

# The enthalpy of the alanine peptide helix measured by isothermal titration calorimetry using metal-binding to induce helix formation

Maria M. Lopez\*<sup>†</sup>, Der-Hang Chin\*<sup>‡§</sup>, Robert L. Baldwin\*<sup>†¶</sup>, and George I. Makhatadze\*<sup>¶</sup>

\*Department of Biochemistry and Molecular Biology, Penn State University, College of Medicine, Hershey, PA 17033; <sup>†</sup>Department of Biochemistry, Beckman Center B400, Stanford University Medical Center, Stanford, CA 94305; and <sup>‡§</sup>Department of Chemistry, National Changhua University of Education, Changhua 50058, Taiwan, Republic of China

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**The goal of this study is to use the model system described earlier to make direct measurements of the enthalpy of helix formation at different temperatures. For this we studied model alanine peptides in which helix formation can be triggered by metal (La<sup>3+</sup>) binding. The heat of La<sup>3+</sup> interaction with the peptides at different temperatures is measured by isothermal titration calorimetry. Circular dichroism spectroscopy is used to follow helix formation. Peptides of increasing length (12-, 16-, and 19-aa residues) that contain a La<sup>3+</sup>-binding loop followed by helices of increasing length, are used to separate the heat of metal binding from the enthalpy of helix formation. We demonstrate that (i) the enthalpy of helix formation is  $-0.9 \pm 0.1$  kcal/mol; (ii) the enthalpy of helix formation is independent of the peptide length; (iii) the enthalpy of helix formation does not depend significantly on temperature in the range from 5 to 45°C, suggesting that the heat capacity change on helix formation is very small. Thus, the use of metal binding to induce helix formation has an enormous potential for measuring various thermodynamic properties of  $\alpha$ -helices.**

The year 2001 marked the 50th anniversary of the  $\alpha$ -helix, the first proposed protein secondary structure (1). However, despite numerous efforts (see refs. 2–5 and references therein), the detailed thermodynamic basis for helix formation and for the helix propensities of the amino acids is not yet well understood. Accurate values for such basic thermodynamic parameters as the changes in enthalpy and heat capacity on helix formation are still under debate. Recently Bierzynski and coworkers (6, 7) developed a peptide system for inducing helix formation by adding a metal ion. They took a 12-residue sequence, analogous to a Ca<sup>2+</sup>-binding loop from calmodulin (peptide P1), which forms a short and very stable C-terminal helix, containing three to four residues, when the peptide binds La<sup>3+</sup>. The C-terminal segment of La<sup>3+</sup>-bound P1 provides a stable helical nucleus for helix propagation when additional residues are added at the C terminus. Bierzynski and coworkers also made a longer peptide (P2) with three additional alanine residues plus one C-terminal glutamine residue, and determined the NMR structure of the La<sup>3+</sup>-ligated form. Their model system should allow accurate determination of the enthalpy of helix formation by using ITC (isothermal titration calorimetry) to monitor stepwise, La<sup>3+</sup>-induced helix formation, and we exploit this potential here.

Helix-coil transitions of short peptides in water cannot usually be described by a two-state model because the helix propagation constant is not large enough. For this reason, and also because the enthalpy change on helix formation is not large, temperature-induced helix unfolding spans a very broad temperature range, which makes it difficult to determine the baseline. Analysis of thermal helix-coil transitions can be based on the Zimm–Bragg or Lifson–Roig theories (e.g., see ref. 2), but the enthalpy values determined in this way must be tested by direct calorimetric measurements. The calorimetric measurements, on the other hand, are hampered by the broad transition curves and uncertain baselines: fully helical structures in typical peptide systems are

never attained in aqueous solutions, even at 0°C. Moreover, the change in heat capacity on helix formation,  $\Delta C_p$ , is a critical parameter in determining the enthalpy change and  $\Delta C_p$  cannot be measured at present by scanning calorimetry because of the broadness of the thermal transition curves.

If a small change in solvent composition can induce helix formation, the enthalpy of helix formation can be measured using ITC, and the calmodulin-derived peptide system (6, 7) offers this possibility. The basic motif (P1-peptide) consists of 12-aa residues analogous to the calcium-binding loop III of rat calmodulin; the residues that coordinate Ca<sup>2+</sup> in calmodulin are 1, 3, 5, 9 and 12 (8). Although P1 does not bind Ca<sup>2+</sup> with high affinity, it does bind La<sup>3+</sup> reasonably well ( $K = \approx 10^5$  M<sup>-1</sup>). In the NMR structure of the 16-residue La<sup>3+</sup>-ligated peptide P2 (1NKF, ref. 7), which contains four additional helix-forming residues at the C-terminal end, seven to eight C-terminal residues are helical (Fig. 1). CD data corroborate the structural results and, furthermore, CD data indicate that P2 does not have a high helical content in the absence of La<sup>3+</sup>, and therefore La<sup>3+</sup> binding triggers helix formation. Consequently, helix formation induced by lanthanum binding can be a useful model system to study the thermodynamics of helix formation at constant temperature. In addition to determining more accurate values of the enthalpy changes on helix formation for various peptides, it should be possible to determine  $\Delta C_p$  by measuring  $\Delta H$  as a function of temperature.

