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CALORIMETRIC STUDIES ON RENATURATION BY $CaCl_2$ ADDITION OF METAL-FREE α-AMYLASE FROM *BACILLUS LICHENIFORMIS* (BLA)

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In our previous work we characterized the Ca binding properties of BLA by analysis of Ca-induced renaturation in the presence of urea. In this study we focused on the renaturing capacity of $CaCl_2$ in the absence of urea and analysed the apparent thermodynamic and kinetic properties of renatured BLA by DSC, CD and dynamic light scattering (DLS) measurements. Ca-free protein did not return fully to the native state even after extensive dialysis against high concentrations of calcium. Thus Ca removal provokes changes in protein structure that can not be reversed completely even by addition of an excess of calcium. However, activity studies performed simultaneously with the DSC experiments showed a significant return of enzymatic activity despite the failure of complete return of native stability. This conclusion is in excellent agreement with findings of Machius *et al.* who suggested on the basis of their X-ray studies that it is not possible to remove CaI and CaII without introducing significant structural changes. It is important to note that all unfolding reactions of metal-free, partially renatured and native protein were found to be irreversible. Therefore the DSC transition curves were fitted using irreversible transition models. It turned out that the apparent heat capacity profiles of partially renatured BLA could be well represented by superposition of two irreversible processes each following the two-state irreversible model.

Keywords: *α-amylase (BLA), DSC, influence of Ca ions, recovery of activity, renaturation, stability*

Introduction

Bacillus licheniformis is a mesophilic bacterium usually found in temperate soil that produces a highly thermostable α-amylase (BLA) which is even more thermostable than the related enzyme produced by thermophilic organisms such as *Bacillus stearothermophilus*. These differences exist although these proteins possess highly homologous primary and tertiary structures [1]. Because of its remarkably high thermal resistance BLA has become an established model system for addressing questions having both fundamental and technological implications regarding protein thermostability [2]. For the very reason of stability this enzyme has been extensively used in biotechnological processes such as starch liquefaction at temperatures of up to 110°C, and in alcohol-, sugar- and brewing industries for the initial hydrolysis of starch [3].

The crystal structure of BLA, shown in Fig. 1, consists of three domains with overall topologies similar to those found in all known structures of α-amylases. The central domain (Domain A) is an $(\alpha/\beta)_8$ TIM barrel which is interrupted by an irregular β-domain (Domain B) inserted between the third β-strand and the third α-helix of the TIM barrel. Domain C is located at the C terminus of the protein and contains a Greek key motif. Domain A is the most strictly conserved domain in the family of α-amylases, domain C is also very well conserved while domain B is the least similar domain in this family of enzymes [4, 5].

It was known that α-amylases require calcium to maintain structural integrity and enzymatic activity. Removal of calcium leads to decreased thermostability and/or decreased enzymatic activity. All known structures of holo α-amylases show a common calcium-binding site stabilizing the interface between the highly homologous central domain A and the more variable B domain (Fig. 1). The distance of this conserved calcium to the catalytic centre precludes a direct involvement in the reaction mechanism so its role is assumed to be more structural. The conserved calcium binding site in BLA is part of a novel metal-binding motif with two calcium ions in close proximity to a central cation which is sodium. The stem of a pronounced loop is wound around this novel CaI–Na–CaII metal triad. The ligands for this metal triad come almost completely from residues in domain B with only two interactions established with residues from domain A. The third calcium ion (Ca III) in BLA is located in the interface between domains A and C. This calcium ion has not been observed previously in the structures of other α-amylases. The ion bridges two loops in domain C with a large loop in domain A. The position of Ca III

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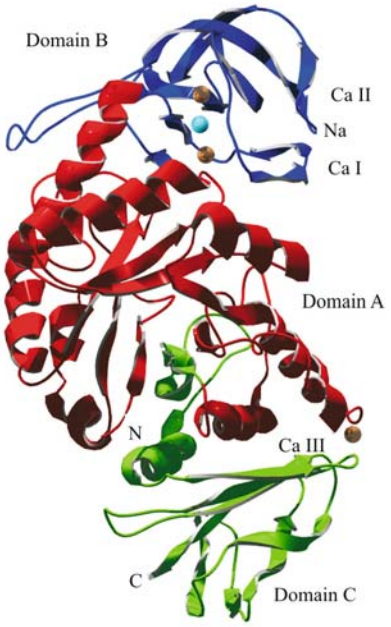

Fig. 1 Tertiary structure of *Bacillus licheniformis* α-amylase (BLA, PDB code: 1BLI). The calcium and sodium ions bound to the protein in its native form are shown. Domain A, shown in red, is an alpha-beta TIM barrel. Domain B is colored blue and domain C green. The three calcium ions are shown in gold and the sodium ion is shown in blue. The figure was made and colored using Swiss PDB viewer and refined using PovRay

on the C-terminal side of the central α/β-TIM barrel is directly opposite to the region where domain A joins domain C, and thus provides a second major anchor point for the interaction between these two domains [5, 6].

