Isolated Calcium-Binding Loops of EF-Hand Proteins Can Dimerize To Form a Native-Like Structure†

Jacek Wójcik, Jakub Góral, Krzysztof Pawłowski, and Andrzej Bierzyński*

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 02-106 Warszawa, ul. Pawińskiego 5a, Poland

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ABSTRACT: Helix−loop−helix fragments of EF-hand proteins are known to dimerize in solution, re-producing the characteristic structure of native protein domains [Shaw, G. S., Hodges, R. S., & Sykes, B. D. (1990) *Science 249*, 280−283]. In this paper we present evidence that isolated calcium-binding loops can also dimerize, when saturated with lanthanide ions, interacting with each other in a similar way as do loops in intact proteins. A synthetic analogue of calcium binding loop III of calmodulin, AcDKDGDGYISAAE-NH_2, has been studied by ^{1}H NMR spectroscopy. For the La^{3+}-saturated peptide, concentration dependent broadenings and shifts of certain signals have been observed indicating dimerization process of intermediate rate on the NMR time scale. Analysis of signal shape and position of the Tyr7 ring protons as a function of concentration makes it possible to determine the association and dissociation rate constants of the process for various temperatures within the range of 10−80 °C. The dimerization constant changes according to van't Hoff relationship with $\Delta S = 233$ J/mol·K and $\Delta H = 62$ kJ/mol. A distance of 11.4 ± 0.4 Å between the ions coordinated by dimer molecules has been determined by measurements of $Tb^{3+} \rightarrow Ho^{3+}$ luminescence energy transfer. This value suggests that the dimer structure is similar to that of two-loop structural elements in native EF-hand proteins. From a thermodynamic cycle it can be shown that La^{3+} ion binding to the peptide dimers must be highly cooperative. Therefore, cooperativity of ion binding to domains of EF-hand proteins is, at least partly, due to local interactions between binding loops.

Calcium binding proteins classified as members of the troponin C (TnC)[1] superfamily control a large variety of biological processes (Kawasaki & Kretsinger, 1994). All those proteins show very high sequence homology (Moncrief et al., 1990) and strikingly similar structural pattern (Strynadka & James, 1989): their molecules are built of repetitive structural elements, so called EF-hand motives (Kretsinger & Nockolds, 1973) connected with each other by short linkers. Each of these elements contains a 12-residue long calcium binding loop flanked at both termini by α-helical structures. The loops are characterized by a strongly conservative sequence pattern (Godzik & Boguta, 1989). As an example, the sequence of loop III from rat testis calmodulin is shown in Figure 1.

Calcium ions are coordinated by the main chain carbonyl group of residue 7 and the side chain carboxyl, carbonyl, and hydroxyl groups of residues 1, 3, 5, 9, and 12. The loops are wrapped around the calcium ions, so that their ligands assume an almost octahedral arrangement occupying $\pm x$, $\pm y$, and $\pm z$ positions in the Cartesian framework. In the same way as calcium, the lanthanide ions are coordinated (Marsden et al., 1989). Because their radii are very close to that of Ca^{2+} ion, and because of similar coordination properties, lanthanides are good substitutes of calcium ligand

	93					104
Cal	-D-	K-	D-	G-	N-	G-	Y-	I	-S-	A-	A-	E-
	x		y		z	-y		-x		-z		
P12	Ac-D-	K-	D-	G-	D-	G-	Y-	I	-S-	A-	A-	E-NH_2
C12	Ac-D-	K-	D-	G-	D-	G-	Y-	C	-S-	A-	A-	E-NH_2
N12	Ac-D-	K-	N-	G-	D-	G-	Y-	C	-S-	A-	A-	E-NH_2
	1		3		5	7		9		12		

FIGURE 1: Sequences of calcium binding loop III of native rat testis calmodulin (Cal) and its synthetic analogues: P12, C12, and N12 peptides. Numbering of residues within the protein and within the loops are indicated along with ligand positions within the Cartesian framework.

(Snyder et al., 1990). Their binding constants are usually higher because of larger positive charge of Ln^{3+} ions (Gariepy et al., 1983). This makes them very useful as fluorescent (Horrocks, 1993) or paramagnetic (Lee & Sykes, 1983) probes in studies of calcium binding sites.

Conformations of EF-hand elements are very similar in all proteins from TnC superfamily (Pawlowski et al., 1996). Domains of these are built of two EF-hand segments arranged with respect to each other in a pseudo-2-fold symmetry. The rigid structure of the domains is maintained by hydrophobic interactions between α-helices of two segments as well as by hydrogen bonds and hydrophobic interactions between their binding loops. At the interface of the loops a short anti-parallel β-sheet is formed. A key role in stabilization of this structure is played by the residues at position 8 in the two loops that are linked to each other by hydrogen bonds between their main chain CO and NH groups and by hydrophobic interactions between their side chains. Residues

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[1] Abbreviations: TnC, troponin C; HPLC, high-performance liquid chromatography; TSP, sodium 2,2-tetradeutero-3-trimethylsilylpropionate; PIPES, piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]; Ln, lanthanide.

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with bulky aliphatic side chain groups are always found in this position: Ile, Leu, Val or, sometimes, Met (Godzik & Boguta, 1989).

