEBS Letters 410 (1997) 280–284 FEBS 18754

Ligand-free form of human α-fetoprotein: evidence for the molten globule state

Vladimir N. Uversky[a,*], Natalya V. Narizhneva[a], Tatyana V. Ivanova[a], Marina D. Kirkitadze[a], Andrey Yu. Tomashevski[b]

[a] *Institute of Protein Research, Russian Academy of Sciences, Pushchino, Moscow Region 142292, Russian Federation*
[b] *Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow Region 142292, Russian Federation*

Received 17 April 1997

Abstract By means of circular dichroism and fluorescence spectroscopy, viscometry and scanning microcalorimetry we have shown that the release of ligands from human α-fetoprotein (AFP) results in a considerable rearrangement of the protein molecule. Ligand-free form is practically as compact as the native molecule and has native-like content of secondary structure but no rigid tertiary structure. This means that the release of ligands transforms the AFP molecule into a molten globule state. Stripping the ligands from AFP is the irreversible process, i.e., native protein molecule cannot be reconstituted from the ligand-free form of AFP by adding back ligands. A possible functional role of such a structural transformation is discussed.

Key words: α-Fetoprotein; Molten globule state; Ligand-free form; Conformational transition; Protein denaturation and unfolding

1. Introduction

α-Fetoprotein (AFP) is a large (~67 kDa) cancero-developmental glycoprotein the content of which is high (~10 mg/ml) in embryonal and fetal sera but decreases to trace amounts soon after birth. Increase of AFP concentration in adults is a sign of the development of some pathological conditions [1–3]. Changes in AFP content during pregnancy is also of diagnostic importance as this indicates the development of many fetal abnormalities [4–6]. The physiological role of AFP is very diverse from binding of various ligands [7–12] to regulation of immune response [12–14].

The molten globule state is an equilibrium state of many proteins whose structural properties are intermediate between those of native and unfolded protein molecules. This state is compact and has a number of important features of the native secondary structure and the overall native architecture, but its rigid tertiary structure is either absent or strongly reduced (for a recent review see Ref. [15] and references therein). It was shown that the molten globule, being separated from the native and unfolded states by 'all-or-none' transitions, represents a third thermodynamic state of protein molecules [16–18]. The functional role of this intermediate state is discussed [19].

In the present paper we investigate the effect of release of natural ligands on structural properties and conformational stability of AFP. We show that the formation of a ligand-free form is an irreversible process which transforms the AFP molecule into a compact denatured intermediate with pronounced secondary structure, i.e., into the molten globule state. A possible functional role of such a structural transformation is suggested.

2. Materials and methods

2.1. Materials

AFP was isolated from human cord serum by the procedure described in Ref. [20]. The protein purity was no less than 98% according to SDS and native polyacrylamide gel electrophoresis (PAGE) with immunoblotting and reversed-phase chromatography.

Hexane and powdered charcoal (Norit, Serva) were of extra pure grade. Buffer compounds, of analytical or extra pure grade, were used without additional purification. The pH values were adjusted by addition of 0.5–1.0 M NaOH or HCl. All solutions were prepared in bidistilled water. Measurements were carried out in 100 mM sodium phosphate buffer (pH 7.2) containing 0.15 M NaCl (PBS buffer).

2.2. Experimental procedure

Hydrophobic ligands were released from the AFP molecules by charcoal [21,22] or hexane treatment. To this end, protein solution at pH 6.0 was mixed with a portion of charcoal or hexane. In the first case, the weight ratio charcoal/protein was equal to 5:1; in the second case, the volume ratio 1:1 was used. The mixture was stirred for 12 h at room temperature, before being centrifuged. The aqueous fraction was collected, filtered and passed through a small gel-filtration column (G-75) equilibrated with PBS buffer. Completeness of ligand release was controlled by gas chromatography. Chromatographic profiles obtained for AFP after the above procedures contain the only peak corresponding to protein molecules.

Addition of arachidonic acid to lipid-free AFP was carried out as follows. An amount of fatty acid (FA) sufficient to provide a 10-fold molar excess over the amount of protein to be employed was dissolved in ethanol. A small aliquot (10 μl) of this solution was then added to 1000 μl of solution of ligand-free AFP in distilled water and the contents were allowed to react for 2 h at room temperature with gentle shaking. Finally the reaction mixture was passed through a small gel-filtration column (G-75) equilibrated with PBS buffer. The presence of fatty acids in the reconstituted samples of AFP was checked by gas and reversed-phase chromatography. The incorporation of arachidonic acid in AFP was not detected.

