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Rapid report

Evidence for the molten globule state of human apo-ceruloplasmin

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Abstract

The conformational features of copper-free ceruloplasmin (CP), as compared to the holo-protein, were evaluated utilizing far- and near-UV circular dichroism and fluorescence spectroscopy. The results obtained indicate that apo-CP maintains the secondary structure of the holo-protein, while the tertiary interactions are much weaker. In addition, the removal of copper from the holo-protein leads to the exposure of hydrophobic patches to solvent, as shown by the fact that apo-CP, at variance from the holo-protein, binds the hydrophobic probe ANS. It is proposed that the CP molecule, upon copper removal, acquires the conformational features typical of a molten globule, which might be the conformational state of CP during its biosynthesis before metal incorporation.

Keywords: Ceruloplasmin; Molten globule; Circular dichroism; Fluorescence; Second derivative spectroscopy

Human serum ceruloplasmin (CP) is a single-chain copper-binding glycoprotein (ferro-O_2-oxidoreductase, EC 1.16.3.1) consisting of 1046 amino acids [1] and containing six copper ions [2]. The crystallographic structure solved at 3.1 Å resolution demonstrates that human CP contains six domains arranged in a triangular array, each domain comprising a β-barrel [2]. Of the six copper ions bound, three occupy mononuclear centers in domains 2, 4 and 6, whereas the remaining three form a trinuclear cluster at the interface between domains 1 and 6. The mononuclear copper ions have typical 'type I' environment [3], with two histidines, a cysteine and a methionine as ligands, except for the copper in domain 2 where the methionine is lacking. In the case of domain 6, the main-chain carbonyl oxygen of Leu-974 appears to point towards the copper ion. The trinuclear cluster is constituted by two 'type III' copper binding sites, where each metal ion is coordinated by three histidines, and by a 'type II' site, where the copper is surrounded by two histidines. Moreover, as evidenced by the 3D structure and amino-acid sequence of CP [1], several Tyr- and Trp-residues are in close vicinity to the copper-binding sites. The analysis of the 3D structure of human CP reveals that the putative active site involves three copper ions in the trinuclear cluster and another copper ion in a 'type I' binding site [2,4].

It has been proposed that human CP is involved in several important biological functions, including copper transport, ferroxidase and amine oxidase activity (see Ref. [2], for citations). Furthermore, mutations in CP gene are related to systemic haemosiderosis [5] and, in particular, to the pathogenesis of Wilson's disease in humans (hepatolenticular degeneration), a severe genetic disease that leads to the accumulation of copper in the liver and brain [5]. The mechanism of copper incorporation in the course of CP biosynthesis in hepatocytes is poorly understood, especially regarding the cellular organelle(s) where metal binding occurs and the particular conformational state of the protein molecule before metal binding [6]. The molecular details of this mechanism are of critical importance for an understanding of the actual physiological role of CP and its possible involvement in the pathogenesis of Wilson's disease.

In previous studies, copper depletion has been used to address the role of copper ions in the enzymatic activity and stability of CP [7–13]. In this respect, apo-CP has

Abbreviations: CP, ceruloplasmin; apo-CP, copper-free ceruloplasmin; CD, circular dichroism; UV, ultraviolet; ANS, 8-anilino-1-naphthalenesulfonic acid; Gdn-HCl, guanidine hydrochloride.

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some peculiar features as compared to the holo-protein, such as lower sedimentation coefficient [14], higher electrophoretic mobility [15] and slightly larger hydrodynamic radius [16]. Moreover, apo-CP exhibits new antigenic determinants with respect to the holo-protein, but is strongly recognized also by the antibodies raised against the holo-enzyme [17]. Overall, these data indicate that removal of copper leads to a new conformational state of the CP molecule [14,16].

In this work we show that apo-CP acquires conformational features typical of the molten globule state of proteins (see Ref. [18], for a recent review), being characterized by significant secondary structure content, much weaker tertiary interactions and an enhanced exposure of hydrophobic groups to solvent. The possible biological implications of these results are also discussed.

