Rigidity of Human α-Fetoprotein Tertiary Structure Is under Ligand Control†

Vladimir N. Uversky,*,‡ Natalya V. Narizhneva,‡ Tatyana V. Ivanova,‡ and Andrey Yu. Tomashevski§

Institutes of Protein Research and of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, 142292 Pushchino, Moscow Region, Russia

Received February 13, 1997; Revised Manuscript Received August 18, 1997⊗

ABSTRACT: Comparative study of the natural ligand effect on structural properties and conformational stability of human α-fetoprotein (AFP) and its homologue, human serum albumin (HSA), was performed using several approaches, including circular dichroism, fluorescence spectroscopy, and scanning microcalorimetry. Here we show that denaturation of AFP, induced by the increase of temperature or urea concentration, is irreversible. We have established the fact that this irreversibility is caused by ligand release from the AFP molecule. Interestingly, the ligand-free form of AFP has no rigid tertiary structure but exhibits substantial secondary structure and high compactness. This means that the rigid tertiary structure of AFP is controlled by interaction with ligands, while their release results in transition of a protein molecule into a molten globule-like intermediate. In contrast, processes of HSA denaturation and unfolding are completely reversible. Release of ligands from HSA results only in a small decrease in stability but not transformation into the molten globule state.

α-Fetoprotein, AFP,[1] is a cancerodevelopmental glycoprotein consisting of 590 amino acid residues (Morinaga et al., 1983) that is produced primarily by fetal liver and yolk sac cells. Synthesis of AFP during fetal development is very high but decreases drastically just after birth. As a result, only trace amounts of AFP are presented in the serum of healthy adults, where AFP is replaced by serum albumin. An increase of AFP content in adults is a sign of the development of some pathological conditions, usually of oncological nature (Abelev, 1971).

AFP is a well-known molecular marker indicating the development of cancer as well as fetal abnormalities such as open neural tube defects (Abelev, 1971; Brock et al., 1975; Seppala, 1975; Smuckler et al., 1976; Purves et al., 1978; Ruoslahti & Seppala, 1979; Lange, 1983). In addition to its substantial diagnostic importance, the physiological functions of AFP are also of great interest. It was established that AFP occurred in pregnancy sera and in the fetal sera of rats was capable of binding estrogens (Soloff et al., 1972; Uriel et al., 1972; Nishi et al., 1991). It was shown that AFP can strongly bind fatty acids (Parmelee et al., 1978; Hsia et al., 1980); material isolated from human fetuses contained 2−3 mol of fatty acid, mainly of unsaturated nature (arachidonic, C20:4, and docosahexaenoic, C22:6, acids). Human hepatoma AFP isolates show a much larger variety in fatty acid content as compared with fetal ones (Parmelee et al., 1978; Nagai et al., 1982). A high affinity of AFP for bilirubin that appears due to a single binding site has been shown (Ruoslahti et al., 1979; Hsia et al., 1980). Binding of both copper and nickel by human AFP has been also noted (Aoyagi et al., 1978). In addition to its transport function, AFP participates in regulation of cell multiplication and metabolism and can effectively interact with microphages (Lu et al., 1984) and T-lymphocytes (Torres et al., 1989). A role of AFP in the suppression of immune response has also been discussed (Calwell et al., 1973; Lu et al., 1984).

Human serum albumin (HSA), the major component in blood plasma, consists of 585 amino acid residues (King & Spences, 1972; Brown, 1975). The importance of HSA stems from the fact that it is involved in bioregulatory and transport phenomena. It was shown that HSA participates in the control of osmotic pressure in blood (Peters, 1985). It binds various metal ions: Ca^{2+} (Pedersen, 1978), Cu^{2+} and Ni^{2+} (Bradshaw et al., 1968; Sarkar, 1983), Zn^{2+} (Gurd & Wicox, 1956), Mg^{2+}, Co^{2+}, and Cd^{2+} (Nandedkar et al., 1973), and many others (Peters, 1985). HSA takes part in transport and storage of different fatty acids (Klopfenstein, 1969; Spector & Fletcher, 1978; Roda et al., 1982), unsaturated arachidonic acid among them (Savu et al., 1981). It also binds bilirubin (Jacobsen, 1977), steroids (Pearlman & Crepy, 1967; Unger, 1972), amino acids (Sollene et al., 1981), and many other ligands, usually with hydrophobic moieties (Kragh-Hansen, 1981; Peters, 1985). This unique property enables HSA to fulfil a fundamental biological role as a universal carrier and reservoir in blood plasma, tissues, and secretions throughtout the mammalian body (He & Carter, 1992).

As it follows from the listed above data, AFP and HSA can interact with the same ligands; that is why AFP has been often regarded as a fetal-type albumin. There is, however, a difference between these two proteins in their binding properties—the number of different ligands transported by HSA is much larger than that of AFP. Additionally, albumin's affinity for many organic ligands is in the range of $\log K_a = 4-6$, while for AFP the corresponding value is considerably larger ($\log K_a = 6-8$) (Deutsch, 1991).

† This work was supported in part by grants from Human Frontier Scientific Foundation Program (RG-331/93), RFBR-INTAS (95-IN/RU-1278), and RFFI (97-04-48044).

* To whom correspondence should be addressed at Institute of Protein Research, Russian Academy of Sciences, 142292 Pushchino, Moscow Region, Russia. Phone/Fax: +7 (095) 924-04-93. E-mail: uversky@sun.ipr.serpukhov.su.

‡ Institute of Protein Research.

§ Institute of Biochemistry and Physiology of Microorganisms.

⊗ Abstract published in *Advance ACS Abstracts,* October 1, 1997.

[1] Abbreviations: AFP, α-fetoprotein; HSA, human serum albumin; CD, circular dichroism; UV, ultraviolet; PBS, phosphate-buffered saline; TFA, trifluoroacetic acid.

S0006-2960(97)00332-2 CCC: $14.00

It is necessary to emphasize that these proteins also show some structural similarity. Indeed, they have about 40% homology in their amino acid sequences and relatively close molecular dimensions (King & Spences, 1972; Brown, 1975; Morinaga et al., 1983). Their structures are stabilized by large number of disulfide bridges, 17 and 15 for HSA and AFP, respectively. At the same time, albumin has only one free sulfhydryl group, while AFP has two; α-fetoprotein, being a glycoprotein, has considerable heterogeneity in the degree of glycosylation, depending on the source of the protein (Morinaga et al., 1983; Deutsch, 1991), while HSA has no carbohydrates (Peters, 1985). The X-ray structure of HSA, solved at 2.8 Å resolution, shows that this protein consists of three quasi-independent domains (He & Carter, 1992).

Considerable structural and functional similarity as well as overlapping lists of natural ligands carried by HSA and AFP show that comparative studies of these two proteins are not meaningless. As for structural properties of HSA, they are relatively well studied (Peters, 1985). In particular, the possibility for this protein to be transformed into a number of different forms (probably with different spatial orientations of its domains) upon pH changes has been discussed (Foster, 1977; Peters, 1985; Oliviery & Craievich, 1995). Intrestingly, this large protein shows a high degree of reversibility in numerous unfolding−refolding studies (Wallewik, 1973; Peters, 1985).