Protein concentrations were measured by absorbance at 280 nm on Cary-219 spectrophotometer (Varian, Australia) using the extinction coefficient ($\varepsilon^{1mg/ml}_{1cm,280nm}$) 0.365. For circular dichroism (CD) and calorimetric measurements, protein concentration was 0.5–1.0 mg/ml, fluorescence investigations were done using 0.01 mg/ml protein solutions, viscometry investigations were carried out at protein concentration 1.0–7.0 mg/ml.

CD spectra were obtained on a Jasko-600 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan) equipped with a temperature-controlled holder. For far- and near-UV measurements 0.148 and 10 mm pathlength cells were used, respectively.

Calorimetric measurements were carried out using DASM-5M precision scanning microcalorimeter (Bureau of Biological Instrumentation, Pushchino, Russia) with a cell volume of 1 ml. The rate of heating was 1 K/min. The excess pressure was kept equal to 3.6 atm.

Intrinsic viscosity [η] was determined as

*Corresponding author. Fax: (7) (095) 924 0493.
E-mail: uversky@sun.ipr.serpukhov.su

PII S0014-5793(97)00606-6

$[\eta] = \lim_{c\to 0} \eta_{red} = \lim_{c\to 0}(\eta-\eta_0)/\eta_0 c$,

where η is the viscosity of the protein solution of concentration c and η_0 is the viscosity of the solvent. Reduced viscosity η_{red} values were measured with a Zimm rotation viscosimeter (Bureau of Biological Instrumentation, Pushchino, Russia) and were calculated as

$\eta_{red} = (t-t_0)/t_0 c$

where t and t_0 correspond to the time of one turn of the rotor in the protein solution of concentration c or in the solvent, respectively.

3. Results

3.1. Release of ligands results in denaturation of AFP molecule

It is well-known that aromatic CD signals are determined by the incorporation of aromatic residues into the rigid chiral environment, i.e., by the presence of unique tertiary structure in a protein molecule. Decrease of elipticity in this region reflects the reduction in ordered tertiary structure upon change of experimental conditions. Near-UV CD spectra of AFP in different conformational states (native, ligand-free and completely unfolded by 9.5 M urea) are presented in Fig. 1A. Ligand-free form of AFP was obtained either by charcoal or hexane treatment (see below). One can see that near-UV CD spectrum of native AFP is not very intense, but is consistent with the presence of asymmetry in the environment of aromatic amino acid residues, i.e., with the presence of rigid tertiary structure. At the same time, spectra of the ligand-free form are much less pronounced and very close to the near-UV CD spectrum of completely unfolded AFP. This means that release of ligands results in destruction of the unique tertiary structure in the protein molecule, i.e., leads to its denaturation.

This suggestion was confirmed by the results of calorimetric measurements which are presented in Fig. 1B. One can see that the temperature course of the excess heat capacity for native AFP is the typical calorimetric curve that has a single heat absorption peak. The presence of such a peak reflects cooperative disruption of rigid tertiary structure of a protein molecule [15,23]. This means that, in the presence of natural ligands, AFP has a rigid tertiary structure which can be cooperatively destroyed by heating. From analysis of the calorimetric curve, it is possible to determine calorimetric (ΔH^{cal}) and van 't Hoff (ΔH^{vH}) enthalpy values. Comparison of these two quantities provides information on the mechanism of a given temperature-induced transition [23]. In our case, $\Delta H^{cal} = 480$ kJ/M, $\Delta H^{vH} = 510$ kJ/M and $\Delta H^{vH}/\Delta H^{cal} = 1.1$. This means that temperature-induced denaturation of the ligand-saturated form of AFP is a two-state process. Interestingly, van 't Hoff enthalpy of AFP melting is considerably lower than the typical ΔH^{vH} values measured for proteins of this molecular weight [24]. Such low ΔH^{vH} values obtained for AFP cannot be explained by the partial denaturation of this protein in the initial samples. Indeed, we have established that the ligand-saturated form of AFP possesses high resistance towards proteolytic digestion (data will be published elsewhere), whereas it is known that partially denatured protein molecules are rather unstable in respect to proteolysis.

