New ice-binding face for type I antifreeze protein

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Abstract **Type I antifreeze protein (AFP) from winter flounder is an alanine-rich, 37 amino acid, single α-helix that contains three 11 amino acid repeats (Thr-X_2-Asx-X_7), where X is generally Ala. The regularly spaced Thr, Asx and Leu residues lie on one face of the helix and have traditionally been thought to form hydrogen bonds and van der Waals interactions with the ice surface. Recently, substitution experiments have called into question the importance of Leu and Asn for ice-binding. Sequence alignments of five type I AFP isoforms show that Leu and Asn are not well conserved, whereas Ala residues adjacent to the Thr, at right angles to the Leu/Asn-rich face, are completely conserved. To investigate the role of these Ala residues, a series of Ala to Leu steric mutations was made at various points around the helix. All the substituted peptides were fully α-helical and remained as monomers in solution. Wild-type activity was retained in A19L and A20L. A17L, where the substitution lies adjacent to the Thr-rich face, had no detectable antifreeze activity. The nearby A21L substitution had 10% wild-type activity and demonstrated weak interactions with the ice surface. We propose a new ice-binding face for type I AFP that encompasses the conserved Ala-rich surface and adjacent Thr.**

Key words: Antifreeze protein; α-Helix; Thermal hysteresis

1. Introduction

Antifreeze proteins (AFPs) are present in many organisms that must survive in sub-zero environments (for reviews, see [1–4]). AFPs depress the non-equilibrium freezing point below the melting point by binding to nucleating ice crystals to prevent their growth. AFPs were first found in fish that live in sub-zero Antarctic waters [5,6]. Five structurally different types of AFPs have been found in fish: the antifreeze glycoproteins and four protein types, I, II, III and IV [3,4,7]. Type I AFP found in shorthorn sculpin, winter flounder and their close relatives is the smallest and simplest of these AFPs [8]. It is a single 3–5 kDa α-helix that exists in solution as a monomer [9]. Helicity is maintained by its high Ala content (>60%), internal salt bridges and N- and C-terminal cap structures [10]. The most extensively studied type I AFP is the 37 amino acid long high performance liquid chromatography (HPLC)-6 isoform from winter flounder [11]. HPLC-6 is typical of the isoforms that contain three 11 amino acid repeats of Thr-X_2-Asx-X_7 where X is generally alanine (Fig. 1) [12–14]. The 11 amino acid repeat places the Thr residues 16.5 Da apart on the same side of the helix to match the 16.7 Da spacing between the oxygen atoms in the ⟨0 1-1 2⟩ direction on the {2 0-2 1} plane of ice. This plane has been defined by ice etching as the type I AFP-binding surface [15]. A number of reports have implicated the Thr hydroxyl in direct hydrogen bonding interactions, either with the ice surface [15–18] or embedded within the ice lattice to increase the number of potential hydrogen bonds [19]. The role of the *i*+3 Asx residue is more ambiguous. Sicheri and Yang [10] have suggested that it acts together with the *i*−1 Leu to stabilize the Thr residue and form an ice-binding motif (IBM) that may make hydrogen bonds [12] and van der Waals (VDW) interactions [16] with the ice surface.

Recently, the contribution of hydrogen bonding to the energetics of binding the ice surface has been questioned. Thr to Val replacements in the internal IBMs of HPLC-6 resulted in retention of near wild-type antifreeze activity, while Thr to Ser replacements produced a dramatic loss of antifreeze activity [20–23]. Conceptually, it has been suggested that the Thr γ-methyl group might interact with the ice surface through VDW interactions and play a greater role in ice-binding than the OH group. Also, recent replacement studies implicate Asn and Leu in helix solubility rather than direct ice-binding [24]. These studies have led us to ask if the traditional ice-binding surface (involving Leu, Asn and the Thr OH group) has been misidentified. Based on the conservation of Ala residues in numerous type I AFP isoforms, and the importance of the Thr methyl group to ice-binding, we hypothesize that the ice-binding surface of type I AFP comprises the Thr side chains and the conserved *i*+4 and *i*+8 Ala residues. To test this hypothesis, we have substituted Leu for Ala at positions around the helix to identify the ice-binding site by steric interference.

