Zinc-Induced Conformational Transitions of Acidic Peptides: Characterization by Circular Dichroism and Electrospray Mass Spectrometry

Odile Hénin, Bernard Barbier, François Boillot, and André Brack*[a]

Abstract: A series of amphiphilic peptides have been synthesized which have a defined chain length and are based either on the dipeptide periodicity Asp-Leu or the tetrapeptide periodicity Leu-Asp-Asp-Leu. Their behavior in the presence of low concentrations of metal ions was studied by circular dichroism spectroscopy. In pure water, the peptides adopt a random coil conformation. The addition of Zn^{2+} specifically induces a β-sheet structure for $(Asp\text{-}Leu)_n$ and an α-helix structure for $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_n$-Asp. The conformational transitions are dependent on the chain length: the critical main chain length for β-sheets and α-helix formation is between 10 and 24 residues, and between 13 and 25 residues, respectively. The addition of NH_4^+ and Mg^{2+} have no effect, whereas Ca^{2+} has only a slight effect on the conformation. The peptide $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_8$-Asp complexed to Zn^{2+} was used in circular dichroism (CD) spectroscopic studies to investigate the self-association of α-helices as a function of the temperature. The Zn^{2+} complexes were analyzed by electrospray mass spectrometry. As expected from the CD studies, the binding of Zn^{2+} is dependent on the chain length. At 0.4 equiv Zn^{2+}/Asp, $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_8$-Asp binds a maximum of five zinc ions, which is less than the theoretical value of eight expected from the peptide sequence. $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_6$-Asp and $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_3$-Asp bind two and one zinc ions, respectively, without the formation of an α-helix.

Keywords: circular dichroism · mass spectrometry · noncovalent interactions · peptides · zinc

Introduction

Metal cations have been used to template and to stabilize secondary structures in de novo metalloprotein design. For example, lanthanides enhanced the capability of a disulfide-bridged peptide containing γ-carboxyglutamic acid to undergo a transition from a random coil to α-helical coiled-coil.[1] It has also been shown that specific geometrically restrictive metal–ligand interactions are required in order to obtain a unique structure for a four-helix bundle peptide.[2] There are several reports on the use of metal compounds as cross-linking agents to stabilize α-helices,[3,4] β-sheets,[5] and β-turns.[6] Most of these examples are based on a rational design of peptides that take into account the affinity of metal ligands for the selected metal ion and the compatibility of the metal ion geometry and coordination sphere with the desired conformation. Moreover, it has been demonstrated that amino acids have various propensities to form an α-helix,[7,8] a β-sheet,[9] or a β-turn[10] and that the sequence periodicity, in particular, is a determinant factor generating distinct conformational preferences.[11]

Many enzymes take advantage of metallic ion cofactors. Among these ions, zinc plays a special role. Alkaline phosphatase, phospholipase C, nuclease P1, and leucine aminopeptidase all belong to the family of *co-catalytic* zinc enzymes.[12] Zinc ions induce the aggregation of the peptide $A\beta_{1-40}$ that accumulates in the brain cortex in patients suffering from Alzheimer's disease.[13, 14] The zinc ions also play an important role in stabilizing the structure of the archaeal ferredoxin from the thermoacidophilic archaeon *Sulfolobus sp.*[15–17] Zinc-finger motifs are known to exhibit various biological activities, such as the capsid formation around genomic RNA by the nucleocapsid protein of the immunodeficiency virus type 1.[18] The particularity of zinc-finger peptides was used to design peptidyl chemosensors.[19, 20]

Enzymatic activity is driven by the nature of the amino acids associated with zinc ions. Histidine and aspartic acid are often present in zinc-finger peptides. Recently, an extracellular protease acidolysin produced by *Clostridium acetobutylicum* was purified. This enzyme contains a high level of acidic and polar amino acids and requires zinc ions for its full activity.[21]

For the de novo design of enzymes, model proteins with biological activities, are synthesized without reference to any known particular protein structure or consensus sequence. In previous publications, we have reported that periodic basic polypeptides such as poly(Lys-Leu) and poly(Leu-Lys-Lys-

[a] Dr. A. Brack, Dr. B. Barbier, O. Hénin, F. Boillot
Centre de Biophysique Moléculaire, CNRS
rue Charles Sadron, F-45071 Orléans Cedex 2 (France)
Fax: (+33) 38-63-15-17
E-mail: brack@cnrs-orleans.fr

0947-6539/99/0501-0218 $ 17.50+.50/0 *Chem. Eur. J.* **1999**, *5*, No. 1

Leu) adopt well-defined secondary structures and exhibit a ribonuclease activity related to their length and conformation.[22] The hydrolytic activity increases with the chain length. The decapeptide $(Leu\text{-}Lys)_5$ is long enough to adopt a β-sheet conformation in the presence of oligoribonucleotides and shows strong hydrolytic activity.[23] The dodecamer Ac-$(Leu\text{-}Lys\text{-}Lys\text{-}Leu)_3$-NHEt exhibits an α-helix structure in the presence of $NaClO_4$ and hydrolyzes oligoribonucleotides.[24]

We have generalized the conformational behavior of basic polypeptides to simple periodic acidic oligopeptides. We have synthesized a series of amphiphilic peptides of increasing chain length which are based on the dipeptide periodicity -Asp-Leu- and the tetrapeptide periodicity -Leu-Asp-Asp-Leu-.

In aqueous solution, short amphiphilic peptides do not generally possess a complete helical structure. Several properties of the α-helix have been utilized to force short peptides to adopt this conformation. Physical factors, such as the action of surfactants[25] or of liposomes,[26] help the formation of the structures of 13- and 12-residue peptides, respectively. Helix-dipole stabilization has been obtained by the modification of the charged groups near the termini of the 13-residue C-peptide of ribonuclease A.[27] The selection of specific amino acid sequences may also favor the helical conformation. Repeats of hydrophilic amino acids every three or four residues generate hydrophobic areas on the helix and allow interchain aggregation as a result of hydrophobic clustering. De Grado and Lear reported that the 14-residue peptide $(Leu\text{-}Lys\text{-}Lys\text{-}Leu\text{-}Leu\text{-}Lys\text{-}Leu)_2$ forms α-helical tetramers in water.[28] Marqusee and Baldwin demonstrated on model peptides with 16 or 17 amino acid residues that salt bridges between Glu and Lys residues in positions i and $i+4$ stabilize the α-helix.[29] The use of salt bridges and the positioning of hydrophobic residues enabled Eisenberg et al. to obtain four-helix bundles with the 12-residue peptide Ac-Glu-Leu-Leu-Lys-Lys-Leu-Leu-Glu-Glu-Leu-Lys-Gly-OH and with 16-residue peptides.[30, 31] Krstenansky et al. reported that the 11-residue peptide Suc-Leu-Leu-Glu-Lys-Leu-Leu-Trp-Leu-Lys-amide, which combines some of the above-mentioned stabilizing factors, showed a very strong tendency to form α-helices (51%).[32] Brack and Spach have shown that segments containing 6 to 7 adjacent residues of the same chirality in alternating poly(leucyl-lysyl) with varying amounts of L and D residues in the chains, are sufficient to obtain a β-sheet configuration.[33]

We have investigated the behavior of the synthesized acidic peptides in the presence of low concentrations of metal ions by means of circular dichroism spectroscopy. These peptide models were used to study the ability of acidic peptides to bind Zn^{2+} ions. $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_8$-Asp was used to study the self-association of α-helices as a function of temperature. Binary complexes obtained with peptides and zinc ions were analyzed by electrospray mass spectrometry.