Although it is obvious that deep insight into the molecular mechanism of AFP function cannot be achieved without detailed investigation of structural properties of the protein, this is still a poorly studied field. In particular, even a brief overview of AFP physiological activities allows one to conclude that the environment of this protein varies considerably with its function, but the only example of investigation of the environmental effect on AFP structural properties is our recent work (Uversky et al., 1995), which shows formation of the molten globule state driven by a decrease in pH. It has been established that AFP possesses considerable microheterogeneity, associated with dissimilar character of glycosylation or ligand contents (Purves et al., 1970; Alpert et al., 1973; Smith & Kelleher, 1980) of the protein molecules, but virtually nothing is known about the effect of prosthetic groups (such as hydrophobic ligands) on AFP structure and stability.

In the present paper we compare the effect of ligands on structural properties and conformational stability of AFP with that of its structural and functional homologue, serum albumin. We show that *any kind of denaturing action leads to irreversible destruction of AFP's unique tertiary structure*, caused by the spontaneous irreversible release of ligands from the protein molecule. Special procedures are required for the removal of ligands from HSA. Formation of a ligand-free form transforms the AFP molecule into a compact denatured intermediate with pronounced secondary structure, i.e., into the molten globule state. For HSA, ligands play only a stabilizing role but do not determine protein conformation.

MATERIALS AND METHODS

Chemicals. Sepharose 4B, epichlorohydrin, iminodiacetic acid, borate hydride, and Cibacron Blue 3Ga used for preparation of Cibacron Blue−Sepharose and metal-chelating Sepharose were from Sigma. Triethylamine, trifluoroacetic acid, and acetonitrile were also from Sigma. Na_2HPO_4, NaH_2PO_4, NaCl, NH_4Cl, $NiCl_2$, and $CuCl_2$ were of analytical or extra-pure grade and were used without additional purification. Ultrapure urea was from BRL. Human serum albumin was from Reanal and was used without additional purification. Electrophoresis reagents were purchased from Bio-Rad.

Bidistilled water was used in the experiments. Desirable pH values were adjusted by the addition of 0.5−1.0 N NaOH or HCl. In the course of structural investigations all solutions contained 0.15 M NaCl.

AFP Purification. To obtain homogeneous AFP samples suitable for structural investigations, a special scheme for a large-scale α-fetoprotein isolation from human cord serum was elaborated. The peculiarities of this purification procedure will soon be published (Tomashevski & Uversky, 1997). We are presenting here a short protocol of the AFP purification. The isolation procedure includes three chromatographic steps, on affinity (Cibacron Blue−Sepharose), metal-chelating (*$NiCl_2$*− and *$CuCl_2$*−Sepharose), and reverse-phase (C-3) columns. At the first stage, human cord serum was centifuged at 40000*g* for 30 min. The supernatant was collected, filtered, and passed through a Cibacron Blue−Sepharose 4B column preequilibrated with phosphate buffer (20 mM potassium phosphate buffer, pH 7.2). At these conditions AFP did not interact with column matrix; the protein eluted within column void volume. The AFP-containing fractions were pooled and passed through a metal-chelating Sepharose 4B column saturated with $NiCl_2$. This column was preequilibrated with PBS (20 mM potassium phosphate and 1.5 M NaCl, pH 7.2) and elution was carried out in the same buffer. There was also no AFP binding to the column. Fractions with AFP (detected by rocket immunophoresis) were cocentrated and applied to a metal-chelating Sepharose 4B column, saturated with $CuCl_2$. Proteins was eluted in PBS (see above) with a linear gradient of NH_4Cl (0−2 M). AFP-containing fractions were pooled and concentrated by $(NH_4)_2SO_4$ precipitation (440 g/L). The precipitate was dissolved in a small amount of PBS containing 1 mM triethylamine and 0.1% TFA. This sample was passed through a reverse-phase C-3 column equilibrated with 1 mM triethylamine/0.1% TFA buffer, pH 2.0. AFP was eluted in the same buffer with a linear gradient of acetonitrile (0−55%). Fractions containing AFP were pooled, concentrated, and passed through a PD-10 column with Sephadex G-25 to substitute buffer with acetonitrile on PBS. As a result, homogeneous AFP samples were obtained: SDS and native polyacrylamide gel electrophoresis, reverse-phase chromatography and the ELISA test with antibodies against human serum albumin, α-antitrypsin, and transferrin showed no less than 98% purity of the isolated protein. Gas chromatography analysis allowed us to conclude that the purified AFP contains at least part of its natural ligands. Indeed, the chromatographic profile showed the existence of two nonprotein peaks, major and minor (data not shown). The major peak was attributed to arachidonic acid, while the minor peak (with intensity $\sim^1/_5$ that of the major one) was due to docosahexaenoic acid. Any other natural ligands were not detected in the purified AFP samples.

Release of Hydrophobic Ligands from AFP and HSA. A standard procedure for the release of hydrophobic ligands

from the protein molecules was used (Chen, 1967; Sogami & Foster, 1968). To this end, protein solution at pH 6.5 was mixed with charcoal. The mixture was kept stirring for 12 h at room temperature, then centrifuged, and passed through a small gel-filtration column (G-75) equilibrated with 100 mM sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl. The completeness of ligand release was monitored by gas chromatography. A chromatographic profile obtained for AFP after the above procedure contains only the peak corresponding to protein molecules.

Add-Back Experiments with the Stripped Form of AFP. Arachidonic acid was dissolved in ethanol. A small amount of this ethanol solution was mixed with a large excess of aqueous solution of ligand-free AFP (to have a final ethanol concentration less than 1%). The AFP/arachidonic acid mixture (molar ratio 1/1) was kept stirring at room temperature for several hours. The effectiveness of resaturation of stripped AFP by fatty acid was monitored using gas chromatography and scanning microcalorimetry.

Experimental Procedures. Protein concentrations were measured by the absorbance at 280 nm on a Cary-219 spectrophotometer (Varian). The values for extinction coefficients ($\epsilon^{1mg/mL}_{1cm,280nm}$) were estimated by the nitrogen determination method (Jaenike, 1974) and were equal to 0.365 and 0.455 for AFP and HSA, respectively. For circular dichroism and calorimetric measurements protein concentration was 0.5−1.0 mg/mL, while fluorescence investigations were performed using 0.01 mg/mL protein solutions.