Human CP (95–96% pure, $A_{610}/A_{280} = 0.046$) obtained from the Pasteur Institute of the Russian Ministry of Health Care (Saint-Petersburg) was used in our study. To prepare apo-CP, 0.1 mM (13.2 mg/ml) CP was dialyzed for 2 h at 20°C against 20 mM ascorbic acid in 0.1 M Tris-HCl (pH 7.0). Subsequently, 0.25 mM KCN and 10 mM EDTA were added. The dialysis proceeded for another 12 h, after which the protein sample was transferred into 0.1 M Tris-HCl buffer (pH 7.0). After two changes of the buffer within 6 h the copper content was measured using a Perkin Elmer (Norwalk, CT, USA) model 4000 atomic absorption spectrometer.

The protein concentration was determined by UV absorption measurements at 280 nm on a Perkin-Elmer double beam model Lambda-2 spectrophotometer. Absorption coefficients of holo- and apo-CP at 280 nm were calculated according to Gill and Von Hippel [19] and both taken as 1.61 mg^{-1} cm^2.

CD spectra were recorded on a Jasco (Tokyo, Japan) model J-710 spectropolarimeter equipped with a thermostatted cell-holder connected to a NesLab (Newington, NH) model RTE-110 water circulating bath. The instrument was calibrated with *d*-(+)10-camphorsulfonic acid. Far- and near-UV CD spectra were recorded at 25°C at a protein concentration ranging from 5 to 50 μM, using 0.1 or 1 cm pathlength quartz cells in the far- and near-UV region, respectively. The results were expressed as mean residue ellipticity $[\theta]_{MRW} = (\theta_{obs}/10) \cdot (MRW/l \cdot c)$, where θ_{obs} is the observed ellipticity at a given wavelength, MRW is the mean residue weight of holo- and apo-CP taken as 126.2 Da, l is the cuvette path length in cm, and c is the protein concentration expressed as g/ml. All CD spectra resulted from averaging four scans and the final spectrum was corrected by subtracting the baseline spectrum taken under identical conditions.

Fluorescence emission spectra were recorded at 25°C on a Perkin-Elmer spectrofluorimeter model LS-50B, exciting protein samples (2 μM) at 280 nm and recording the emission fluorescence in the wavelength range 285–500 nm. The binding of the ANS to holo- and apo-CP was measured by adding 50 μl of ANS stock solution (100 μM) to 450 μl of protein dissolved (2 μM) in the specified buffer (ANS/protein molar ratio $\approx$ 5:1). After 2 h incubation, samples were excited at 390 nm and the emission fluorescence recorded in the wavelength range 400–700 nm. Emission spectra were corrected for the base line spectra recorded under identical conditions. The concentration of ANS was determined spectrophotometrically using an extinction coefficient of 5000 cm^{-1} M^{-1} at 350 nm [20].

The second-derivative ultraviolet absorption spectra of holo- and apo-CP (80–100 μg) were taken after 1 h equilibration at 25°C in the proper buffer (300 μl). The average exposure of tyrosine residues to solvent (α) was calculated according to Ragone et al. [21]. The error on the determination of tyrosine solvent exposure was determined in different experiments on hen egg white lysozyme (Sigma) at pH 7.5 and found as low as 2%.

Treatment of ascorbate-reduced CP with KCN and EDTA allows to obtain a sample of apo-CP containing less than 1% of copper still bound [13]. This protein prepartion was used for the spectroscopic studies herein reported.