To test the usefulness of this model system, we measured the enthalpies of La<sup>3+</sup> binding to P1, P2 (P1 with additional four residues at the C terminus), and P3 (P1 with additional seven residues at the C terminus) using ITC. These experiments, when combined with CD-based estimates of the changes in fraction helix on La<sup>3+</sup> binding, give estimates of the enthalpy of helix formation from 5–45°C.

## Materials and Methods

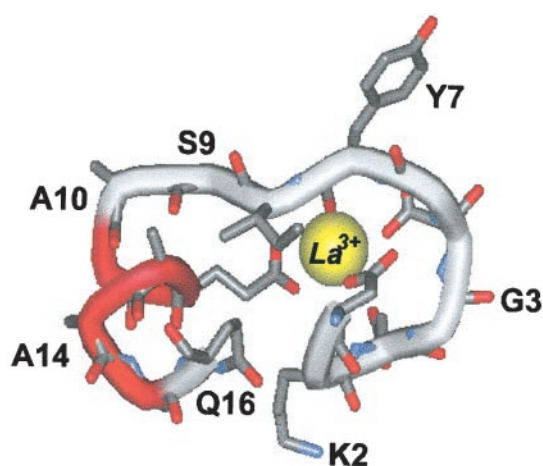
**Peptide Synthesis, Purification, and Sample Preparation.** Peptides P1 (Ac-DKDG DG YISAAE-NH<sub>2</sub>), P2 (Ac-DKDG DG YISAAE-AAAQ-NH<sub>2</sub>), and P3 (Ac-DKDG DG YISAAEAAAAAAQ-NH<sub>2</sub>) were synthesized by the solid phase method, using fluorenylmethoxycarbonyl (Fmoc) chemistry as described (9). P6N5 peptide (Ac-AAKAAY-NH<sub>2</sub>) was a gift from J. M. Scholtz (Texas A&M University, College Station, TX). The peptides were purified by C18 reverse-phase column chromatography. The identity of peptides was confirmed by mass spectrometry and purity was shown to be better than 95% by analytical C18

Abbreviation: ITC, isothermal titration calorimetry.

<sup>†</sup>M.M.L. and D.-H.C. contributed equally to this work.

<sup>¶</sup>To whom reprint requests may be addressed. E-mail: bbaldwin@cmgm.stanford.edu or makhatadze@psu.edu.

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**Fig. 1.** Cartoon representation of the structure of P2-peptide in  $\text{La}^{3+}$ -ligated form. Selected amino acid residues are labeled.  $\text{La}^{3+}$ -ion shown in the CPK representation. One representative structure of 30 models in 1NFK entry (7) to Protein Data Bank was used for illustration.

reverse-phase column chromatography. For the  $\text{La}^{3+}$  titration experiments, the stock solutions of peptides were dialyzed extensively against at least two changes of corresponding buffer containing either 1 mM sodium cacodylate or Tris, and 100 mM NaCl (pH 6.9). Spectrapor CE dialysis membranes with a 1,000-Da molecular weight cutoff were used for dialyses. The peptide stock solution was diluted to a working concentration of  $\approx 50 \mu\text{M}$  in the last change of the dialysis buffer. The peptide concentration was measured spectrophotometrically by using a molar extinction of  $1,450 \text{ M}^{-1}\text{cm}^{-1}$  at 275 nm determined as described (9). The  $\text{LaCl}_3$  (Alfa Aesar, Ward Hill, MA) solutions for ITC experiments in the last change of corresponding buffer were prepared by weight. The  $\text{LaCl}_3$  concentration was kept around 2 mM.

