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Conformational aspects of the Cu^{2+} binding to α-lactalbumin. Characterization and stability of the Cu-bound state

H. Van Dael, E. Tieghem, P. Haezebrouck and F. Van Cauwelaert

Interdisciplinair Research Centrum, K.U. Leuven Campus Kortrijk, B-8500 Kortrijk (Belgium)

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Abstract

By circular dichroism experiments the existence of a typical Cu^{2+}-bound state is demonstrated for bovine- and for goat α-lactalbumin. As in the near-UV region an important ligand to metal charge-transfer band overlaps with the aromatic band of the protein, a subtraction method is developed in order to determine the nett effect of Cu^{2+} ions on the protein conformation. The Cu^{2+}-bound state, characterized by a vanishing tertiary structure and a substantial loss of secondary structure, clearly differs from the well-known Ca^{2+}-, apo-, and acid conformers. At room temperature, the Cu^{2+} binding has already decreased the α-helix content of bovine α-lactalbumin to the extent that further unfolding by thermal or guanidine hydrochloride denaturation behaves in a non-cooperative way. Since for goat α-lactalbumin the Cu^{2+} binding to His-68 is much less important than for bovine α-lactalbumin, we observe a somewhat different conformational behaviour for goat α-lactalbumin. The results of this conformational circular dichroism study are confirmed by isothermal calorimetric data.

Keywords: α-Lactalbumin; Secondary structure; Tertiary structure; Cupric ion; Protein binding; Circular dichroism

1. Introduction

In the studies on the binding of metal ions to α-lactalbumin, traditionally most attention was paid to the Ca^{2+}-binding site. Although different values for the binding constant were proposed (10^6–10^9 M^{-1}), there is general agreement on a Ca^{2+}-binding substructure, consisting of two helical segments (residues 76 to 82 and 86 to 99) and an "elbow" loop that connects the helices [1]. Different techniques were used to describe the conformational changes that accompany the Ca^{2+}-binding process [2–6]. It was also demonstrated that the stability of the folded state is remarkably enhanced by the presence of Ca^{2+} ions [7,8].

Murakami et al. introduced a distinct zinc-binding site and showed that Cu^{2+}, Co^{2+} and Al^{3+} ions bind at that specific site [9]. In a previous study [10] we were able to determine the groups involved in Cu^{2+} binding. We also proved that Cu^{2+}- and Ca^{2+} binding are totally independent processes and that Cu^{2+} does not expel Ca^{2+} from its binding site. By tryptophan fluorescence measurements, it was shown by Permyakov

Correspondence to: H. Van Dael, Interdisciplinair Research Centrum, K.U. Leuven Campus Kortrijk, B-8500 Kortrijk, Belgium.

et al. [11] that binding of Cu^{2+} to α-lactalbumin significantly lowers the thermostability and the stability towards urea denaturation, so that its conformational state is characterized as "apo-like" [11,12], i.e. similar but not identical to the apo-state. This conclusion was based on the shift of the tryptophan fluorescence spectrum to longer wavelengths as is observed by the removal of Ca^{2+} ions. It must be remarked, however, that this red-shift was not accompanied by the normal increase of the quantum yield. Further or more direct information on the conformation of the Cu^{2+}-bound state and its stability is not available in the literature.

Therefore, in the present work we have examined the secondary and tertiary structure of Cu^{2+}-loaded bovine α-lactalbumin by circular dichroism experiments. We were able to prove and characterize a distinct Cu^{2+}-bound state whose conformation significantly differs from both the Ca^{2+}- and the apo-state. Furthermore, we have studied the stability of this conformation towards thermal and guanidine hydrochloride (GdnHCl) denaturation. As the binding of Cu^{2+} ions to bovine and goat α-lactalbumin proceeds in a different way [13], we have also determined the conformational properties of Cu^{2+}-loaded goat α-lactalbumin. Finally, we performed calorimetric experiments, providing direct thermodynamic information on the conformational transitions. These results are interpreted in the same framework as we used for the CD data.

2. Materials and methods

Bovine α-lactalbumin is from Sigma Chemical Co and goat α-lactalbumin was prepared from fresh milk as described previously [14]. The preparation of the apo-form of both these α-lactalbumins was carried out following our earlier described methods [14] and it was stored in Hepes solution (10 m*M*, pH 7.5).