Urea concentrations were measured by the refractive index. Unfolding and refolding transitions were monitored by the characteristic shift of the intrinsic tryptophan fluorescence, using an Aminco SPF-100cs corrected spectrofluorometer (American Instrument Co.) with a 10.0 mm cell. To investigate urea-induced unfolding, AFP or HSA was initially dissolved in buffer A to a final protein concentration of about 1 mg/mL. Then 10 μL of this stock (stock 1) was mixed with 990 μL of the same buffer, containing urea in the desired concentration. Solutions were incubated at 23 °C for 24 h before measurements. The same procedure was used in the refolding experiments, but protein solutions with 9.5 M urea (stock 2) were used as the initial sample. The stock for the repetition unfolding scans was obtained by dilution of stock 2 to a final urea concentration of about 0.2−0.4 M.

Circular dichroism spectra were obtained on a Jasco-600 spectropolarimeter (Japan Spectroscopic Co.) equipped with a temperature-controlled holder. Cells with 0.148 and 10 mm path lengths were used for far- and near-UV measurements, respectively. Temperature-induced conformational transitions were monitored by changes in ellipticity at 220 and 265 nm. The rate of heating was 0.9 °C/min.

Calorimetric measurements were carried out by a precision scanning microcalorimeter DASM-5M (Bureau of Biological Instrumentation) 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. The excess heat capacity of AFP and HSA in solutions was determined as described earlier (Privalov & Potekhin, 1979). The protein partition volume of 0.72 cm^3/g was used for calculations (Uversky et al., 1995).

Reverse-phase chromatography was carried out on a C-3 column (Beckman) connected to an HPLC system (LKB-Pharmacia).

RESULTS AND DISCUSSION

As was already mentioned, α-fetoprotein and serum albumin share structural homology. Most of all, both of them participate in transport of hydrophobic ligands; that is, they belong to the class of carrier proteins. To understand the role of hydrophobic ligands in stabilization of protein structure, the structural properties and conformational stabilities of AFP and HSA in the presence and absence of ligands were studied.

Structural Properties and Conformational Stabilities of AFP and HSA in the Presence of Their Natural Ligands

Heat-Induced Denaturation. Protein conformational changes, induced by the increase of temperature, were investigated using several structural methods. Disruption of tertiary structure was studied by scanning microcalorimetry and circular dichroism (CD) in the near-UV region, while far-UV CD spectra were used to follow the changes in the protein secondary structure.

Figure 1 (solid lines) shows typical excess heat capacity curves for ligand-containing forms of AFP and HSA at pH 7.2. The curve for AFP has a single heat absorption peak with maximum at ∼70 °C (Figure 1A). The excess heat capacity curve for HSA has more than one peak, as shown in Figure 1B. The presence of heat absorption peak(s) in a calorimetric curve is usually considered as evidence for the cooperative disruption of rigid tertiary structure of a protein molecule (Privalov, 1979; Ptitsyn, 1995). This means that in the presence of their natural ligands both proteins have unique tertiary structure, which is cooperatively destroyed by heating. It should be also emphasized that *thermal denaturation of HSA is a reversible process, while the disruption of AFP's tertiary structure is completely irreversible.* This was confirmed by reheating of protein solutions in the calorimetric cell just after cooling after the first run (see Figure 1, dashed lines).

The existence of more than one peak in a calorimetric curve reflects the domain denaturation mechanism (Privalov, 1982). Thus, the results for HSA melting (see Figure 1B) clearly show that this protein consists of at least two quasi-independent domains, rather than a single cooperative unit. This observation is in good agreement with data obtained earlier for bovine and human serum albumins (Privalov, 1982; Leybman et al., 1975).

The values of calorimetric (ΔH^{cal}) and van't Hoff (ΔH^{vH}) enthalpies for AFP and HSA were obtained directly from the calorimetric curves and are summarized in Table 1. One can see that calorimetric enthalpy of AFP is practically equal to the effective one, which indicates that temperature-induced denaturation of this protein is a two-state process (Privalov, 1979). The value of calorimetric enthalpy for HSA is typical of proteins of this molecular weight (Privalov & Potekhin, 1986), while the corresponding value for AFP is approximately 4 times lower. Such low ΔH^{cal} values cannot be explained by the partial denaturation of AFP in the initial samples. It is known that partially denatured protein molecules show poor resistance to proteolysis. We have established that the ligand-saturated form of AFP possesses high resistance toward proteolytic digestion (data will be published elsewhere).

The complementary lines of evidence of the temperature-induced disruption of AFP and HSA tertiary structure can

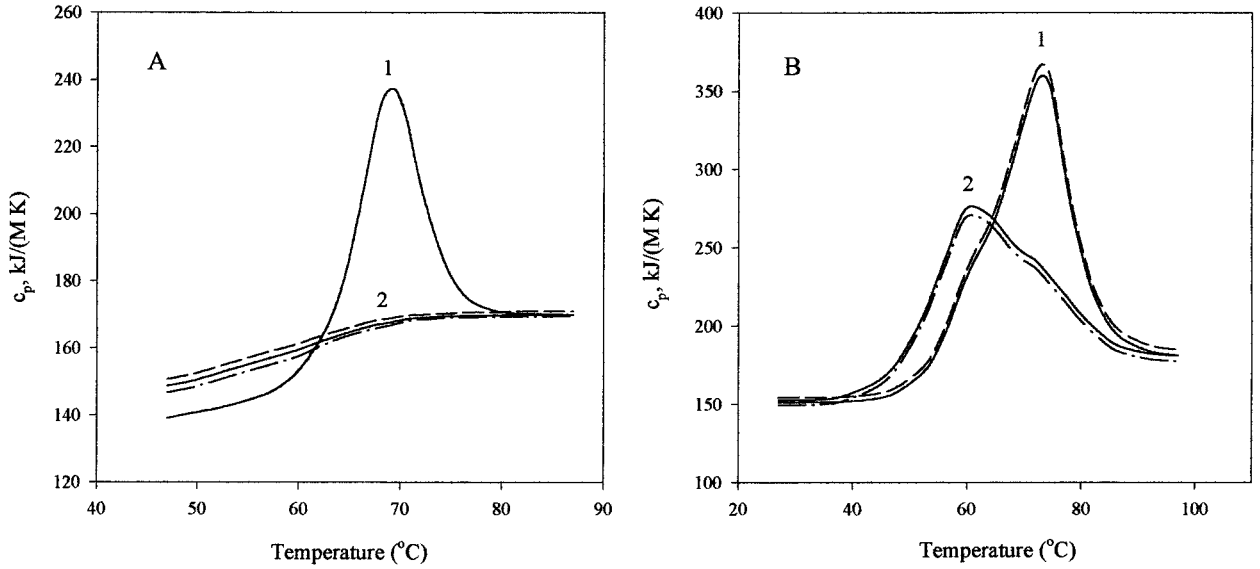

FIGURE 1: Temperature dependence of partial molar heat capacity of human α-fetoprotein (A) and serum albumin (B) at pH 7.2. Curve 1, calorimetric recordings of proteins in the presence of ligands; curve 2, melting curves of the defatted samples. Dashed lines present the results of repeated temperature scans for natural AFP and HSA, while dashed-and-dotted lines show results of the same experiments for the ligand-free proteins. All measurements were done in 100 mM sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl. Protein concentration was 1.0 mg/mL.