Because of the vast biotechnological usage of α-amylases, a number of studies have aimed at the elucidation of the molecular determinants of their thermostability [7–15]. Particularly BLA has been subject of much research activity due to availability of high resolution X-ray studies [4, 16] and the characteristic dependence on Ca^{2+}-concentration of both activity and conformational stability [17–19].

In general most mesophilic and thermophilic proteins unfold irreversibly. They unfold into inactive but kinetically stable structures (scrambled structures), and they often form aggregates. In this regard α-amylases are not unusual [7, 20]. It had been known that in cases where temperature induced transitions were monitored practically all α-amylases unfold irreversibly [20–22]. The only known exception is a psychrophilic α-amylase from *Alteromonas haloplanctis*, that exhibits a remarkable degree of reversibility [23]. Possible sources of irreversibility of thermal unfolding are high temperature chemical modifications, such as deamidation, cysteine oxidation, or peptide bond hydrolysis that take place once the protein is unfolded. The increased aggregation tendency results from hydrophobic residues that become exposed to the solvent and interact preferably with hydrophobic residues from other unfolded protein molecules to minimize their exposure to the solvent [20, 24].

Differential scanning calorimetry has been instrumental in characterizing reversible and irreversible conformational changes of proteins [25–29, 30, 31, 38, 39].

In the present DSC study we characterised the unfolding behaviour of BLA as a function of both heating rate and Ca concentration. It could be demonstrated that the C_p profiles can be well fitted to two kinetically determined irreversible two-state transitions. The present works are an extension of our previous investigations the interaction of Ca^{2+}-ion with BLA where we employed Ca-binding to refold the urea denatured protein [32].

Experimental

Materials

Protein purification

Bacillus licheniformis α-amylase (BLA) was generously provided by Novozymes (Termamyl 300 L Type DX). The sample was of industrial grade and it was purified for the present studies. Purification was done in two steps: 1 – by using Butyl Sepharose (Amersham Bioscience) which is a separation medium for hydrophobic interaction chromatography and 2 – by applying Q Sepharose (Amersham Bioscience) which is a strong anion exchanger. Usually 10 mL of the industrial grade protein sample containing 10 mg mL^{-1} were dialyzed *vs.* 20 mM Tris–HCl pH 8.0 for 24 h. Ammonium sulphate was added to a final concentration of 1 M and the protein solution was then applied to a butyl-Sepharose column equilibrated with 20 mM Tris–HCl/1 M ammonium sulphate (Merck), pH 8.0. Elution was done with a 0 to 100% gradient of 5 mM Tris–HCl, pH 8.0, 30% glycerol.

Amylase fractions were pooled and dialyzed against 5 mM Tris–HCl, pH 9.0 and applied to a Q-Sepharose column equilibrated with 20 mM Tris–HCl, pH 9.7. Amylase was eluted by increasing NaCl concentration from 0 to 1 M in the same buffer (20 mM Tris–HCl/1 M NaCl, pH 9.7). Amylase fractions were pooled and concentrated using an Amicon stirred ultrafiltration cell, using 30 kD membranes (YM30) Amicon. Typical yield from 10 mL of starting solution was 7 mL purified protein of about 8 mg mL^{-1}.

Preparation of Ca^{2+}-free protein

After the purification steps, the protein sample was dialyzed against Tris–HCl 20 mM, pH 8.0. To obtain Ca-depleted samples, 4 mL of the purified protein were dialyzed against 20 mM Tris/20 mM EGTA overnight with at least two changes of the equilibrium dialysis buffer. EGTA was subsequently removed from the samples by dialysis against 20 mM Tris/ pH 8.0. The dialyses were carried out at 4°C. All Ca-removal and following steps were performed in plastic vessels to avoid any Ca^{2+} contamination from glass. Protein concentration was determined at 280 nm using an IKS – XDAP-diode array UV-Vis Spectrophotometer. The molar extinction coefficient used was 139690 M^{-1} cm^{-1}. It was determined according to Pace *et al.* [25].

Methods

BLA activity test

For activity tests the Phadebas assay was used. The Phadebas α-amylase test kit is based on the release of blue colour from the substrate (blue-colored starch) upon cleavage. A solution of 40 mg mL^{-1} Phadebas tablet (Pharmacia Diagnostics) was prepared in 50 mM Britton–Robbinson buffer containing 0.1 mM $CaCl_2$ (50 mM sodium borate, 50 mM sodium acetate, 50 mM sodium phosphate, pH 8.0).