**Circular Dichroism Spectroscopy.** The CD spectra of peptides in the presence or absence of  $\text{LaCl}_3$  were recorded using 1-mm path-length cells in an Aviv 62 DS spectropolarimeter equipped with a Peltier temperature control unit (Aviv Associates, Lakewood, NJ) or in a Jasco J-715 spectropolarimeter (Jasco, Easton, MD) equipped with a Neslab RTE-140 temperature control unit (Neslab Instruments, Portsmouth, NH) as described (9). CD samples contained 100  $\mu\text{M}$  of peptide in 1 mM sodium cacodylate/100 mM NaCl (pH 6.9) buffer, with or without 2.5 mM  $\text{LaCl}_3$ . The thermal transition data were recorded every  $1^\circ\text{C}$  with an equilibration time of 2 min (Aviv 62 DS) or 6–8 min (Jasco J-715). The CD spectra of these short helices will be reported elsewhere (D.H.C., R. W. Woody, C. A. Rohl, and R.L.B., unpublished work), and compared with theoretical calculations by R. W. Woody. In comparing CD results with theory, ellipticity is expressed per mole of peptide groups rather than amino acid residues (10), and the same convention is used here.

**Isothermal Titration Calorimetry.** The overall procedure for ITC experiments was similar to that described (11). The VP-ITC from Microcal (Northampton, MA) was used in all experiments. Briefly, 3  $\mu\text{l}$  of the  $\text{LaCl}_3$  solution at concentrations  $\approx 2 \text{ mM}$  were injected into the cell containing peptide solution at concentration  $\approx 50 \mu\text{M}$ . The blank injections of solution into corresponding buffer were used to account for the heats of mixing and dilution. In each experiment 40 to 45 injections are made, so that the final molar ratio of  $\text{LaCl}_3$  to peptide is 3.5 to 4. The heat of the reaction,  $Q$ , is obtained by integrating the peak after each injection of peptide ligand using ORIGIN software provided by

the manufacturer (Microcal, Northampton, MA). The heat of the reaction at each injection is related to the calorimetric enthalpy of binding,  $\Delta H_{\text{cal}}$ , and the other thermodynamic parameters as (12):

$$Q = \Delta H_{\text{cal}} \frac{A - \sqrt{A^2 - 4[\text{La}^{3+}]_t[\text{pep}]_t}}{2[\text{pep}]_t} \quad [11]$$

where  $A = [\text{pep}]_t + [\text{La}^{3+}]_t + 1/K_a$ ,  $n$  is the stoichiometry of the  $\text{La}^{3+}$ /peptide complex,  $K_a$  is the association constant,  $[\text{pep}]_t$  is the concentration of a peptide, and  $[\text{La}^{3+}]_t$  is the total concentration of  $\text{LaCl}_3$ . Alternatively,  $\Delta H_{\text{cal}}$  was calculated by summing individual heat increments corrected for dilution and dividing it by the total number of moles of peptide in the cell.

## Results

**Isothermal Titration Calorimetry.** Fig. 2A shows representative ITC titration of the solution of P1-peptide with  $\text{LaCl}_3$  at  $25^\circ\text{C}$ . The interaction is endothermic and the titration of  $\text{La}^{3+}$  goes to completion within 30 injections. Fig. 2B plots the sum of the measured heat effects after each injection, normalized by the concentration of P1-peptide in the cell, for two independent experiments performed in sodium cacodylate buffer and for one experiment performed in Tris·HCl buffer. The agreement between titration profiles performed in the two different buffers (sodium cacodylate and Tris·HCl) is remarkably good. These two buffers have significantly different ionization enthalpies:  $-0.5 \text{ kcal/mol}$  for cacodylate and  $11.3 \text{ kcal/mol}$  for Tris (13). Agreement of the titration profiles performed in buffers with different ionization enthalpies provides experimental evidence for the absence of linked protonation effects when  $\text{La}^{3+}$  binds to P1-peptide (11, 14). Similar results have been obtained for peptides P2 and P3 (data not shown). Consequently, the measured heat effects do not include any contribution from buffer ionization linked to protonation of P1-peptide.

Fig. 2B shows also the results of the simultaneous fit of all three experiments to Eq. 1. The stoichiometry of binding is  $0.99 \pm 0.03$ , indicating that one molecule of  $\text{La}^{3+}$  binds to P1-peptide, as expected. The dissociation constant is 5  $\mu\text{M}$ , similar to earlier estimates for the synthetic peptide analogues of calcium-binding loops (7, 8).

Fig. 3 shows the temperature dependence of the experimental enthalpy,  $\Delta H_{\text{cal}}$ , that accompanies  $\text{La}^{3+}$  binding to P1, P2, and P3 peptides. The enthalpy values differ not only in magnitude but also in the sign of the observed heat effect, which is endothermic for the P1 peptide but exothermic for P2 and P3 peptides.