Table 1: Thermodynamic Parameters Obtained by Scanning Microcalorimetry of Human α-Fetoprotein and Serum Albumin[a]

protein	T_m (°C)	ΔH^{vH} (kJ K^{-1} M^{-1})	ΔH^{cal} (kJ K^{-1} M^{-1})	ratio $\Delta H^{vH}/\Delta H^{cal}$
AFP	69 ± 2	500 ± 20	480 ± 20	1.05 ± 0.08
HSA (natural form)	59 ± 2		410 ± 20	
	67 ± 2	1100 ± 50	310 ± 20	
	80 ± 3		340 ± 20	
HSA (defatted form)	59 ± 2		440 ± 20	
	64 ± 2	1260 ± 50	440 ± 20	
	72 ± 3		370 ± 20	

[a] Table represents results averaged over four independent experiments. Conditions were 100 mM sodium phosphate and 0.15 M NaCl, pH 7.2. Protein concentration was 1.0 mg/mL for all calorimetric measurements.

be obtained by following the changes of protein spectra in the near-UV CD region. Indeed, it is known that incorporation of aromatic residues into a rigid chiral environment gives rise to aromatic CD signals (Adler et al., 1973). The decrease of ellipticity in this region reflects reduction in ordered tertiary structure. Solid lines (1) in Figure 2 represent the near-UV CD spectra of ligand-saturated forms of AFP (Figure 2A) and HSA (Figure 2B) at 23 °C. Solid lines (3) refer to the proteins at 87 °C. The spectra for the proteins unfolded completely by 9.5 M urea are presented for comparison (see Figure 2, dotted lines). One can see that at room temperature the spectra of both proteins show the existence of asymmetry in the environment of aromatic amino acid residues. At 87 °C the spectra are much less pronounced and close to those obtained for randomly coiled AFP and HSA. Such considerable reduction in spectrum intensity reflects melting of the unique protein structure. The temperature-induced changes in ellipticity at 265 nm for both proteins are presented in the insets in Figure 2 (lines 1). The transition midpoints are at 69 and 66 °C for AFP and HSA, respectively. *The data obtained by CD measurements confirm reversibility of HSA denaturation and irreversibility of AFP denaturation.* Indeed, cooling of the HSA solution leads to complete restoration of the near-UV CD spectrum (see dashed curve in Figure 2B). Moreover, the inset in Figure 2B shows that in this case reheating leads to the same changes in ellipticity at 265 nm as those observed upon the first heating. On the contrary, the near-UV CD spectrum of AFP is not restored upon the decrease of temperature (see Figure 2A, dashed curve). The repetition temperature scan for AFP shows the absence of any cooperative changes in ellipticity at 265 nm (see Figure 2A, inset, dashed line).

Far-UV CD spectra of ligand-saturated forms of AFP and HSA are presented in Figure 3, panels A and B, respectively. Spectra were measured at 23 °C (solid lines (1), 87 °C (solid lines 3) and 23 °C just after cooling after the heating run (dashed lines). The spectra of both proteins in the presence of 9.5 M urea are also presented. It is seen that even at 87 °C proteins have pronounced CD spectra. It means that heating does not lead to the complete unfolding of both proteins. After cooling, *the far-UV CD spectrum of HSA is completely restored, whereas in the case of AFP it is slightly different from the initial one*. Insets in Figure 3 represent the melting curves for AFP and HSA, followed by the changes in ellipticity at 220 nm. The transition midpoints are at 69 and 66 °C for AFP and HSA, respectively. These values are in good correlation with calorimetric and near-UV CD data (see above). The *temperature course of* $[\Theta]_{220}$ *for HSA was completely reversible, while this was shown to be irreversible for AFP* (see Figure 3).

Urea-Induced Unfolding. It is known that solvated tryptophan residues have a fluorescence maximum at 350 nm. Embedding of Trp in the nonpolar core of globular proteins results in the characteristic blue shift of its fluorescence spectrum. This means that the position of the tryptophan fluorescence spectrum, being sensitive to the polarity of the environment, allows us to study protein unfolding (Stryer, 1968). Panels A and B of Figure 4 show the Trp fluorescence spectra of AFP and HSA in the presence and absence of 9.5 M urea. The spectra of renatured proteins are also presented (see dashed lines). One can see that the addition of urea results in the well-defined shift of the fluorescence spectral maximum to 350 nm. The decrease