25 μL of α-amylase solution of known concentration was added to 575 μL of the Phadebas solution and incubated at 50, 55, 60 and 70°C for 15 min. After the incubation time the reaction was stopped using 100 μL of 1 M NaOH. The suspensions were centrifuged for 15 min at 15000 rpm and the absorbance of the supernatant was measured at 620 nm. For each measurement the absorbance was corrected for baseline absorption. For that purpose all activity test procedures were identical with the exception of enzyme addition. The OD at 620 nm of the reference solution was found negligible (<0.001 OD) [6].

DSC measurements

DSC experiments were performed using a CSC 6100 Nano II DSC. BLA solutions were dialyzed against the desired buffer, and this dialysis buffer was used as reference. Prior to scanning all solutions were degassed by stirring under vacuum to avoid formation of air bubbles. An excess pressure of 3 atm was applied to the cells during scanning. Buffer scans were always run prior to protein scans. DSC data were analyzed using CpCalc software from CSC (version 2.1), supplied with the instrument. For the analysis of the kinetic effects on the heat capacity profile we varied heating rates from 0.125 to 2°C min^{-1}. Typical protein concentrations for the DSC measurements were between 0.5 to 1.5 mg mL^{-1} unless otherwise mentioned.

The two-state irreversible model

Thermal denaturation of BLA has been analysed in terms of the simple two-state irreversible denaturation model [26–28]. The overall denaturation process is approximated by a first order irreversible process:

$$N \xrightarrow{k} F \tag{1}$$

in which only the native and final states are significantly populated and the conversion from N to F is determined by a strongly temperature-dependent, first order rate constant (k) [29, 33]. This model has been used successfully to describe various irreversible unfolding processes of different proteins [30, 31, 34, 35]. The rate constant (k) is assumed to change strongly with temperature according to the Arrhenius equation:

$$k = \exp\left[-\frac{E_A}{R}\left(\frac{1}{T}-\frac{1}{T^*}\right)\right] \tag{2}$$

where E_A is the activation energy and T^* is the temperature at which k=1 min^{-1}. According to this model the excess heat capacity can be described as:

$$C_p^{ex} = \frac{\Delta_N^F H E_A}{R T_m^{*2}} \exp\left(\frac{E_A (T - T_m^*)}{R T_m^{*2}}\right) \cdot \exp\left(-\exp\left(\frac{E_A (T - T_m^*)}{R T_m^{*2}}\right)\right) \tag{3}$$

where C_p^{ex} is the excess heat capacity, E_A is the activation energy, $\Delta_N^F H$ is the fit parameter corresponding to the overall enthalpy of the irreversible process and T_m^* is the temperature of the maximum of the heat capacity profile. A fit of the data to Eq. (3) permits to obtain all three parameters, $\Delta_N^F H$, T_m^*, and E_A [27].

Heat denaturation studies using CD spectroscopy

For heat denaturation studies, self-constructed CD cuvettes that allow for pressure regulation (1–4 bar) on the sample were used to extend studies up to 100°C. The path length of the cuvettes was chosen according to the concentration of the protein; typically, 0.1 cm cuvettes were used for concentrations between 0.5–1 mg mL^{-1} and 1 cm cuvettes for concentrations below 0.1 mg mL^{-1}. The ellipticity of the samples was measured at 222 nm using a Circular Dichroism spectrometer from Jobin Yvon CD6, Paris, France. For temperature dependent studies heating rates of typically 0.25 to 2°C min^{-1} were used. The value of [Θ], the Mean Residue Ellipticity (MRE) [deg cm^2 $dmol^{-1}$], was calculated using:

$$[\Theta]=\frac{\Theta M_w}{10ncl}$$

where Θ is ellipticity in mdeg, M_w=58549 g mol^{-1} is the molar mass of the protein, n=512 is the number of amino acids in the protein sequence, c is the protein concentration in g/L, and l is the path length in cm.

In all experiments ellipticity values were corrected for buffer absorption. CD spectra were usually averages over five scans.

DLS measurements

DLS measurements were performed using the dynamic light scattering system DynaPro equipped with the temperature controlled micro sampler (Protein Solutions, Charlottesville VA, U.S.A.). Isothermal DLS measurements were performed at 20°C on both native and Ca-depleted enzyme. Heating measurements were only performed on the metal-free protein due to temperature limitations of the instrument. Data were collected every 3°C. Temperature increase between measurements was fast, however, data collection was only started after an equilibration period of 5 min.

Results and discussion

Purification

The purity and the monomeric state of the protein were controlled by SDS-PAGE and DLS measurements, respectively. The purified sample appeared as a single band in SDS-PAGE. Using Dynamic Light Scattering on 0.05 mg mL^{-1} protein solutions in Tris–HCl we determined a radius of 3.13±0.39 nm for the purified native enzyme while the unpurified industrial sample exhibited an average radius of 6.39±1.02 nm. The radius obtained from the DLS measurements for the purified native BLA is in good agreement with the value of r=3.2±0.2 nm reported by Fitter *et al.* [11].