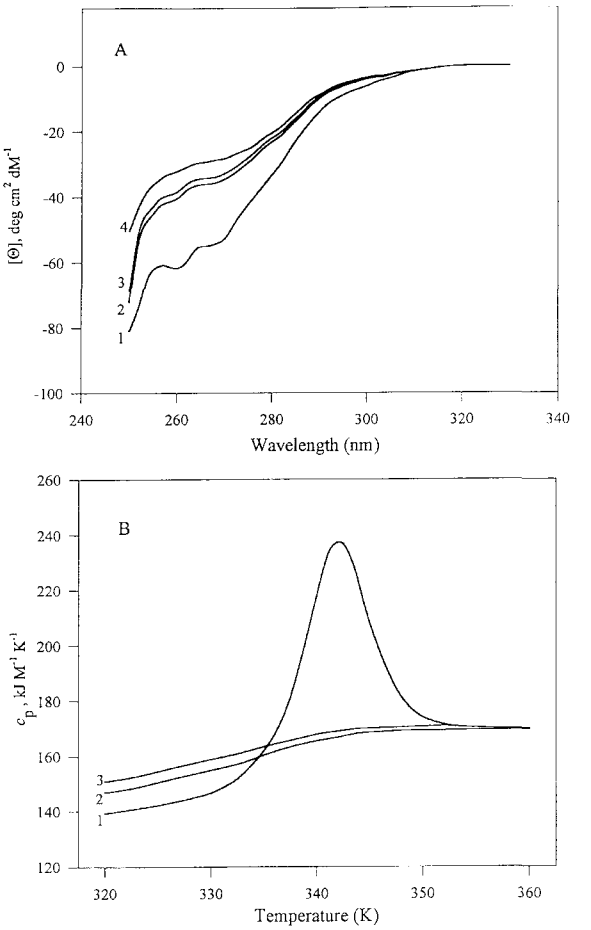

Fig. 1. A: Near-UV CD spectra of human AFP in native (1), ligand-free (2,3) and completely unfolded by 9.5 M urea (4) states. Defatted forms of the protein were obtained by charcoal (2) or hexan (3) treatment. Measurements were done at 23°C. B: Temperature dependence of partial molar heat capacity of AFP in the presence (1) or absence (2,3) of hydrophobic ligands. Ligand-free forms of the protein were obtained by charcoal (2) or hexane (3) extraction. All measurements were done in 100 mM sodium phosphate buffer (pH 7.2) containing 0.15 M NaCl. The protein concentration was 1.0 mg/ml throughout the experiments.

It is necessary to emphasize that temperature-induced denaturation of AFP molecules is the irreversible process. We have ascertained that such behaviour is due to the irreversible release of ligands from AFP and this is also the case for other types of denaturing action (increase of urea concentration or decrease of pH value, see Ref. [25] for details).

In the case of ligand-free form of AFP, we did not obtain any visible heat absorption (see Fig. 1B). Thus, we can con-

Table 1
Structural properties of human AFP in different conformational states

Conformational state	Melting peak	$[\Theta]_{265}$ (deg cm^2 dM^{-1})	$[\Theta]_{220}$ (deg cm^2 dM^{-1})	$[\eta]$ (ml/g)	R_s(Å)	R_s/R_s^N
Native	+	55 ± 3	15 200 ± 200	3.3 ± 0.5	32.3	1.00
Ligand-free	−	35 ± 3	14 600 ± 200	5.6 ± 0.8	38.5	1.19
Unfolded	−	30 ± 3	700 ± 100	33.7 ± 0.8	70.0	2.17

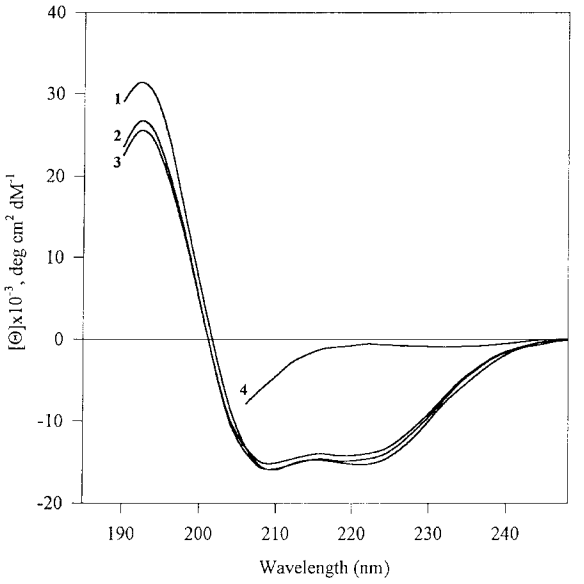

Fig. 2. Far-UV CD spectra of AFP in natural (1), defatted (2,3) and completely unfolded (4) forms. Measurements were done in 100 mM sodium phosphate buffer (pH 7.2) containing 0.15 M NaCl at 23°C. Protein concentration was 0.5 mg/ml for all the experiments.

clude that the ligand-free form of AFP has no cooperatively melted rigid tertiary structure, i.e., it is denatured.