Some proteins from the TnC superfamily, like calbindins D_{9k}, contain only one domain, while troponins C and calmodulins are built of two domains. The domains in the latter behave, in many respects, as autonomous units and separated from each other bind calcium in a similar way as when being parts of intact protein (Boguta et al., 1988; Marsden et al., 1990). On the contrary, the two EF-hand elements of each domain are not only structurally, but also functionally linked together. Binding of a single metal ion induces conformational changes in the whole domain, so that an adjacent EF-hand element assumes virtually the same conformation as its metal-saturated neighbor (Carlstrom & Chazin, 1993; Akke et al., 1991; Skelton et al., 1992; Shaw et al., 1991). Functional linkage between the pairs of EF-hand elements is most strikingly manifested by metal binding cooperativity proved for many domains. The second Ca^{2+} ion binds to calbindin D_{9k} with 7-fold higher binding constant than the first one (Linse et al., 1987, 1991). Both domains of calmodulin (Linse et al., 1991; Tsalkova & Privalov, 1985), at least C-terminal domain of troponin C (Drakenberg et al., 1987; Tsalkova & Privalov, 1985), and a single two-calcium binding domain of calerythrin (Bylsma et al., 1992), all coordinate Ca^{2+} ions in a highly cooperative way.

Separated EF-hand fragments can then be expected to have a different affinity for calcium than when they are part of a native protein. Indeed, it was shown that they coordinate Ca^{2+} ions with a few orders of magnitude higher dissociation constants (Boguta et al., 1988; Marsden et al., 1990). A further drop in Ca^{2+} binding affinity is observed for isolated calcium binding loops devoid of N- and/or C-terminal helices (Marsden et al., 1990).

By studying the conformational and metal binding properties of small fragments of proteins from the TnC superfamily, it has been possible to address a number of questions concerning not only the mechanism of metal binding by these proteins but also the more general problem of electrostatic interactions within protein molecules and the basic problem of protein folding process.

From the latter point of view a series of papers published recently by Shaw et al. (1990, 1991, 1992) and Kay et al. (1991) are particularly important. They show that isolated EF-hand fragments III and IV of TnC associate in solution forming structures mimicking the C-terminal domain of the native protein. It has been shown, therefore, that fragments of proteins from the TnC superfamily can be used to study, in an elegant way, a crucial step on protein folding pathway, namely, the formation of tertiary interactions between preformed secondary structures.

Association of protein fragments into a native-like molecule with full biological activity was observed long ago in RNase S. The S-peptide−S-protein system has been studied extensively and provided valuable information concerning the mechanism of RNase folding (Kato & Anfinsen, 1969; Labhardt & Baldwin, 1979; Labhardt et al., 1983). It seems that calcium binding fragments of proteins from the TnC superfamily provide yet another promising system for similar studies.

In this paper we present results of our ^{1}H NMR studies of a 12-residue long synthetic peptide, an analogue of calcium binding loop III of rat testis calmodulin. We show that even such a short peptide, when saturated with lanthanum ions, can dimerize forming a native like two-loop structure. We show, then, that local interactions between two loops can be sufficient, in some cases, to stabilize such a structure even in the absence of interhelical interactions. These observations provide an insight into the mechanism of metal binding cooperativity characteristic for domains of proteins from TnC superfamily.

MATERIALS AND METHODS

Peptide Synthesis and Purification

Peptides P12, N12, and C12 (Figure 1), synthesized using Fmoc chemistry, were generous gifts from Dr. P. S. Kim. The peptides were cleaved using standard procedures, desalted on G-10 Sephadex column in 5% acetic acid, and purified by reverse-phase HPLC chromatography on Vydac C18 semipreparative column using linear acetonitrile−water gradient with both solutions containing 0.1% of trifluoroacetic acid. On standing in solution, we observed that the peptides isomerise. Three different impurities accumulate, which can be separated on HPLC. This reaction is observed only in metal-free peptides and depends strongly on the temperature. In the absence of calcium or lanthanide ions the ^{1}H NMR spectra of the impurities are nearly identical as those of the original peptides, but unlike the latter they do not show any shifts induced by metal binding. We believe that these impurities are products of transamidation of one or both Asp-Gly groups (Borin et al., 1989) present in the peptide sequences (see Figure 1).

Dimers of C12 peptide molecules linked covalently by S−S bonds between their Cys8 residues were prepared by overnight incubation of C12 peptide at 25 °C in air-saturated 0.01% solution of $CuSO_4$ in 0.2 M Tris, pH = 8.6, and purified by HPLC.

NMR Experiments

All samples of P12 and N12 peptides were prepared in 99.98% D_2O or in 20% D_2O containing 100 mM NaCl and 50 mM imidazole as a buffer. pH or pD, measured with glass electrode without correction for isotope effect, was adjusted to 6.9. Peptide concentration was determined by fitting tyrosine UV absorbance within the range 230−280 nm (Fasman, 1976) to sample spectra measured on a Cary 3E spectrometer. A program was written by us for IBM PC computer using the least-squares fitting procedure. This method eliminates, in large extent, errors due to base line deviations. We estimate the accuracy of determination of peptide concentration at about 2%. Fractions of transamidated species were estimated from NMR spectra, and concentration corrections were introduced when necessary.