Far-UV CD spectra of holo- and apo-ceruloplasmin (Fig. 1A) share common features with a minimum centered at 219 nm and a maximum at about 200 nm, characteristic of a protein containing predominantly β-like secondary structure [22]. This is in agreement with previous CD studies conducted on holo-CP [23] and with the low-resolution crystallographic structure of CP [2], showing that each of the six domains in CP comprises β-barrel typically organized as in azurin and plastocyanin [24]. The far-UV CD spectra of holo- and apo-CP reported in Fig. 1A suggest that the secondary structure content of the native CP is not dramatically changed upon copper removal. However, the spectrum of apo-CP displays significant differences with respect to that of the holo-form in the 200 nm region. These differences could reflect conformational changes occurring in the CP molecule upon copper depletion, such as partial unfolding of the β-structure and/or a variation of the twisting angle between the β-strands [25] that form the six domains of CP. Moreover, the possibly different contribution of aromatic residues [26] to the far-UV CD spectra of holo- and apo-CP (96 Tyr, 70 Phe and 25 Trp) should also be taken into account. These contributions arise from the coupling between electronic transitions of aromatic moieties with those involving other groups possessing π-orbitals, such as side-chain amide groups or peptide bonds [27]. The intensities of these bands depends on the intrinsic rotational strength of the functional groups involved, as well as on the distance and orientation between interacting groups [26,27]. The far-UV CD spectra of model dipeptides containing Phe or Tyr show a prominent positive band in the 200 nm region with ellipticity values of about 30000 and 40000 deg cm^2 $dmol^{-1}$ for Phe and Tyr, respectively, while the spectrum of the Trp-model peptide shows a broad negative absorp-

tion centered at 200 nm ($[\theta]_{200\ nm} = -12000$ deg cm^2 dmol^{-1}) [22]. These contributions are more significant for proteins displaying a poor CD signal in the far-UV region, such as that often observed for β-proteins, and in most cases they prevent a correct estimation of the secondary structure content from CD data [27].

The near-UV CD spectrum of holo-CP (Fig. 1B) shows a predominant negative signal at ≈ 285 nm, assigned to Tyr- and Trp-absorption, and a fine structure in the 260–270 nm range, due to the contribution of Phe-residues [28]. The spectrum of apo-CP is characterized by a substantial decrease (~ 50%) of the CD signal in the 280 nm region and by the appearance of a distinct fine structure at 296 and 288 nm, assigned to the vibronic contribution of Trp-residue(s) [28]. The appearance of this band may result

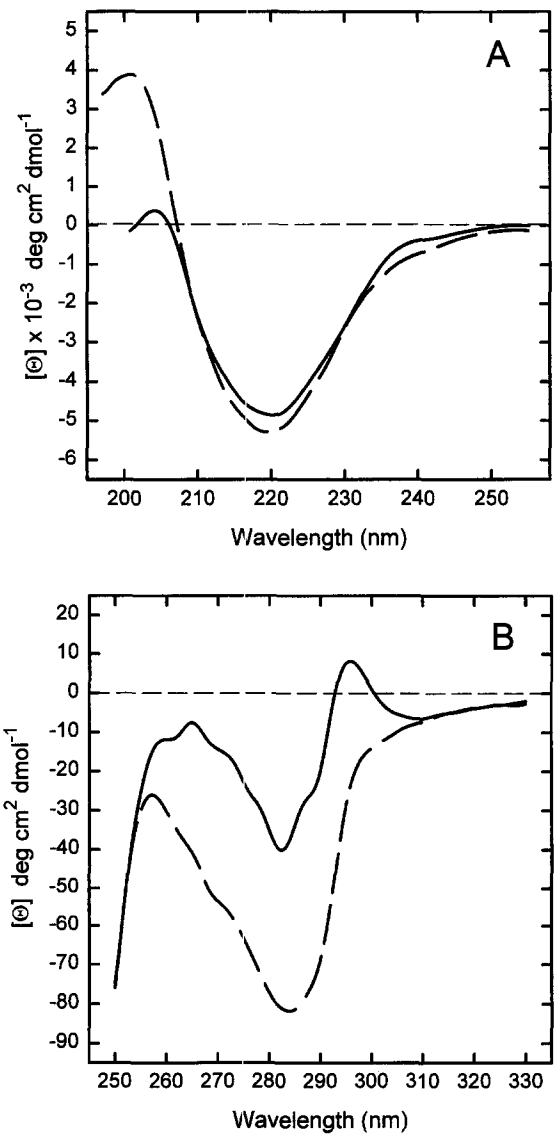

Fig. 1. Far- (A) and near-UV (B) CD spectra of holo- (---) and apo-CP (————). The spectra were recorded at 25°C in 10 mM Tris-HCl/0.1 M NaCl (pH 7.0). Protein concentration was 2 and 8 μM in the far- and near-UV region, respectively.