Circular dichroism experiments were carried out as described previously [10,13]. Unlike indicated otherwise, the experimental conditions were always: protein concentration about 25 μ*M*, pH 7.5 in 10 m*M* Hepes. The results were expressed as the ellipticity θ (mdeg.), or as the mean residue ellipticity [θ] (deg.cm^2/dmol) at 222 nm or 270 nm. Baseline normalization was made at 250 nm for secondary structure measurements and at 308 nm for tertiary structure measurements respectively. In the calculation of [θ], we used 115.21 and 115.47 as the mean residue weights for bovine- and goat α-lactalbumin. Control circular dichroism experiments showed that Cu^{2+} ions do not interact with Hepes nor with GdnHCl. The microcalorimetry data were obtained as described elsewhere [4].

3. Results and discussion

3.1 Tertiary structure

At room temperature the CD spectrum of Ca^{2+}-loaded α-lactalbumin in the spectral region 250–310 nm is characterized by a deep negative aromatic band near 270 nm [2,10]. This band reflects the pronounced tertiary structure of the protein and it largely disappears when the protein is demetallized, submitted to thermal or chemical denaturation or is brought to acid pH. The addition of Cu^{2+} ions also leads to the dissappearance of this negative band but that effect is accompanied by the simultaneous occurrence of a strong positive band with maximum at 258 nm. That large band, characteristic for a ligand to metal charge-transfer with nitrogen as the principal ligand, was attributed [10] to the consecutive binding of Cu^{2+} ions to His-68, and at higher Cu^{2+} concentration also to the *N*-terminal amine and to peptide groups. The occurrence of this charge-transfer band, that overlaps with the aromatic band [10], has temporarily prevented us from determining the nett effect of Cu^{2+} on the protein conformation. The questions, which binding is responsible for the conformational changes and to what extent,were still unsolved.

In order to separate the charge-transfer from the aromatic contribution, we developped the following procedure. In addition to the measurement in 10 m*M* Hepes [10], the Cu^{2+}-loaded samples were also examined in 7 *M* GdnHCl; in

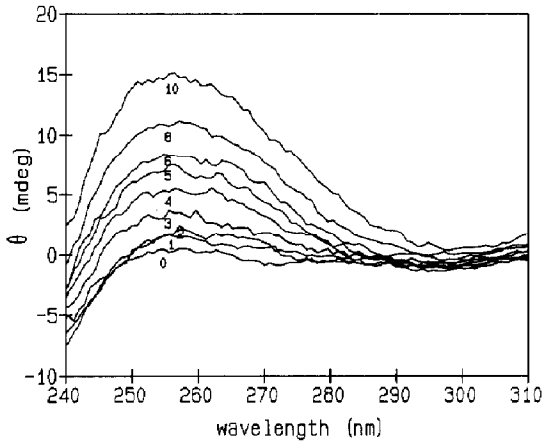

Fig. 1. The ligand to metal charge-transfer band in the CD spectrum (240–300 nm) for the Ca^{2+}-form of bovine α-lactalbumin (25 μ*M* protein, 30 μ*M* Ca^{2+}, 7 *M* GdnHCl, pH 7.4) at room temperature. The figures refer to the molar ratio (M.R.) of Cu^{2+} ions added to the solution.

this medium the tertiary structure has completely disappeared and the whole CD signal can unambiguously be attributed to charge-transfer effects (Fig. 1). Subtraction of these two signals provides the nett effect of Cu^{2+} on the protein conformation (Fig. 2). As a function of Cu^{2+} concentration, the nett $[\theta]_{270}$ of bovine α-lactalbumin increases steeply up to saturation around a molar