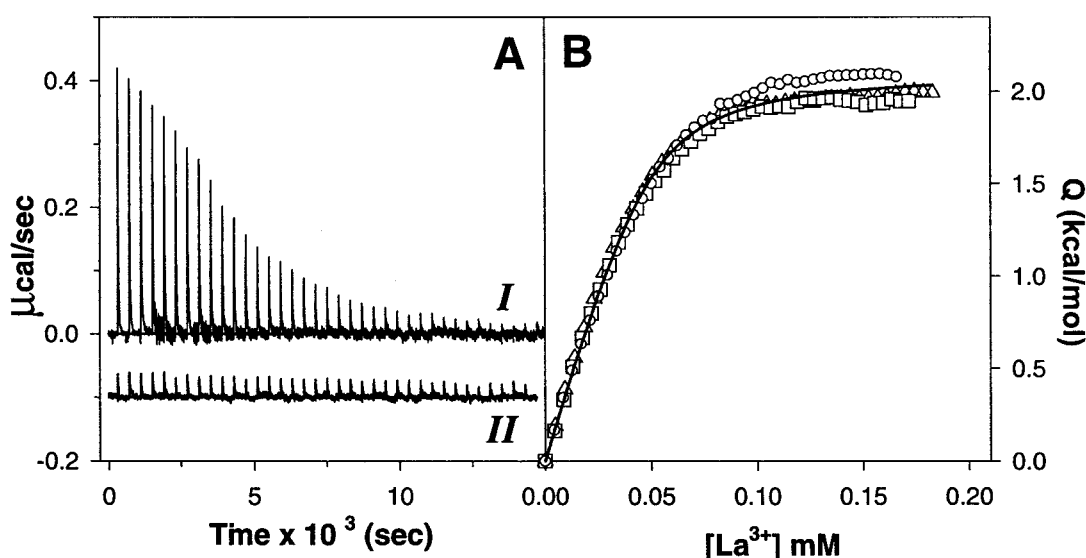
The enthalpy measured experimentally for each peptide on adding  $\text{La}^{3+}$  [ $\Delta H_{\text{cal}}(\text{P1})$ ,  $\Delta H_{\text{cal}}(\text{P2})$ ,  $\Delta H_{\text{cal}}(\text{P3})$ ] consists of several terms:

$$\Delta H_{\text{cal}}(\text{P1}) = \Delta H_{\text{La}^{3+}} + \Delta H_{\text{ns}} + \Delta H_{\text{ion}} \quad [2]$$

$$\Delta H_{\text{cal}}(\text{P2}) = \Delta H_{\text{La}^{3+}} + \Delta H_{\text{ns}} + \Delta H_{\text{ion}} + \Delta H_{\alpha}(\text{P2}) \quad [3]$$

$$\Delta H_{\text{cal}}(\text{P3}) = \Delta H_{\text{La}^{3+}} + \Delta H_{\text{ns}} + \Delta H_{\text{ion}} + \Delta H_{\alpha}(\text{P3}) \quad [4]$$

where  $\Delta H_{\text{La}^{3+}}$  represents the enthalpy of binding  $\text{La}^{3+}$  to the calcium binding loop;  $\Delta H_{\text{ion}}$  is the enthalpy linked to protonation effects, caused by changes in pKa values on complex formation;  $\Delta H_{\text{ns}}$  is the enthalpy of reorganization of the calcium binding loop (i.e., first 12 residues); and  $\Delta H_{\alpha}$  is the enthalpy of helix formation in each of the peptides. Of these four terms, the enthalpy of ionization appears to be very small—i.e.,  $\Delta H_{\text{ion}} \approx 0$ . The enthalpy of rearrangement of the first 12 residues involves conformational change in the calcium-binding loop on  $\text{La}^{3+}$  binding and also helix formation by three to four C-terminal residues; it is represented by the sum of  $\Delta H_{\text{La}^{3+}}$  and  $\Delta H_{\text{ns}}$ . This term is assumed to be the same for all three peptides and



**Fig. 2.** Isothermal titration calorimetry. (A) Representative ITC experiment. Heat effects recorded as a function of time during 40 successive 3- $\mu$ l injections of 2.01 mM solution of  $\text{LaCl}_3$  into the cell containing either 50  $\mu\text{M}$  solution of P1-peptide (profile I) or buffer solution (profile II) at 25°C. Buffer conditions: 1 mM sodium cacodylate, 100 mM NaCl, pH 6.9. (B) Comparison of three independent  $\text{LaCl}_3$  titration profiles for P1-peptide at 25°C. The buffer conditions are sodium cacodylate ( $\square$  and  $\circ$ ) and Tris ( $\triangle$ ). The solid line represents the nonlinear regression of all three experiments to Eq. 1 with the parameters  $n = 0.99 \pm 0.03$ ;  $K_d = (5.3 \pm 0.6) \times 10^{-6}$  M;  $\Delta H = 2.1 \pm 0.1$  kcal/mol.