The peptides were titrated by Ca^{3+} or Ln^{3+} ions by adding small volumes of concentrated solutions of $CaCl_2$ or $LnCl_3$. When necessary, corrections for peptide concentration changes were introduced. Concentrations of $LnCl_3$ solutions ($LaCl_3$ in the case of NMR experiments) were determined by titration with EDTA in the presence of xylene orange (Lee & Sykes, 1980).

For assignment of proton signals two-dimensional ^{1}H NMR experiments were performed on a Varian Unity Plus 500 MHz instrument. Data were processed using Varian VNMR software (version 5.1). The real data matrix was

typically 512 points in ω_1 and 2048 in ω_2. Water signal suppression was achieved by presaturation during recycle delay. TSP was used as an internal reference. TOCSY spectra were recorded using a MLEV17 (Bax & Davis, 1985) with mixing times of 100 and 150 ms. ROESY experiments were performed with 50 and 100 ms mixing times.

1D ^{1}H NMR spectra were recorded on a Bruker AM 500 spectrometer equipped with a BVT-1000 temperature control unit. The spectra were acquired using 64K data points to achieve a digital resolution of 0.16 Hz/point.

Line shape analysis (Binsch, 1969; Szymanski et al., 1978) of aromatic tyrosine signals in 1D ^{1}H NMR spectra was performed using the SYMULANT software on a 486 PC computer.

Fluorescence Measurements

For energy transfer measurements stock solutions containing 200 μM peptides were prepared in 0.1 M NaCl, 20 mM PIPES, pH 6.9. Peptide concentration was determined as described above. Adequate volumes of concentrated $TbCl_3$ or $HoCl_3$ solutions were added to two separate samples of peptide solution to achieve a peptide/metal ratio of 1/1. Then the two samples were mixed together in various proportions. In this way a series of samples was prepared containing the same concentration of peptide fully saturated with lanthanide ions with various $[Tb^{3+}]/[Ho^{3+}]$ proportions. The samples were put into a 1 mm quartz cell, rapidly frozen with liquid nitrogen, placed into a 10 mm quartz cell filled with acetonitrile, and kept in a temperature control unit at -22 ± 1 °C.

Terbium luminescence was excited at 283 nm using a xenon−mercury lamp (Hamamatsu L2482) equipped with a double quartz prism monochromator (M3, Cobrabid). The excitation beam was modulated by a mechanical chopper giving 5 ms periods of excitation and 5 ms dark periods with a shut-off time of 150 μs. The emission from the surface of frozen samples was measured using a glass filter (OG4, Schott, Jena) with transmission less than 1% below 480 nm, and R585 (Hamamatsu) photomultiplier working in the single photon counting regime. Data were recorded with Nucleus Personal Computer Analyzer PCA-II (Tennelec/Nucleus Inc.) in microchannel scaler mode with time resolution of 10 μs.

Luminescence decay lifetimes were determined as described in Dadlez et al., (1991) by fitting data collected during dark periods to a monoexponential function describing accumulation as well as decay of excited Tb^{3+} ions during the excitation and decay periods, respectively.

The equilibrium constants of Ca^{2+} and Ln^{3+} ion coordination by N12 peptide have been determined from titration measurements of Tyr7 fluorescence intensity and least-square fit of theoretical curve to experimental data. Tyrosine fluorescence was measured using an apparatus described previously (Dadlez et al., 1991).

Molecular Modeling

Molecular models of Ln^{3+}-saturated P12 and C12 dimers were generated on a 486 PC computer using AMBER program (Pearlman et al., 1991) supplemented by simple self-made procedures. The loop structures were assumed to be identical to that of loop III of rat testis calmodulin (Babu et al., 1988). P12 dimer was constructed by linking two loops together by two hydrogen bonds between CO and NH groups of their Ile residues of exactly the same length and angle as those linking loops III and IV in rat testis calmodulin (Babu et al., 1988). Various structures of C12 dimer were obtained corresponding to a set S−S bond configurations found in proteins (Richardson, 1981).

Calculations of Electrostatic Interactions

Electrostatic interactions between P12 and N12 peptide molecules in a dimer were calculated using the DelPhi program (Sharp & Nicholls, 1989; Gilson et al., 1988). This program calculates the electrostatic potential for a system of low dielectric constant containing charges (the protein) immersed in a region of high dielectric constant (the solvent). The program accomplishes this by numerically solving the Poisson−Boltzmann equation.

The calculations were performed using dielectric constants of 2 and 4 relative to the permittivity of vacuum for the peptide region. For the outside (solvent) region the value of 80 was used. Partial electric charges for all atoms were adapted from the AMBER package (Weiner et al., 1984). Dimer coordinates were modeled on the basis of the crystal structure of calmodulin as described above.

The interaction between two peptides forming a dimer was calculated as follows: the total coulombic energy (E_{AB}) was calculated for the dimer with all partial charges present, for the dimer with partial charges present only in the first peptide (E_A) and for the dimer with partial charges present only in the second peptide (E_B). Thus the electrostatic interaction between the two peptides forming the dimer can be expressed as $E_{inter} = E_{AB} - E_A - E_B$.

