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A comparative CD and fluorescence study of a series of model calcium-binding peptides*°

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Lanthanide-saturated peptides analogous to calcium-binding loops of EF-hand proteins can be used to stabilize the α-helical structure of peptide or protein segments attached to their C-termini. To study conformational properties of such loop-containing hybrids it is necessary to produce them in bacteria. In peptides obtained in this way the helix will be destabilized by the negatively charged C-terminal α-carboxyl groups. We propose to block them by the homoserine lactone. The results presented in this paper indicate that the presence of the lactone even at the C-terminus of the loop does not have any negative effect on the loop helix-nucleation ability. On the other hand, the presence of the α-NH_3^+ at the loop N-terminus leads to a drop of metal-binding constant and loss of the rigid structure of the α-helical segment of the loop. The α-amino group separated by one glycine residue from the loop N-terminus should also be avoided because it perturbs the conformation of the N-terminal part of the loop and may reduce the loop affinity to lanthanide ions.

Twelve residue long calcium-binding loops of EF-hand proteins attain a very rigid structure upon metal binding with their three C-terminal peptide groups fixed in the α-helical conformation. It has been shown by us [1] that the peptide with the sequence analogous to the IIId calcium-binding loop of calmodulin AcDKDGDGYIAAE- saturated with lanthanide ions, stabilizes very effectively the α-helical conformation of a polypeptide segment attached to its C-terminus. Thus, using model peptides containing this loop sequence it is

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possible to study conformational properties, dynamics, thermodynamics, and spectroscopic properties of very short α-helices, formed by only a few amino-acid residues. Such studies can provide important data characterizing the very first steps of α-helix propagation – one of the processes determining the mechanism of protein folding.

The loop can be used also to stabilize, in a La^{3+}-dependent manner, structures of proteins or protein fragments containing N-terminal α-helices; e.g. the native structure of RNase A modified by attachment of the loop sequence to its N-terminus should be much more stable in the presence of lanthanide ions.

Well known, high-efficiency expression systems of *Escherichia coli* can be used to obtain the modified proteins as well as ^{15}N- and ^{13}C-enriched peptides indispensable for advanced NMR experiments. The polypeptide chains produced in this way will contain free α-amino and carboxyl groups at their N- and C-termini, respectively.

AcP	Ac-D-K-D-G-D-G-Y-I-S-A-A-E-NH$_2$
	1 3 5 7 9 12
PA	D-K-D-G-D-G-Y-I-S-A-A-E-A-NH$_2$
AcPA	Ac-D-K-D-G-D-G-Y-I-S-A-A-E-A-NH$_2$
PAA	D-K-D-G-D-G-Y-I-S-A-A-E-A-A-NH$_2$
AcPAA	Ac-D-K-D-G-D-G-Y-I-S-A-A-E-A-A-NH$_2$
GPhSl	G-D-K-D-G-D-G-Y-I-S-A-A-E-CO-NH-CH-COO-CH$_2$ (ring closed by CH$_2$)

Figure 1. Amino-acid segments of the peptides studied in this work; hSl denotes the homoserine lactone: HN-CH-(CO)-O-CH$_2$-CH$_2$

The presence of the charged NH_3^+ group at the N-terminus of the loop can possibly have a detrimental effect on its conformation making it much less efficient as the helix nucleation site. The negatively charged COO^- group situated at the C-terminus destabilizes the α-helix. Thus, it is not tolerable in model peptides designed to study the helix formation.

We decided, therefore, to block the α-carboxyl group of the peptides by homoserine lactone and to separate the α-amino group from the loop by introduction of an additional N-terminal glycine. A gene coding for the peptide with the following sequence: leader-M-G-DKDGDGYIAAE-M has been cloned in *E. coli*. During the reaction of the gene product with CNBr the leader to inclusion bodies is cut off and the C-terminal methionine residue of the peptide is converted into the homoserine lactone (see peptide GPhSl in Fig. 1).

The La^{3+}-binding ability and conformation of met-GPhSl have been compared with those of the reference peptide AcP. The effects of the presence of the α-amino group on conformational properties of the loop have been determined by comparative studies of PA and PAA peptides and their amino-acylated counterparts: AcPA and AcPAA, respectively.

MATERIALS AND METHODS

Peptide synthesis. Peptides AcP, PA, AcPA, PAA, and AcPAA, synthesized using Fmoc chemistry, were a generous gift from Dr. W. Wiczk (Department of Chemistry, Gdańsk University, Poland). The peptides were purified by reverse-phase HPLC chromatography on Vydac C18 semipreparative column.