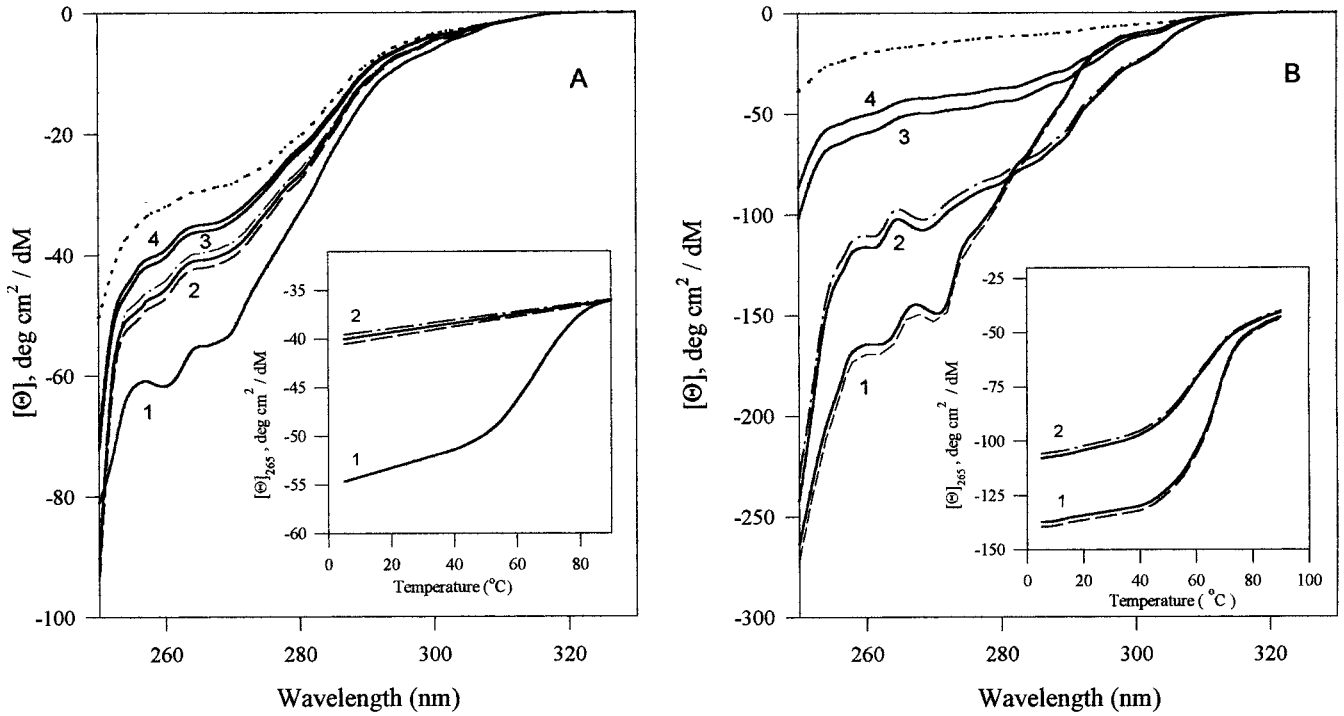

FIGURE 2: Near-UV CD spectra of AFP (A) and HSA (B). Curves 1 and 2, spectra of natural and ligand-free forms of proteins, respectively, measured at 23 °C; curves 3 and 4,- spectra of natural and ligand-free forms, respectively, measured at 87 °C. Reversibility of thermal denaturation was checked by recording of the spectra just after cooling of protein solutions from 90 to 23 °C. These results are depicted by dashed and dashed-and-dotted lines for natural and ligand-free forms, respectively. Spectra of the proteins completely unfolded by 9.5 M urea are given by dotted lines. Insets present the temperature-induced increase in $[\Theta]_{265}$, measured for natural proteins (1) and their ligand-unsaturated forms (2). Dashed and dashed-and-dotted lines represent the corresponding repeated temperature runs. All measurements were done in 100 mM sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl. Protein concentration was 1.0 mg/mL.

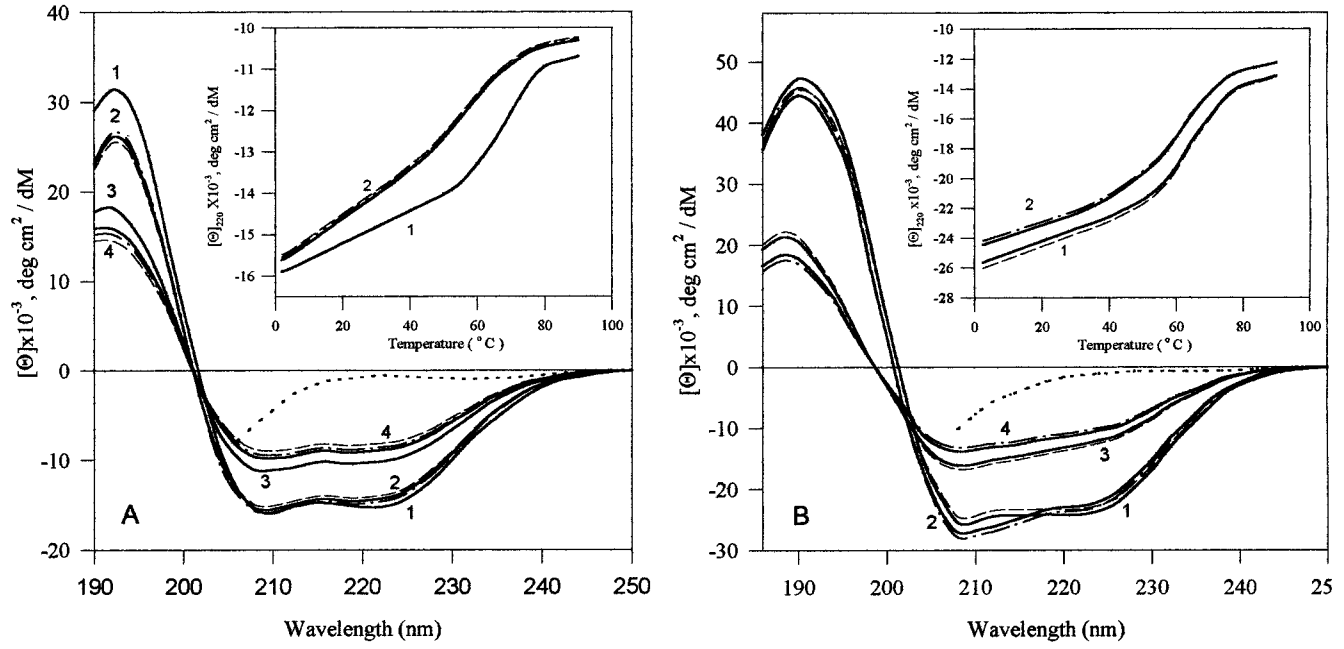

FIGURE 3: Far-UV CD spectra of AFP (A) and HSA (B). Curves 1 and 2, spectra of natural and ligand-free forms of proteins, respectively, measured at 23 °C; curves 3 and 4, spectra of natural and ligand-free forms, respectively, measured at 87 °C. Dashed and dashed-and-dotted lines represent the spectra measured just after cooling of protein solutions from 90 to 23 °C for ligand-saturated and ligand-free forms, respectively. Spectra of the proteins completely unfolded by 9.5 M urea are shown by dotted lines. Insets present the temperature-induced changes in $[\Theta]_{220}$, observed for the natural proteins (1) and their ligand-free forms (2). Results of the corresponding repeated temperature runs are given by dashed and dashed-and-dotted lines, respectively. All measurements were done in 100 mM sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl. Protein concentration was 0.5 mg/mL.

in urea concentration leads to practically complete restoration of the fluorescence spectra position. This allows us to suggest that both proteins are considerably unfolded in 9.5 M urea, while the removal of denaturant results in the restoration of their compact structure. As also follows from Figure 4, the urea-induced unfolding of both proteins is accompanied by the decrease of the fluorescence intensity, while renatured and native proteins are relatively close according to this parameter. It can be seen that the effect of intensity reduction is more pronounced in the case of AFP.