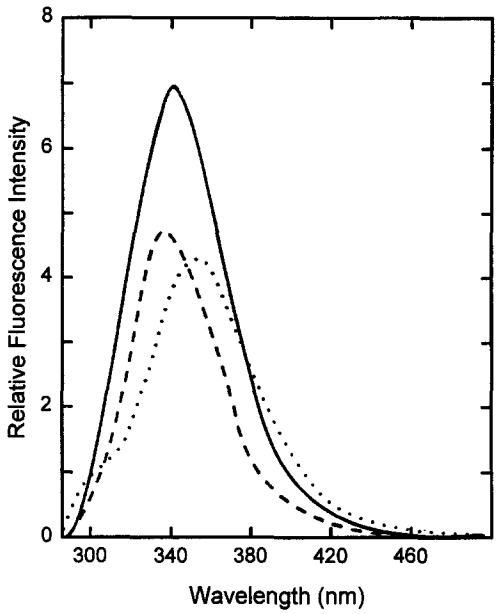

Fig. 2. Fluorescence emission spectra of holo- (---) and apo-CP (————) (2 μM) taken at 25°C, after excitation at 280 nm in 10 mM Tris-HCl/0.1 M NaCl (pH 7.0), and apo-CP (2 μM) in the presence of 6 M Gdn-HCl (· · ·).

from the reduced negative absorption of some Tyr- and Trp-residues that, in the partly folded state of apo-CP, become more flexible and thus, contribute less to the near-UV CD signal. Overall, the CD spectra of holo- and apo-CP, shown in Fig. 1B, suggest that removal of copper ions induces in apo-CP a looser and more flexible environment nearby aromatic residues. On the other hand, the presence of fine structure in the near-UV CD spectrum of apo-CP suggests that significant tertiary interactions do exist even in the copper-free CP molecule.

The fluorescence spectrum of apo-CP shows a 5 nm red-shift of λ_{max} (from 335 to 340 nm) and a substantial increase of the emission intensity with respect to the holo-protein (Fig. 2). This latter feature can be explained considering that, in general, bound metal ions can effectively quench the fluorescence of both Tyr- and Trp-residues located in the vicinity of the metal binding-sites [29]. In the case of CP, upon copper depletion, this quenching effect is abolished to a great extent, with a consequent enhancement of the fluorescence quantum yield. The red-shift of λ_{max}, observed in apo-CP, is in line with the results of near-UV CD measurements (Fig. 1B). In fact, in the more relaxed structure of the copper-free CP molecule, some Trp-residues may become more exposed to the polar solvent, shifting the λ_{max} value to longer wavelengths [29]. On the other hand, the presence of an efficient Tyr-to-Trp energy transfer, observed in the 280 nm spectrum of apo-CP, is taken as an indication that the copper free protein still retains significant amount of residual tertiary structure [29], at variance from the fully unfolded protein in 6 M Gdn-HCl, where a distinct tyrosine band at ≈ 303 nm and a Trp-fluorescence at 355 nm are observed.