3.2. *Stripped form of AFP has native-like content of secondary structure*

Fig. 2 shows far-UV CD spectra of AFP in the native state (100 mM phosphate buffer (pH 7.2), 0.15 M NaCl) in the ligand-free form (i.e., after charcoal or hexane treatment) and unfolded state (in the presence of 9.5 M urea). CD spectrum of a protein in 9.5 M urea is typical of random coils (completely unfolded state) while the CD spectra of the ligand-free form of AFP are very close to that of the native state, which excludes protein unfolding under these conditions. As stripping the ligands does not affect considerably the shape and intensity of far-UV CD spectra of AFP (Fig. 2), we can conclude that the protein has native-like content of secondary structure in the ligand-free form.

3.3. *Ligand-free form of AFP is compact*

To determine hydrodynamic dimensions of the AFP molecule in different conformational states (native state, ligand-free form and random coil with intact SS bonds) intrinsic viscosity [η] values were measured. These values were used in calculations of the Stokes radius (R_s) values according to ref. [26]

$$[\eta] = (2.5\ N_a/M)(4/3\pi R_s^3)$$

where N_a is Avogadro's number and M is protein molecular weight. The corresponding [η] and R_s values are presented in Table 1. One can see that the presence of 9.5 M urea leads to considerable increase of AFP's hydrodynamic dimensions while the protein molecule in the ligand-free form is practically as compact as in the native state (see Table 1).

3.4. *Stripping the ligands from AFP is an irreversible process*

It is known that AFP possesses substantial heterogeneity connected mainly with different level of saturation with hydrophobic ligands (saturated and/or unsaturated fatty acids) [7] and/or different degree of glycosylation [7,12]. On the other hand, it was established that the composition of hydrophobic ligands bound to AFP depends considerably on the source of protein and method of its isolation and purification [7,12]. To avoid the effect of heterogeneity, a special rather harsh procedure of AFP isolation was elaborated [26]. As a result, homogeneous AFP samples were obtained. Gas chromatography analysis allows to conclude that the major non-protein component of our AFP samples (>90%) is arachidonic acid (data not shown).

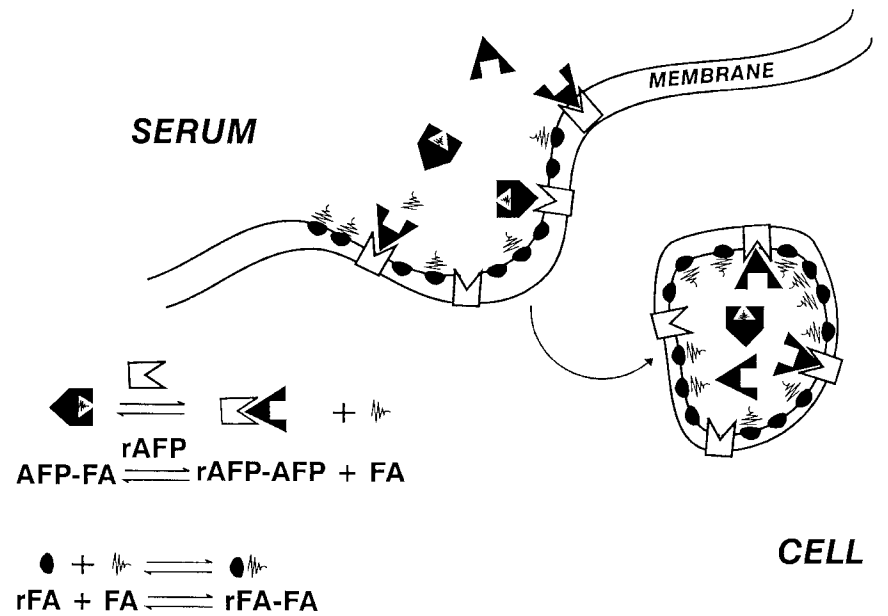

Fig. 3. Schematic representation of a two-receptor model for AFP endocytosis and FA transfer into target cell. rAFP, AFP receptors; rFA, FA receptors; AFP–FA, ligand-saturated form of AFP; rAFP–AFP, complex between rAFP and AFP; rFA–FA, complex between rFA and FA. Adapted from Ref. [32].