Abstract in French: *Une série de peptides amphiphiles de longueur croissante constitués par la répétition de séquences Asp-Leu et Leu-Asp-Asp-Leu a été synthétisée. Les conformations obtenues en présence d'ions métalliques en faible concentration ont été étudiées par dichroisme circulaire. Dans l'eau pure, les peptides ne sont pas structurés. Les ions Zn^{2+} induisent spécifiquement une structure en feuillets β pour $(Asp\text{-}Leu)_n$ et une structure en hélice α pour $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_n$. Les transitions conformationnelles dépendent de la longueur des chaînes peptidiques: la formation des feuillets β requiert entre 10 et 24 résidus et celles d'hélices α entre 13 et 25 résidus. L'addition d'ions Ca^{2+} n'agit que très faiblement sur la structure tandis que NH_4^+ et Mg^{2+} sont sans effet. L'auto-association des hélices α de $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_8$-Asp induite par les ions Zn^{2+} a été étudiée à différentes températures. Les peptides complexés aux ions Zn^{2+} ont été analysés par spectrométrie de masse en mode électrospray. En présence de 0.4 équivalent d'ions Zn^{2+} par résidu Asp, le peptide $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_8$-Asp fixe un maximum de 5 ions Zn^{2+}, alors que la neutralisation complète des résidus Asp nécessite théoriquement 8 ions Zn^{2+} par peptide. $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_6$-Asp et $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_3$-Asp fixent 2 et 1 Zn^{2+} par chaîne, respectivement.*

Results

It has been previously shown that peptides with repeats of hydrophobic (ho) and hydrophilic (hi) amino acids adopt well-defined secondary structures depending on their repeating pattern.[11, 34–35] Strict alternation of hydrophilic and hydrophobic amino acids induces a β-sheet structure, while tetrapeptide periodicity (-hi-hi-ho-ho-) induces an α-helix conformation in basic polypeptides containing leucine and lysine[36] and in acidic peptides based on leucine and glutamic acid.[37–38]

We designed periodic peptides with well-defined chain lengths based on leucine and aspartic acid with either a dipeptide periodicity $(Asp\text{-}Leu)_n$ or a tetrapeptide periodicity $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_n$-Asp. Seven peptides have been synthesized; their primary sequences and molecular weights are summarized in Table 1.

The peptides exhibit CD spectra of a random coil in pure water at pH 7; this is caused by charge repulsion. If the charges are screened by cations, then the peptides tend to adopt a regular conformation. The cation-induced transitions in the peptide conformations were monitored by circular dichroism spectroscopy. Monovalent (NH_4^+), divalent (Mg^{2+},

Table 1. Molecular weights of peptides determined by ES-MS[a]

Peptides	Calculated mass (a.m.u)	Experimental mass (a.m.u)
$(Asp\text{-}Leu)_5$	1159.24	1158.99 ± 0.12
$(Asp\text{-}Leu)_{12}$	2756.97	2755.77 ± 0.53
$(Asp\text{-}Leu)_{15}$	3441.71	3441.60 ± 0.52
Leu-Asp-Asp-Leu-Asp	589.58	589.30 ± 0.1
$(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_3$Asp	1502.57	1502.23 ± 0.21
$(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_6$Asp	2872.06	2871.76 ± 0.42
$(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_8$Asp	3785.05	3784.68 ± 0.5

[a] Carrier solvent was water except for $(Asp\text{-}Leu)_{12}$, $(Asp\text{-}Leu)_{15}$, and $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_8$Asp which were carried by acetonitrile/water (20%). a.m.u. = atomic mass unit.

Ca^{2+}, and Zn^{2+}), and trivalent cations (Fe^{3+}) were used. Cationic metal ions were expected to establish electrostatic interactions with acidic side chains in order to inhibit charge repulsions. Isopropyl alcohol was also added in some cases in order to create a more hydrophobic environment.

***β*-Sheet structure**: Alternating $(Asp\text{-}Leu)_5$ and its higher oligomers $(Asp\text{-}Leu)_{12}$ and $(Asp\text{-}Leu)_{15}$ were expected to undergo a coil-to-*β*-sheet transition in the presence of metal ions. No transition was observed if NH_4Cl, $CaCl_2$, or $MgCl_2$ were added to $(Asp\text{-}Leu)_5$ and $(Asp\text{-}Leu)_{12}$; however, $(Asp\text{-}Leu)_{15}$ underwent a partial *β*-sheet transition in the presence of 0.5 equiv Ca^{2+}/Asp, 1 equiv Mg^{2+}/Asp, or 0.3 equiv Fe^{3+}/Asp (data not shown).

Zn^{2+} was the only salt which induced important conformational changes. Figure 1 shows the CD spectra of $(Asp\text{-}Leu)_{15}$ as a function of the amount of $ZnCl_2$. In pure water, $(Asp\text{-}Leu)_{15}$ exhibited a random coil structure. Addition of

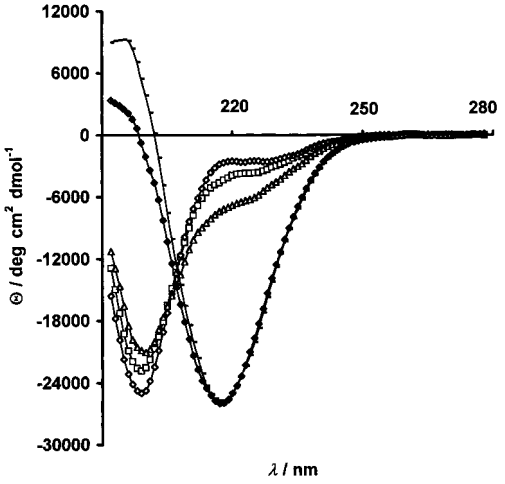

Figure 1. CD spectra of $(Asp\text{-}Leu)_{15}$ as a function of the amount of $ZnCl_2$: (◇) in water; (□) 0.1 equiv Zn^{2+}/Asp; (△) 0.2 equiv Zn^{2+}/Asp; (◆) 0.4 equiv Zn^{2+}/Asp; (–) 0.5 equiv Zn^{2+}/Asp. Experimental conditions: peptide 0.8 mM (expressed as peptide bond molarity); pH 7.0; 1 mm pathlength; 20 °C.

0.4 equiv Zn^{2+}/Asp induced a complete coil-to-*β*-sheet transition, characterized by a negative maximum at 218 nm. The isodichroic point at 207 nm is characteristic of an equilibrium between coils and *β*-sheets. IR spectra of a film of $(Asp\text{-}Leu)_{15}$, obtained in the presence of 0.6 equiv Zn^{2+}/Asp, exhibits an amide I band at 1627 cm^{-1} with a shoulder at 1684 cm^{-1} which is characteristic of an antiparallel *β*-sheet.[39] Above 0.5 equiv Zn^{2+}/Asp, which is the amount of zinc necessary to totally neutralize the aspartic acid side chains, the peptide precipitates. The propensity to form *β*-sheets in the presence of Zn^{2+} decreases if the peptide chains are shorter. In the presence of 0.4 equiv Zn^{2+}/Asp, $(Asp\text{-}Leu)_5$ remains as random coil, while $(Asp\text{-}Leu)_{12}$ undergoes only a partial transition (Figure 2). Interestingly the addition of up to 50% isopropyl alcohol reinforced the *β*-sheet character. This suggests that hydrophobic interactions in association with ionic interactions contribute to the stabilization of the *β*-sheet structure.