The contributions of individual groups to this energy were calculated by computing the electrostatic potential in the dimer with partial charges present only in the first peptide. Then energy of interaction between an atom group in the second peptide (J) and the whole first peptide was calculated as $E_J = \Sigma q_i \phi_i$ where q_i is the partial charge of atom i, ϕ_i is the potential at this atom, and summation is performed over the atom group of interest (J), viz. a side chain or a main chain of a residue.

CD Measurements

CD spectra were acquired on an Aviv 62DS spectrometer. Samples were prepared in 2 mM cacodylic buffer, pH 7.0, and 100 mM NaCl by dilution of concentrated stock solutions of the peptides. Peptide concentrations in the stock solutions were determined by tyrosine absorbance, as described above. The peptides were freshly purified by HPLC and did not contain any detectable transamidation products.

RESULTS

NMR Measurements

The ^{1}H NMR spectra of P12 peptide are characteristic for random coil conformation (Bundi & Wuthrich, 1979). With increasing concentration of calcium, most signals shift either upfield or downfield (not shown) by at least 0.01 ppm and some of them by as much as 0.1 ppm. Such spectral changes are characteristic of the coordination of metal ions with fast association−dissociation kinetics on NMR time scale (Gariepy et al., 1983; Marsden et al., 1988). The value of the binding constant for Ca^{2+} at 25 °C, determined by best-fit analysis of titration shifts is about 400 M^{-1}. A similar value

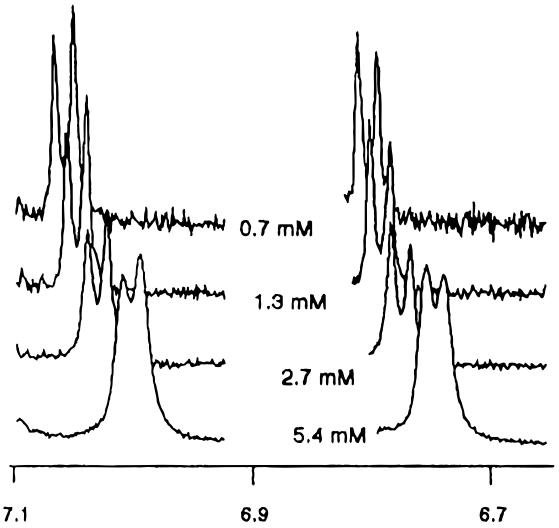

FIGURE 2: Concentration dependence of Tyr7 ring proton signals of La^{3+}-saturated P12 peptide measured at 80 °C in D_2O, 100 mM NaCl, 50 mM imidazole, pD 6.9. Small signals that do not shift come from impurities (see Materials and Methods).

(200 M^{-1}) was obtained from CD and fluorescence titration experiments (Dadlez, 1991).

At low peptide concentrations (0.7 mM) the spectral changes induced by La^{3+} ions are very similar to those produced by calcium. All observed shifts are strictly linear with increasing La^{3+} concentration up to [La^{3+}]/[peptide] = 1, and then remain constant. Since the lowest peptide concentration was 0.3 mM, the coordination constant for lanthanum must exceed 10^5 M^{-1}. The value of 1.5×10^5 M^{-1} has been determined from peptide fluorescence titration experiments (Dadlez, 1991).

There is an important difference, between the spectral behavior of the calcium- and lanthanum-saturated peptides. While the spectra of the former do not show any concentration dependence, some resonances of the latter shift considerably with concentration increasing above 1 mM and they become much broader. The resonances most affected are the aromatic, Hα, and one of the Hβ protons of Tyr7, the Hα and Hβ of Ser9, the Hα of Gly6 and Ala10 residues. A number of signals do not shift, e.g., those of the Hβ of both alanines, and remain sharp, indicating that the observed effect is not due to nonspecific peptide aggregation.

The smallest concentration effects are observed at low temperature. The line broadening is the most pronounced at about 40 °C. Then, with increasing temperature, the signals become narrower again, although they shift even more. As an example the temperature changes of Tyr7 ring proton signals in concentrated peptide samples (10.8 mM) are shown in Figure 3.

The observed phenomenon strongly suggest that P12, when saturated with La^{3+} ions, associates, most probably dimerizes, with association−dissociation kinetics that is intermediately fast on the NMR time scale. At high temperatures and concentrations the peptide is almost completely associated, its NMR signals are quite sharp and their positions are close to those characteristic for peptide dimers. The association constant drops with temperature, so that the NMR spectrum at 10 °C, of even quite concentrated peptide (10 mM), is rather similar to that measured in diluted solutions.

We attribute line broadening to transition of peptide molecules between monomeric and dimeric forms. Broadening is most pronounced when both forms are about equally populated and the dimerization kinetics not too fast, viz. at about 40 °C.

