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Volume Changes in the Binding of Lanthanides to Peptide Analogues of Loop II of Calmodulin†

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ABSTRACT: The solution expansion accompanying coordination of lanthanide ions to synthetic peptide analogues of a metal-binding loop in calmodulin was determined by a density method. This study was designed to further test the hypothesis that the nonlinear expansions observed upon sequential addition of Ca^{2+} to intracellular calcium-binding proteins reflect principally upon the coordination event at specific binding sequences. Three peptides of 13 residues each were synthesized as analogues of binding loop II in mammalian calmodulin: Peptide I was the native analogue; peptide II contained an aspartyl in place of an asparaginyl residue at position 5 from the N-terminus; for peptide III, the aspartyl residue in position 3 of the native analogue was interchanged with the asparaginyl residue in position 5. Thus, the number of charged-oxygen donor atoms for coordination was the same in I and in III, but the latter peptide could permit two pairs of acidic groups to converge toward the metal ion as in some loops of these proteins. The observed expansions with different lanthanide ions to the same peptide varied appreciably, suggesting dissimilar structures [Gariépy et al. (1983) *Biochemistry 22*, 1765–1772]; coordination to the simpler tetracarboxylate sequestrants, on the other hand, generated an expansion profile approximately as expected from the properties of the lanthanide series. The largest expansions were generated with peptide II (having the additional acidic group) for all lanthanides tested. The smaller expansions seen with peptide III as compared to those for peptide I indicate that the free peptides do not adopt configurations like that comprising the apo-binding cavities in this class of proteins, wherein a putative overlap of electrostatic fields from converging carboxylate groups is frozen in. The distinctive volume changes attending these coordinations support the proposition that the volume property reflects small differences in coordinating sequences. Thus, volume change may be applied efficaciously to those proteins in which a sequential uptake of metal ion to multiple sites is manifested.

Considerable information on the intracellar calcium-binding proteins has been generated by studying metal-ion binding to synthetic peptide analogues of selected sequences within these proteins. A particular aspect has dealt with spectral studies on the binding of various lanthanide ions to peptide analogues (12–13 residues) of the calcium-binding loops within these proteins (Reid et al., 1980; Marchiori et al., 1983; Kanellis et al., 1983; Gariépy et al., 1983, 1985; Pavone et al., 1984; Buchta et al., 1986; Malik et al., 1987). These peptides do

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not take up Ca^{2+} appreciably (except in hydrophobic solvents) owing to the much smaller charge to ionic radius ratio of this ion; longer sequences comprising helical regions flanking these loops are required for Ca^{2+} to bind with affinities approaching those in the native proteins (Reid et al., 1981; Gariépy et al., 1982; Reid, 1987; Malik et al., 1987).

The above studies have presented an opportunity to test whether the nonuniform volume increases observed upon sequential addition of Ca^{2+} to this class of proteins reflect primarily the coordination event to specific binding loops (Kupke & Dorrier, 1986; Kupke, 1986). Our initial studies with Ca^{2+} added to model compounds bearing coordination resemblances to these binding loops have shown that the EGTA[1] class of sequestrants generate similar large volume increases (Kupke & Shank, 1989). In proceeding to synthetic peptide analogues of binding loops, which form the basis for this paper, we have observed little or no volume change upon adding Ca^{2+} or Cd^{2+} to these substances. Although volume changes do not necessarily parallel binding affinities, a zero volume change indicates no appreciable interaction. Upon addition of lanthanide ions, however, large volume increases were measured (30–60 mL/mol of metal ion). To ascertain whether significant differences in these expansions attend the addition of metal ion to different peptide analogues, expansions such as observed when Ca^{2+} was added sequentially to the proteins, we have undertaken a volume study of the binding sequences in calmodulin. For this paper, we have focused upon binding loop II (the second loop from the N-terminus) of mammalian calmodulin. Herein, we have compared a synthetic, 13-residue analogue of this binding loop with two similar peptides that were designed to test the effect of charge and of possible stereoelectronic influences of coordinating donor oxygens.

The physical principle underlying the relatively large expansions observed when di- and trivalent metal ions are coordinated to multiple-donor atoms of a liganding molecule is believed to be that of electrostriction. In aqueous media, water dipoles are highly compressed in the strong electric fields immediately adjacent to ions, such that the local collapse of the open, quartzlike structure of normal water leads to a reduction of as much as one-third in the molar volume [Conway, 1981; see also Millero (1971, 1972) on refinements to the original theory by Drude and Nernst (1894)]. Hence, upon coordination of a metal ion, a decompression, if not actual release of electrostricted water molecules, accompanies the bonding interaction. If donor atoms of the ligand molecule are also charged (negatively in this case), the expansion should be even larger. (Theoretical work on electrostriction and the volume property for multicoordination of metal ions has not been addressed appreciably; therefore, this work with models offers some substance for such studies.)