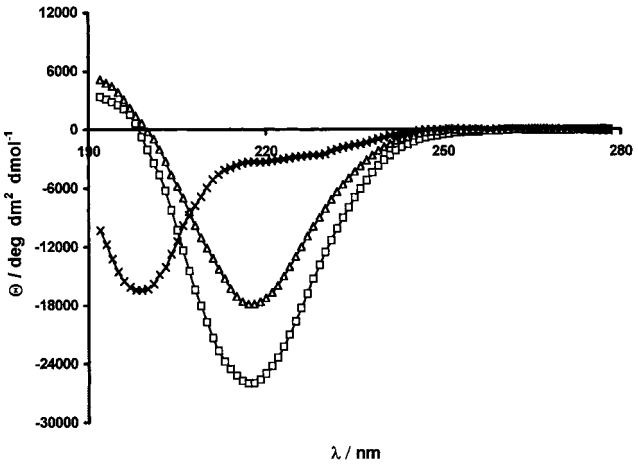

Figure 2. CD spectra of $(Asp\text{-}Leu)_{15}$, $(Asp\text{-}Leu)_{12}$, and $(Asp\text{-}Leu)_5$ in the presence of 0.4 equiv Zn^{2+}/Asp: (□) $(Asp\text{-}Leu)_{15}$; (△) $(Asp\text{-}Leu)_{12}$; (×) $(Asp\text{-}Leu)_5$. Experimental conditions: see Figure 1.

***α*-Helical conformations**: It has been previously reported that *α*-helices are obtained when doublets of hydrophobic and hydrophilic amino acids are repeated along the chain.[36–38] We have found that the peptide series $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_n\text{-}Asp$ with $n = 1$, 3, 6, and 8 had a tendency to form *α*-helices specifically with Zn^{2+} ions, whereas NH_4Cl and $MgCl_2$ had no effect on the conformation. Figure 3 A shows the increase of

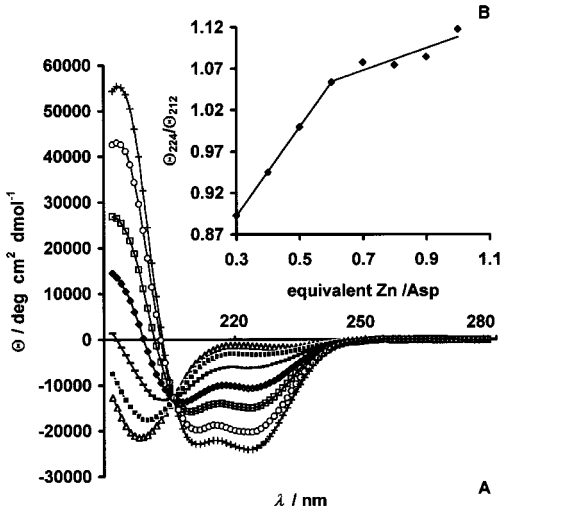

Figure 3. A: CD spectra of $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_8\text{-}Asp$ as a function of the amount of Zn^{2+}: (△) in water; (■) 0.1 equiv Zn^{2+}/Asp; (–) 0.2 equiv Zn^{2+}/Asp; (◆) 0.3 equiv Zn^{2+}/Asp; (□) 0.4 equiv Zn^{2+}/Asp; (○) 0.5 equiv Zn^{2+}/Asp; (+) 0.6 equiv Zn^{2+}/Asp. B: Ratio of $\Theta_{222}/\Theta_{212\,nm}$ as a function of equiv Zn^{2+} per aspartic acid for $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_8\text{-}Asp$.

$(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_8Asp$ helicity as a function of increasing Zn^{2+} concentration. The percentage of helicity, calculated by the method of Zhong and Johnson[40] with a 26 protein data set, varied linearly with the amount of $ZnCl_2$ added. A plateau is obtained for 0.6 equiv Zn^{2+}/Asp (Figure 4 B). The formation of *α*-helices is dependent on the chain length: for 0.6 equiv Zn^{2+}/Asp, 73% helicity is obtained for $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_8\text{-}Asp$, but only 43% helicity is observed for $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_6\text{-}Asp$. No *α*-helix formation could be obtained

0947-6539/99/0501-0220 $ 17.50+.50/0 *Chem. Eur. J.* **1999**, *5*, No. 1

for (Leu-Asp-Asp-Leu)-Asp and for (Leu-Asp-Asp-Leu)$_3$-Asp (Figures 4A and 4B). Even at 20 equiv Zn^{2+}/Asp, (Leu-Asp-Asp-Leu)-Asp and (Leu-Asp-Asp-Leu)$_3$-Asp are unordered.

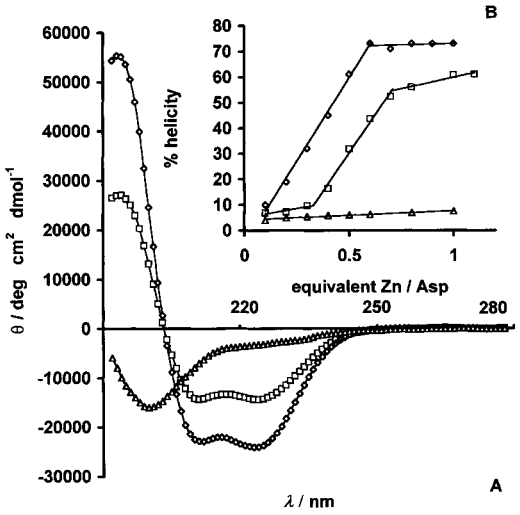

Figure 4. A: CD spectra of (Leu-Asp-Asp-Leu)$_8$-Asp, (Leu-Asp-Asp-Leu)$_6$-Asp, and (Leu-Asp-Asp-Leu)$_3$-Asp in the presence of 0.6 equiv Zn^{2+} per aspartic acid: (◇) (Leu-Asp-Asp-Leu)$_8$-Asp; (□) (Leu-Asp-Asp-Leu)$_6$-Asp; (△) (Leu-Asp-Asp-Leu)$_3$-Asp. B: Percentage of helicity determined by the equation: $\Theta_{222\,nm} \times (-10)/3298$ as a function of the amount of Zn^{2+}.

Zinc-induced self-association of α-helices: The highly helical 33-residue peptide (Leu-Asp-Asp-Leu)$_8$-Asp was used to study the self-association of α-helices. As the concentration of $ZnCl_2$ was increased, the molar ellipticity increased more at 222 nm than at 212 nm. The 222 nm CD band, which corresponds to the $n-\pi^*$ transition, is only related to the helical content. The $\pi-\pi^*$ excitation band at 212 nm, on the other hand, polarizes parallel to the helix axis and is sensitive to whether the α-helix is monomeric or is engaged in tertiary contacts, as in self-aggregated α-helices. The ratio $\Theta_{222}/\Theta_{212}$ is therefore characteristic of the degree of coiling.[41, 42] The ratio $\Theta_{222}/\Theta_{212}$ becomes >1 when the concentration of Zn^{2+} exceeds 0.5 equiv Zn^{2+}/Asp (Figure 3B); at this concentration all the carboxylic charges of the aspartic acid side chains are potentially neutralized. This suggests that self-association of the α-helices occurs at this concentration. The peptide concentration was decreased from 0.8 mM to 0.08 mM (expressed in peptide bonds), while the Zn^{2+}/Asp ratio was kept constant at 0.6 (Figure 5). Below 0.32 mM, a significant random coil contribution appeared at the expense of the α-helices; this suggests that, as found in the case of the β-sheets, concentration-dependent hydrophobic interactions play a role in the conformational stability.