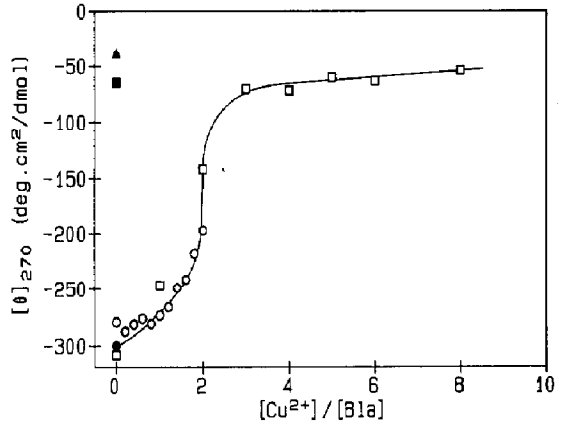

Fig. 2. The nett effect of Cu^{2+} on the tertiary structure of Ca^{2+}-loaded bovine α-lactalbumin, derived from the subtraction of the results in 7 *M* GdnHCl from the results in Hepes buffer. Samples with an integer as molar ratio (□) are measured simultaneously in both conditions. Additional results on independent samples (○) were obtained after subtraction of the CT contribution interpolated from the former results. The filled symbols are data taken from ref. [2] for the native (●), the apo- (■) and the acid state (▲) of bovine α-lactalbumin.

ratio (M.R.) of 3. This represents the cooperative unfolding of the tertiary structure to the same extent as by Ca^{2+} removal or by acidification. This result is important as it demonstrates that the complete change in tertiary structure is already induced by the primary binding of Cu^{2+} ions to the histidine group and no additional effect on the conformation can be observed by further Cu^{2+} binding to other residues.

A correct subtraction procedure, however, implies that, while Cu^{2+} remains bound to the protein, the charge-transfer contribution itself is unaffected by changes in the protein's tertiary structure. In order to check the validity of this presumption, we investigated the properties of the charge-transfer band for various conformational states. We compared the charge-transfer (CT) band of bovine α-lactalbumin in the native state, in the completely unfolded state (7 *M* GdnHCl) and in the thermal unfolded apo-state at 35°C. In all these conformational states the CT-band has the same shape with maximum at 258 nm and upon Cu^{2+} titration, the increase of the CT-intensity proceeds in nearly the same way. Moreover, the CD spectrum of a Cu^{2+}-loaded α-lactalbumin sample (M.R. = 6), shows no changes upon GdnHCl titration. This proves that GdnHCl does not interfere with the interaction between Cu^{2+} and the protein. If GdnHCl interacted with Cu^{2+}, the CT-band would have collapsed in the way observed upon addition of some millimoles of Tris buffer.

The conformational change concomitant with the binding of the Cu^{2+} ions to His-68 is also easily confirmed by microcalorimetry. The reaction M^{2+} + apo-protein → M–protein is accompanied by an exothermic molar enthalpy change, the size of which depends on the cation M^{2+} and the species [15]. The largest negative value is obtained for the binding of Ca^{2+} to bovine α-lactalbumin. The molar enthalpy change ΔH is the sum of two contributions resulting from a conformational and a binding process [14], the latter being the smallest part. The microcalorimetric results are plotted in Fig. 3. The binding of Cu^{2+} to the Ca^{2+} form of bovine- and goat α-lactalbumin is strongly endothermic: this suggests that a conformational change occurs which

is the reverse of the apo- $\rightarrow M^{2+}$-bound state and whereby tertiary structure is lost. The enthalpy change upon binding Cu^{2+} to the apo-state is slightly negative suggesting some small gain in tertiary structure compared to the apo-state.

3.2 Secondary structure

In secondary structure analysis, there is no need for such a complex subtraction method. At 220 nm no important charge-transfer contribution nor $[Cu^{2+}]$ dependence has been detected. The CD signal of Ca^{2+}-loaded bovine α-lactalbumin in 7 *M* GdnHCl is small compared with the signal in Hepes and a Cu^{2+} titration in the former circumstances leaves the ellipticity practically unchanged (Fig. 4).

Addition of successive amounts of Cu^{2+} ions to bovine α-lactalbumin induces a decrease in ellipticity that takes place in two steps: after a considerable but gradual loss in ellipticity in the molar range of 1 to 3, it further declines in a linear way (Fig. 4). This result demonstrates that