Peptide GPhSl was expressed in *E. coli*. The 57 bp DNA fragment coding for the peptide was obtained by enzymatic assembly of two chemically synthesized DNA oligonucleotides designed according to the amino-acid sequence and codon usage preferences of *E. coli*. Oligonucleotides were annealed in one step and ligated. The gene for GPhSl was ligated into pAED4 plasmid. The sequence of the gene was under control of *lac UV5* promoter

and contained a leader sequence to inclusion bodies. The ligation product was transformed to competent *E. coli* MM294 cells and the resulting plasmid was used to transform competent *E. coli* BL21(OE3)pLysS cells grown in LB media. After CNBr cleavage of the expression product GPhSl peptide was purified by reverse phase HPLC.

Sequences of all peptides were checked by NMR.

Fluorescence measurements. Terbium, as well as peptide tyrosine luminescence was measured using an apparatus described previously [2]. Terbium luminescence was excited at 280 nm and measured using a glass filter (GG10, Schott, Jena, Germany). Data were recorded with Nucleus Personal Computer Analyzer PCA-II (Tennelec/Nucleus Inc., U.S.A.). Terbium luminescence lifetimes were determined as described in [2].

The equilibrium constants of La^{3+} ion coordination by the peptides were determined from titration measurements of tyrosine fluorescence intensity and least-square fitting of a theoretical curve to experimental data.

CD measurements. CD spectra were acquired on an Aviv Model 202 spectrometer. Samples were prepared in 2 mM Tris buffer, 0.1 M NaCl, pH 6.9, by dilution of concentrated stock solutions of the peptides. Peptide concentrations in the stock solutions were determined by tyrosine UV absorbance. The peptides were freshly purified by HPLC and did not contain any detectable impurities.

RESULTS AND DISCUSSION

Whereas the CD spectra of La^{3+}-saturated AcP and GPhSl are, within error, the same, large differences can be seen between the spectra of peptides containing the free α-amino group – PA and PAA – and their acylated derivatives – AcPA and AcPAA, respectively (Fig. 2). The former show a smaller contribution of an ordered structure that is manifested by the less pronounced minimum at about 215 nm. Evidently, the structures of the unacylated peptides are less rigid, most probably due to weaker binding of La^{3+} ions to these peptides [3]. Indeed, as follows from the data shown in Fig. 3, the binding constant of La^{3+} ions to PA ($2 \pm 0.5 \times 10^5$ M^{-1}) is about three times lower than to AcPa ($5.4 \pm 0.2 \times 10^5$ M^{-1}). On the other hand, as could be expected from CD data, GPhSl binds lanthanum

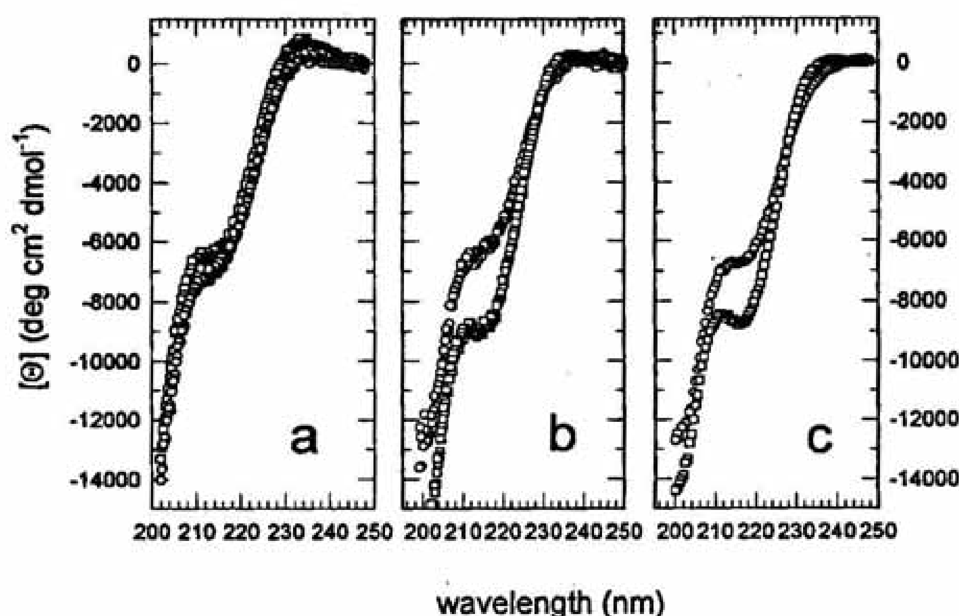

Figure 2. Comparison of CD spectra at 1°C, pH 6.8.

a: GPhSl (o) and AcP (□); b: PA (o) and AcPA (□); c: PAA (o) and AcPAA (□). All peptides were saturated with La^{3+} ions.

ions as tightly as AcP – the binding constants for both peptides are, within error, the same: $1.1 \pm 0.3 \times 10^5\ M^{-1}$ and $1.4 \pm 0.2 \times 10^5\ M^{-1}$, respectively.