DSC results

To characterize the thermodynamic properties of native BLA (i.e. BLA after the purification without addition or removal of Ca-ions), thermal denaturation of the native sample was studied using differential scanning calorimetry. The results showed that native BLA has a melting temperature of 77.5°C. To examine the reversibility of the unfolding reaction a second run was always performed on the same sample after cooling. The results showed that native BLA has a melting temperature of 77.5°C. The second run with the same sample showed essentially no apparent excess heat capacity suggesting that the denaturation process is practically irreversible. Aggregation of the protein after the scan was evident by the turbidity of the solution.

It was emphasized in the Introduction that calcium ions assume an important role in maintaining structure, stability and function of BLA. As one of the aims of the present study was to investigate the effect of Ca^{2+} on structure and stability of BLA, DSC measurements were also performed on Ca^{2+}-free BLA. Ca^{2+} was removed by extensive dialysis against Tris HCl 20 mM/20 mM EGTA, pH=8.0. To avoid interaction of EGTA with the DSC cells, EGTA was removed from the protein sample before the DSC measurements by dialysis against EGTA-free buffer, with at least 2 changes of the equilibrium buffer.

The results showed that Ca^{2+}-removal decreases T_m of the protein by 18°C (Fig. 2A) in comparison to T_m of native BLA (Fig. 2B).

As in the industrial protein sample the Ca^{2+}-binding sites were not saturated, the purified protein was dialyzed against 3 mM $CaCl_2$ to accomplish saturation of all the binding sites. As expected, the DSC results showed an increase in T_m of about 20 degrees relative to native protein (Fig. 2C).

Because irreversible reactions, such as aggregation, are kinetically determined processes, the relation between the rate constants and the experimental heating rates is decisive for the shape of the apparent heat capacity peaks. Furthermore, due to the fact that aggregation is related to an interaction of at least two particles, the process depends on protein concentration. To study these effects DSC measurements on Ca-free BLA were made using different heating rates and different protein concentrations. The results of these measurements are shown in Figs 3 and 4.

When plotting the T_m values *vs*. heating rate it is evident from Fig. 3 that T_m tends to get independent

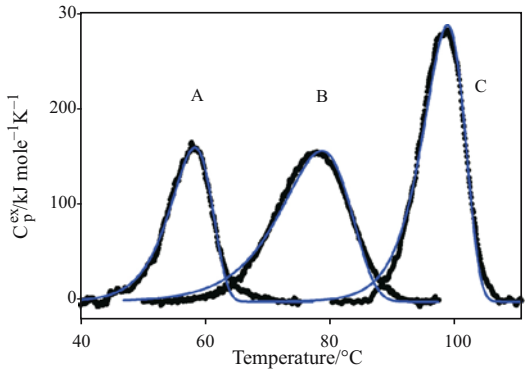

Fig. 2 Experimental excess molar heat capacities of BLA. Lines refer to fits according to Eq. (3). A – Ca-free BLA; pH 8.0, heating rate 1°C min^{-1}. B – purified native BLA; pH 8.0, heating rate 1°C min^{-1}. C – native BLA after equilibrium dialysis against 3 mM $CaCl_2$ over-night (0.5 mg mL^{-1}), pH 8.0, heating rate 2°C min^{-1}

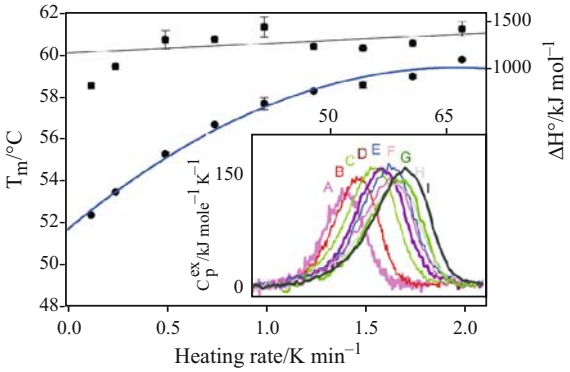

Fig. 3 Variation of ● – apparent transition temperature T_m and ■ – apparent transition enthalpy ΔH_0 of Ca-free BLA with heating rate. A, B, C, D, E, F, G, H, I are measurements performed using 0.125, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75 and 2°C min^{-1}. Protein concentration was 0.84 mg mL^{-1}, pH 8.0. The resulting thermodynamic values are summarized in Table 1