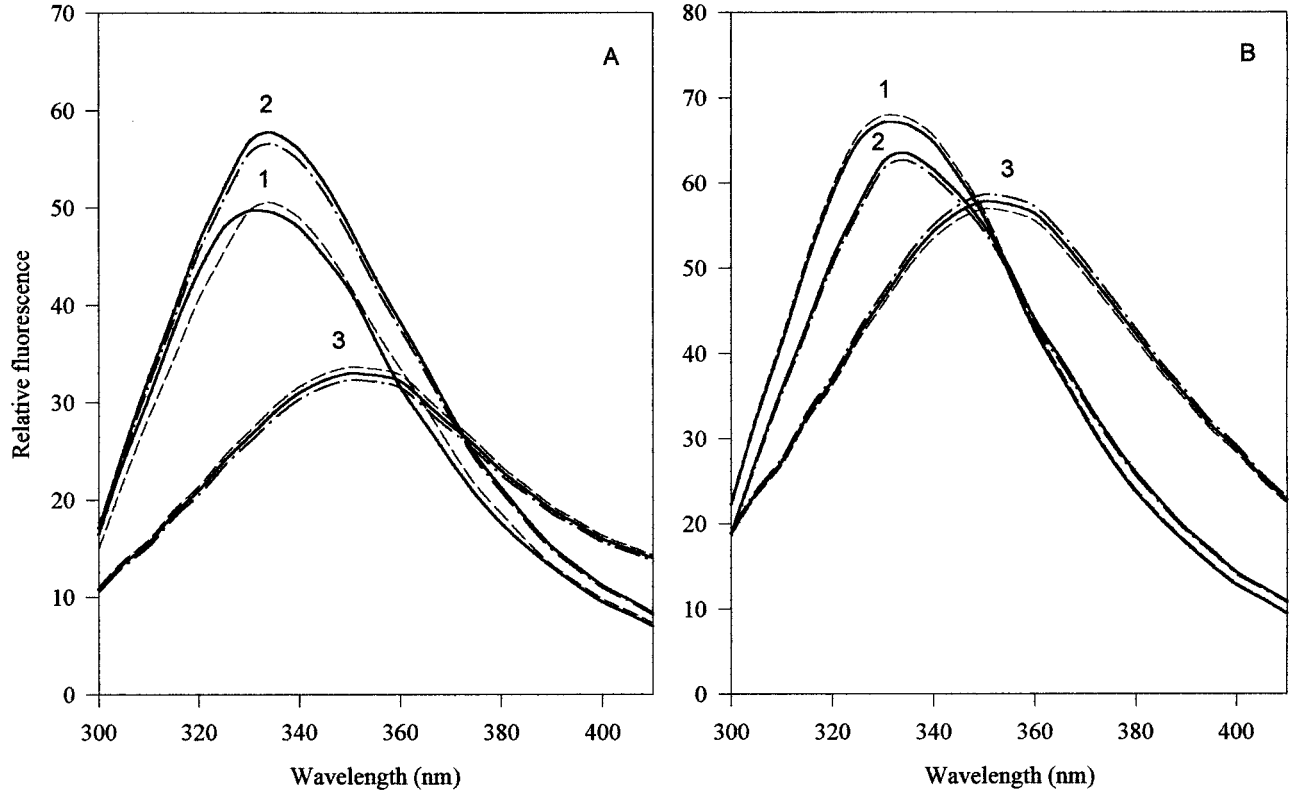

FIGURE 4: Tryptophan fluorescence spectra of AFP (A) and HSA (B) in natural (1), defatted (2), and completely unfolded by 9.5 M urea (3) states. Dashed (natural forms) and dashed-and-dotted (ligand-free forms) lines are attributed to the spectra of renatured proteins and the results of repeated unfolding. All measurements were done at 23 °C in 100 mM sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl. Protein concentration was 0.01 mg/mL.

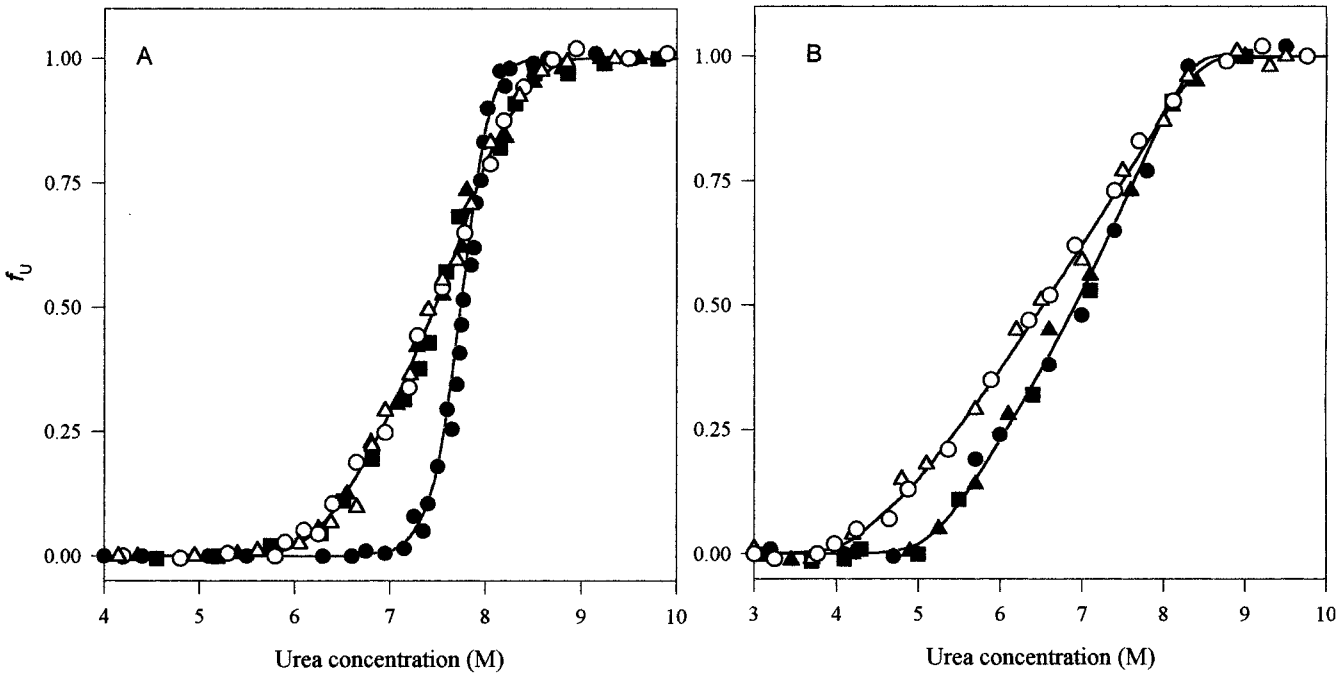

FIGURE 5: Equilibrium urea-induced unfolding (circles and triangles) and refolding (squares) of AFP (A) and HSA (B), monitored by the characteristic red shift of intrinsic Trp fluorescence. Solid symbols refer to the natural forms of proteins, while the open ones describe the unfolding of their ligand-free species obtained either by charcoal treatment (circles, see Materials and Methods) or by overnight dialysis of a protein solution against 9.5 M urea (triangles, see text). Fraction of unfolded molecules, f_U, was calculated from $f_U = (\theta_N - \theta)/(\theta_N - \theta_U)$, where θ is λ_{max}^{Trp} at given conditions, while θ_N and θ_U are the values of this parameter at given urea concentration extrapolated from baselines for native and unfolded molecules. All measurements were done at 23 °C in 100 mM sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl. Protein concentration was 0.01 mg/mL.