The self-association of α-helices was confirmed by the thermal treatment of (Leu-Asp-Asp-Leu)$_8$-Asp in the presence of $ZnCl_2$ (0.8 mM, 0.6 equiv Zn^{2+}/Asp). When the solution was heated from 5.5 °C to 35 °C, the helicity increased from 66% to 71%. It then decreased linearly to 67% as the temperature was raised to 82 °C (Figure 6A). Between 5.5 °C and 82 °C, the ratio $\Theta_{222}/\Theta_{212}$ decreased from 1.06 to 0.90. This data suggests a thermal melting of the aggregates between

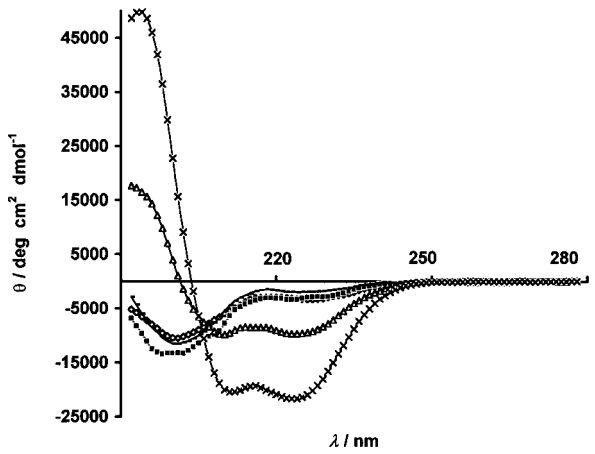

Figure 5. CD spectra of various concentrations of (Leu-Asp-Asp-Leu)$_8$-Asp with a constant ratio of Zn^{2+}/Asp = 0.6 equiv: (◇) 0.08 mM, (–) 0.106 mM, (■) 0.16 mM, (△) 0.32 mM, (×) 0.8 mM. Peptide concentrations are expressed as peptide bond molarity.

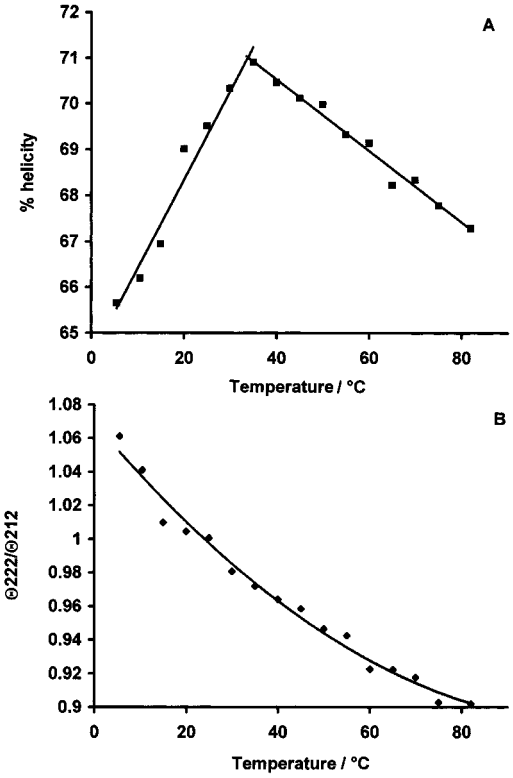

Figure 6. A: Percentage of helicity versus temperature for (Leu-Asp-Asp-Leu)$_8$-Asp, 0.8 mM, in the presence of 0.6 equiv Zn^{2+}/Asp. B: Ratio of $\Theta_{222}/\Theta_{212\,nm}$ versus temperature for (Leu-Asp-Asp-Leu)$_8$-Asp, 0.8 mM, in the presence of 0.6 equiv Zn^{2+}/Asp.

5.5 °C and 82 °C accompanied by an alteration of the individual helices above 35 °C (Figure 6B).

Interhelical aggregates were subjected to analytical ultracentrifugation. A complete loss of the initial absorbance was indicative of a highly associated system.[5] This was confirmed by standard centrifugation followed by UV inspection of the supernatants. The linear absorbances of the supernatant decreased markedly, even at low speeds (Figure 7); this indicates that aggregates of very high molecular weight are formed.

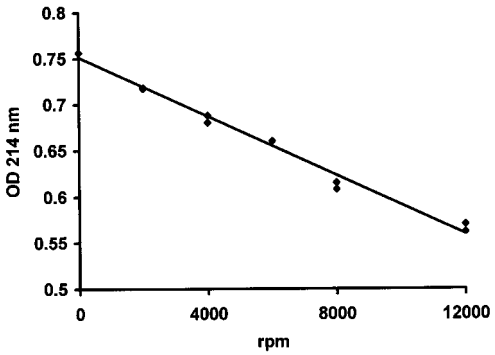

Figure 7. Optical density of the supernatant after centrifugation of (Leu-Asp-Asp-Leu)$_8$-Asp (0.8 mM, 0.6 equiv Zn^{2+}/Asp) solutions at varying speeds.

Analysis of Zn complexes by electrospray mass spectrometry: The technique of electrospray mass spectrometry (ES-MS) was used to measure the molecular weight of the synthetic peptides and to study the binary complexes formed with zinc. Increasing amounts of $ZnCl_2$ were added to (Leu-Asp-Asp-Leu)$_3$-Asp, (Leu-Asp-Asp-Leu)$_6$-Asp, and (Leu-Asp-Asp-Leu)$_8$-Asp solutions. The peptide concentrations were identical to those used for the CD experiments. The pH was fixed at 7.0 to allow the complete ionization of the carboxylic charges of Asp ($pK_a = 3$). Dilutions were made in the carrier solvent prior to the ES-MS studies. Acetonitrile/water (20%) was used to improve the solubility of the complexes and to preserve non-denaturating conditions. Mass spectra were well-defined up to 0.4 equiv Zn^{2+}/Asp. With higher salt concentrations, no defined mass spectra could be obtained.

As shown in Figure 8 A and Table 2, the ES-MS spectrum of (Leu-Asp-Asp-Leu)$_3$-Asp in the presence of 0.4 equiv Zn^{2+}/Asp is characterized by four major ion peaks (A) with charges of 5, 4, 3, and 2; these are identical to those obtained in pure water. A molecular mass of 1502 was determined. Since the

Table 2. Ionization states and molecular weights determined with addition of $ZnCl_2$ to (Leu-Asp-Asp-Leu)$_n$-Asp.

Zn^{2+}/Asp (equiv)	Observed N_{max}[a]	Observed N_{mean}[b]	Peptides	Calculated mass (a.m.u.)	Name
0	A5	A5	(Leu-Asp-Asp-Leu)$_3$-Asp	1502	A
0.4	A5	A5	(Leu-Asp-Asp-Leu)$_3$-Asp	1502	A
	B5	B4	(Leu-Asp-Asp-Leu)$_3$-Asp, Zn^{2+}	1565	B
0	A10	A7	(Leu-Asp-Asp-Leu)$_6$-Asp	2872	A
0.4	A9	A7	(Leu-Asp-Asp-Leu)$_6$-Asp	2872	A
	B9	B7	(Leu-Asp-Asp-Leu)$_6$-Asp, Zn^{2+}	2935	B
	C9	C7	(Leu-Asp-Asp-Leu)$_6$-Asp, 2 Zn^{2+}	2998	C
0	A12	A8	(Leu-Asp-Asp-Leu)$_8$-Asp	3785	A
0.4	C9	C8	(Leu-Asp-Asp-Leu)$_8$-Asp, 2 Zn^{2+}	3911	C
	D9	D8,D7	(Leu-Asp-Asp-Leu)$_8$-Asp, 3 Zn^{2+}	3974	D
	E9	E6	(Leu-Asp-Asp-Leu)$_8$-Asp, 4 Zn^{2+}	4037	E
	F7	F6	(Leu-Asp-Asp-Leu)$_8$-Asp, 5 Zn^{2+}	4099	F

[a] N_{max} is the maximum number of charges observed. [b] N_{mean} is the average number of charges observed.