Second-derivative UV absorption spectroscopy can be used to estimate the solvent exposure of tyrosines in proteins. This technique takes advantage of the fact that the peak-to-trough distances in the 280–295 nm region, expressed as a/b ratio (see Fig. 3), are related to the polarity of the medium in which Tyr-residues are embedded [21]. The second derivative spectra of holo- and apo-CP (Fig. 3) share common features in the 240–350 nm range, suggesting that the microenvironment of the aromatic residues (96 Tyr, 70 Phe and 25 Trp) is not dramatically altered upon removal of copper. However, the estimated average exposure (α) of Tyr-residues to solvent is higher for the holo-CP ($\alpha_{holo} = 65\% \pm 2$) than for the apo-protein ($\alpha_{apo} = 52\% \pm 2$). These results can be explained considering that the value of α increases for increased values of dielectric constant of the medium [21]. In the case of holo-CP, in fact, the crystallographic structure shows that the side-chain hydroxyl group of Tyr-107 closely interacts with a Cu^{2+} ion in a 'type II' copper-binding site [2], while several other tyrosines along the amino-acid sequence of human CP are close to copper binding-sites [1]. Therefore, upon removal of copper ions, those tyrosine residues that in holo-CP are located in the vicinity of copper binding-sites become embedded in a less polar environment in the apo-protein, with a consequent decrease of the α value. These results parallel those previously reported for the holo- and apo-form of another β-protein, like the Cu/Zn-superoxide dismutase (SOD). In this case, in fact, metal depletion causes a decrease in the accessibility of tyrosines, from 75% of the holo-SOD to about 50% of the apo-protein [30].

8-Anilino-1-naphthalenesulfonic acid (ANS) was used as a non-polar probe to identify the presence of equilibrium and kinetic intermediates in protein folding, commonly known as molten globule states [18]. The binding of ANS to proteins is associated with a strong enhancement of fluorescence quantum yield and with a blue-shift of λ_{max}, reflecting the burial of ANS molecule(s) in a more hydrophobic environment [31]. As shown in Fig. 4, the fluorescence intensity of ANS in the presence of apo-CP is much higher than that obtained in the presence of holo-CP or with the fully unfolded protein in 6 M Gdn-HCl (not shown). Moreover, the λ_{max} of the ANS fluorescence emission is shifted from $\approx$ 505 nm of holo-CP to 480 nm of the apo-form. These data clearly indicate that ANS binds to CP in its apo-form only, and suggest that copper depletion, weakening tertiary interactions, induces the exposure to solvent of hydrophobic surfaces amenable to interact with ANS molecule(s).

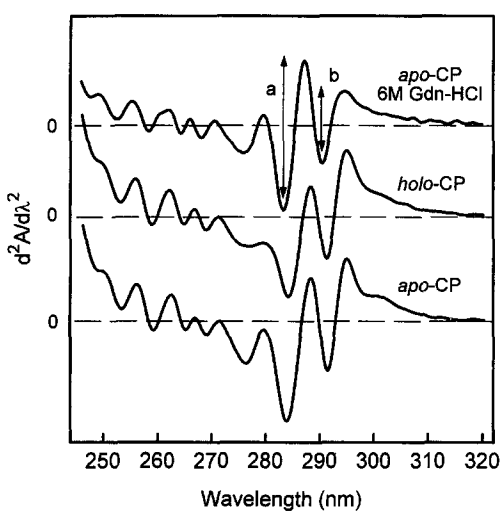

Fig. 3. Second-derivative ultraviolet absorption spectra of holo- and apo-CP in 10 mM Tris-HCl/0.1 M NaCl (pH 7.0) and apo-CP in the presence of 6 M Gdn-HCl. The peak-to-trough distance between the maximum at 287 nm and the minimum at 283 nm (a) and that between the maximum at 295 nm and the minimum at 290.5 nm (b) were used to calculate the Tyr-exposure to solvent, according to Ragone et al. [21].