Figure 5 represents unfolding curves obtained for AFP and HSA in the presence of ligands (solid circles in panels A and B, respectively). Transitions were provoked by the increase of urea concentration and followed by the changes in the position of the Trp fluorescence spectral maximum. One can see that the transitions in both proteins are

cooperative. The midpoints are at 7.7 and 6.9 M urea for AFP and HSA, respectively. This means that both proteins show great stability toward the urea action. The reversibility of urea-induced unfolding was checked by equilibrium renaturation and repetition of the unfolding scan (see Materials and Methods). Results of these investigations are presented in Figure 5 as solid squares and triangles, respectively. The figure clearly shows that the structural changes in ligand-saturated form of HSA observed upon protein unfolding, refolding, and reunfolding (see Figure 5B, solid circles, squares, and triangles) can be described by one transition curve. This is not the case for AFP. Indeed, as follows from Figure 5A, data for this protein represent two, rather than one, unfolding curves. One of these curves describes the changes in AFP structure upon its unfolding (solid circles, Figure 5A), while the other is attributed to the refolding and repeated unfolding of AFP (solid squares and triangles, Figure 5A). This means that *urea-induced unfolding of HSA is a completely reversible process. The renatured form of AFP, being practically as compact as the natural state of this protein, shows lower conformational stability.*

Structural Properties and Conformational Stabilities of Ligand-Free Forms of AFP and HSA

The data presented above show that α-fetoprotein and serum albumin, having about 40% homology in their amino acid sequences, have quite different conformational stability. In particular, any kind of denaturing action on AFP (heating or increase of urea concentration) results in the irreversible destruction of its unique tertiary structure. As for HSA, its urea-induced unfolding and melting show high reversibility. To explain these results, let us assume that (1) rigidity of AFP tertiary structure is controlled by interactions with ligands, while for HSA these interactions are not so crucial, and (2) denaturation of AFP is accompanied by irreversible transformation of a protein molecule to the ligand-free form, while HSA preserves the possibility for ligand binding.

The second suggestion was partially confirmed by gas-chromatography experiments, which show that renatured AFP molecules almost do not contain their natural ligands. This means that AFP irreversibly loses ligands during denaturation. To have more complete information about the ligand effect on AFP and HSA, the structural properties and conformational stabilities of ligand-free forms of both proteins were studied. A standard procedure for the release of hydrophobic ligands from the protein molecules was employed (see Materials and Methods).

Results of these investigations are presented in Figures 1−5 by solid lines (2). It can be seen that the release of ligands leads to the appearance of much more considerable differences between two proteins studied.

Tertiary Structure of Ligand-Free Forms. Figure 1A shows that there is no visible heat absorption upon the increase of temperature in the solution containing AFP in the ligand-free form. It is in good agreement with the fact that the temperature scan, followed by changes in $[\Theta]_{265}$, shows the absence of any cooperative transition (Figure 2A, inset). Figure 2A demonstrates that the release of ligands leads to considerable change in the AFP near-UV CD spectrum: it is close to that of a protein fully unfolded by 9.5 M urea. Thus, we can conclude that the *AFP ligand-free form has no unique tertiary structure*.

It follows from Figures 1B and 2B that this is not the case for the defatted HSA. Indeed, the calorimetric curve in this case consists of at least three heat absorption peaks with the averaged maximum position at about 60 °C (Figure 2B). The shape of this calorimetric curve is quite different from that for HSA in the presence of ligands. This means that the release of ligands destabilizes the HSA molecule and leads to rearrangement (but not to disruption!) of its unique tertiary structure. This conclusion is also confirmed by near-UV CD measurements (see Figure 2B).

Secondary Structure and Compactness. Release of ligands from both proteins does not affect considerably either the shape of their far-UV CD Spectra (Figure 3) or the position of their Trp fluorescence maxima (Figure 4). This means that *in the ligand-free form the proteins are rather compact and have secondary structure which is close to that of the native state*. On the other hand, insets in Figure 4 clearly show that the stability of secondary structure decreases. It is interesting that temperature-induced changes in $[\Theta]_{220}$ of AFP ligand-unbounded form coincide with those monitored upon repetition of the heating scan of natural AFP (see Figure 4). This close similarity is also observed for the renatured and ligand-free forms of AFP in their far-UV CD and fluorescence spectra (Figures 3 and 4).

Urea-Induced Unfolding. Stability toward the urea-induced unfolding of α-fetoprotein and serum albumin ligand-free forms were compared with the data presented above. In these investigations two ligand-unbounded forms were studied. One of these forms was the result of charcoal treatment, described in Materials and Methods. The other one was obtained by overnight dialysis of a protein solution against 9.5 M urea with subsequent refolding by dilution to 0.1−0.2 M urea. Results of this study are presented in Figure 5 by open circles and triangles, respectively. It can be seen that unfolding of the AFP ligand-free forms follows the same urea dependence as was observed upon the refolding and repeated unfolding scan of the natural protein. The unfolding curve for ligand-unbounded species of HSA is quite different from that obtained for this protein in the presence of ligands.

Add-Back Experiments. Direct evidence on the irreversibility of ligand release from AFP was obtained from add-back experiments described in Materials and Methods. As was already mentioned, the effectiveness of resaturation of the ligand-free form of AFP by arachidonic acid was monitored using gas chromatography and scanning microcalorimetry. It was established that neither chromatographic nor calorimetric behavior of a stripped protein was changed after its coincubation with free fatty acid for 12 h (data not shown).

CONCLUSIONS

Results presented above allow us to conclude that two homologous proteins, human α-fetoprotein and serum albumin, show a large difference in their conformational stabilities. It has been established that this difference is attributed mainly to different effect of ligands on AFP and HSA structure. In the case of HSA, the protein molecule after the release of ligands, being destabilized and considerably structurally rearranged, preserves rigid tertiary structure. Quite another situation is observed for AFP. This protein in ligand-free form has no unique tertiary structure but retains nativelike secondary structure and compactness. This means

that the release of ligands results in the transformation of the AFP molecule into the molten globule state [see Uversky et al. (1997) for a more detailed investigation of structural properties of the ligand-free form of AFP]. We cannot exclude the possibility that such a difference between two homologous proteins can be associated with the difference in their functioning. Indeed, it is known that the most important activity (if not the only function) of HSA is nonspecific binding of a wide variety of organic and inorganic ligands and their trafficking (see introduction). As for AFP, its activities are more diverse (see introduction). We assume that the structural transformations in the AFP molecules induced by the release of ligands might be important for further functioning of this protein, e.g., as immunoregulator.

Another fact that should be pointed out here is the irreversibility of the ligand release from AFP and complete reversibility of this process for HSA. It was already noted that AFP is a glycoprotein, while HSA has no covalently attached carbohydrates. We assume that the glycosylation of AFP is responsible for the above irreversibility. Indeed, it is difficult to exclude that the site of ligand (arachidonic acid?) binding and site of the glycosyl chain attachment are relatively close in space. In this case the oligosaccharide component of AFP can screen the hydrophobic pocket of the ligand binding site and prevent normal penetration of ligand to this pocket.

We understand that our suggestions are rather courageous and do not follow directly from the presented results. We also realize that further investigations are required to clarify this point.

ACKNOWLEDGMENT

We express our deepest gratitude to Professors G. I. Abelev, A. L. Fink, A. V. Finkelstein, E. A. Nunez, S. A. Potekhin, and O. B. Ptitsyn for valuable discussions. We thank Dr. M. D. Kirkitadze for some preliminary calorimetric measurements. We also thank A. S. Karnoup for critically reading and editing the manuscript.