It was revealed that stripping the FA ligands from human AFP by charcoal or hexane extraction is an irreversible process. This means that native protein molecule cannot be reconstituted from the ligand-free form by adding back the major FA ligand of AFP — (in our samples) arachidonic acid.

4. Discussion

The results presented in this paper show that the release of ligands transforms molecules of human AFP into the molten globule state. Indeed, as it follows from near-UV CD and scanning microcalorimetric data (see Fig. 1 and Table 1) the ligand-free form of this protein has no rigid tertiary structure. On the other hand, at these conditions, the protein preserves native-like secondary structure (see Fig. 2 and Table 1). Finally, viscometry measurements clearly show (see Table 1) that the AFP molecule in the defatted (ligand-free) form is practically as compact as in the native state — its R_s exceeds the corresponding native value in $\sim$1.2 times, which is the usual case for molten globules [15,27–29]. Recently we have shown that decrease of pH also leads to transformation of native AFP molecules into the molten globule state [30].

We have also established that the release of AFP by charcoal or hexane extraction is an irreversible process. This correlates well with the earlier observation that "the delipidation of AFP by charcoal treatment at acid pH resulted in a loss of the subsequent FA-binding capacity of the protein" [31]. It is necessary to emphasize here that, from our point of view, the irreversibility of AFP delipidation is not connected with stripping procedures used but determined mainly by the structural rearrangement of the protein molecule in ligand-free form. This suggestion is confirmed by our recent work [25] where we have established that any kind of AFP denaturation (induced either by decrease of pH or increase of temperature or urea concentration) is an irreversible process connected with irreversible ligand release from the AFP molecule.

It should be emphasized that such a structural transformation, induced by the release of ligands, can be associated with biological functioning of AFP. Indeed, one of the most important functions of AFP is the transport of various hydrophobic ligands [7–12]. A special two-receptor model for AFP endocytosis and FA transfer into a target cell was suggested [32]. This model was based on the results of microbiological investigations and proposed two different receptors on the cell surface–AFP- and FA-binding sites. Interaction of ligand-saturated AFP molecules with the cell surface areas where AFP receptors are located leads to a conformational change in the protein accompanied by dissociation of the AFP–FA complex (see Fig. 3). The fatty acids are then translocated to nearby FA-binding sites on the cell surface. An FA-free AFP molecule can dissociate from an AFP receptor or can be transferred into the cell by endocytosis along with fatty acids (see Fig. 3).

An intriguing possibility is that the ligand-free form of AFP studied in our paper might be considered as an in vitro counterpart of the defatted AFP from the above model. As mentioned above, biological activities of AFP are rather diverse — from binding of various ligands [7–12] to regulation of immune response [12–14]. We assume that structural transformations in the AFP molecule which occur after release of ligands might be important for the further functioning of this protein, for example, as immunoregulator.

As for the driving forces of protein denaturation in a living cell, it is necessary to emphasize that membrane can cause partial unfolding of protein molecules [33] due to the negative charges on its surface and through decrease of the effective dielectric constant [19,34–36]. It has been established that the membrane-induced transition of a protein molecule into the molten globule state is of functional importance (see refs. [15] and [19] for recent reviews and references therein). This facilitates transmembrane protein transport [33] and toxin penetration into membranes [37]. A similar mechanism was suggested for membrane-mediated release of retinol from retinol-binding protein [38] and we cannot exclude that an analogous mechanism may also apply to AFP.

Acknowledgements: The authors express their deepest gratitude to Profs. G.I. Abelev, A.V. Finkelstein, A.L. Fink, E.A. Nunez and O.B. Ptitsyn for valuable discussions. We also thank A.S. Karnoup for critically reading and editing the manuscript. This work was supported in part by grants from RFBR-INTAS (95-IN/RU-1278) and RFBR (97-04-48044).