In the binding loops of the intracellular calcium-binding proteins, often three to four of the six (or more) oxygen donor atoms are charged. In some cases there are five coordinating acidic groups. [Marsden et al. (1988) have supplied a count summary of the coordinating carboxylate groups in 201 naturally occurring loops for this class of proteins.] In some of these binding loops, pairs of coordinating carboxylates appear more or less aligned toward the metal ion along a common axis according to earlier crystallographic studies; recent refinements of these, however, provide less support for narrowly confined alignments as a general trend (Szebenyi & Moffat, 1986; Satyshur et al., 1988). Convergent carboxylate pairs in synthetic organic compounds have been found to greatly enhance the stability of coordination (Rebek, 1987). These and other stereoelectronic influences may be presumed to also affect electrostriction in view of the volume studies by Kauzmann et al. (1962) on the protonation of constrained carboxylate groups. Accordingly, we have first focused upon binding loop II of beef calmodulin which contains four charged donor oxygens and a putative, convergent pair of carboxyl groups. Peptide I is designated in this paper as the analogue of the native binding loop II with the following amino acid sequence: Ac-Asp-Ala-Asp-Gly-Asn-Gly-Thr-Ile-Asp-Phe-Pro-Glu-Phe-NH_2, wherein the N-terminal amino group is acetylated and the carboxyl group of the C-terminal phenylalanine is amidated. These modifications remove charges that are not present in the native protein. The addition of the amide of phenylalanine as a 13th residue, while technically not of the binding loop, allows for the penultimate residue, glutamate, to coordinate via its λ-carboxyl group as in the native proteins. The oxygen donor atoms frequently found in this class of proteins are derived from residues 1, 3, 5, 7, 9, and 12, numbered from the N-terminus of the binding-loop sequence. Residues 1 and 9 are assigned to the $X,-X$ coordinates, 3 and 7 to the $Y,-Y$ coordinates, and 5 and 12 to the $Z,-Z$ coordinates in the Cartesian system that defines an approximate octahedral cavity containing the metal ion (Kretsinger & Nockolds, 1973; Reid & Hodges, 1980). For peptide II, an aspartyl residue was substituted for the asparaginyl residue in position 5, the peptide being otherwise the same as peptide I. This substitution allows for two pairs of convergent carboxylate donor groups at the $X,-X$ coordinates and the $Z,-Z$ coordinates by analogy to binding-loop configurations in some of the calcium-binding proteins. This peptide is analogous to that reported for loop II in scallop calmodulin (Toda et al., 1981). In the construction of the third peptide, peptide III, the purpose was to remove the additional charge given to peptide II without affecting the putative, two convergent pairs of carboxylates along the X and Z axes, if an octahedral-like configuration indeed is formed. Thus, peptide II has five possible charged-oxygen donor atoms compared to four in peptides I and III. Peptide III was synthesized to contain an asparaginyl residue at position 3 in place of the aspartyl residue (as occurs in peptides I and II), but was otherwise the same as peptide II. The complete sequences of these three peptides are shown in Table I. Since different lanthanide ions have been shown by ^1H NMR to not complex identically with a similar peptide analogue (Gariépy et al., 1983), a number of the ions encompassing the span of the lanthanide series ($La^{3+} \rightarrow Lu^{3+}$) were tested on each peptide.

For additional insight, lanthanide ions were coordinated also to smaller tetracarboxylate liganding molecules, the EGTA class of sequestrants. These sequestrants have been found to generate relatively large volume increases with Ca^{2+} (Kupke & Shank, 1989). For this comparison with the peptides, we have avoided the use of EGTA itself because the complication attended by the release of protons upon coordination requires additional corrective experiments, which reduces the overall precision. Hence, only the derivatives known as BAPTA and QUIN-2, which do not release protons during the complexation with metal ions at neutral pH (Tsien, 1980), were studied with

[1] Abbreviations: t-Boc, *tert*-butyloxycarbonyl; BAPTA, 1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid; EF, a designation commonly given to the characteristic sequence type and structure of metal-binding regions in many calcium-binding proteins (Kretsinger & Nockolds, 1973); EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HPLC, high-performance liquid chromatography; QUIN-2, *N*-[2-[[8-[bis(carboxymethyl)amino]-6-methoxy-2-quinolinyl]methoxy]-4-methylphenyl]-*N*-(carboxymethyl)glycine; TFA, trifluoroacetic acid.

the lanthanides. The X-ray structure of the Ca^{2+} complex with difluoro-BAPTA was reported recently (Gerig et al., 1987) and shown to follow an 8-fold coordination pattern similar to that found for the Ca^{2+} complex with EGTA (Schauer & Anderson, 1987).