2. Materials and methods

2.1. AFP synthesis and purification

Type I AFP and its variants were synthesized by solid-phase peptide synthesis as described previously [9,25]. Peptide concentrations were determined by amino acid analysis. Samples were hydrolyzed (6 N HCl at 160°C for 1.5 h) and analyzed in a Beckman Model 6300 amino acid analyzer (San Ramon, CA, USA). Norleucine was used as an internal reference to correct the amount of each identified amino acid.

2.2. Circular dichroism (CD) spectroscopy

CD spectra were collected as previously described [26] using a Jasco

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Abbreviations: AFP, antifreeze protein; CD, circular dichroism; HPLC, high performance liquid chromatography; IBM, ice-binding motif; VDW, van der Waals

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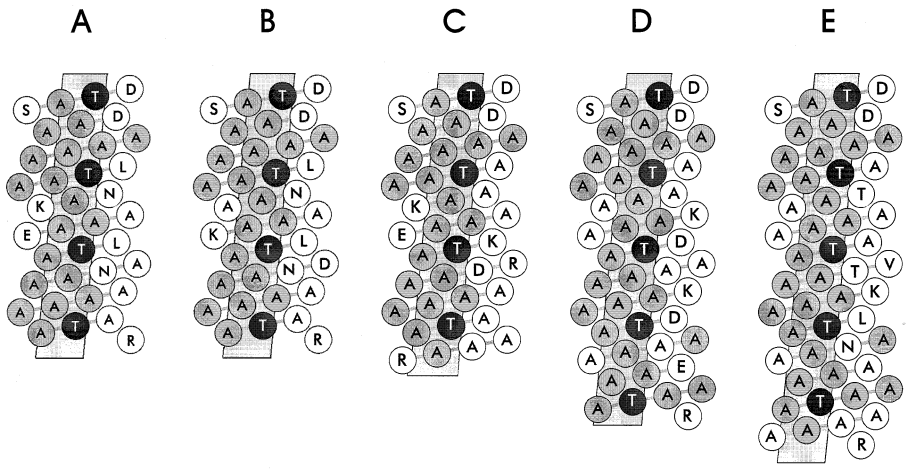

Fig. 1. Helical net representations of right-eye flounder type I AFP isoforms. (A) HPLC-6 from winter flounder [11]; (B) HPLC-8 from winter flounder [36]; (C) Alaskan plaice [10]; (D) yellow-tail flounder [37]; (E) AFP-9 from winter flounder [26]. Conserved Ala and Thr residues are shown in gray and black, respectively. The light gray parallelogram in the background represents the new putative ice-binding face of the helix.

J-500C spectropolarimeter (Jasco, Easton, MD, USA). The helicity of the protein was monitored at 222 nm during temperature denaturation studies. Each data point was the average of a minimum of 24 readings. The buffer used was 50 mM potassium phosphate (pH 7.0) in 50 mM KCl.

2.3. Sedimentation equilibrium ultracentrifugation

Sedimentation equilibrium ultracentrifugation experiments were carried out on a Beckman XL-I ultracentrifuge as previously described [20].

2.4. Antifreeze activity and photomicroscopy

Thermal hysteresis was measured using a nanoliter osmometer (Clifton Technical Physics, Hartford, NY, USA) as described by Chakrabartty and Hew [27]. Thermal hysteresis is defined as the temperature difference (°C) between the melting point and the non-equilibrium freezing point of a solution. Ice growth of more than 0.2 μm/s signifies that the solution freezing point has been reached or exceeded. Ice crystal morphology was observed using a Leitz 22 microscope and recorded by a Panasonic CCTV camera linked to a JVC Super VHS video recorder. Still images were obtained from a Silicon Graphics INDY terminal using IRIS Capture version 1.2.