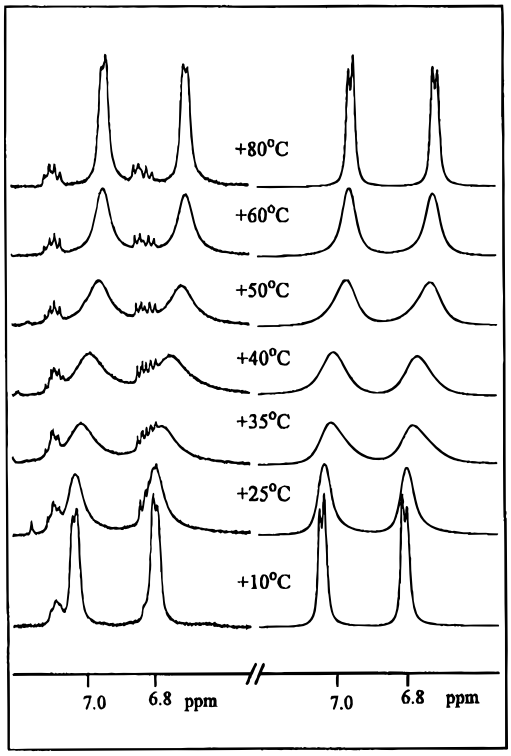

FIGURE 3: Temperature dependence of Tyr7 ring proton signals of P12 peptide saturated with La^{3+} ions. Left, experimental spectra. Peptide concentration 10.8 mM in D_2O, 100 mM NaCl, 50 mM imidazole, pD, 6.9. Small signals that do not shift come from impurities (see Materials and Methods). Right, spectra symulated using the dimerization equilibrium and rate constants shown in Table 1 and the following NMR parameters: chemical shifts for the monomer, 3532, 3532, 3404, and 3404 Hz; for the dimer, 3466, 3466, 3335, and 3335; the coupling constants for both forms, J_{meta} = 2.0, J_{ortho} = 8.0, and J_{para} = 0.5 Hz.

At constant temperature the signal half-widths and chemical shifts depend on their chemical shifts in monomer and dimer, δ_1 and δ_2, respectively, on the dimerization equilibrium constant (K), and on k_a, the association rate constant. δ_1 can be measured directly at low peptide concentration. The other parameters can be found by total line shape analysis fitting the calculated signals to those measured at various peptide concentrations. We have performed this analysis on the Tyr7 ring proton signals as described in Materials and Methods, for various temperatures ranging from 10 up to 80 °C. Parameters K and k_a were determined independently for each temperature, but it was assumed that the signal chemical shifts were temperature-independent and the same δ_2 best-fit values were used in all simulations. The Tyr7 ring proton signals have been chosen since they are well separated from the rest of the spectrum and their half-widths and positions can be determined with high precision.

Results of this calculation are presented in Figure 4 and in Table 1. The association equilibrium (K) and rate (k_a) constants change with temperature according to van't Hoff relationship. For the unitary process of monomer → dimer transition we obtain ΔH^* = 49 kJ/mol and ΔS^* = 188 J/mol·K. The thermodynamic parameters of the dimer formation are ΔH = 62 kJ/mol. and ΔS = 233 J/mol·K.

^{1}H NMR spectra of N12 peptide provide no evidence of dimerization neither of its Ca^{2+}- nor La^{3+}-saturated forms.

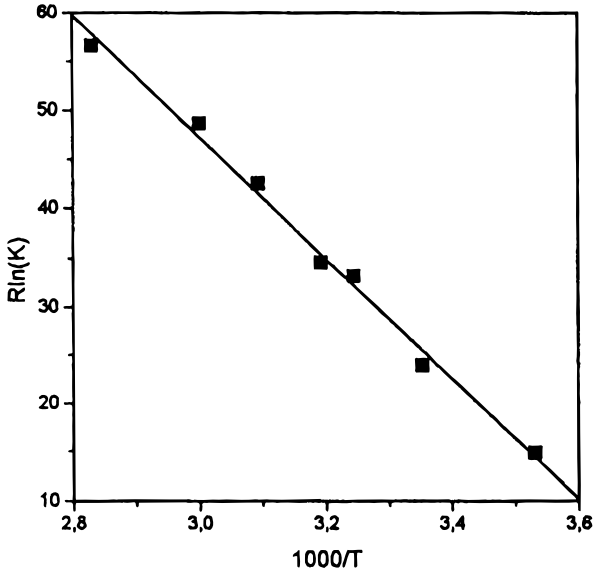

FIGURE 4: Van't Hoff plot of dimerization constant of La^{3+}-saturated P12 peptide. Interpolation line corresponds to $\Delta H = 62$ kJ/mol and $\Delta S = 233$ J/mol·K.

Table 1: Dimerization Equilibrium (K) and Rate (k_a) Constants of La^{3+}-Saturated P12 Peptide Determined by Analysis of Its Tyr7 Ring Proton NMR Signals

t (°C)	k_a (10^3 mol^{-1} s^{-1})	K (mol^{-1})
10	9	6
25	11	18
35	23	55
40	30	65
50	58	170
60	120	360
80	510	930

The dimerization constants for this peptide must be at least one order of magnitude lower than those of La^{3+}-saturated P12 peptide.