Experimental Procedures

Volume changes were determined by density as described (Kupke, 1986), using here the batch protocol since the amounts of test materials were not so limiting as with the proteins. The electronics and certain other aspects of the magnetic-suspension instrument were revised and updated recently (Gillies & Kupke, 1988). The precision for replicate determinations on the same test sample was within 0.3 nL in the 100-μL portions used in the densimeter. These portions contained 0.1–0.2 μmol of peptide or sequestrant. Usually, 0.4–0.5 mL of sample was prepared by weight for each determination to allow for triplicate measurements and rinsings. Since samples were arranged to generate 10 nL or more in volume expansion per 100 μL for a measurement, an overall variation in the volume change, ΔV (mL/mol of complex), of no worse than 3% was observed routinely. The ΔV values from separately prepared samples containing the same materials corresponded to within 2 mL/mol except in a few worst cases. The ΔV values in this study ranged upward from 30 mL/mol for all lanthanide complexes; hence, the overall uncertainty in raw data translated usually to ±3% or better about the mean value. When lanthanide ions were added to these ligands, constant stirring with a slow rate of addition of ion was required for best precision. With the peptides, a pH of 6.0–6.5 was found to give the most consistent results [cf. Gariépy et al. (1985)]. With the sequestrants, a pH of 7 was utilized so that proton release during the complexation was minimal. The solvent throughout these experiments was 0.1 M KCl after treatment with Chelex. The ΔV values, in milliliters per mole of metal–ion complex, were calculated by subtracting the initial volume sum of the two phases prior to mixing from the final volume after mixing the phases. This volume difference was then reduced to that per mole of assumed complex. With these ligands, only 1:1 molar complexes were indicated by ΔV when the molar ratios of the metal ion and liganding compound were varied. Approximately the same volumes of ligand or metal ion (in the same solvent) were mixed to hold the correction for dilution effects to that within the accepted level of uncertainty for these studies.

Materials. The lanthanide ions (REacton grade) were obtained as the chloride hexahydrates from Johnson-Matthey Inc. (Seabrook, NH). Lanthanide concentrations were assayed by the xylenol-orange method (Lee & Sykes, 1980) using EDTA, complexometric grade, from Fluka (Ronkonkoma, NY) for the titration. This procedure often gave somewhat larger molar values than those deduced from the weighed-in amounts assuming constant hydration. BAPTA was obtained as the tetrapotassium salt from Fluka. QUIN-2 was the tetrapotassium salt from CalBiochem (La Jolla, CA).

Preparation of Peptide Analogues. The three peptides were synthesized by stepwise solid-phase peptide synthesis on a BioSearch 9500 automated peptide synthesizer. All amino acid amino termini were protected by t-Boc. The syntheses were performed according to the general method of Stewart and Young (1984). The first amino acid of each peptide was double coupled with diisopropylcarbodiimide onto methylbenzhydrylamine polystyrene resin. The extent of coupling of all amino acid derivatives was monitored by a quantitative ninhydrin reaction (Virender et al., 1981). The peptides were cleaved from the resin with HF containing 5% anisole.

The crude peptides were first desalted and partially purified on a Sephadex G-10 column (2 × 30 cm) with 50% acetic acid in distilled water as the eluant. The desalted peptides were further purified by reverse-phase HPLC. The column used was a Dynamax C-18 (21.4-mm i.d. × 300 mm; Ranin, CA). Buffer A was 0.1% TFA in distilled water; buffer B was 0.1% TFA in acetonitrile. The peptides were injected on the column in 10% buffer A/90% buffer B. A linear gradient of 10% buffer A to 100% buffer B was developed at 1%/min with a flow rate of 21 mL/min. The column effluent was monitored at 230 nm. The major peak from the chromatography of each peptide was collected and lyophilized. An aliquot of this product was rechromatographed and appeared as a single peak. The concentration and the composition of the pure peptides were determined by amino acid analysis (Yokote et al., 1986).