3. Results and discussion

Comparison of the primary sequences of five type I isoforms from right-eye flounders shows that Leu and Asx of the putative IBMs are not well conserved (Fig. 1). Leu occurs in the $i-1$ position (relative to Thr) only 22.5% of the time, while Asp and Asn make up 32% and 26% of the residues in the $i+3$ position (Fig. 2). In contrast, there is 100% conservation of the Thr residues and of Ala residues at several positions ($i+4$, $i+7$, $i+8$) along one face of the α-helix. The conservation of this face is especially evident when viewed as a helical wheel (Fig. 2). The importance of the conserved Ala residues has largely been overlooked prior to this study. These observations, along with recent evidence indicating that hydrogen bonding is less important than previously thought [19–22], have focussed our attention on the conserved Ala-rich face. In order to test the importance of this face for ice-binding, we made a series of Ala to Leu replacements in the HPLC-6 isoform that would produce steric effects at different positions around the α-helix (Fig. 2) to disrupt ice-binding interactions. This strategy of introducing steric mutations was previously used to identify the ice-binding faces of type II and type III AFPs [28,29]. For type I AFP, the Ala to Leu replacements were chosen (at positions 17, 19, 20 and 21) to fall between the two central IBMs of the HPLC-6 isoform and hence maximize potential disruption to ice-binding. Amongst the five isoforms, there is 100% conservation of Ala at positions 17, 20 and 21, and 72% conservation at position 19.

To verify that any loss of activity observed with the replacements was not due to aggregation or conformational changes caused by the Ala to Leu substitutions, sedimentation equilibrium ultracentrifugation and CD spectroscopy were carried out [20] (Table 1). Sedimentation equilibrium centrifugation showed that the peptides were not aggregating or forming higher order oligomers at the concentrations used for thermal hysteresis measurements. All of the samples fit best to a single-species (monomer) and the experimental molecular weights of the peptides were close to the calculated values. CD spectroscopy produced spectra with molar elipticity minima at 222 nm for all the peptides. Based on molar elipticity values, peptides with Ala to Leu substitutions had helicity equivalent to, or greater than, the wild-type (Table 1). These results indicate the Ala to Leu substitutions do not disrupt the α-helical conformation of the peptides. Therefore, any deviations observed in activity were presumably due to the Leu

Table 1
Molecular weight determination by sedimentation equilibrium analysis and the observed molar elipticity at 1°C

Sample	Molecular weight		$[\Theta]_{222\,nm}$ (° cm^2 dmol^{-1})
	Observed	Calculated	
A17L	3189 (±58)	3285	−33820
A19L	3011 (±53)	3285	−39770
A20L	3107 (±65)	3285	−38700
A21L	2956 (±55)	3285	−40650
Wild-type (HPLC-6)	3021 (±61))	3243	−37220