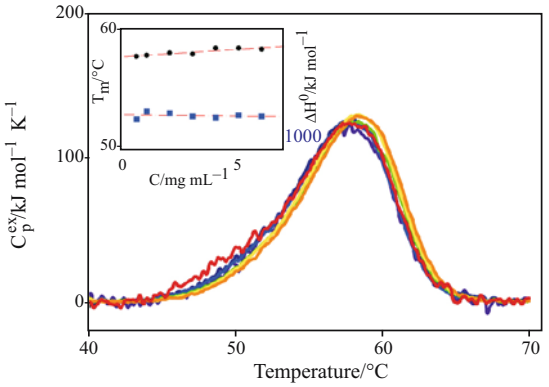

Fig. 4 Test of concentration dependence of heat capacity profiles of Ca-free BLA; concentrations: 0.55, 1, 2, 3, 4, 5, and 6 mg mL^{-1}; heating rate: 1°C min^{-1}; pH 8.0. The inset shows ● $-T_m$ and ■ $-\Delta H^0_{cal}$ as function of BLA concentration

of heating rates at rates above approximately 1.5°C min^{-1}. Due to instrument limitations we could not use heating rates higher than 2°C min^{-1}. The scan-rate dependence of the DSC transition curves is clearly suggestive of kinetic control governing the overall denaturation process.

Table 1 summarizes the apparent thermodynamic parameters obtained from integrations and fits of the heat capacity curves.

The concentration dependence of T_m and ΔH^0_{cal} was tested using Ca-depleted BLA in DSC studies employing a heating rate of 1°C min^{-1}. Inspection of Fig. 4 shows that the C_p transition curves are practically independent of concentration between 0.5 and 6 mg mL^{-1}. The differences in the apparent thermodynamic values of T_m and ΔH^0_{cal} are within the margins of the experimental error.

Therefore we can conclude that the aggregation occurring during the heat denaturation of BLA does not have any significant effect on the T_m – or enthalpy values at 1 °C min^{-1}.

Transition behavior of BLA as a function of Ca-ion concentration

To investigate whether Ca-depleted BLA could be fully renatured by Ca^{2+}-ion addition the protein was dialyzed extensively against different Ca^{2+} concentrations over two nights with three changes of the buffer prior to the DSC measurements. Ca^{2+} concentrations between 0.1 and 100 mM were chosen for the experiments.

Figure 5 shows the corresponding excess molar heat capacity curves. It is obvious that even after extensive dialysis against high concentrations of Ca^{2+} the enzyme does not fully resume native-state stability.

The following observations are intriguing: First, increasing Ca-concentration results in the appearance

Table 1 Heating rate dependence of apparent thermodynamic parameters of Ca-free BLA. DSC measurements were performed in Tris–HCl 20 mM, pH 8.0. Protein concentration was 0.84 mg mL^{-1} for all measurements. Columns 2 and 3 show the apparent calorimetric T_m values and the enthalpy obtained from integration of the C_p peak. Columns 4 to 6 show the parameters obtained from fitting the data to Eq. (3). Error limits of $\Delta^F_N H$ and E_A are 5%

Heating rate/ °C min^{-1}	T_m/°C	ΔH_{cal}/kJ mol^{-1}	T_m/°C	$\Delta^F_N H$/kJ mol^{-1}	E_A/kJ mol^{-1}
0.125	52.4	811	52.5	718	365
0.250	53.5	1021	54.2	986	335
0.500	55.3	1314	56.2	1211	297
0.750	56.7	1318	57.3	1230	294
1.00	57.7	1449	58.2	1429	276
1.25	58.3	1241	58.2	1183	278
1.5	58.6	1218	59.2	1159	289
1.75	59.0	1273	58.9	1151	272
2.0	59.8	1429	60.1	1367	267

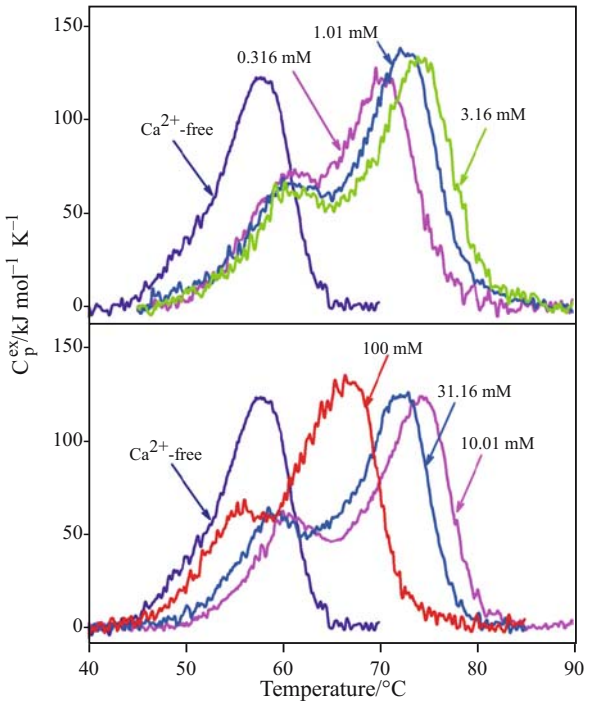

Fig. 5 Excess molar heat capacity of Ca-depleted BLA after equilibrium dialysis against different concentrations of $CaCl_2$, pH 8.0. The Ca^{2+} concentrations are indicated by the numbers in the graph. All measurements were performed using a scan rate of 1°C min^{-1}

of a low temperature shoulder and a high temperature C_p peak. Second, the complete peak is shifted to higher temperatures with increasing Ca concentration up to about 3 mM. Third, above 3 mM Ca the trend is reversed and the C_p profile shifts back to lower temperatures.