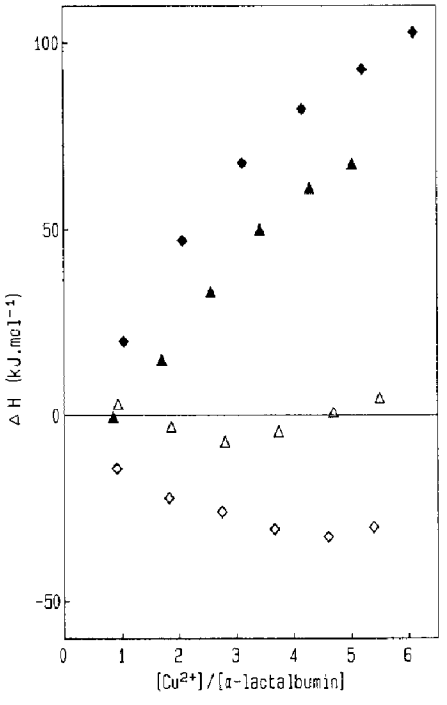

Fig. 3. The molar enthalpy change as a function of $[Cu^{2+}]$, added to a solution of the apo- (open symbols) and the Ca^{2+} (filled symbols) form of bovine (diamond) and goat (triangle) α-lactalbumin.

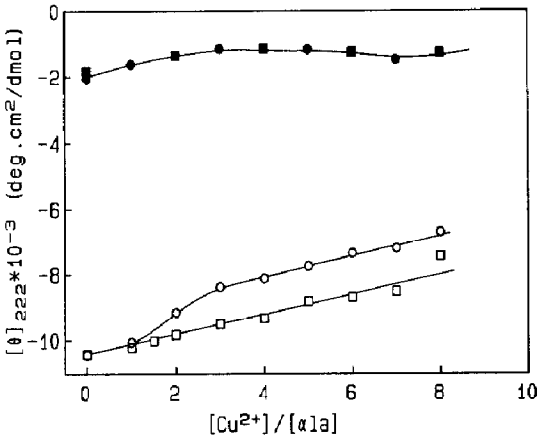

Fig. 4. The effect of Cu^{2+} on the secondary structure of bovine (circle) and goat (square) α-lactalbumin. Open symbols for the results in 10 m*M* Hepes, filled symbols for results in 7 *M* GdnHCl.

binding of up to three Cu^{2+} ions is accompanied not only by a total loss of tertiary structure (Fig. 2) but also by an important reduction of secondary structure. Above this molar ratio, additional Cu^{2+} ions give only rise to a small linear solvent effect. These observations are in agreement with our earlier phenomenological description on Cu^{2+} binding to bovine α-lactalbumin [10].

3.3 Effect of GdnHCl

The GdnHCl concentration dependence of $[\theta]_{222}$ for bovine α-lactalbumin with Cu^{2+} (M.R. = 6) and for the native protein (pH 7.5, $T = 25°C$) is illustrated in Fig. 5. For the latter form, the unfolding of the native (N) state in a cooperative way is followed by a linear concentration dependence at high GdnHCl concentration. This linear behaviour is the result of the solvent effect on the randomly coiled polypeptide. Ikeguchi et al. [16] have already shown that the shape of the unfolding curve is $[Ca^{2+}]$ dependent: addition of Ca^{2+} ions increases the stability of the protein and enhances the cooperativity of the unfolding transition. The unfolding curve of the secondary structure in the acid (A) state in urea, although it has been obtained in different physical conditions (0.1 *M* KCl, pH 2, $T = 4.5°C$), principally shows the same characteristics [17]. Comparison between both results suggests that

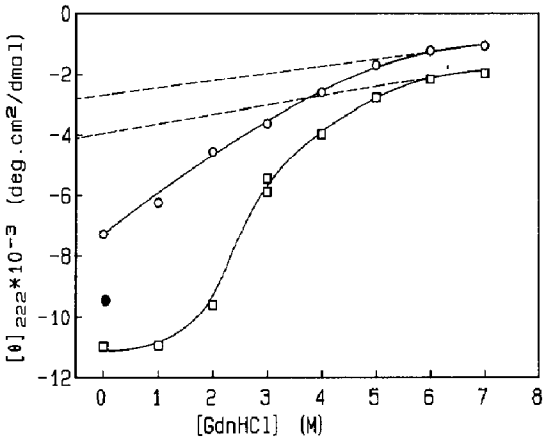

Fig. 5. Changes in secondary structure by GdnHCl titration of bovine α-lactalbumin (25 μM) without (□) and with M.R. = 6 Cu^{2+} (○). The dotted lines are the postulated ellipticities for the unfolded states. The filled circle is the result on disulfide reduced α-lactalbumin from [17].

the unfolding of the secondary structure in the A- and in the N-state happens in nearly the same way.