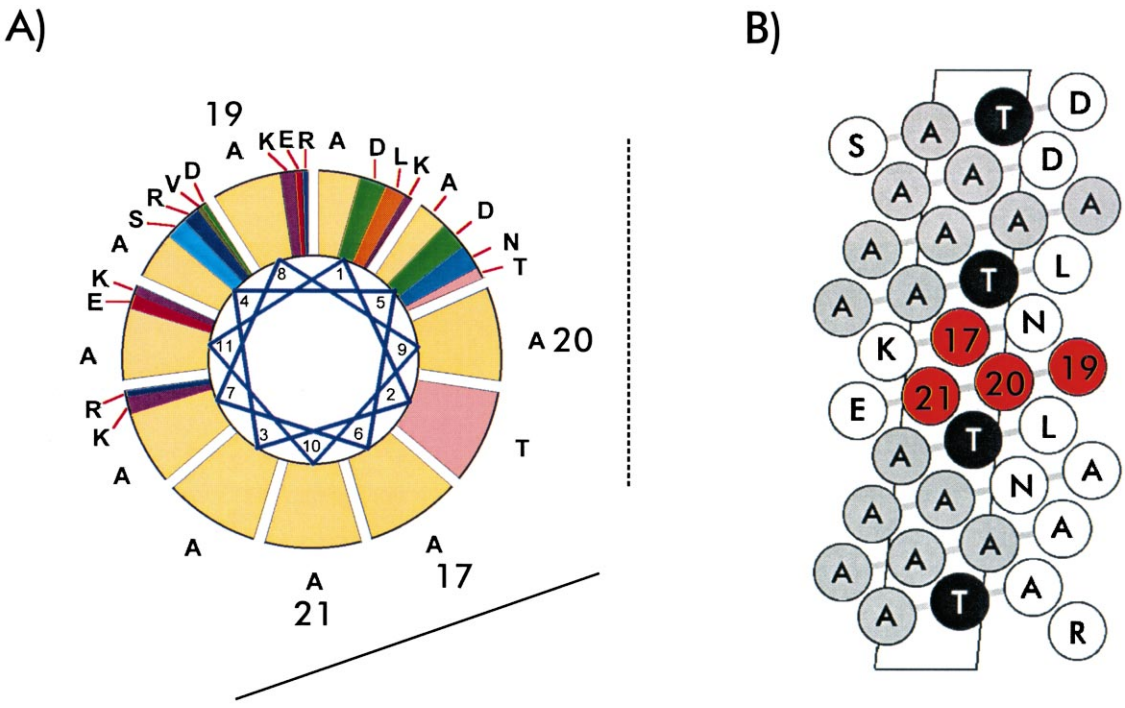

Fig. 2. (A) Helical wheel representation of the five right-eye flounder isoforms superimposed to form a pie diagram. The area of shading indicates the frequency of naturally occurring amino acid substitutions. The relative positions of the Ala to Leu replacements around the α-helix are denoted by amino acid number 17, 19, 20 or 21. The putative ice-binding face via the IBM [13] is denoted by a dashed line, the new ice-binding face as mapped by the Ala to Leu mutations in this study is indicated by a solid line. (B) Helical net representation of the HPLC-6 isoform. The positions of the Ala to Leu replacements are shown in orange.

residues sterically interfering with ice-binding and not due to solubility or conformational changes caused by the substitutions.

Antifreeze activity of the mutant and wild-type peptides was analyzed at concentrations up to 8.0 mg/ml (Fig. 3). Variants A19L and A20L had close to wild-type activity across the entire concentration range. These mutant peptides produced small, hexagonal bipyramidal ice crystals that did not grow in size prior to the end point of the assay (Fig. 4). In this regard, they were identical to ice crystals produced by wild-type HPLC-6. In contrast, A17L had no thermal hysteresis activity over the entire concentration range assayed and no influence on ice crystal morphology. Ice crystals formed in its presence grew as disks perpendicular to the *c*-axis as the solution was cooled (Fig. 4). This ice crystal morphology is seen with solutions lacking AFPs, indicating that the A17L substitution abolished all interactions with the ice surface. Unlike A17L, A21L showed weak thermal hysteresis activity and did produce hexagonal bipyramids. These grew continuously when the temperature was lowered beyond the freezing point and maintained a c:a ratio of 3.2:1 (Fig. 4). This is the same ratio seen with wild-type HPLC-6, which suggests that the A21L peptide was interacting with the same ice plane as wild-type AFP. Thus, although A21L was interacting with the ice surface, it could not prevent ice crystal growth under these conditions.