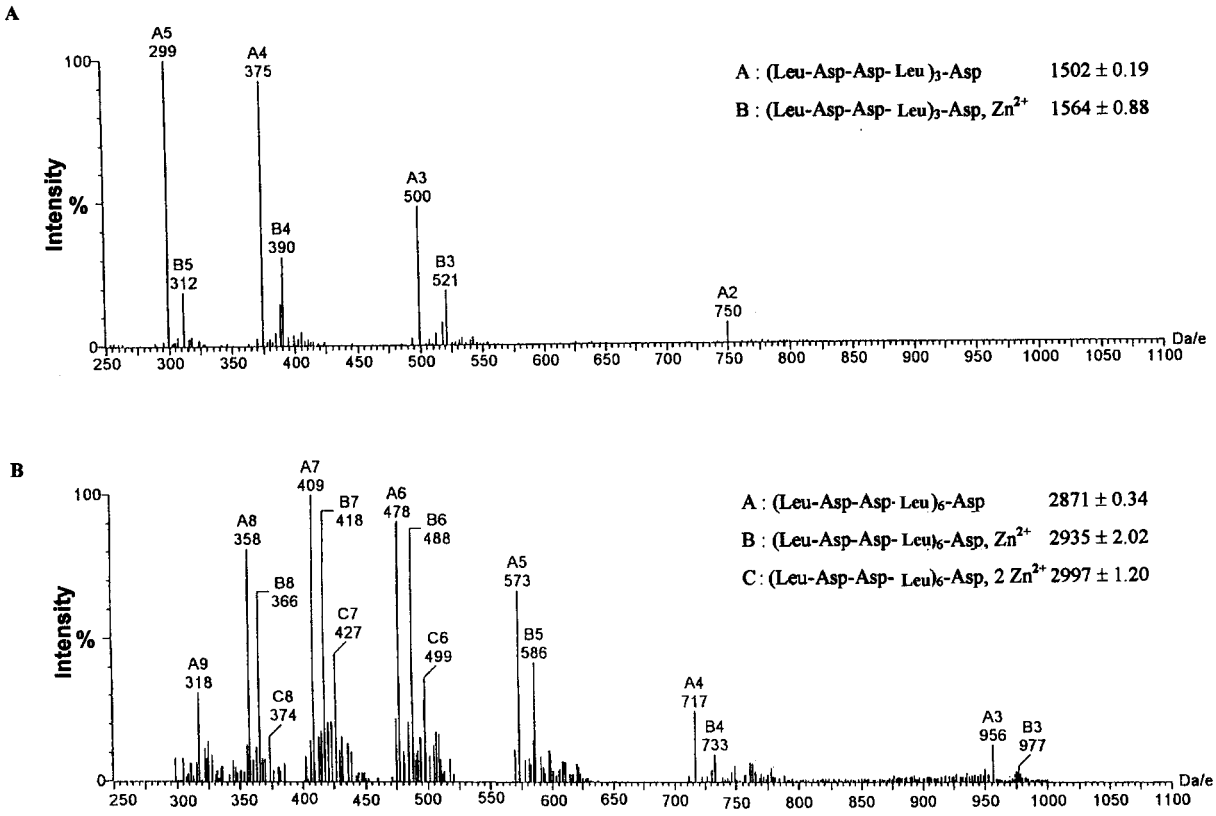

Figure 8. A: ES-MS spectrum of (Leu-Asp-Asp-Leu)$_3$-Asp with 0.4 equiv Zn^{2+}/Asp. B: ES-MS spectrum of (Leu-Asp-Asp-Leu)$_6$-Asp with 0.4 equiv Zn^{2+}/Asp.

0947-6539/99/0501-0222 $ 17.50+.50/0 *Chem. Eur. J.* **1999**, *5*, No. 1

mean number of charges (5) coincides with the maximum number of charges observed, the peptide seems to be largely ionized (charges 6 and 7 cannot be seen on the scan under the conditions used). A series of minor peaks (B), corresponding to a molecular mass higher by 63 a.m.u., was observed. This indicates that two hydrogen atoms have been substituted by one zinc ion per peptide.

In pure water, (Leu-Asp-Asp-Leu)$_6$-Asp is characterized by an envelope of nine peaks centered around seven charges (Figure 8B and Table 2) whereas the theoretical maximum number of charges is 13. The addition of $ZnCl_2$ up to 0.4 equiv Zn^{2+}/Asp produced an equilibrum of three forms (A, B, and C) which are assignable to (Leu-Asp-Asp-Leu)$_6$-Asp without zinc (A, $[M]=2872$), associated with one zinc ion (B, $[M]=2935$), and associated with two zinc ions (C, $[M]=2998$) and a peak shape centered around seven charges. The number of Zn^{2+} ions fixed to a peptide is smaller than the theoretical value of six expected from the peptide sequence.

(Leu-Asp-Asp-Leu)$_8$-Asp in pure water exhibits a series of peaks with charges of ranging from 12 to 4 centered around eight charges (Figure 9A and Table 2). Again, the mean number of charges is much lower than that expected from the sequence. The addition of $ZnCl_2$ induces the formation of complexes, the number of which increases with increasing $ZnCl_2$ concentration. At 0.4 equiv Zn^{2+}/Asp, four complexes were obtained (Table 3 and Figure 9B), (Leu-Asp-Asp-Leu)$_8$-Asp associated with two zinc ions (C, $[M]=3911$), with three zinc ions (D, $[M]=3974$), with four zinc ions (E, $[M]=4036$), and with 5 zinc ions (F, $[M]=4099$). The number of Zn^{2+} fixed to each peptide is smaller than the theoretical value of eight expected from the peptide sequence. Table 3 shows the variation of the charge number as a function of the amount of $ZnCl_2$ for (Leu-Asp-Asp-Leu)8-Asp.

For the β-sheet-forming series (Asp-Leu)$_{5,12,15}$, no soluble binary complexes could be obtained under the standard conditions used for electrospray. Additions of >50 %

Table 3. Variation of the charge number as a function of the concentration of $ZnCl_2$ added to (Leu-Asp-Asp-Leu)$_8$-Asp

Zn^{2+}/Asp (equiv)	Observed N_{max}	Observed N_{mean}	Peptides	Calculated mass (a.m.u.)	Name
0	A12	A8	(Leu-Asp-Asp-Leu)$_8$-Asp	3785	A
0.1	A10	A6	(Leu-Asp-Asp-Leu)$_8$-Asp	3785	A
	B10	B8	(Leu-Asp-Asp-Leu)$_8$-Asp, 1 Zn^{2+}	3846	B
	C10	C8	(Leu-Asp-Asp-Leu)$_8$-Asp, 2 Zn^{2+}	3911	C
0.2	A7	A6	(Leu-Asp-Asp-Leu)$_8$-Asp	3785	A
	B10	B8	(Leu-Asp-Asp-Leu)$_8$-Asp, 1 Zn^{2+}	3846	B
	C9	C8	(Leu-Asp-Asp-Leu)$_8$-Asp, 2 Zn^{2+}	3911	C
0.3	B9	B8	(Leu-Asp-Asp-Leu)$_8$-Asp	3846	B
	C10	C8	(Leu-Asp-Asp-Leu)$_8$-Asp, 2 Zn^{2+}	3911	C
	D9	D8,D7	(Leu-Asp-Asp-Leu)$_8$-Asp, 3 Zn^{2+}	3974	D
	E8	E7,E6	(Leu-Asp-Asp-Leu)$_8$-Asp, 4 Zn^{2+}	4037	E
0.4	C9	C8	(Leu-Asp-Asp-Leu)$_8$-Asp, 2 Zn^{2+}	3911	C
	D9	D8,D7	(Leu-Asp-Asp-Leu)$_8$-Asp, 3 Zn^{2+}	3974	D
	E9	E6	(Leu-Asp-Asp-Leu)$_8$-Asp, 4 Zn^{2+}	4037	E
	F7	F6	(Leu-Asp-Asp-Leu)$_8$-Asp, 5 Zn^{2+}	4099	F