To exclude the possibility that the reason for the lower T_m value in the presence of 100 mM Ca might be slow kinetics of Ca incorporation, the DSC experiment was repeated using an equivalent sample that was dialyzed 7 days longer. There was essentially no difference between the results of the two experiments. This shows unequivocally that equilibrium has been attained after 24 h (Data not shown). The obvious trend of decreasing transition temperatures with increasing Ca^{2+} concentrations above 3 mM must therefore be due to an unspecific Ca^{2+}-ion effect.

To identify the origin of the low temperature shoulder of the heat capacity profile the following strategy was assumed. First the protein was heated from 35 to 65°C in the presence of 10 mM $CaCl_2$, as 65°C is approximately the final temperature of the heat capacity peak of the first transition. Then the sample was cooled to 10°C for maximal renaturation, and a second heating was performed, this time to a final temperature of 90°C. Figure 6 shows the results of these experiments along with a reference experiment performed on an equivalent fresh sample of protein dialyzed against 10 mM $CaCl_2$ and heated to 85°C.

This experiment suggests the presence of two populations of the protein differing by their Ca-ion incorporation. Therefore the denaturation profiles shown in Fig. 5 can be assumed to result from a superposition of two irreversible transitions, each of which can be fitted to the $N \rightarrow F$ model.

The heat denaturation studies on Ca-free BLA reconstituted with Ca^{2+}-ions show an important result. Ca-depleted protein does not return to the native state even after excessive dialysis *vs.* high concentrations of calcium. Thus removal of Ca-ions provokes obviously changes in protein structure that can not be reversed completely.

This conclusion is in excellent agreement with findings of Machius *et al.* [5] who suggested on the basis of X-ray studies that it is not possible to remove CaI and CaII without introducing significant structural changes. Our studies demonstrated that these changes are obviously irreversible.

Although alignment of the structures of Ca-free (PDB # 1BPL) and native BLA (PDB # 1BLI) shows an overall similarity, there is one significant difference in that a loop (amino acids 178–189) is missing in the Ca-free 1BPL structure [32].

Further differences between the structures of the metal-free form and the metal-containing form of BLA can be found in the regions around the ligand ions. The most striking of these differences is the ordering of the segment between residues 182 and 192, which contains the metal-liganding residue (Asp183) and a *cis* peptide bond between residues Trp184 and Glu185. *Cis* peptide bonds are rare and, if

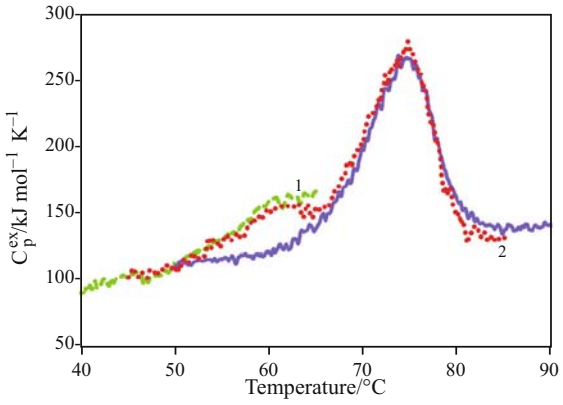

Fig. 6 Heating cycles for the identification of the low temperature shoulder. 1 – grene dashed curve: first heating to 65°C of Ca-free BLA equilibrated with 10 mM $CaCl_2$; blue solid curve: second heating of the same sample to 90°C after renaturation at 10°C. The low temperature peak has disappeared. 2 – Red dots: reference DSC measurement on a fresh sample of Ca-free BLA equilibrated with 10 mM $CaCl_2$ to 85°C. pH 8.0, protein concentration: 0.95 mg mL^{-1}, heating rate 1°C min^{-1}

Table 2 Summary of thermodynamic values obtained from deconvolution of the DSC curves shown in Fig. 5. Error limits of $\Delta^F_N H$ and E_A are 5%

	$T^*_{m_1}$/°C	$\Delta^F_N H_1$/kJ mol^{-1}	E_{A_1}/kJ mol^{-1}	$T^*_{m_2}$/°C	$\Delta^F_N H_2$/kJ mol^{-1}	E_{A_2}/kJ mol^{-1}
Ca-free	58.2	1299	283	–	–	–
0.3 mM	59.7	361	307	70.2	1379	231
1.01 mM	60.2	482	259	72.5	1474	245
3.16 mM	60.8	439	271	74.0	1481	235
10.01 mM	60.9	418	287	73.9	1269	257
31.16 mM	59.3	455	262	71.9	1255	264
100 mM	54.9	283	333	66.2	1502	230