Although several laboratories have in total synthesized over 40 variants of HPLC-6 to probe its structure-function relationships [20–23,30–33], as reviewed by Harding et al. [8], only a few substitutions have caused the profound activity loss seen with A17L and A21L. With one exception [30], the substitutions can all be linked to one surface of the helix, approximately 100° from the traditional ice-binding face comprised of Thr, Asx and Leu of the IBM [10]. In the one exception [30], the loss of helicity caused by a proline substitution (A20P) provides a trivial explanation for the inactivity of this variant.

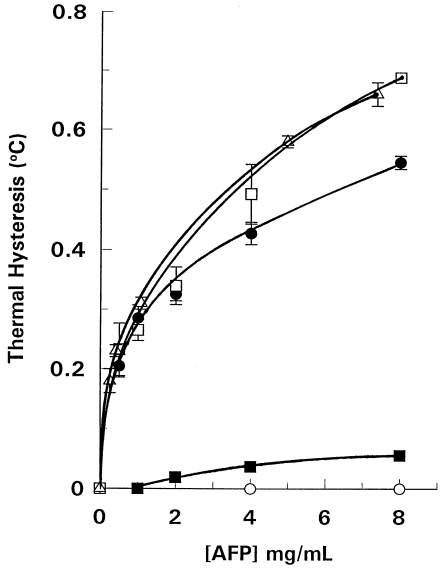

Fig. 3. Thermal hysteresis activity as a function of the concentration of A17L (open circles), A19L (closed circles), A20L (open squares), A21L (closed squares) and wild-type (open triangles). Each data point represents the mean of at least three determinations and the vertical bars represent the S.D.

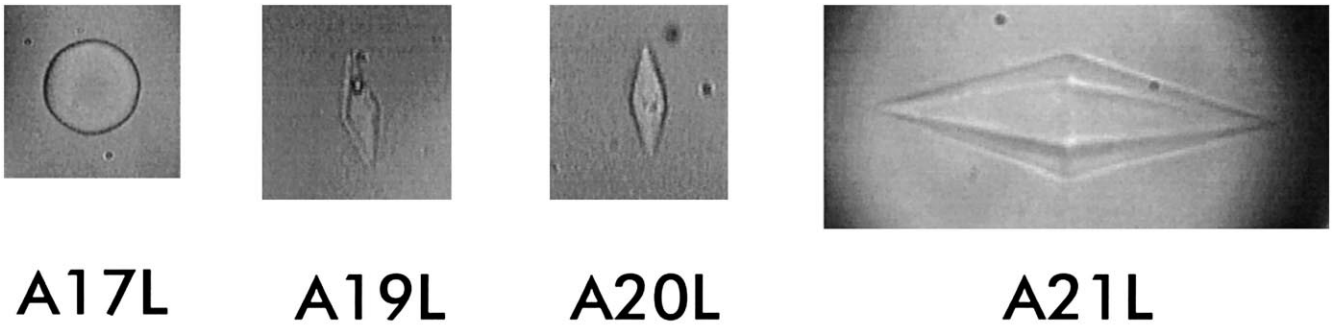

Fig. 4. Ice crystal morphology of the type I AFP variants. Ice crystals were formed in the presence of 9 mg/ml A17L and A21L, or 1 mg/ml A19L and A20L in 0.1 M NH_4HCO_3 (pH 7.9). A17L and A21L images were taken after 0.02°C undercooling; the A19L and A20L images were taken after 0.2°C undercooling.

Previous work by Wen and Laursen [31] produced Ala to Leu or Gln substitutions at position 17 and 28 in a background of other amino acid substitutions (peptides S41, S42 and S52). Like the A17L and A21L single substitutions, all three of these mutant peptides had no antifreeze activity. The explanation provided for the activity loss by Wen and Laursen was that the bulky substituents prevented side-by-side associations of the type I AFP, which were felt to be essential for cooperative binding to ice [16]. However, it has been subsequently shown that AFPs can bind independently to ice [34]. Although the effect of additional substitutions complicates interpretation of Wen and Laursen's results, we suggest that the loss of activity seen with the Leu- or Gln-substituted type I AFP reflects direct steric interference with ice-binding at position 17 and its equivalent, 28.