Fluorescence Measurements

The equilibrium constants of Ca^{2+} and La^{3+} ion coordination by N12 peptide determined from Tyr7 fluorescence titration experiments are 10^2 and 10^3 M^{-1}, respectively. The latter value is 2 orders of magnitude lower than for P12 peptide (10^5 M^{-1}).

If lanthanide-saturated molecules of P12 really form dimers it should be possible to observe an energy transfer from terbium to holmium ions (Horrocks et al., 1980; Snyder et al., 1981; Rhee et al., 1981) coordinated to the same dimer molecule, provided that the distance between them is not too large. As a result, in the presence of Ho^{3+}, shortening of luminescence lifetime of peptide-bound Tb^{3+} should be observed. Unfortunately, the results obtained in liquid solutions would be uninterpretable for two reasons. Firstly, even at high temperatures and concentrations the peptide is not fully dimerized. Secondly, dissociation of Tb^{3+} ion from the peptide is fast relative to its luminescence lifetime (Dadlez et al., 1991). Therefore, we decided to make the measurements in frozen solutions, expecting that the peptide molecules would be frozen out of the crystallizing solvent and be forced to dimerize because of their very high local concentration.

Samples of the peptide fully saturated with Tb^{3+} and Ho^{3+} mixtures with variable molar fraction of terbium (x) were prepared, and the Tb^{3+} luminescence was measured as described in Materials and Methods.

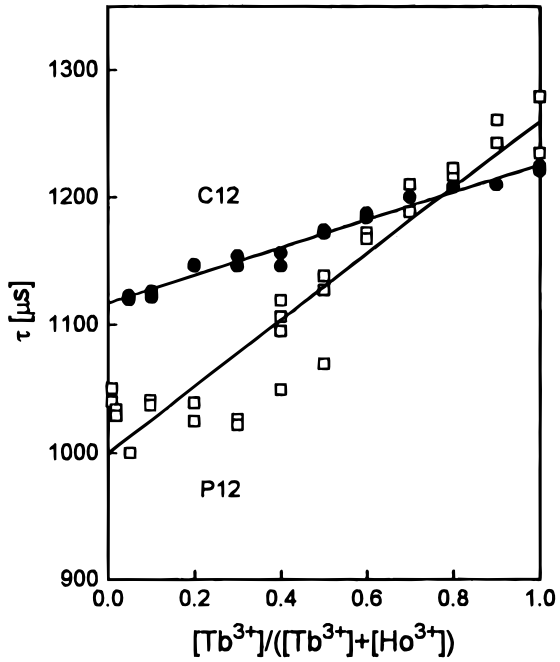

FIGURE 5: Luminescence lifetimes (τ) of Tb^{3+} ions bound to P12 and C12 peptides, as indicated, as a function of Tb^{3+} molar fraction (x) in $TbCl_3$ + $HoCl_3$ mixture in frozen H_2O solutions, 100 mM NaCl, 20 mM PIPES, pH 6.9, $t = -22$ °C. The least-squares interpolation lines are shown. The τ_h values have been calculated by extrapolation to $x = 0$.

In principle, two-exponential decays should be expected since the luminescence lifetimes of a Tb^{3+} ion equals either τ_h or τ_0 depending on whether its neighbor in the dimer is Ho^{3+} or another Tb^{3+} ion. The normalized amplitudes of both exponents should be equal to the respective probabilities of both events, namely, $1 - x$ and x, respectively. Therefore, the observed luminescence decay S(t) should be described by the following expression:

$$S(t) = x \exp(-t/\tau_0) + (1 - x)\exp(-t/\tau_h) \qquad (1)$$

In practice, the τ_0 and τ_h values are so close to each other that the observed luminescence decay can be described by a single exponent with the time constant τ being a weighted value of τ_0 and τ_h:

$$\tau = x\tau_0 + (1 - x)\tau_h \qquad (2)$$

Equation 2 was verified by simulating experimental data for various x values using eq 1 and fitting them to a monoexponential curve.

In Figure 5 the τ values determined for various $[Tb^{3+}]/[Ho^{3+}]$ ratios are shown. As expected, the terbium luminescence is quenched in the presence of holmium and its apparent lifetime changes linearly with Tb^{3+} molar fraction (x).

Quenching of terbium luminescence by energy transfer to holmium is described by the standard Forster relation (Forster, 1948, 1965):

$$\tau_0/\tau_h = 1 + (R_0/r)^6 \qquad (3)$$

where r is the donor−acceptor distance and R_0 the critical distance for 50% energy transfer.

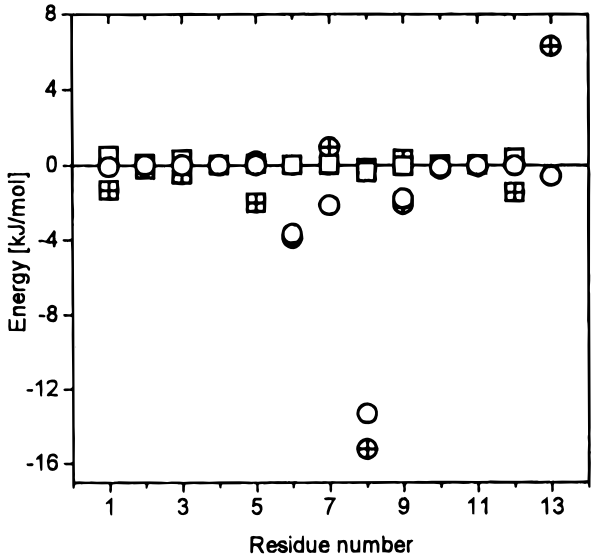

FIGURE 6: Contributions to the energy of electrostatic interactions between peptides in a P12 dimer ($\epsilon = 2$). Open and crossed symbols represent Ca^{2+}- and Ln^{3+}-saturated dimer, respectively. Circles and squares represent main chain and side chain contributions, respectively. "Residue" 13 is the bound metal ion.