For the peptide sequence and amino acid analysis, weighed samples of the peptides were dissolved into acetic acid. An aliquot of these solutions, containing about 500 pmol of the peptide, was lyophilized in a hydrolysis tube to remove the acid. The samples were then subjected to gas-phase hydrolysis at 100 °C with 5.7 M HCl containing 1% phenol for 24 h. Following hydrolysis, the acid from the samples was removed and the samples were derivatized with phenyl isothiocyanate. This material was subjected to reverse-phase chromatography (Yokote et al., 1986; Bidlingmeyer et al., 1984). The peptide hydrolysate chromatograms were compared with hydrolyzed standard solutions of amino acids (Pierce, Rockford, IL) for identification and quantitation. Aliquots of the acetic acid solutions of the peptides were subjected to amino acid sequence determination on an Applied Biosystems 440A sequencer, equipped with an on–line 120 PTH analyzer (Hunkapillar et al., 1983).

Samples of lyophilized peptides were prepared for density analysis by dissolving 10–20 mg/mL in 0.1 M KCl and adjusting to the desired pH with 10 N KOH. The solutions were passed through demetalized and buffer-washed 0.22-μm Microstar filtering assemblies (Costar, Cambridge, MA). All samples were tested for heavy metals by atomic absorption and found to contain <50 ppm by weight. On occasion, the peptide solutions were stirred with Chelex 100 resin, centrifuged, and filtered; these gave identical values for the ΔV of complexation as those that had not been treated with Chelex. Final concentrations of peptide, ~5 mM, were calculated from the absorption at 257.4 nm related to the content of phenylalanine determined by amino acid analysis as noted above.

Results and Discussion

The volume increases observed upon coordination of selected lanthanide ions to the peptide analogue of binding loop II in beef calmodulin and to the two variants of this analogue are shown in Table I; values for Ca^{2+} and for Cd^{2+} complexations are also shown for comparison. From these data, it is clear that significant differences in ΔV of complexation can attend a single interchange of carboxyl and amide groups in a residue of the 13-member peptides. Moreover, it is obvious that substantial differences in ΔV arise from the coordination with individual members of the lanthanide series to a common liganding peptide. The immediate purpose for these experiments was to test the proposition that the similar, but nonidentical, binding loops in the intracellular calcium-binding proteins possess the potential for generating measurably distinct volume changes upon complexation with metal ions. These results provide a more firm basis for interpreting the nonlinear increases in volume observed when Ca^{2+} is added sequentially to calmodulin and to troponin C (Kupke & Dorrier, 1986; Kupke, 1986).

Table I: ΔV for Complexation of Metal Ions to Synthetic Peptides[a,b]

metal ion	ΔV[c] (mL/mol) peptide I	peptide II	peptide III
La^{3+}	34.1	48.5	33.1
Nd^{3+}	38.7	54.3	34.2
Eu^{3+}	39.0	53.6	29.8
Tb^{3+}	34.0	52.5	29.9
Yb^{3+}	40.3	58.5	30.2
Lu^{3+}	36.5	53.8	30.6
Cd^{2+}	1.1	6.6	2.6
Ca^{2+}	0.8	0.9	−2.1

[a] Peptide I: Ac-Asp-Ala-Asp-Gly-Asn-Gly-Thr-Ile-Asp-Phe-Pro-Glu-Phe-NH_2. Peptide II: Ac-Asp-Ala-Asp-Gly-Asp-Gly-Thr-Ile-Asp-Phe-Pro-Glu-Phe-NH_2. Peptide III: Ac-Asp-Ala-Asn-Gly-Asp-Gly-Thr-Ile-Asp-Phe-Pro-Glu-Phe-NH_2. Underlined residues reflect changes from the analogue of beef calmodulin binding loop II (peptide I) that contains residues 56–68, numbered from the N-terminus of the native protein (Roberts et al., 1986). [b] Conditions: 0.1 M KCl, pH 6.0–6.5, 20 °C. [c] All ΔV values are within ±1.5 mL/mol of the mean raw data for assumed 1:1 molar complexes upon independent determinations except for the Nd^{3+}–peptide II complex (±2.5 mL/mol).