Concerning the Cu^{2+}-containing sample (Fig. 5), two important considerations have to be made: (1) Even without GdnHCl, the ellipticity of the Cu^{2+}-loaded sample has considerably decreased (7.3 10^3 deg. cm^2/dmol) compared to the Ca^{2+}-protein without Cu^{2+} (11 10^3 deg. cm^2/dmol). Here it should be remarked that the ellipticity of native bovine α-lactalbumin even after reduction and carboxymethylation of the four disulfide bridges still exceeds 9 10^3 deg. cm^2/dmol at 218 nm [17]. 2) This Cu^{2+}-bound state unfolds in a gradual but non cooperative way. The parallelism of the tangents on the GdnHCl titration curve at high concentration, proves that the solvent effect is not altered whether Cu^{2+} is bound to the protein or not. The small difference in ellipticity that remains between these two states is a direct Cu^{2+} contribution. The absorption peak (maximum = 239 nm) in the copper protein spectrum has not yet vanished at 222 nm and is responsible for this small ellipticity contribution.

The study of the effect of GdnHCl on the conformation of Cu^{2+}-containing samples is limited to considerations on the secondary structure. As shown above, the tertiary structure of the Cu^{2+}-bound protein is already broken up by the specific effect of Cu^{2+} ions so that GdnHCl titration cannot give additional information.

3.4 Effect of temperature

By monitoring the ellipticity at 270 nm, it has been shown that apo- and holo α-lactalbumin exhibit the thermal unfolding of the tertiary structure [2,7]. As the addition of Ca^{2+} ions to α-lactalbumin is known to stabilize the native structure extensively, it provokes an upward shift in thermal transition temperature [7,8]. For the native protein, a concomitant cooperative transition in the secondary structure was observed by CD measurements at 222 nm [18,19].

The unfolding behaviour of the apo- and Ca^{2+}-form of bovine α-lactalbumin and their Cu^{2+}-bound counterparts is presented in Fig. 6. The transition temperature deduced from the

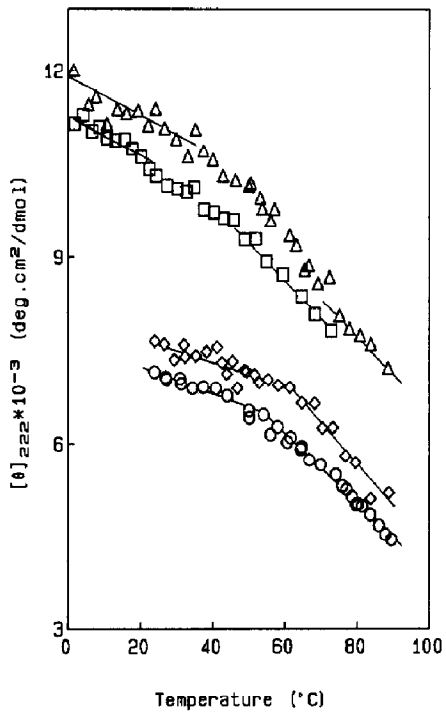

Fig. 6. Temperature dependence of the ellipticity at 222 nm for bovine α-lactalbumin in the Ca^{2+}-form (△), in the apo-form (□), and for the Cu^{2+}-loaded protein with Ca^{2+} (◇) and without Ca^{2+} (○). Protein concentrations were 26.7 μM, Ca^{2+} and Cu^{2+} concentrations respectively M.R. = 1.2 and M.R. = 6. Full lines correspond with the ellipticities in the folded state before the transition and in the unfolded state at high temperature.

curves for the apo- and the Ca^{2+}-protein correspond with former values for the secondary structure transition by Kuwajima et al. [19] and also with the transition temperature observed with aromatic CD [18]. Comparison of the ellipticity of the Ca^{2+}- and apo-protein with and without Cu^{2+} at room temperature, shows a much lower value for the Cu^{2+}-containing sample (Fig. 6). The conformational state of the Cu^{2+}-loaded samples before unfolding, clearly differs from the Ca^{2+}- or the apo-state and the fact that the α-helicity has significantly decreased, agrees with our former statement [10] that the helices in bovine α-lactalbumin have to unwind to become chelated.