References

[1] Abelev, G.I. (1971) Adv. Cancer Res. 14, 295–358.
[2] Smuckler, E.A., Koplitz, M. and Sell, S. (1976) Cancer Res. 36, 4558–4561.
[3] Purves, L.R., Bersohn, I. and Geddes, E.W. (1978) Cancer 25, 1261–1270.
[4] Ruoslahti, E. and Seppala, M. (1979) Adv. Cancer Res. 29, 275–346.
[5] Brock, D.J., Scrimgeour, J.B. and Nelson, M.M. (1975) Genetics 7, 163–169.
[6] Seppala, M. (1975) Ann. NY Acad. Sci. 259, 59–73.
[7] Parmelee, D.C., Evenson, M.A. and Deutsch, H.F. (1978) J. Biol. Chem. 253, 2114–2119.
[8] Uriel, J., de Nechaud, B. and Dupiers, M. (1972) Biochem. Biophys. Res. Commun. 46, 1175–1180.
[9] Nishi, S., Matsue, H., Yoshida, H., Yamamoto, R. and Sakai, M. (1991) Proc. Natl. Acad. Sci. USA 88, 3102–3105.
[10] Hsia, J.C., Er, J.S., Tan, C.T., Ester, T. and Ruoslahti, E. (1980) J. Biol. Chem. 255, 4224–4227.
[11] Ruoslahti, E., Ester, T. and Seppala, M. (1979) Biochim. Biophys. Acta 578, 511–519.
[12] Deutsch, H.F. (1991) Adv. Cancer Res. 56, 253–312.
[13] Lu, C.Y., Changelian, P.S. and Unanue, E.R. (1984) J. Immunol. 132, 1722–1727.
[14] Calwell, J.L., Severson, C.D. and Thompson, J.S. (1973) Fed. Proc. 32, 979.
[15] Ptitsyn, O.B. (1995) Adv. Prot. Chem. 47, 83–229.
[16] Uversky, V.N., Semisotnov, G.V., Pain, R.H. and Ptitsyn, O.B. (1992) FEBS Lett. 314, 89–92.
[17] Ptitsyn, O.B. and Uversky, V.N. (1994) FEBS Lett. 341, 15–18.
[18] Uversky, V.N. and Ptitsyn, O.B. (1996) Folding Design 1, 117–122.
[19] Bychkova, V.E. and Ptitsyn, O.B. (1993) Chemtracts Biochem. Mol. Biol. 4, 133–163.
[20] A.Yu. Tomashevski, V.N. Uversky, submitted.
[21] Chen, R.F. (1967) J. Biol. Chem. 242, 173–181.
[22] Sogami, M. and Foster, J.F. (1968) Biochemistry 7, 2172–2182.
[23] Privalov, P.L. (1979) Adv. Protein Chem. 33, 167–244.
[24] Privalov, P.L. and Potekhin, S.A. (1986) Methods Enzymol. 131, 4–51.
[25] V.N. Uversky, N.V. Narizhneva, T.V. Ivanova, A.Yu. Tomashevski, submitted.
[26] Tanford, C. (1970) Adv. Prot. Chem. 24, 1–95.
[27] O.B. Ptitsyn, in: T.E. Creighton (Ed.), Protein Folding, W.H. Freeman, New York, NY, 1992, pp. 245-302.
[28] Uversky, V.N. (1993) Biochemistry 32, 13288–13298.
[29] Uversky, V.N. (1994) Int. J. Bio-Chromatogr. 1, 103–114.
[30] Uversky, V.N., Kirkitadze, M.D., Narizhneva, N.V., Potekhin, S.A. and Tomashevski, A.Yu. (1995) FEBS Lett. 364, 165–167.

[31] Uriel, J., Naval, J. and Laborda, J. (1987) J. Biol. Chem. 262, 3579–3585.
[32] J. Uriel, J. Laborda, J. Naval, M. Geuskens, in: G.J. Mizejewski, H.I. Jacobson (Eds.), Biological Activities of alpha$_1$-Fetoprotein, Vol. 2, CRC Press, Boca Raton, FL, 1989, pp. 103-117.
[33] Eilers, M., Endo, T. and Schatz, G. (1988) EMBO J. 7, 1139–1145.
[34] Ptitsyn, O.B., Bychkova, V.E. and Uversky, V.N. (1995) Phil. Trans. R. Soc. Lond. B 348, 35–41.
[35] Bychkova, V.E., Dujsekina, A.E., Klenin, S.I., Tiktopulo, E.I., Uversky, V.N. and Ptisyn, O.B. (1996) Biochemistry 35, 6058–6063.
[36] Uversky, V.N., Narizhneva, N.V., Kirschstein, S.O., Winter, S. and Löber, G. (1997) Folding Design 2 N3, 163–173.
[37] Van der Goot, F.G., Gonzalez-Manas, J.M., Lakkey, J.H. and Pattus, F. (1991) Nature 354, 408–410.
[38] Bychkova, V.E., Berni, R., Rossi, G.-L., Kutyshenko, V.P. and Ptitsyn, O.B. (1992) Biochemistry 31, 7566–7571.