Single-residue changes at the same positions as those seen in Table I have recently been studied on a peptide analogue of binding loop III in rabbit skeletal troponin C. These residue changes were found to produce distinctive ^1H NMR signals from which substantial differences in the binding affinities with the lanthanum ion (La^{3+}) were calculated (Marsden et al., 1988); a detailed comparison of those results with our volume changes is given presently. It is not possible from our data alone to relate the volume changes to binding affinities. ΔV contributes only trivially to the total energy change in condensed systems such as these. An analysis by Hepler (1965) on volume changes accompanying protonation of aqueous acid anions suggests that ΔV relates most closely to the entropy change of the system [cf. also Kauzmann et al. (1962)]. If we assume that the overriding contribution to the total volume change during the multicoordination of metal ion is from decompression of local water dipoles or partial dehydration of the inner hydration sphere of ions, an increase in the partial molar entropy of the solvent component is to be expected. As yet, no heat-transfer experiments have come to our attention on lanthanide complexation with similar peptide analogues by which to deduce the entropic contribution for possible correlation with these relatively large volume increases.

The following sections are presented as foundational material for correlating ΔV with available structure information that relates to the coordination events which take place during metal-ion uptake by the intracellular calcium-binding proteins.

Effect of Different Lanthanides. The variable volume increases induced by the coordination of different lanthanide ions to the same peptide accord with the ^1H NMR observations of a structural dissimilarity between complexes when a peptide analogue of binding loop III from rabbit skeletal troponin C is used (Gariépy et al., 1983). These authors showed that the peptide conformation and binding affinity differed appreciably between the complexes with the two lanthanides La^{3+} and Lu^{3+}, which ions comprise the extreme ends of this series. In those studies, the binding affinity with La^{3+} was found to be larger than that for the lutetium complex. In our study, the expansions found for the lutetium complexes were, unexpectedly, not greatly different from those for the lanthanum ones. The ΔV results for the various lanthanide ions complexed to our peptides show no correlation with the decrease in ionic radius as the atomic number increases. In electrostriction theory, the volume contraction when ions are added to a solvent of dipoles is inversely proportional to the ionic radius if charge and the other governing variables (compressibility and dielectric behavior) are constant [Drude & Nernst, 1894; Conway, 1981; but see Rashin and Honig (1985)]. Thus, the variations in Table I for a given peptide suggest structural differences for these lanthanide complexes in quasi agreement with the ^1H NMR findings noted above. We emphasize, however, that the volume property reflects upon the structures of both the solvent and the solute.

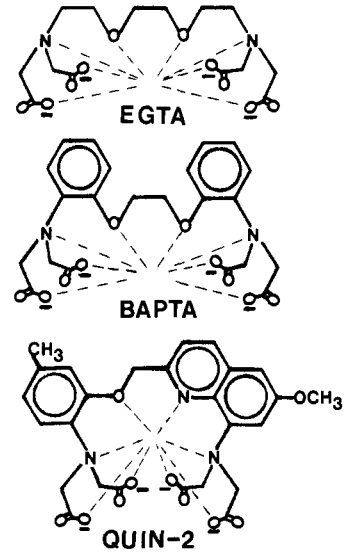

FIGURE 1: Skeletal outlines in two dimensions of BAPTA and QUIN-2 relative to EGTA. Hydrogen and carbon atoms (except as noted) are not shown; the latter are indicated at vertices. Dashed lines indicate the donor atoms coordinating to metal ion: EGTA donor atoms are from Schauer and Anderson (1987, 1989); BAPTA donor atoms are from Gerig et al. (1987); QUIN-2 donor atoms are assumed. Compound names represented by the preceding acronyms are given in footnote 1.

Table II: ΔV for Metal Ion Complexation to Tetracarboxylate Sequestrants[a]

metal ion	ΔV[b] (mL/mol) BAPTA	QUIN-2
La^{3+}	51.0	61.2
Nd^{3+}	55.5	66.4
Eu^{3+}	53.3	61.1
Gd^{3+}	55.3	57.5
Tb^{3+}	56.1	56.0
Er^{3+}	59.2	65.0
Yb^{3+}	62.2	75.2
Lu^{3+}	67.9	75.6
Cd^{2+}	28.1	37.5
Ca^{2+}	40.0	44.7[c]

[a] Conditions: 0.1 M KCl, pH 7, 20 °C. [b] All ΔV values are within ±1.0 mL/mol of the mean raw data upon independent determinations. [c] Reported previously (Kupke & Shank, 1989).