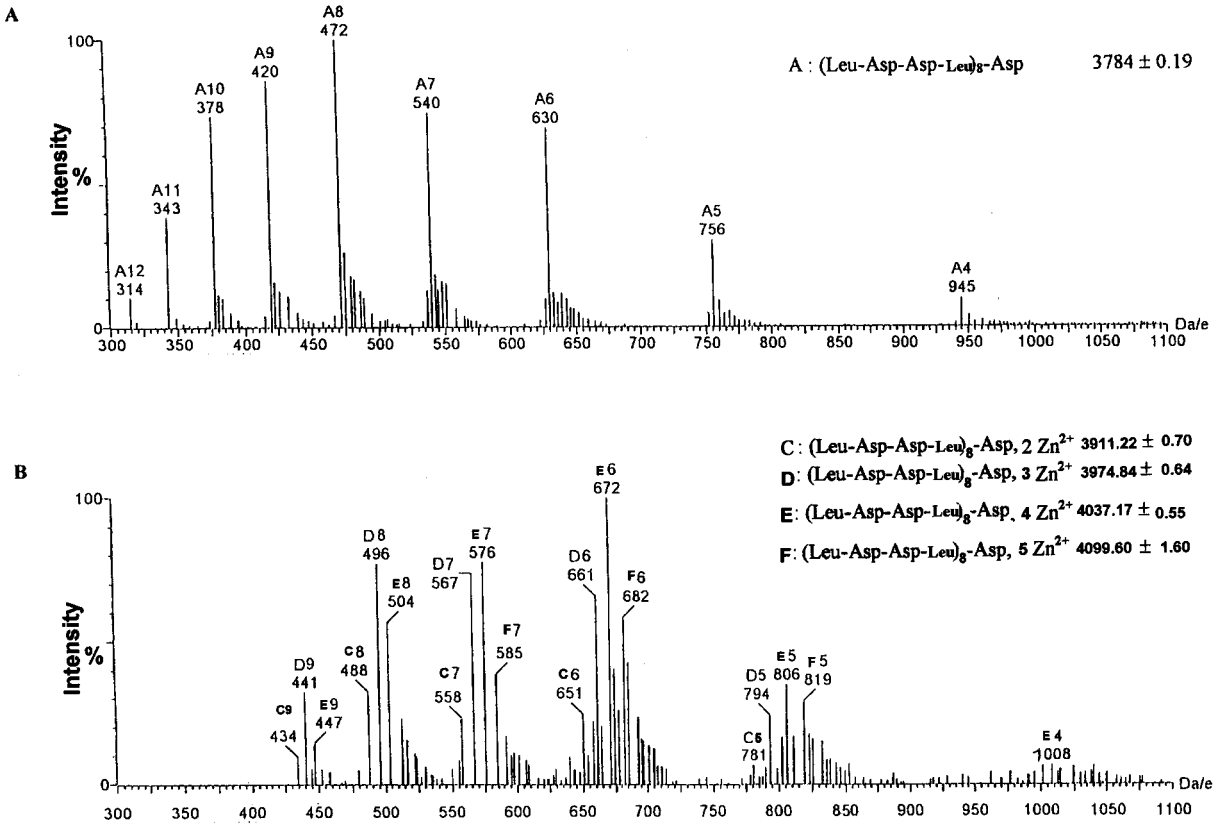

Figure 9. A: ES-MS spectrum of (Leu-Asp-Asp-Leu)$_8$-Asp in pure water. B: ES-MS spectrum of (Leu-Asp-Asp-Leu)$_8$-Asp with 0.4 equiv Zn^{2+}/Asp.

acetonitrile were necessary in order to solubilize the complexes. Under these denaturing conditions, it was not possible to observe any ES-MS spectra of binary complexes.

Discussion

Metal–peptide interactions intervene structurally and functionally in many biological processes. As an extension of our previous work on the enzyme mimetics by de novo design of alternating basic polypeptides, we have synthesized two series of acidic peptides with the dipeptide periodicity $(Asp\text{-}Leu)_n$ and the tetrapeptide periodicity $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_n$. As expected, the peptides adopt a random coil conformation in pure water, whereas addition of low concentrations of metallic cations, specifically Zn^{2+}, induces ordering into β-sheets and α-helices, respectively. As previously shown, sequential peptides containing glutamic acid and leucine adopt a β-sheet or an α-helix structure in the presence of various metallic cations.[37, 38] Aspartic acid side chains are shorter than those of glutamic acid and are therefore less flexible. The lack of flexibility of the aspartic acid side chains has been found to be an advantage in the case of electrostatic interactions across a β-sheet[9] and a drawback for the α-helix.[8] In this respect, the highest helicity obtained in our study for the 33-residue peptide $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_8$-Asp in the presence of 0.6 equiv Zn^{2+}/Asp, that is $-24200\ deg\,cm^2\,dmol^{-1}$, is lower than the expected value of $-35700\ deg\,cm^2\,dmol^{-1}$ for an infinite α-helix.[43] The specificity of Zn^{2+} could also be understood by the small radius of Zn^{2+} (0.74 Å): the higher charge density probably induces a tighter binding of Zn^{2+}, as observed by Hodges and coworkers for a coiled-coil peptide.[1] The Zn^{2+} ion has a full 3d orbital and can therefore form tetradentate complexes with the 4s and 4p orbitals. Since its magnetic moment is 0 μ_B, the complexes must also exhibit covalent character. The two specific features of Asp and zinc could explain the high thermal stability of the α-helices described in this study.

Electrospray mass spectrometry has become a standard technique for the accurate determination of the molecular weight of small proteins and peptides.[44, 45] This technique has been recently extended to the study of noncovalent interactions between proteins and their metallic cofactors.[46, 47] We have used ES-MS to study the binary complexes obtained by the addition of $ZnCl_2$ to $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_n$-Asp ($n = 3$, 6, 8) solutions. Although the signal is weaker in the negative mode detection, our results show that the addition of increasing amounts of $ZnCl_2$ induces the formation of binary complexes containing up to five zinc ions per peptide in the most favorable case.