Table 2: Energy of Electrostatic Interactions between Metal-Saturated Peptide Molecules in a Dimer (kJ mol^{-1})

	$\epsilon = 2$	$\epsilon = 4$
Ln^{3+}-saturated P12	−19.2	−10.4
Ca^{2+}-saturated P12	−21.4	−11.3
Ln^{3+}-saturated N12	−17.8	
Ca^{2+}-saturated N12	−21.2	

The τ_0 value (1.26 ± 0.03 ms) was measured directly at $x = 1$, and that of τ_h was obtained by linear extrapolation of eq 2 to $x = 0$ (see Figure 5). The critical distance for terbium and holmium ions coordinated by proteins has been determined by Horrocks and his collaborators (Snyder et al., 1981; Rhee et al., 1981). Since R_0 depends on the acceptor lifetime we calculated R_0 from Horrocks's data for $\tau_0 = 1.26 \pm 0.03$ ms ($R_0 = 9.12 \pm 0.06$ Å). In this way the distance $r = 11.4 \pm 0.4$ Å has been determined from eq 3. This is the same, within the error limits, as the distance of 11.2 Å determined from molecular modeling of a dimer corresponding to the two-loop segment of native calmodulin (see Materials and Methods).

For comparison, similar measurements have been made for dimers of C12 peptide (see Figure 1) linked covalently by S8−S8 bonds. An analogous linear function of $\tau = f(x)$ is obtained in this case (see Figure 5). The distance r between Tb^{3+} and Ho^{3+} ions coordinated to the C12 dimer calculated in the same way as for P12 dimers is greater and equals 13.9 ± 1.5 Å, in agreement with the value obtained from molecular modeling (11.8−14.5 Å, depending on S−S bond conformation).

Electrostatic Interactions within the Dimer

The global energy of electrostatic interactions is favorable for dimer formation (see Table 2). Its calculated absolute values are not trustworthy, because they strongly depend on a rather arbitrarily chosen ϵ value of the peptide. Nevertheless, the calculation results provide a firm enough basis for qualitative analysis of the electrostatic interactions within the dimer. The major contribution to dimer stability comes from interactions between main chain groups (NH and CO) of residue 8 of both peptide molecules that are hydrogen-bonded with one another (see Figure 6). Substitution of calcium by lanthanide ions leads to a substantial increase of the dimerization electrostatic energy of P12 molecules due to strong repulsion between the trivalent charges (see position 13 in Figure 6). This unfavorable effect is almost completely compensated by favorable interactions of negatively charged side chain groups of Asp1, Asp5, and Glu12, as well as main chain carbonyl group of Ile8 of one peptide molecule with the lanthanide ion coordinated by the other. Interestingly, the positive charges of both Lys2, distant from the rest of the molecule and surrounded by water, do not contribute significantly to the total electrostatic energy of the dimer. Intermolecular interactions of the side chain group of Asp3 are also negligeably small. In consequence, the electrostatic energy of dimer formation is almost the same for P12 and N12 molecules saturated with calcium ions (see Table 2).

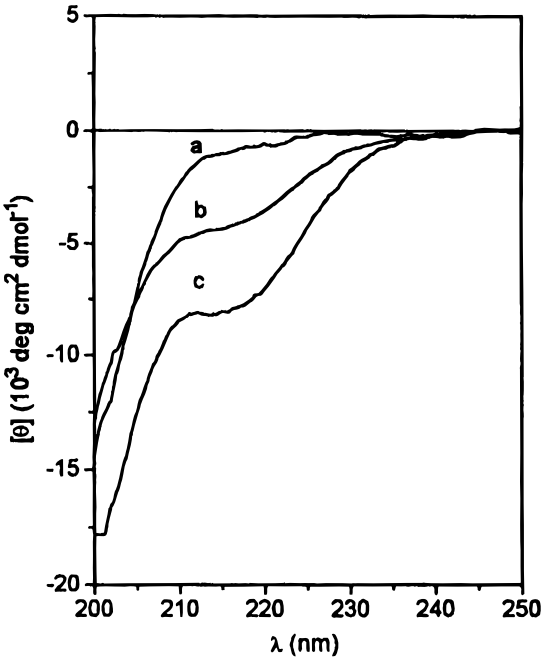

FIGURE 7: CD spectra of peptides P12 and N12 at 1 °C. (a) In the absence of Ca^{2+} or Ln^{3+} ions the spectra of both peptides are indistinguishable from one another. (b) In the presence of 200 mM $CaCl_2$ the both spectra are close to each other and are presented by a common curve. (c) P12 peptide in the presence of 2 mM $TbCl_3$.