Quite unexpectedly, though, the effect of the La^{3+} ion binding on fluorescence of Tyr-8 residue of the two peptides is quite different. The large, about 50% drop, of AcP fluorescence intensity upon La^{3+} binding (Fig. 3) is a result of fixation of the Tyr-8 ring predominantly in the *gauche*⁻ conformation by the rigid structure of the peptide backbone (Góral *et al.*, in preparation). A much smaller drop of the GPhsSl tyrosine fluorescence (about 30%) proves that the backbone structure of this peptide is different, perhaps less rigid, than that of met-AcP.

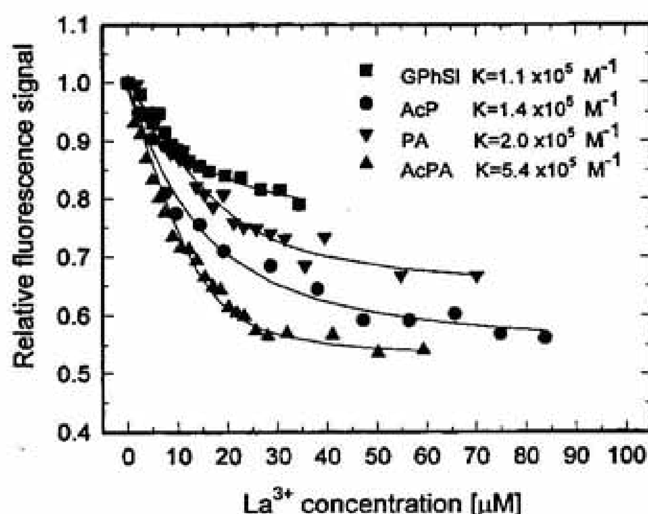

Figure 3. Fluorescence of peptides titrated with La^{3+} ions at 25°C, pH 6.8, peptide concentration about 15 μM.

The best-fit values of the ion binding constants *K* are listed.

That latter conclusion has been confirmed by the observation that different numbers of water molecules are coordinated by a lanthanide ion bound to GPhsSl or AcP. These numbers (q) can be determined from luminescence lifetimes of Tb^{3+} ion coordinated to the peptides in H_2O (τ_H) and D_2O (τ_D), according to the expression [4]: $q = 4.2\ (1/\tau_H - 1/\tau_D)$. At 25°C, pH 6.8, $\tau_D = 2.7$ ms and 2.5 ms; $\tau_H = 1.3$ ms and 0.95 ms for AcP and GPhSl, respectively. As calculated from these data, the number of water molecules coordinated by Tb^{3+} bound to AcP equals 1.8 and is exactly the same as for the terbium ion bound to a typical calcium-binding loop of EF-hand proteins [4]. One more water molecule is coordinated by terbium bound to GPhSl (q = 2.8). Clearly, then, the conformation of met-GPhSl must be different from that of a typical, metal-saturated calcium-binding loop.

Yet, as shown in Fig. 2, these conformational differences remain undetectable by CD. This proves that they are restricted to the non-helical part of the peptide backbone, i.e. to the peptide groups formed by residues Asp1–Ser9. The CD spectrum of this segment is very close to that of an unordered "random coil" conformation and remains unaffected by conformational changes, provided that no structure with repetitive Φ and Ψ angles, such as α-helix or β-strand is formed.

Since differences in the loop conformations in peptides GPhSl and AcP are restricted to their N-terminal parts it seems that they arise from the presence of Gly-NH_3^+ group in GPhSl. It is not clear, though, how the La^{3+}-binding constant is affected by the amino group separated by one glycine residue from the loop terminus. Maybe, its negative effect is compensated by the presence of the lactone which additionally stabilizes the helical structure of the C-terminal loop segment.

Thus, studies on conformational properties of loop-containing hybrids showed that the presence of the free α-NH_3^+ group bound to

the first residue of the loop is not acceptable. It causes a drop of lanthanide binding constants and destabilizes the rigid structure of the α-helical segment of the loop. The free α-amino group separated by one Gly residue from the loop should also be avoided since it perturbs the conformation of the N-terminal part of the loop and, perhaps, reduces the loop affinity to lanthanide ions.

Homoserine lactone can be used to block the C-terminal α-carboxyl group of peptides produced in bacteria. The lactone does not destabilize the helical structure. It is even possible that it increases its stability.

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