Recently, Zhang and Laursen produced synthetic antifreeze peptides based on type I AFP, each of which contained six lysines in a poly-alanine helix [35]. Two of the peptides, AKAAK and LKAAK, had (L)KAAK motifs recurring at 11 amino acid intervals, which produced an amphipathic helix with Lys-rich and Ala-rich faces. The other peptide, poly-AK had Lys residues projecting from every face of the helix. Both AKAAK and LKAAK showed weak activity, which supports our contention that the Ala-rich face of the helix is a critical component of the ice-binding surface. In contrast, poly-AK was completely inactive, which is consistent with the evidence reported here that long side chains projecting from the Ala-rich surface can sterically prevent AFP-binding to ice.

The Ala to Leu substituents have redefined the ice-binding face of type I AFP, which we propose involves Thr-13, Ala-17 and Ala-21, and equivalent residues at 11 amino acid intervals along the helix. The Thr γ-methyl group has shown to be essential from the retention of activity in Thr to Val substituents [20–23]. Ala-17 is also a critical residue, but Ala-21 likely lies on the periphery of the new ice-binding face as the A21L peptide still interacts with the ice surface. This plane coincides with the helix face that is conserved amongst the flounder type I isoforms. We propose that this conserved Ala- and Thr-rich helix quadrant binds to the {2 0-2 1} planes of ice in the ⟨0 1-1 2⟩ directions largely because of surface to surface complementarity. In this regard, it should be noted that the argument for a steric match between residues with an 11 amino acid repeat distance (16.5 Da) and the 16.7 Da ice lattice repeat on this pyramidal plane applies equally well to any residue or structural feature of the helix with 11 amino acid spacing. Molecular modeling using Sybyl 6.5 (Tripos Associates) has been carried out to simulate binding of the Ala- and Thr-rich face to the {2 0-2 1} ice planes (personal communication, Dr. M. Kuiper). The peptide was successfully placed in the ⟨0 1-1 2⟩ directions using manual docking followed by energy minimization using the Tripos force field with Gasteiger and Marsali charges and a distant-dependent dielectric constant, gradient terminated at 0.05 kcal/mol. Although the nature and relative contribution of the forces binding AFP to ice are still being debated, they likely involve VDW interactions, hydrogen bonds and the displacement of water from the protein and ice surfaces into the bulk solvent. Identifying the correct ice-binding surface is a critical step towards modeling the interaction and deducing contributions to binding energetics.

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References

[1] Cheng, C.C. and DeVries, A.L. (1991) in: Life Under Extreme Conditions (di Prisco, G., Ed.), pp. 1–14, Springer-Verlag, Berlin.
[2] Yeh, Y. and Feeney, R.E. (1996) Chem. Rev. 96, 601–617.
[3] Davies, P.L. and Sykes, B.D. (1997) Curr. Opin. Struct. Biol. 7, 828–834.
[4] Ewart, K.V., Lin, Q. and Hew, C.L. (1999) Cell. Mol. Life Sci. 55, 271–283.
[5] DeVries, A.L. and Wohlschlag, D.E. (1969) Science 163, 1073–1075.
[6] DeVries, A.L., Komatsu, S.K. and Feeney, R.E. (1970) J. Biol. Chem. 245, 2901–2913.
[7] Deng, G., Andrews, D.W. and Laursen, R.A. (1997) FEBS Lett. 402, 17–20.
[8] Harding, M.M., Ward, L.G. and Haymet, A.D.J. (1999) Eur. J. Biochem. 264, 653–665.
[9] Gronwald, W., Chao, H., Reddy, D.V., Davies, P.L., Sykes, B.D. and Sönnichen, F.D. (1996) Biochemistry 35, 16698–16704.
[10] Sicheri, F. and Yang, D.S.C. (1995) Nature 375, 427–431.
[11] Fourney, R.M., Fletcher, G.L. and Hew, C.L. (1984) Can. J. Zool. 62, 28–33.
[12] DeVries, A.L. and Lin, Y. (1977) Biochem. Biophys. Acta 495, 388–392.
[13] Davies, P.L., Roach, A.H. and Hew, C.L. (1982) Proc. Natl. Acad. Sci. USA 79, 335–339.