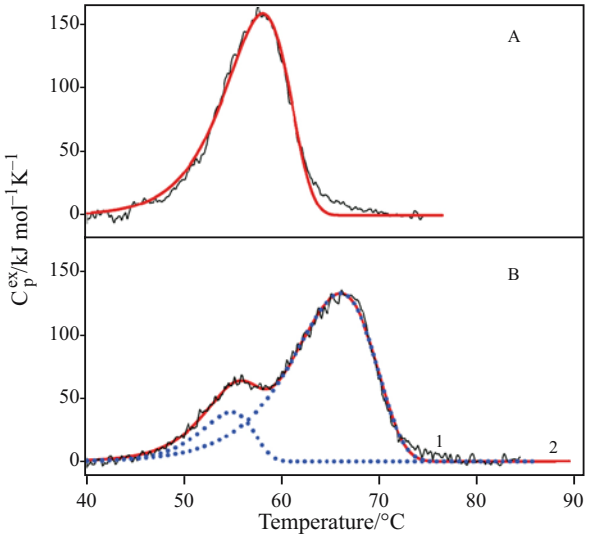

Fig. 7 Deconvolution of transition profiles A – DSC curve of Ca-free BLA and fit. B – DSC experiment and fit curves of Ca-free BLA in presence of 100 mM $CaCl_2$. 1 – refers to the original data and 2 – is the superposition of the two independent irreversible transitions (dotted curves) analyzed according to Eq. (3). The thermodynamic and kinetic information obtained from these fits is shown in Table 2

they occur, generally assume an important structural function. The ordered residues form a large loop-like structure which is positioned between domain A and the complex β-sheet in domain B, partially filling the large space between these two domains. In addition to the disorder-to-order transition, the segments adjacent to the newly ordered region exhibit drastically different conformations depending on whether metal ions are bound or not. The segment comprising residues 178–182 is characterized by a dislocation of the main chain so that an ionic interaction between Lys180 and the metal-liganding Asp202 can be established, indicating the importance of this interaction for the stabilization of the metal-ligand sphere [5].

Residues 193–199 adopt a helical conformation in Ca-free BLA, but become unwound and wrapped around the metal ions in a rather irregular fashion in native BLA. This extended conformation, which is presumably energetically less favourable compared to the helical conformation, is stabilized by the presence of the metal ions and a strong hydrogen bond between the hydroxyl group of Tyr195 and Tyr198. In the metal-free form of BLA, the corresponding region is completely disordered [5].

Other studies on BLA have shown that mainly two features contribute to BLA thermostability: a) the region between domain A and domain B which is stabilized by the triadic Ca–Na–Ca metal binding site surrounded by a network of electrostatic interactions and b) additional salt bridges in other regions of the structure [2, 17].

We conclude on the basis of our results that removal of Ca from native BLA changes the structure of the triad binding site in an irreversible manner. Even addition of Ca concentration to a value ten-times the enzyme concentration does either not saturate all binding sites or the triad does not form completely. This interpretation corroborates with the high number of binding sites observed by ITC (17 binding sites, our results not shown) which has also been reported previously [36], and the significantly smaller number of two structurally relevant sites obtained by our Ca-induced denaturation-renaturation studies [32]. Na-ions might also play but a less important role in thermostability of BLA. In principle Ca^{2+}-removal together with the structural changes involved could cause the Na^+-ions to dissociate from the enzyme in an irreversible manner. This hypothesis needs of course further investigation.

The rationalization of the irreversibility of the unfolding transition by taking into account the presence of a *cis*-peptide bond at a critical structural site sheds new light on possible mechanisms of irreversibility of the protein. Besides chemical changes such as deamidation, the restoration of a *cis*-peptide bond after partial folding associated with Ca binding could be responsible for the irreversibility of the reaction. It is well known that activation energies for the *cis-trans* equilibrium are in the order of 90–100 kJ mol^{-1} [37] and they might be even

higher in partially folded proteins if the misfolded structures assume energetic local minima.

CD and DLS unfolding studies

CD measurements permit simultaneous estimates of changes of secondary structure at 222 nm and static light scattering (OD) changes resulting from aggregation. The size of such aggregates can be determined by dynamic light scattering and we monitored their development as a function of temperature. Figs 8A–C show the results of CD temperature scans and dynamic light scattering measurements. Obviously aggregation occurs simultaneously to unfolding, however, the CD changes appear not to be affected by this reaction. The DLS results support the conclusion and demonstrate the strong increase in aggregate size once unfolding has started.