therefore equals the experimentally measured enthalpy of P1-peptide,  $\Delta H_{\text{cal}}(\text{P1})$ . The enthalpies of helix formation in the P2 and P3 peptides are written as follows:

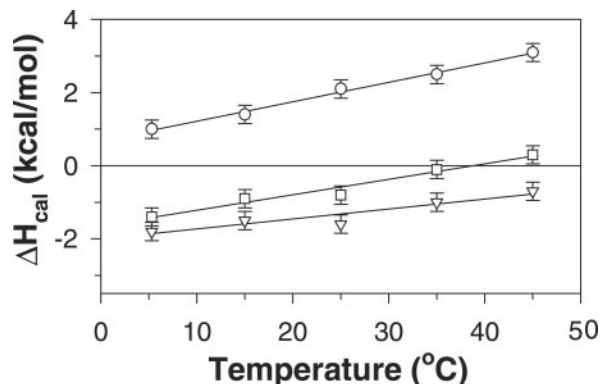
$$\Delta H_{\alpha}(\text{P2}) = 4 \cdot [1 - F_{\alpha}^{-}(\text{P2})] \cdot \Delta h_{\alpha} \quad [5]$$

$$\Delta H_{\alpha}(\text{P3}) = 7 \cdot [1 - F_{\alpha}^{-}(\text{P3})] \cdot \Delta h_{\alpha} \quad [6]$$

where  $F_{\alpha}^{-}$  represents the fraction of each peptide that is already in helical conformation before adding  $\text{La}^{3+}$  and  $\Delta h_{\alpha}$  represents the enthalpy of helix formation per residue, which becomes helical upon  $\text{La}^{3+}$  binding. Note that peptides P2 and P3 contain four and seven additional residues, respectively, that are not included into the  $\Delta H_{\text{ns}}$  term.

From Eqs. 2–6 it follows that the enthalpy of helix formation, on a per residue basis,  $\Delta h_{\alpha}$ , can be calculated as:

$$\Delta h_{\alpha} = \frac{\Delta H(\text{P2}) - \Delta H(\text{P1})}{4 \cdot [1 - F_{\alpha}^{-}(\text{P2})]} \quad [7]$$



**Fig. 3.** Temperature dependence of the calorimetric enthalpy of interaction of peptides P1 ( $\circ$ ), P2 ( $\square$ ), and P3 ( $\nabla$ ) with  $\text{La}^{3+}$ -ions. Error bars represent the standard deviation of multiple experiments. The lines through the points are linear fits of the data and are shown to guide the eye.

$$\Delta h_{\alpha} = \frac{\Delta H(\text{P3}) - \Delta H(\text{P1})}{7 \cdot [1 - F_{\alpha}^{-}(\text{P3})]} \quad [8]$$

provided that the fraction of the peptide forming helical structure upon  $\text{La}^{3+}$  binding is known. This fraction can be measured using circular dichroism spectroscopy.

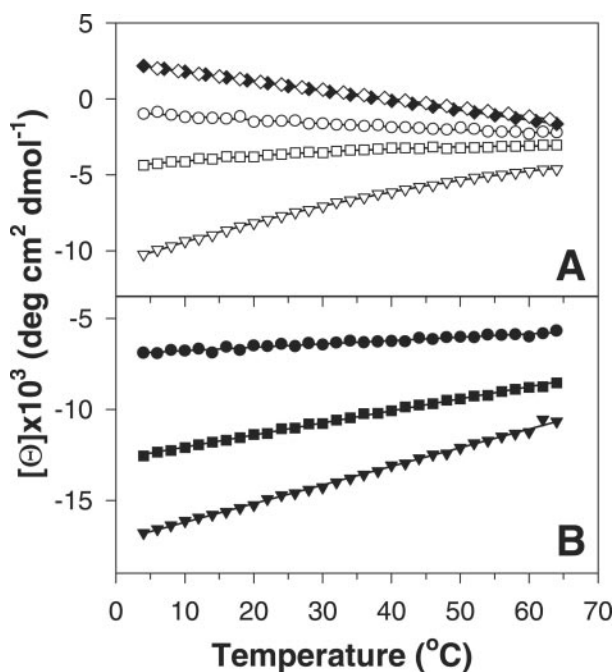
**Circular Dichroism Spectroscopy.** The fraction of helical residues in peptides P2 and P3 in the absence of  $\text{La}^{3+}$  can be calculated from the experimentally measured ellipticity of these peptides,  $[\Theta^{-}(\text{P2})]$  and  $[\Theta^{-}(\text{P3})]$ , as