[14] Pickett, M., Scott, G.K., Davies, P.L., Wong, N., Joshi, S. and Hew, C.L. (1984) Eur. J. Biochem. 143, 35–38.
[15] Knight, C.A., Cheng, C.C. and DeVries, A.L. (1991) Biophys. J. 59, 409–418.
[16] Wen, D. and Laursen, R.A. (1992) Biophys. J. 63, 1659–1662.
[17] Chou, K.-C. (1992) J. Mol. Biol. 223, 509–517.
[18] Cheng, A. and Merz, K.M. (1997) Biophys. J. 73, 2851–2873.
[19] Knight, C.A., Driggers, E. and DeVries, A.L. (1993) Biophys. J. 64, 252–259.
[20] Chao, H., Houston Jr., M.E., Hodges, R.S., Kay, C.M., Sykes, B.D., Loewen, M.C., Davies, P.L. and Sönnichsen, F.D. (1997) Biochemistry 36, 14652–14660.
[21] Haymet, A.D.J., Ward, L.G., Harding, M.M. and Knight, C.A. (1998) FEBS Lett. 430, 301–306.
[22] Zhang, W. and Laursen, R.A. (1998) J. Biol. Chem. 273, 34806–34812.
[23] Haymet, A.D.J., Ward, L.G. and Harding, M.M. (1999) J. Am. Chem. Soc. 121, 941–948.
[24] Loewen, M.C., Chao, H., Houston Jr., M.E., Baardsnes, J., Hodges, R.S., Kay, C.M., Sykes, B.D., Sönnichsen, F.D. and Davies, P.L. (1999) Biochemistry 38, 4743–4749.
[25] Hodges, R.S., Semchuck, P.D., Taneja, A.K., Kay, C.M., Parker, J.M.R. and Mant, C.T. (1988) Pept. Res. 1, 19–30.
[26] Chao, H., Hodges, R.S., Kay, C.M., Gauthier, S.Y. and Davies, P.L. (1996) Protein Sci. 5, 1150–1156.
[27] Chakrabartty, A. and Hew, C.L. (1991) Eur. J. Biochem. 202, 1057–1063.
[28] Loewen, M.C., Gronwald, W., Sönnichsen, F.D., Sykes, B.D. and Davies, P.L. (1998) Biochemistry 37, 17745–17753.
[29] Chao, H., Sonnichsen, F.D., DeLuca, C.I., Sykes, B.D. and Davies, P.L. (1994) Protein Sci. 3, 1760–1769.
[30] Wen, D. and Laursen, R.A. (1992) J. Biol. Chem. 267, 14102–14108.
[31] Wen, D. and Laursen, R.A. (1993) J. Biol. Chem. 268, 16396–16400.
[32] Wen, D. and Laursen, R.A. (1993) J. Biol. Chem. 268, 16401–16405.
[33] Chakrabartty, A., Ananthanarayanan, V.S. and Hew, C.L. (1989) J. Biol. Chem. 264, 11307–11312.
[34] DeLuca, C.I., Comley, R. and Davies, P.L. (1998) Biophys. J. 74, 1502–1508.
[35] Zhang, W. and Laursen, R.A. (1999) FEBS Lett. 455, 372–376.
[36] Pickett, M., Scott, G.K., Davies, P.L., Wang, N., Joshi, S. and Hew, C.L. (1984) Eur. J. Biochem. 143, 35–38.
[37] Scott, G.K., Davies, P.L. and Fletcher, G.L. (1987) Eur. J. Biochem. 168, 629–633.