A more detailed study of the temperature dependence of the ellipticity at 222 nm (Fig. 6) also provides good arguments for a peculiar Cu^{2+}-bound state that has lost a substantial part of its helix structure. The sigmoidal behaviour typical of a helix-to-random coil transition does not occur in the unfolding curves of the Cu^{2+}-loaded samples. The helix structure that must transform to random coil has already disappeared by Cu^{2+} binding. This reasoning also explains the absence of a cooperative GdnHCl transition for Cu^{2+}-loaded samples (preceding paragraph). The temperature unfolding curve proceeds linearly but suddenly changes its slope. The exact meaning of this breakpoint is still unclear. It is unlikely that it indicates the temperature whereby Cu^{2+} binding begins to release by denaturation of the protein, as this phenomenon is not observed in tertiary structure measurements. In the latter case at 270 nm, Cu^{2+}-containing samples show a constant CD signal as a function of temperature up to 80°C (data not shown). Therefore we rather believe that at high temperature, the ligation of amide groups is enhanced, which leads to a perturbation of the interactions that stabilize the secondary structure.

3.5 *The Cu^{2+}-bound state of goat α-lactalbumin*

The changes in secondary structure of goat α-lactalbumin as a function of Cu^{2+} content can be derived from Fig. 4. The ellipticity decreases in a linear way in the whole concentration range and the conformational change coupled at the

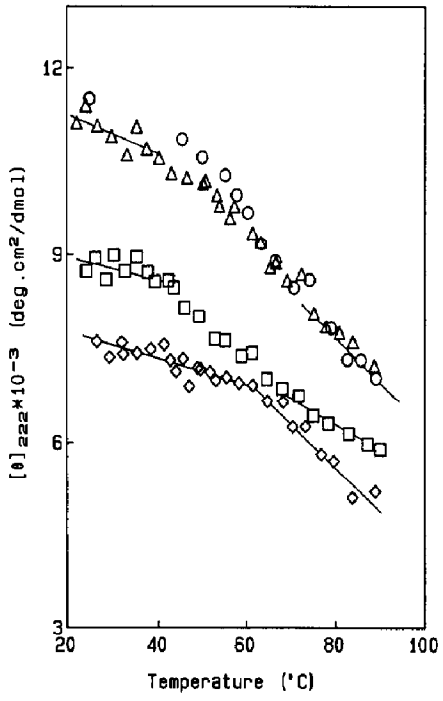

Fig. 7. Temperature dependence of the ellipticity at 222 nm for goat α-lactalbumin in the Ca^{2+}-form without (○) and with Cu^{2+} (□). For comparison the results on the Ca^{2+}-form (△) and the Ca^{2+}, Cu^{2+}-form (◇) of bovine α-lactalbumin from Fig. 6 are plotted once again.

binding on the histidyl site that occurs in bovine α-lactalbumin, is absent here. This is in agreement with our earlier CD results in the visible region that this type of binding contributes only in a minor way in the goat species [13]. The ellipticity of Cu^{2+}-bound goat α-lactalbumin (M.R. = 7) at room temperature (Fig. 7) is obviously reduced compared to the ellipticity of the other conformers. This reduction, however, is less pronounced than for bovine α-lactalbumin. Therefore,it is also interesting to compare the thermal transition of bovine- and goat α-lactalbumin. By monitoring the tertiary structure, it has been shown [2] and it was confirmed recently by our aromatic CD measurements [14] that in comparison with bovine α-lactalbumin, the native to unfolded state transition of apo-goat α-lactalbumin is shifted upwards with at least 10°C. Harushima and Sugai [20] calculated that the stability at 25°C is in the order goat α-lactalbumin > bovine α-lactalbumin both in the presence and the absence of 1 m*M* Ca^{2+}. As

goat- and bovine α-lactalbumin have the same enthalphy change of the thermal unfolding, they attributed the difference in stability to the difference in the entropy change of the thermal unfolding. When the secondary structure is followed by the residue ellipticity at 222 nm, such an explicit shift of the transition temperature is not observed and the transition curves of both species practically coincide (Fig. 7). Although local structural differences give rise to a change of the aromatic CD signal, they do not affect the global nature of the protein so that bovine and goat α-lactalbumin show the same unfolding behaviour in secondary structure. This effect is observed in 6 *M* GdnHCl as well as by thermal treatment.