$$F_{\alpha}^{-}(\text{P2}) = \frac{[\Theta(\text{P2})]_{\text{c}} - [\Theta^{-}(\text{P2})]}{[\Theta(\text{P2})]_{\text{c}} - [\Theta(\text{P2})]_{\text{h}}} \quad [9]$$

$$F_{\alpha}^{-}(\text{P3}) = \frac{[\Theta(\text{P3})]_{\text{c}} - [\Theta^{-}(\text{P3})]}{[\Theta(\text{P3})]_{\text{c}} - [\Theta(\text{P3})]_{\text{h}}} \quad [10]$$

where  $F_{\alpha}^{-}(\text{P2})$  and  $F_{\alpha}^{-}(\text{P3})$  are fraction helix in the absence of  $\text{La}^{3+}$ ,  $[\Theta(\text{P2})]_{\text{c}}$  and  $[\Theta(\text{P3})]_{\text{c}}$  are the ellipticities of peptides P2 and P3 in the unfolded (“coil”) state, respectively, and  $[\Theta(\text{P2})]_{\text{h}}$  and  $[\Theta(\text{P3})]_{\text{h}}$  are the ellipticities of peptides P2 and P3 in the fully helical state, respectively. Note that  $F_{\alpha}^{-}(\text{P2})$  and  $F_{\alpha}^{-}(\text{P3})$  refer only to those additional residues that are forming helical structure besides the first 12 residues of peptide P1.

Fig. 4 A and B shows the dependence on temperature of the ellipticity of peptides P1, P2, and P3 in the presence and absence of  $\text{La}^{3+}$ . Note that the ellipticity of each peptide is larger in the presence of  $\text{La}^{3+}$  (Fig. 4B) than in its absence (Fig. 4A) and that the plots with  $\text{La}^{3+}$  present are straight lines, whereas the plots with  $\text{La}^{3+}$  absent curve downward for peptides P2 and P3. The temperature dependence of the ellipticity of a short model peptide P6N5 (see sequence in *Materials and Methods*) is also given in Fig. 4A. Analysis of these results (D.H.C., R. W. Woody, C. A. Rohl, and R.L.B., unpublished work) indicates that, in the presence of  $\text{La}^{3+}$ , the helix contents of peptides P1, P2, and P3 are independent of temperature from 4–65°C, and are presumed to be >95% helical in this range. The NMR structure of P2 at 2°C indicates that residues Ser-9 to Ala-15 are helical and that



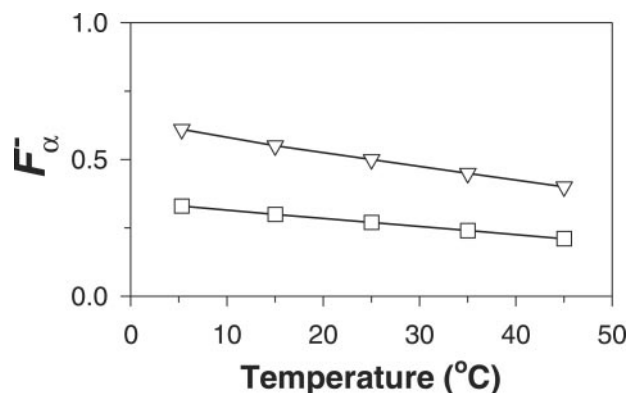
**Fig. 4.** Temperature dependence of the ellipticity of peptides in the presence and in the absence of  $\text{LaCl}_3$ : P1 at 215 nm ( $\circ$ , no  $\text{La}^{3+}$ ;  $\bullet$ , with  $\text{La}^{3+}$ ); P2 at 217 nm ( $\square$ , no  $\text{La}^{3+}$ ;  $\blacksquare$ , with  $\text{La}^{3+}$ ); P3 at 219 nm ( $\nabla$ , no  $\text{La}^{3+}$ ;  $\blacktriangledown$ , with  $\text{La}^{3+}$ ). The opened and closed diamonds ( $\diamond$ ,  $\blacklozenge$ ) in A show the temperature dependence of the ellipticity of P6N5 peptide (Ac-AAKAAAY-NH<sub>2</sub>) monitored at 219 and 217 nm, respectively.