Activity measurements as a function of Ca replenishment

It is of great importance for industrial application to know whether activity of BLA can be reconstituted once the enzyme has lost Ca. From a scientific point of view the question arises whether the observed conformational and stability changes parallel activity changes. Therefore we performed activity tests on Ca^{2+}-depleted BLA samples which were treated with calcium in a similar manner as the DSC samples. To test the effect of Ca ions on the activity of BLA the original test designed for native BLA had to be modified. Unlike the common BLA activity test which has 1 mM $CaCl_2$ in the Brithon Robbinson buffer to saturate the binding sites, the test buffer was made without $CaCl_2$. However, $CaCl_2$ was added to different final concentrations before the overnight incubation step at room temperature. Tests were performed at 60, and 70°C.

The results are shown in Fig. 9. It is evident that maximal activity occurs in the presence of about 1 mM $CaCl_2$. At both higher and lower Ca concentrations the activity decreased.

These experiments show also that activity of Ca-depleted BLA is not fully recovered after addition of Ca to the protein. At best the reconstituted enzymes reach 70% of the activity of the native protein. This conclusion is in agreement with the DSC results. Native protein had always higher T_m values than Ca-depleted BLA in presence of identical Ca concentrations.

Previously Fitter *et al.* [7] had reported that Ca does not have a direct impact on the catalytic mechanism. It was assumed to preserve only enzymatic activity at higher temperatures via a structure stabilizing effect. They also reported that addition of calcium to Ca-free amylase had no effect on the activity at room temperature. Our results are in contrast to their findings. Our finding show clearly that, although overall activity of the native enzyme decreases irreversibly after removal of calcium ions, the addition of extra Ca

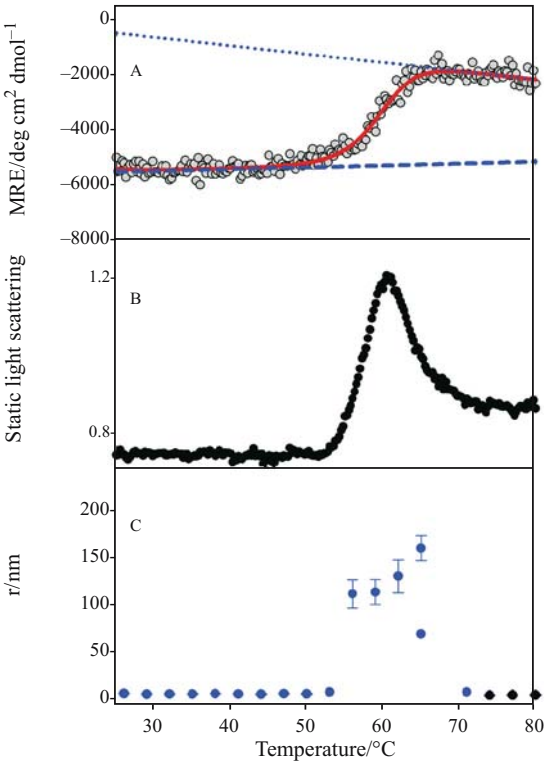

Fig. 8 Changes in A – MRE, B – static light scattering and C – dynamic light scattering of Ca-free BLA as a function of temperature. Protein concentration was 0.5 mg mL^{-1} in the CD scans. The radius of the ● – native protein in the DLS measurements is 3.13±0.39 nm. At temperatures above 75°C the protein is practically fully precipitated and the *r* value drops to that of buffer (◆)

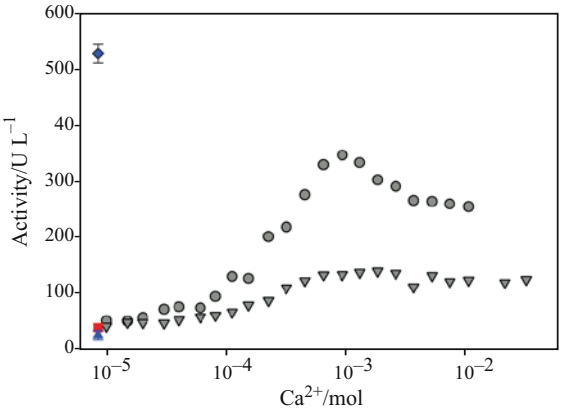

Fig. 9 Activity of Ca-depleted BLA as function of Ca concentration at 60°C (circles), and 70°C (triangles); ■ and ▲ show the reference activity of Ca-free BLA and Ca-free BLA in presence of EGTA in the test solution (±Std.dev.), respectively; ◆ – average activity of native BLA (537±42 UL^{-1})

in the activity test can partially recover BLA activity in the temperature range between 50 and 70.

The reduced activity in the presence of extra Ca ions of reconstituted BLA compared to the activity of the native protein suggests that Ca removal might induce changes in the 3D structure that interfere with re-attachment of Ca ions. Alternatively, the structural changes associated with Ca removal might induce changes in the active site of the enzyme. Although the detailed mechanism of Ca action remains unsolved, the important conclusion can be drawn that changes in the apparent thermodynamic parameters of reconstituted BLA are reflected in the activity of the enzyme.

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