In pure water at pH 7.0, the mean peak envelope for $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_6$-Asp is centered around seven charges (Table 2), whereas the maximum value expected from the sequence is 13. The same observation can be made for $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_8$-Asp with a mean peak centered around eight charges for a maximum number of charges of 17. These shapes can be explained by the fact that seven and eight-charges, respectively, are the most populated ionization states on account of the electrostatic repulsive effects present between the charges of two adjacent side chains, as was observed for the tripeptide $(Arg)_3$ and for protamines which appear to be only half-protonated in the positive-ion mode.[48,49]

The addition of 0.4 equiv Zn^{2+}/Asp has practically no effect on the shorter peptide $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_3$-Asp (Table 2 and Figure 8A), as already observed by circular dichroism. The longer peptides, $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_6$-Asp (Table 2 and Figure 8B) and $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_8$-Asp (Table 2 and Figure 9B), bind Zn^{2+} ions. In the case of the latter peptide, the complexation of Zn^{2+} ions shifts the mean peak envelope from eight to six charges, as a result of the neutralization of the carboxylate charges by Zn^{2+} ions (Table 3). Moreover, when four and five Zn^{2+} ions are fixed per peptide, the theoretical remaining charges become visible, that is nine charges and seven charges, respectively. This is probably caused by the screening of the electrostatic repulsions by the Zn^{2+} ions. The addition of 0.4 equiv Zn^{2+}/Asp should lead to a maximum complexation of seven Zn^{2+} ions per peptide, a degree of complexation which was never observed. At 0.4 equiv Zn^2/Asp, the extent of helicity was 45%, as shown by CD measurements. Ionic interactions are likely to be established between one Zn^{2+} ion and two aspartic acid side chains in positions i and $i+4$. Our results suggest that only 10 aspartic acid residues per peptide are able to establish such ionic bridges, the remaining seven uncomplexed charges are exposed to the solvent, probably at the chain ends. ES-MS allowed us to study single-helix complexes with Zn^{2+} and to follow the influence of the chain length and zinc concentration. Above 0.4 equiv Zn^{2+}/Asp, CD measurements give the $\Theta_{222}/\Theta_{212}$ ratios higher than 1, which suggests the formation of high molecular weight aggregates by means of interhelical interactions which could not be analyzed by ES-MS. Dynamic computer modeling (Sybyl software TRIPOS, St. Louis, MO) has been undertaken in an attempt to understand the main factors controlling the observed conformations. The preliminary results are in good agreement with the experimental data: $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_3$-Asp is too short to retain Zn^{2+} in its environment, whereas $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_8$-Asp in the presence of 0.4 equiv Zn^{2+}/Asp adopts an α-helix structure with all charges exposed to the solvent. These encouraging results will be refined further.

Conclusions

Acidic periodic peptides of well-defined chain length adopt ordered structures specific to zinc ions, as demonstrated by circular dichroism spectroscopy. According to their repeating pattern, $(Asp\text{-}Leu)_n$ adopt a β-sheet structure and $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_n$-Asp an α-helical conformation, both structures are dependent on the chain length. Interestingly, longer polydisperse $(Asp\text{-}Leu)_n$ sequences are not specific to zinc ions but also adopt a β-sheet structure in the presence of several different divalent cations.[38] They have been recently demonstrated by Addadi et al. to be capable of specifically inducing aragonite formation, one of the two most stable polymorphs of calcium carbonate.[51] In our study, the α-helices formed are thermally stable. This may support the

0947-6539/99/0501-0224 $ 17.50+.50/0 *Chem. Eur. J.* **1999**, *5*, No. 1

suggestion by Oshima et al. that, in the course of evolution, archea have evolved special adaptation mechanism to extreme environments by means of complexes formed between ferredoxins and zinc.[15] Since metallic cations are involved in the catalysis of many biological reactions, a development of this study will aim at testing these peptides as artificial ribonucleases or inorganic pyrophosphatases and as zinc sensors.

Experimental Section

All sequential oligopeptides were synthesized by solid-phase peptide synthesis with a 431 Applied Biosystem synthesizer with Fmoc protection for N^{α}-functions, dicyclohexylcarbodiimide/1-hydroxybenzotriazole (HOBt) for the coupling steps and acid-labile preloaded Wang resins. Fmoc-Asp(O*t*Bu)-Wang resin was purchased from Novabiochem (Switzerland) and Fmoc-Leu–Wang resin from Neosystem (France). Aspartic acid side chains were protected by the *tert*-butyl group. Fmoc-Asp(O*t*Bu)-OH and Fmoc-Leu-OH were purchased from Senn Chemicals AC (Germany). Unreacted amino groups were capped with acetic anhydride after each coupling step. Fmoc groups were cleaved in piperidine/*N*-methylpyrrolidone (20%) and HOBt (0.1M) to avoid the formation of aspartimide.[50] The peptides were deprotected and cleaved from the resin by trifluoroacetic acid/water (95/5).

The purity of $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_n$-Asp with $n = 1, 3$, and 6, was checked by analytical reversed-phase HPLC on a HPLC Merck-Hitachi L-6200A system equipped with a C_{18} (5 μm) Lichrospher Merck column, a 655A variable wavelength UV monitor, and a Merck Hitachi integrator D-7500. Peptides were eluted with linear gradients of acetonitrile/water/0.1% trifluoroacetic acid. Peptide $(Asp\text{-}Leu)_5$ was eluted with a linear gradient of methanol/water/ammonium acetate (0.05M) at pH 7.6, on a C_8 semipreparative Vydac column. $(Asp\text{-}Leu)_{12}$, $(Asp\text{-}Leu)_{15}$, and $(Leu\text{-}Asp\text{-}Asp\text{-}Leu)_8$-Asp were analyzed by FPLC (Pharmacia Biotech) equipped with a strong anion exchanger column monoQ HR 5/5 (Pharmacia Biotech) and a UV detector (Gilson Holochrom). The eluant was a linear gradient of NaCl (1M) in Tris-HCl (50mM) at pH 7.6. Acetonitrile and methanol (HPLC grade) were purchased from Carlo Erba, milli-Q water was obtained from a Milli-RO 15 water purification system from Millipore. The seven peptides were found to be pure by chromatography and did not require further purification.

UV spectra were recorded on a Perkin–Elmer Lambda 15 UV/Vis spectrophotometer with 1 mm quartz cells.

Centrifugation experiments were performed in eppendorf tubes in a Sigma Bioblock Scientific 1 K15 apparatus thermostatted at 20°C with rotation speeds varying from 2000 to 13000 rpm.

Circular dichroism spectra were recorded at 190–280 nm on a Jobin–Yvon Mark IV dichrograph with quartz cells of 1 mm pathlength at 20°C. In order to study the effect of temperature, spectra were run between 5.5°C and 82°C. At each temperature, solutions were equilibrated over a period of 15 minutes. Peptides were obtained in their free carboxylic form after cleavage from the resin. They were dissolved in water by the addition of a stoichiometric amount of sodium hydroxide (0.1M). Peptide concentrations, expressed in peptide bond molarity, were fixed at 0.8mM at pH 7. Zinc ions were added as a solution of $ZnCl_2$ (0.01M). Cation concentrations are expressed as metal-ion equivalent per aspartyl residue. The solutions were left overnight before recording the CD spectra.

The molecular weight of the peptides was determined by electrospray mass spectrometry. Analyses were performed on a VG Quattro II quadrupole mass spectrometer (Fison Instrument) attached to an electrospray source and controlled with the Masslynx software (version 1.6, Micromass, UK). Carrier delivery was fixed at 10 $\mu L\,min^{-1}$ by means of a Harvard Syringe pump (Harvard Apparatus, South Natick, MA). The instrument was calibrated in the positive-ion mode with horse heart myoglobin. The source temperature was set at 70°C. For molecular weight determinations, peptides were dissolved in the presence of a stoichiometric amount of ammonium carbonate (0.1M) with concentrations ranging between 20 μM and 50 μM in peptides. Water was used as the carrier solvent and no specific voltage was required. The negative-ion mode was used for detection. For the determination of binary complexes between peptides and zinc, the peptide was dissolved in ammonium carbonate, to which increasing amounts of $ZnCl_2$ were then added. The samples were diluted with acetonitrile/water (20%) prior to injection. All experiments were performed with identical voltages: a capillary voltage of 2.47 kV, an extraction cone voltage of 0 kV, the focussing cone voltage was offset by 60 V. Scanning was performed from $m/z = 250$ to 1100.

Acknowledgments

We thank Dr. Y. Trudelle and Dr. A. Delmas for their very helpful advice and Prof. D. Sy for the computer modeling.