Gln-16 is partly helical, whereas chemical shift data indicate that residues Ala-10 to Gln-16 are helical (7). In analyzing our ITC data for both peptides P2 and P3 in the presence of  $\text{La}^{3+}$ , residues past the calcium-binding loop are treated as being fully helical. Therefore, it is assumed that  $[\Theta(\text{P2})]_{\text{h}} = [\Theta^{\oplus}(\text{P2})]$  and  $[\Theta(\text{P3})]_{\text{h}} = [\Theta^{\oplus}(\text{P3})]$ , where  $[\Theta^{\oplus}(\text{P2})]$  and  $[\Theta^{\oplus}(\text{P3})]$  represent the ellipticity of P2 or P3 peptides in the presence of  $\text{La}^{3+}$ . The curved plots for peptides P2 and P3 in the absence of  $\text{La}^{3+}$  (Fig. 4A) suggest that some helical structure is present. The ellipticities for the unfolded states of P2 and P3 peptides can be obtained as follows:

$$[\Theta(\text{P2})]_{\text{c}} = \frac{13[\Theta^-(\text{P1})] + 4[\Theta(\text{P6N5})]}{17} \quad [11]$$

$$[\Theta(\text{P3})]_{\text{c}} = \frac{13[\Theta^-(\text{P1})] + 7[\Theta(\text{P6N5})]}{20} \quad [12]$$

where  $[\Theta^-(\text{P1})]$  is the ellipticity of P1 in the absence of  $\text{La}^{3+}$ , and  $[\Theta(\text{P6N5})]$  is the ellipticity value for the model peptide. The reason for using the CD spectra of two peptides (P1 and P6N5) to represent the unfolded CD spectra of peptides P2 and P3 is that “unfolded” peptides do in fact have CD spectra, and therefore it is desirable to model the unfolded CD spectrum of a peptide by using matching sequences. The P1 peptide matches the sequence of the first 12 residues (or 13 peptide groups) of peptides P2 and P3, whereas the alanine-based peptide P6N5 matches approximately the remaining alanine-based sequence. It is possible that the CD spectrum of unfolded P1 contains a small contribution from partly helical residues. We assume that any partly helical residues are contained in residues 1–8, which do not become helical on adding  $\text{La}^{3+}$ . The fraction helix for peptides P2 and P3 in the absence of  $\text{La}^{3+}$  is shown in Fig. 5. As expected, both peptides P2 and P3 have some residual helix



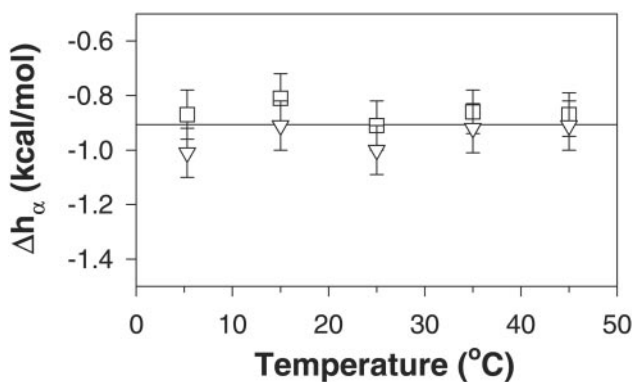
**Fig. 5.** Temperature dependence of the fraction helix of peptides P2 ( $\square$ ) and P3 ( $\nabla$ ) in the absence of  $\text{La}^{3+}$ -ions. The lines through the points are shown only to guide the eye.

content in the absence of  $\text{La}^{3+}$  at low temperature. This residual helix content gradually melts with increasing temperature.

**Enthalpy of Helix Formation.** The enthalpy of helix formation per residue,  $\Delta h_{\alpha}$ , can now be calculated by combining Eqs. 9 and 10 and 7 and 8. The results of these calculations at different temperatures are presented in Fig. 6. There are three important results. First, the values of  $\Delta h_{\alpha}$  calculated from peptide P2 are not very different from the values calculated for peptide P3. This fact indicates that the assumptions made in calculating  $\Delta h_{\alpha}$  values are probably correct. Second, the value of  $\Delta h_{\alpha}$  is  $-0.9 \pm 0.1$  kcal/mol of amino acid residues, which is close to previous estimates of the enthalpy of helix formation (see *Discussion*). Third, the  $\Delta h_{\alpha}$  value does not change significantly with temperature, according to these data. This result means that  $\Delta C_p$  is too small to be detected by enthalpy measurements as a function of temperature in this model system.

## Discussion

The most recent calorimetric measurements of the enthalpy of helix formation are those of Richardson *et al.* (15) for a 31-residue peptide with the sequence Ac-Y(MEARA)<sub>6</sub>-NH<sub>2</sub>. They give  $\Delta H$  equal to  $-0.84 \pm 0.1$  kcal per mole of amino acid residues. CD measurements of the thermal helix-coil transition curves for a set of six alanine-based peptides, varying in chain length from 14–50 residues, gave an estimate of  $-1.0$  kcal/mol (2) when analyzed by either the Zimm-Bragg or Lifson-Roig theories (see also ref. 9). The thermal unfolding curve of the



**Fig. 6.** Temperature dependence of the enthalpy of helix formation  $\Delta h_{\alpha}$  calculated per mole residue found for peptide P2 ( $\square$ ) and P3 ( $\nabla$ ). The solid horizontal line shows the average of all data points.