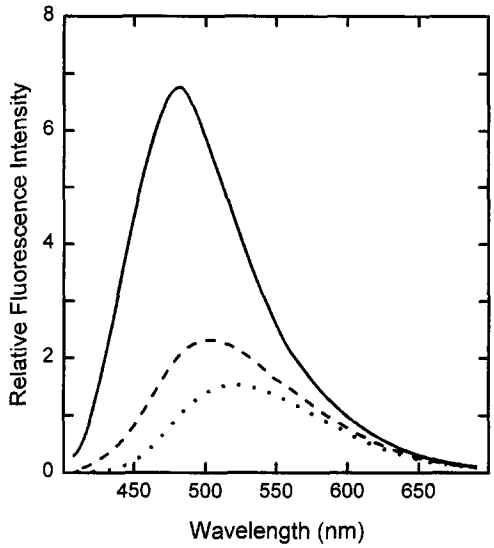

Fig. 4. Fluorescence emission spectra of ANS (10 μM) taken at 25°C in 10 mM Tris-HCl/0.1 M NaCl, pH 7.0 (· · ·), in the presence of holo- (---) and apo-CP (————) (2 μM). Fluorescence spectra were obtained by exciting the samples at 390 nm.

The results of this study indicate that, upon removal of copper ions, human CP retains the secondary structure elements of the holo-protein, while partial unfolding leads to weaker tertiary interactions and an enhanced solvent exposure of hydrophobic groups. These structural features are consistent with the observation that apo-CP has a slightly larger hydrodynamic radius in respect to that of the holo-protein [16] and provide evidence that copper depletion induces in human CP a partly folded state possessing the conformational properties most typical of the molten globule state [18]. These partly folded states of proteins can be generated by alteration of their covalent structure, by exposure to specific solvent conditions (i.e., extreme pH, low concentrations of denaturants) [32] or by depletion of bound metal ions, as in the case of the most studied apo-α-lactalbumin [33]. Although a wide structural variability has been observed [34], general consensus char-

acteristics of the molten globule state include a significant amount of secondary structure and an expanded tertiary structure, lacking the well-defined packing of the native protein and possessing a greater surface accessibility to solvent [18,35].

An increasing body of evidence suggests that the molten globule state represents a general intermediate in the protein folding process [36,37]. In the case of human CP, recent crystallographic data [2] have demonstrated that some of the copper ions in the catalytic center of CP are coordinated by ligands located in the N- and C-terminal domains. This fact, together with the overall complexity of the active center [2], suggests that copper incorporation should occur with the protein in a conformational state intermediate between the native and unfolded state, i.e., the molten globule, that could represent the conformational state of CP during its biosynthesis before metal incorporation. This is in line with the experimental results previously reported for the binding of calcium along the folding pathway of staphylococcal nuclease [38]. In this case, the calcium binding-site is formed by residues that are far apart in the sequence and, in fact, the metal does not bind to the protein until a native-like conformation is reached [38].

The possible implications of the results of this study (conducted on human CP from normal subjects) in the molecular mechanism of Wilson's disease remain to be established.