CD Measurements

The CD spectra (see Figure 7) of free peptides P12 and N12 at 1 °C are characteristic of random coil conformation. The spectrum of P12 peptide saturated with lanthanide ions shows a relatively strong minimum at 215 nm. Moreover, this spectrum does not change with temperature increase up to 80 °C. The spectra of the calcium-saturated peptides are intermediate in character. They change slightly, but noticeably, with temperature approaching the random coil conformation (data not shown).

DISCUSSION

The luminescence energy transfer experiments provide an evidence that when lanthanide-saturated solutions of P12 peptide are frozen the peptide dimers are formed. In no other case, viz. random aggregation or formation of higher

associates, the linear dependence of terbium luminescence lifetime on terbium molar fraction (see Figure 5) could be observed. It should be expected that similar dimers are formed in liquid solutions.

NMR signal broadening analysis has been used extensively to investigate internal mobility in proteins, such as phenylalanine ring flipping motions (Wuthrich & Wagner, 1978). In this work we present a rare example of an intermolecular process, namely a dimerization, that can be studied using this method.

As indicated by temperature dependence of dimerization constants, the process is entropy-driven. This suggests that the dimer is stabilized by hydrophobic interactions, similarly as the two-loop structural motives in native EF-hand proteins (see introduction). The entropy of hydrophobic interactions changes with temperature. Therefore, the ΔS value of P12 dimerization determined by us from the van't Hoff relationship (233 J/mol·K) must be treated only as a rough estimation of its value at room temperature. Our data are not precise enough for a more sophisticated analysis.

Why is it that only lanthanide ions, and not calcium ones, induce dimerization of P12 molecules? Electrostatic repulsion between the loops is not responsible albeit the net global charge equals 0 and -1 for La^{3+} and Ca^{2+} ion complexes, respectively. If it were dimerization should have been observed for calcium-saturated loops of N12 peptide, the net charge of which is 0. Moreover, our calculations show that not the global charge of the peptides but local interactions between the charged groups determine the electrostatic energy of the dimer. This energy is slightly lower in the case of Ca^{2+}-saturated loops and it is the same for P12 and N12 molecules. We believe, then, that the answer lies in the much higher rigidity of the loop structure induced by lanthanide ions in P12. Because calcium is coordinated much less strongly—its binding constant is 3 orders of magnitude lower—it is not able to lock down the liganding groups as tightly as lanthanides do and, consequently, the structure of calcium-saturated loops is much more flexible. The observed differences in CD spectra of P12 saturated with Ca^{2+} and Ln^{3+} ions (see Figure 7) strongly support this hypothesis. Dimerization of flexible loops is not favorable because it leads to considerable decrease in entropy resulting from formation of a rigid interloop β structure. Since N12 peptide coordinates lanthanides with a low binding constant, 2 orders of magnitude lower than P12 peptide, even if it is saturated with Ln^{3+} ions its loop structure is not rigid enough to allow for effective dimerization.

Consider now the thermodynamic cycle shown in Figure 8. It follows from the cycle that the ratio of equilibrium constants K_3/K_1 describing the binding of La^{3+} ions to monomers (K_1) and partly saturated dimers (K_3) of the peptide must be equal to K_2/K_4, the ratio of equilibrium constants describing dimerization of metal-saturated molecules (K_2) and association between metal-saturated and metal-free molecules (K_4). The K_3/K_1 ratio can be treated as a measure of cooperativity similar to that describing the binding of metal ions to two-EF-hand-motive domains in native proteins.

The necessary condition of loop dimerization is their very rigid structure maintained by a tightly bound metal ion. It seems certain, then, that completely flexible metal-free peptide molecules cannot associate either between themselves, or with their metal-saturated counterparts. Therefore,

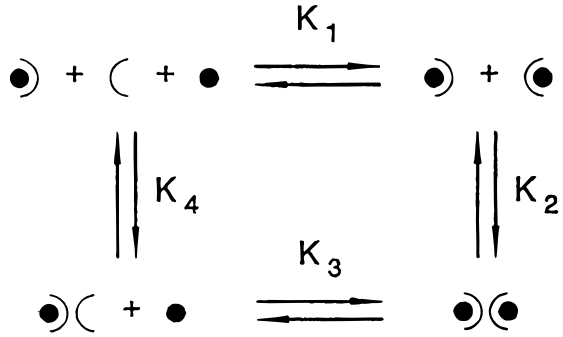

FIGURE 8: Dimerization and metal binding equilibria in solution containing peptide molecules shown as thin half-circles and metal ions indicated as small full circles.

$K_2 \gg K_4$, and La^{3+} binding to P12 dimers is a highly cooperative process with K_3/K_1 value much greater than 1. A few years ago a theoretical analysis of dipoles induced in calbindin D_{9k} upon calcium binding (Wesolowski et al., 1990) led us to conclude that local interactions between system of dipoles induced in calcium-binding loops alone can be responsible, at least to some extent, for the observed cooperativity of Ca^{2+} binding to EF-hand protein domains. The results of this work strongly support this hypothesis.

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