REFERENCES

Abelev, G. I. (1970) *Adv. Cancer Res. 14*, 295−358.
Adler, A. J., Greenfield, N. J., & Fasman, G. D. (1973) *Methods Enzymol. 27*, 675−735.
Alpert, E., Drysdale, J. W., & Isselbacher, K. J. (1973) *Ann. N.Y. Acad. Sci. 209*, 387−391.
Aoyagi, Y., Ikenaka, T., & Ichida, F. (1978) *Cancer Res. 38*, 3483−3486.
Bradshaw, R. A., Shearer, W. T., & Gurd, F. R. N. (1968) *J. Biol. Chem. 243*, 3817−3825.
Brock, D. J., Scrimgeour, J. B., & Nelson, M. M. (1975) *Clin. Genet. 7*, 163−169.
Brown, J. R. (1975) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34*, 591.
Calwell, J. L., Severson, C. D., & Thompson, J. S. (1973) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32*, 979.
Chen, R. F. (1967) *J. Biol. Chem. 242*, 173−181.
Foster, J. F. (1977) in *Albumin Structure, Function and Uses* (Rosenoer, V. M., Oratz, M., & Rothshild, M. A., Eds.) pp 53−84. Pergamon Press, Oxford, England.
Gurd, F. R. N., & Wilcox, P. E. (1956) *Adv. Protein Chem. 11*, 311−427.
He, X. M., & Carter, D. C. (1992) *Nature 358*, 209−211.
Hsia, J. C., Er, J. S., Tan, C. T., Ester, T., & Ruoslahti, E. (1980) *J. Biol. Chem. 255*, 4224−4227.
Jacobsen, J. (1977) *Int. J. Pept. Protein Res. 9*, 235−239.
Jaenike, L. (1974) *Anal. Biochem. 61*, 623−627.
King, T. P., & Spencer, E. M. (1972) *Arch. Biochem. Bioophys. 153*, 627−640.
Klopfenstein, W. F. (1969) *Biochim. Biophys. Acta 187*, 272−274.
Kragh-Hansen, U. (1981) *Pharmacol. Rev. 33*, 17−52.
Lange, P. H. (1983) in *Onco-developmental markers: Biological, diagnosis and monitoring aspects* (Fishman, W. H., Ed.) pp 241−255, Academic Press, New York.
Leybman, D. Ya., Tiktopulo, E. I., & Privalov, P. L. (1975) *Biophysics (Moscow) 20*, 376−379.
Lu, C. Y., Changelian, P. S., & Unanue, E. R. (1984) *J. Immunol. 132*, 1722−1727.
Morinaga, T., Sakai, M., Wegmann, G., & Tamaoki, T. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4604−4608.
Nagai, M., Becker, J. D., & Deutsch, H. F. (1982) *Oncodev. Biol. Med. 3*, 343−350.
Nandedkar, A. K., Nurse, C. E., & Friedbreg, F. (1973) *Int. J. Pept. Protein Res. 5*, 279−281.
Nishi, S., Matsue, H., Yoshida, H., Yamamoto, R., & Sakai, M. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 3102−3105.
Oliviery, J. R., & Craievich, A. F. (1995) *Eur. Biophys. J. 24*, 77−84.
Parmelee, D. C., Evenson, M. A., & Deutsch, H. F. (1978) *J. Biol. Chem. 253*, 2114−2119.
Pearlman, W. F., & Crepy, O. (1967) *J. Biol. Chem. 242*, 182−189.
Pedersen, K. (1978) *Scand. J. Clin. Lab. Invest. 38*, 659−667.
Peters, T., Jr. (1985) *Adv. Protein Chem. 37*, 161−245.
Privalov, P. L. (1979) *Adv. Protein Chem. 33*, 167−244.
Privalov, P. L. (1982) *Adv. Protein Chem. 35*, 1−104.
Privalov, P. L., & Potekhin, S. A. (1986) *Methods Enzymol. 131*, 4−51.
Ptitsyn, O. B. (1995) *Adv. Protein Chem. 47*, 83−229.
Purves, L. R., Vender, M., & Bersohn, I. (1970) *Lancet 2*, 464−465.
Purves, L. R., Bersohn, I., & Geddes, E. W. (1978) *Cancer 25*, 1261−1270.
Roda, A., Cappelleri, G., Clute, O. L., & Peters, T., Jr. (1982) *J. Lipid Res. 23*, 490−495.
Ruoslahti, E., & Seppala, M. (1979) *Adv. Cancer Res. 29*, 275−346.
Ruoslahti, E., Ester, T., & Seppala, M. (1979) *Biochim. Biophys. Acta 578*, 511−519.
Sarkar, B. (1983) *Life Chem. Rep. 1*, 165−207.
Savu, L., Benassasyag, C., Vallette, G., Christeff N., & Nuney, E. (1981) *J. Biol. Chem. 256*, 9414−9418.
Seppala, M. (1975) *Ann. N.Y. Acad. Sci. 259*, 59−73.
Smith, J. P., & Kelleher, P. C. (1980) *Biochim. Biophys. Acta 605*, 1−32.
Smuckler, E. A., Koplitz, M., & Sell, S. (1976) *Cancer Res. 36*, 4558−4561.
Sogami, M., & Foster, J. F. (1968) *Biochemistry 7*, 2172−2182.
Sollene, N. P., Wu, H.-L., & Means, G. E. (1981) *Arch. Biochem. Biophys. 207*, 264−269.
Soloff, M. S., Swartz, S. K., Pearlmutter, F., & Kithier, K. (1972). *Biochim. Biophys. Acta 427*, 644−651.
Spector, A. A., & Fletcher, J. E. (1978) in *Disturbance in Lipid and Lipoprotein Metabolism*, pp 229−248, American Physiology Society, Rockville, MD.
Stryer, L. (1968) *Science 162*, 526−540.
Tomashevski, A. Yu., & Uversky, V. N. (1997) *Russ. J. Bioorg. Chem. 23*, in press.
Torres, J. M., Laborda, J., Naval, J., Darracq, N., Calvo, M., Mishal, Z., & Uriel, J. (1989) *Mol. Immunol. 26*, 851−857.
Unger, W. G. (1972) *J. Pharm. Pharmacol. 24*, 470−477.
Uriel, J., de Nechaud, B., & Dupiers, M. (1972) *Biochem. Biophys. Res. Commun. 46*, 1175−1180.
Uversky, V. N., Kirkitadze, M. D., Narizhneva, N. V., Potekhin, S. A., & Tomashevski, A. Yu. (1995) *FEBS Lett. 364*, 165−167.
Uversky, V. N., Narizhneva, N. V., Ivanova, T. V., Kirkitadze, M. D., & Tomashevski, A. Yu. (1997) *FEBS Lett. 410*, 280−284.
Wallevik, K. (1973) *J. Biol. Chem. 248*, 2650−2655.

BI970332P