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References

[1] Takahashi, N., Ortel, T.L. and Putnam, F.W. (1984) Proc. Natl. Acad. Sci. USA 81, 390–394.
[2] Zaitseva, I., Zaitsev, V., Card, G., Moshkov, K., Bax, B., Ralph, A. and Lindley, P. (1996) J. Biol. Inorg. Chem. 1, 15–23.
[3] Malmstrom, B.G. (1978) in New Trends in Bioinorganic Chemistry (Williams, R.J.P. and Da Silva, J.R.F., eds.), pp. 59–77, Academic Press, New York.
[4] Ryden, L. (1982) Proc. Natl. Acad. Sci. USA 79, 6767–6771.
[5] Yoshida, K., Furihata, K., Takeda, S., Nakamura, A., Yamamoto, K., Morita, H., Hiyamuta, S., Ikeda, S., Shimizu, N. and Yanagisawa, N. (1995) Nature Genet. 9, 267–273.
[6] Holtzman, N.A. and Gaumnitz, B.M. (1970) J. Biol. Chem. 245, 2354–2358
[7] Morell, A.G. and Scheinberg, I.H. (1957) Science 127, 588–590.
[8] Aisen, P. and Morell, A.G. (1966) J. Biol. Chem. 240, 1974–1978.
[9] Jackson, G.E., May, P.M. and Williams, D.R. (1978) FEBS Lett. 90, 173–177.
[10] Morpurgo, L., Graziani, M.T., Avigliano, L., Desideri, A. and Mondovi, B. (1981) Israel. J. Biochem. 21, 26–29.
[11] Herve, M., Garnier-Suillerot, A., Tosi, L. and Steinbuch, M. (1985) J. Inorg. Biochem. 25, 121–130.
[12] Gunnarson, P.-O., Nylen, U. and Petterson, G. (1972) Eur. J. Biochem. 27, 572–577.
[13] Vassiliev, V.B, Kachurin, A.M., Rocco, G.-P., Beltramini, M., Salvato, B. and Gaitskhoki, V.S. (1996) Biochemistry (Moscow) 61, 221–229.
[14] Kasper, C.B. and Deutsch, H.F. (1963) J. Biol. Chem. 238, 2325–2337.
[15] Zakharova, E.T., Vassiliev, V.B., Gorbunova, V.N. and Shavlovski, M.M. (1983) Biokhimia (Russ.) 48, 1709–1720.
[16] Ryden, L. (1984) in Copper Proteins and Copper Enzymes (Lontie, R., ed.), Vol. 3, pp. 37–100, CRC Press, Boca Raton, FL.
[17] Carrico, R.J., Deutsch, H.F., Beinert, H. and Orme-Johnson, W.H. (1969) J. Biol. Chem. 44, 4141–4146.
[18] Ptitsyn, O.B. (1995) Adv. Protein Chem. 47, 83–229.
[19] Gill, S.G. and Von Hippel, P.H. (1989) Anal. Biochem. 182, 319–326.
[20] Weber, G. and Young, L.B. (1964) J. Biol. Chem. 239, 1415–1423.
[21] Ragone, R., Colonna, G., Balestrieri, C., Servillo, L. and Irace, G. (1984) Biochemistry 23, 1871–1875.
[22] Brahms, S. and Brahms, J. (1980) J. Mol. Biol. 138, 149–178.
[23] Noyer, M. and Putnam, F.W. (1981) Biochemistry 20, 3536–3542.
[24] Adman, E.T. (1991) Adv. Protein Chem. 32, 145–197.
[25] Manning, M.C. and Woody, R.W. (1987) Biopolymers 26, 1731–1752.
[26] Manning, M.C. and Woody, R.W. (1989) Biochemistry 28, 8609–8613.
[27] Vuilleumier, S., Sancho, J., Loewenthal, R. and Fersht, A.R. (1993) Biochemistry 32, 10303–10313.
[28] Strickland, E.H. (1974) CRC Crit. Rev. Biochem. 3, 113–175.
[29] Lakowicz, J.R. (1986) in Principles of Fluorescence Spectroscopy (Lakowicz, J.R., ed.), pp. 341–381, Plenum Press, New York.
[30] Mach, H., Dong, Z., Middaugh, C.R. and Lewis, R.V. (1991) Arch. Biochem. Biophys. 287, 41–47.
[31] Stryer, L. (1965) J. Mol. Biol. 13, 482–495.
[32] Fink, A.L. (1995) Annu. Rev. Biophys. Biomol. Struct. 24, 495–522.
[33] Kuwajima, K. (1977) J. Mol. Biol. 114, 241–258.
[34] Miranker, A.D. and Dobson, C.M. (1996) Curr. Opin. Struct. Biol. 6, 31–42.
[35] Haynie, D.T. and Freire, E. (1993) Proteins Struct. Funct. Genet. 16, 115–140.
[36] Jennings, P.A. and Wright, P.E. (1993) Science 262, 892–896.
[37] Balbach, J., Forge, V., Van Nuland, N.A., Winder, S.L., Hore, P.J. and Dobson, C.M. (1995) Nature Struct. Biol. 2, 865–870.
[38] Sugawara, T., Kuwajima, K. and Sugai, S. (1991) Biochemistry 30, 2698–2706.