The similar behaviour of bovine and goat α-lactalbumin is removed upon the addition of Cu^{2+} ions. As remarked before, the loss of secondary structure induced by Cu^{2+} addition is less pronounced for goat α-lactalbumin. The fraction of α-helicity that is still present at room temperature gradually disappears in the temperature range 45–65°C. In contrast to the unfolding behaviour of bovine α-lactalbumin,an obvious helix-to-random coil transition is observed. Compared to the native protein,the size of the ellipticity change during the transition is reduced but the transition temperature itself has hardly changed. The titration of goat α-lactalbumin with GdnHCl (data not shown) also shows that Cu^{2+} ions affect the protein structure so that the denaturation of the protein starts at lower concentration. The near-UV spectra of Cu^{2+}-loaded goat α-lactalbumin samples also demonstrate a diminishing tertiary structure at 270 nm, accompanied by a positive charge-transfer band at 258 nm (Fig. 8). For this species, the observed effect of the bound Cu^{2+} ions is obviously smaller than in the case of bovine α-lactalbumin. A small rest of tertiary structure is left and the charge-transfer from the ligand group to the metal seems to be less effective. In contrast to what happens on bovine α-lactalbumin (Fig. 8), addition of GdnHCl has further effects on the spectrum of goat α-lactalbumin. Tertiary structure is now totally lost and the charge-transfer effect largely exceeds former values. From this it is clear that for the goat species, the subtraction method cannot be used and therefore separation of the charge-transfer and the conformational contribution cannot be done in an unambiguous way.

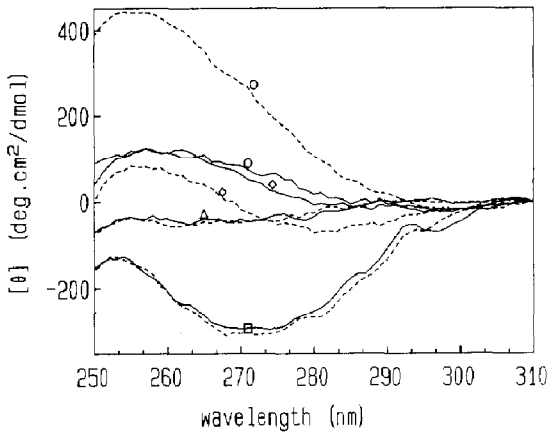

Fig. 8. Near-UV CD spectra of the Ca^{2+}-form of bovine (full line) and the goat (dotted line) α-lactalbumin. Spectra were taken for samples without Cu^{2+} in 10 m*M* Hepes (square) and in 6 *M* GdnHCl (triangle), and for samples with Cu^{2+} ions (M.R. = 6) in Hepes (diamond) and in GdnHCl (circle).

All these observations lead to the conclusion that also for goat α-lactalbumin, a distinct Cu^{2+}-bound state exists. The conformational properties of that state, however, to some extent deviate from the corresponding state of bovine α-lactalbumin.

4. Conclusions

The disappearance of the pronounced tertiary structure of calcium bovine α-lactalbumin upon decalcification occurs with no or minor changes in the secondary structure. We demonstrated the existence of a distinct Cu^{2+}-bound state that has lost not only tertiary structure but also a substantial part of the secondary structure. This Cu^{2+}-bound state thus cannot be described as "apo-like" [11,12]. Cu^{2+} ions act as a moderate chemical denaturant on α-lactalbumin. The change in secondary structure induced by copper bonds, is independent of the Ca^{2+} content. This intermediate Cu-bound state unfolds further by thermal or by GdnHCl treatment. At room temperature, the helicity of Cu^{2+}-loaded bovine α-lactalbumin is reduced to such extent that thermal unfolding is

diffuse without significant cooperativity. The differences in Cu^{2+} binding to goat- and bovine α-lactalbumin, previously established [13], manifest themselves in a different conformational behaviour. At room temperature, goat α-lactalbumin still possesses α-helicity that, upon heating, shows a cooperative transition to a random coil conformation.

Acknowledgements

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