50-residue peptide was also measured calorimetrically and the measured enthalpy agreed within error with the value found from the CD-monitored unfolding curves (16). Although the calorimetric enthalpy measurement is direct, it is less precise than estimates of  $\Delta H$  obtained by using helix-coil theory to analyze thermal unfolding curves, because the unfolding curve even of a 50-residue peptide is very broad and fitting the baseline is problematic. Early estimates of the enthalpy of helix formation using homopolymers of poly-L-lysine and poly-L-glutamate gave values between  $-1.1$  and  $-0.88$  kcal/mol residues (17, 18). All these values are similar to each other and somewhat higher than a recent estimate of  $-0.65$  kcal/mol per residue by Taylor *et al.* (19). These authors studied a 29-residue helical peptide, which contains  $i$  to  $i + 4$  chemical crosslinks (lactam bridges) at both the N and C termini to stabilize the helix covalently. Their results appear to fit the two-state model and they interpreted them by using this model, and by assuming that the cross-linked end residues induce more cooperative unfolding behavior.

Most of these results are consistent with the assumption that the enthalpy of helix formation depends only on the peptide backbone and is the same for different amino acids, except perhaps for glycine and proline. However, the relative helix propensities of the nonpolar amino acids change with temperature (20), and the effect appears to be enthalpic, because it has the opposite sign to that expected for the  $\Delta C_p$  arising from burial of nonpolar surface area. The changes of the relative helix propensities with temperature have the sign expected if side chains reduce the access of water to the peptide backbone and interfere with backbone solvation (20, 21). According to this proposal, other nonpolar amino acids should have smaller (negative) enthalpies of helix formation than alanine and the free-energy differences between the helix propensities provide an upper limit to the enthalpy differences between alanine and other nonpolar amino acids.

Because of the broad thermal unfolding curves shown by peptide helices, it has been particularly difficult to obtain the temperature dependence of the enthalpy change and the partial heat capacity of the fully helical peptide. It is also difficult to obtain accurately the temperature dependence of the partial heat capacity of the fully unfolded peptide. The latter obstacle has been overcome recently, however, by calculating the heat capacity of the unfolded state from an additivity approach (22). Direct measurements of the heat capacities of fully unfolded peptides support the validity of the additivity approach (J. M.

Richardson and G.I.M., unpublished work). The importance of knowing the value of  $\Delta C_p$  is illustrated by the work of Richardson *et al.* (15) who, by using the calculated heat capacity for the unfolded state, were able to measure the enthalpy of helix formation of the 31-residue peptide sequence Y(MEARA)<sub>6</sub>-NH<sub>2</sub>. Their value is based on the assumption that the heat capacity change on helix formation,  $\Delta C_p$ , is zero. Not only the value but even the sign of  $\Delta C_p$  for helix formation has been controversial. A structure-based thermodynamic calculation predicts that  $\Delta C_p$  for alanine-based helix folding should be small and positive (23). The opposite conclusion was reached from a calorimetric study of a short peptide with covalently crosslinked terminal residues (19), and also from a theoretical calculation (A. Garcia, personal communication). Our data demonstrate that the  $\Delta C_p$  of helix formation cannot be large. Using the maximal and minimal values of  $\Delta h_\alpha$  shown in Fig. 6 at the highest and lowest experimental temperatures (5 and 45°C) places limits on the allowed value of  $\Delta C_p$ , which then must be between  $-8$  and  $8$  cal/K per mol residue. Fitting their DSC data, Taylor *et al.* (19) gave a value for  $\Delta C_p$  of  $-3.8 \pm 0.7$  cal/K per mole residue. A computational estimate gave a negative value of  $-6 \pm 3$  cal/K per mol residue (A. Garcia, personal communication). An early structural thermodynamics prediction by Ooi and Oobatake (24) gave a smaller value of  $-1.9$  cal/K per mol residue.

**Concluding Remarks.** The feasibility of obtaining thermodynamic information about helix formation by using metal binding to induce helix formation in peptide sequences based on calmodulin was demonstrated using isothermal titration calorimetry. The enthalpy of alanine helix formation is found to be  $-0.9 \pm 0.1$  kcal per mol residue and does not depend on temperature in the range 5–45°C. This model system has a great potential for measuring more detailed thermodynamic properties, such as the enthalpy of helix formation for various amino acid residues, the enthalpies of interactions between amino acid side chains, and their temperature dependencies.

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