[1] W. D. Kohn, C. M. Kay, B. D. Sykes, R. S. Hodges, *J. Am. Chem. Soc.* **1998**, 120, 1124–1132.
[2] T. M. Handel, S. A. Williams, W. F. DeGrado, *Science* **1993**, 261, 879–885.
[3] M. R. Ghadiri, C. Choi, *J. Am. Chem. Soc* **1990**, 112, 1630–1632.
[4] F. Ruan, Y. Chen, P. B. Hopkins, *J. Am. Chem. Soc.* **1990,** 112, 9403–9404.
[5] J. P. Schneider, J. W. Kelly, *J. Am. Chem. Soc.* **1995**, 117, 2533–2546.
[6] R. P. Cheng, S. L. Fisher, B. Imperiali, *J. Am. Chem. Soc*. **1996**, 118, 11349–11356.
[7] J. K. Myers, C. N. Pace, J. M. Scholtz, *Biochemistry* **1997**, 36, 10923–10929.
[8] G. Merukta, E. Stellwagen, *Biochemistry* **1991**, 30, 1591–1594.
[9] C. A. Blasie, J. M. Berg, *Biochemistry* **1997**, 36, 6218–6222.
[10] K. Gunasekaran, C. Ramakrishnan, P. Balaram, *Prot. Engin.* **1997**, 10, 1131–1141.
[11] H. Xiong, B. L. Buckwalter, H.-M. Shieh, M. H. Hecht, *Proc. Natl. Acad. Sci. USA* **1995**, 92, 6349–6353.
[12] B. L. Vallee, D. S. Auld, *Biochemistry* **1993**, 32, 6493–6500.
[13] X. Huang, C. S. Atwood, R. D. Moir, M. Hartshorn, J.-P. Vonsattel, R. E. Tanzi, A. I. Bush, *J. Biol. Chem.* **1997**, 272, 26464–26470.
[14] R. Balakrishnan, R. Parthasarathy, E. Sulkowsky, *J. Peptide Res.* **1998**, 51, 91–95.
[15] T. Fujii, Y. Hata, H. Moriyama, T. Wakagi, N. Tanaba, T. Oshima, *Biochemistry* **1997**, 36, 1505–1513.
[16] T. Fujii, Y. Hata, T. Wakagi, N. Tanaka, T. Oshima, *Nature: Struct. Biol.* **1996**, 3, 834–837.
[17] T. Iwasaki, T. Suzuki, T. Kon, T, Imai, A. Urushiyama, D, Ohmori, T. Oshima, *J. Biol. Chem.* **1997**, 272, 3453–3458.
[18] H. de Rocquigny, D. Ficheux, M.-C. Fournié-Zaluski, J.-L. Darlix, B. P. Roques, *Biochem. Biophys. Res. Commun.* **1991**, 180, 1010–1018.
[19] G. K. Walkup, B. Imperiali, *J. Am. Chem. Soc.* **1996**, 118, 3053–3054.
[20] H. A. Godwin, J. M. Berg, *J. Am. Chem. Soc*. **1996**, 118, 6514–6515.
[21] C. Croux, V. Paquet, G. Goma, P. Soucaille, *Appl. Environ. Microbiol.* **1990**, 56, 3634–3642.
[22] B. Barbier, A. Brack, *J. Am. Chem. Soc.* **1992**, 114, 3511–3515.
[23] A. Brack, B. Barbier, *Origins Life Evol. Biosphere*, **1990**, 20, 139–134.
[24] M. Perello, B. Barbier, A. Brack, *Int. J. Peptide Protein Res.* **1991**, 38, 154–160.
[25] C.-S. C. Wu, J. T. Yang, *Biopolymers* **1988**, 27, 423–430.
[26] H. Mihara, T. Kanmera, M. Yoshida, S. Lee, A. Aoyagi, T. Kato, N. Izumiya, *Bull. Chem. Soc. Jpn.* **1987**, 60, 697–706.
[27] K. R. Shoemaker, P. S. Kim, E. J. York, J. M. Stewart, R. L. Baldwin, *Nature* **1987**, 326, 563–567.
[28] W. F. De Grado, J. D. Lear, *J. Am. Chem. Soc*. **1985**, 107, 7684–7689.
[29] S. Marqusee, R. L. Baldwin, *Proc. Natl. Acad. Sci. USA* **1987**, 84, 8898–8902.
[30] D. Eisenberg, W. Wilcox, S. M. Eshita, P. M. Pryciak, S. P. Ho, W. F. De Grado, *Proteins* **1986**, 1, 16–22.
[31] S. P. Ho, W. F. De Grado, *J. Am. Chem. Soc*. **1987**, 109, 6751–6758.
[32] J. L. Krstenansky, T. J. Owen, K. A. Hagaman, L. R. McLean, *FEBS Lett*. **1989**, 242, 406–413.
[33] A. Brack, G. Spach, *J. Mol. Evol*. **1979**, 13, 35–46.
[34] A. Brack, L. E. Orgel, *Nature* **1975**, 256, 383–387.

[35] A. Brack, G. Spach, *J. Am. Chem. Soc*. **1981**, 103, 6319 – 6323.
[36] B. Barbier, M. Perello, A. Brack, *Collect. Czech. Chem. Commun*. **1988**, 53, 2825 – 2831.
[37] M. Bertrand, A. Brack, *Origins Life Evol. Biosphere* **1997**, 27, 585 – 595.
[38] M. Bertrand, D. Sy, A. Brack, *J. Peptide Res.* **1997**, 49, 269 – 272.
[39] T. Miyazawa in *Poly-α-amino acids: Protein Models for conformational studies*, *Vol. 1* (Ed.: G. D. Fasman), Marcel Dekker, New York, **1967**, pp. 92.
[40] L. Zhong, W. C. Johnson, *Proc. Natl. Acad. Sci. USA* **1992**, 89, 4462 – 4465.
[41] S. Y. M. Lau, A. K. Taneja, R. S. Hodges, *J. Biol. Chem.* **1984**, 259, 13253 – 13261.
[42] T. M. Cooper, R. W. Woody, *Biopolymers* **1990**, 30, 657 – 676.
[43] N. Greenfield, G. D. Fasman, *Biochemistry* **1969**, 8, 4108 – 4116.
[44] B. T. Chait, S. B. H. Kent, *Science* **1992**, 257, 1885 – 1863.
[45] J. B. Fenn, M. Mann, C. K. Meng, S. F. Wong, C. M. Whitehouse, *Science* **1989**, 246, 64 – 70.
[46] A. Surovoy, D. Waidelich, G. Jung, *FEBS Lett*. **1992**, 311, 259 – 262.
[47] S. M. Fundel, D. L. Pountney, R. Bogumil, P. M. Gehrig, D. W. Hasler, P. Faller, M. Vašák, *FEBS Lett.* **1996**, 395, 33 – 38.
[48] K. Hirakoa, I. Kudaka, *Anal. Chem.* **1992**, 64, 75 – 81.
[49] P. Schindler, F. Bitsch, K.Klarskov, P. Roepstorff, G. Briand, D. Wouters-Tyrou, P. Sautière, A. Van Dorsselaer, *Eur. J. Biochem.* **1992**, 195, 621 – 629.
[50] R. Dölling, M. Beyermann, J. Haenel, F. Kernchen, E. Krause, P. Franke, M. Brudel, M. Bienert, *J. Chem. Soc. Chem. Commun.* **1994**, 853 – 854.
[51] Y. Levi, S. Albeck, A. Brack, S. Weiner, L. Addadi, *Chem.Eur. J.* **1998**, 4, 389 – 396.

Received: May 28, 1998
Revised version: August 24 [F1177]

0947-6539/99/0501-0226 $ 17.50+.50/0