As a further test for the existence of structure differences in the above systems when using different lanthanide ions, we have carried out a comparison on the expansions attending the multicoordination of certain lanthanide ions to two smaller, tetracarboxylate model compounds, BAPTA and QUIN-2 (derivatives of EGTA). For ease in relating the designated acronyms to the differences in structure relative to EGTA, the 2-dimensional, skeletal outlines of these three sequestrants are shown in Figure 1. The ΔV values for lanthanide ion interactions with BAPTA and QUIN-2 are presneted in Table II. A clear trend toward larger volume increases with decreasing ionic radii of the lanthanide series ($La^{3+} \rightarrow Lu^{3+}$) is apparent, especially in the case of BAPTA. These model sequestrants are considerably more constrained for caging a metal ion than

are the 13-member peptides in solution, and they offer fewer options for contributing to an average structure with each lanthanide ion. Nonetheless, a break in this trend is evident between Nd^{3+} and Er^{3+} flanking the entries for Eu^{3+}, Gd^{3+}, and Tb^{3+} in Table II. The anomalous behavior, however, may reflect a change in the coordination number rather than to experimental error or to a departure from the Drude–Nernst relation for the effect of ionic radius on electrostriction. Horrocks and Sudnick (1979) noted that the solution properties of the $La^{3+} \rightarrow Nd^{3+}$ group and of the $Tb^{3+} \rightarrow Lu^{3+}$ group fall on two different smooth curves. A shift in the properties of Sm^{3+}, Eu^{3+}, and Gd^{3+} of intermediate ionic radii form a transitional region that connects the two smooth curves on either side for this projection of the lanthanide series. These authors proposed that this transition region reflects a changeover toward a decrease of one coordination number as the radius decreases with increasing atomic number. (A reduction in the coordination number conforms with the reduced expansions observed over this transition span.) Interestingly, the recent crystallographic data on Nd^{3+} and Er^{3+} complexes with EGTA (Schauer & Anderson, 1989) show clearly that the former ion is 10-coordinate and that the latter is 9-coordinate in these systems. We propose that these ions present the same coordination profiles with BAPTA and also that EGTA follows a similar nonlinear pattern in ΔV with the lanthanide series as found for BAPTA because (a) we have determined previously that BAPTA and EGTA generate virtually the same ΔV values upon complexation with Ca^{2+} (Kupke & Shank, 1989) and (b) the X-ray structures of EGTA (Schauer & Anderson, 1987) and of difluoro-BAPTA (Gerig et al., 1987) when complexed with Ca^{2+} exhibit the same 8-fold coordination to donor atoms of these ligands and show a similar overall geometry. As noted before, sufficiently accurate measurements of ΔV for lanthanides to EGTA are very difficult to accomplish in the appropriate alkaline region.

The larger ΔV values observed for the complexations with QUIN-2 as compared to those for BAPTA with the lanthanides were also found with the use of Ca^{2+} (Kupke & Shank, 1989). QUIN-2 has a quinoline moiety substituted for one of the ether oxygens in EGTA and BAPTA (Figure 1), and this modification could significantly affect the structure of the metal–sequestrant complex. The putative nitrogen donor from this moiety instead of the neutral ether oxygen should not contribute much difference in ΔV, but guidelines are not in hand to account for the presence of the large nonpolar quinoline group.

ΔV versus Affinity. The singular nature of the volume property is also highlighted by comparing the results on lanthanum complexes in Table I with affinity constants calculated from ^1H NMR experiments on similar lanthanum–peptide complexes. The latter studies were conducted on synthetic peptides based on binding loop III of rabbit skeletal troponin C (Marsden et al., 1988). The "native" peptide analogue of this binding loop had the following sequence: Ac-Asp-Arg-Asn-Ala-Asp-Gly-Tyr-Ile-Asp-Ala-Glu-Glu-Leu-NH_2. Additional peptides were prepared by substituting residues at certain coordinating positions in this analogue. The three peptides used in the ΔV experiments corresponded with three of the peptides studied by the latter authors as to positioning of the same nonconserved acidic residues. These residues, which were varied in both studies, were at the $+X$, $+Y$, and $+Z$ coordinating positions (i.e., residues 1, 3, and 5, numbered from the N-terminus; the two conserved acidic residues at coordinating positions $-X$ and $-Z$, or residues 9 and 12 from the N-terminus, were the same for all peptides in both studies as well as the peptide backbone carbonyl-donor oxygen at the $-Y$ coordinating position, residue 7, which is common to EF loops). Residue substitution was carried out by interchanging aspartyl and asparaginyl residues in both of the investigations. Marsden et al. (1988) found that the largest association constant with La^{3+} was generated with the peptide having aspartyl residues at the $+X$ and $+Z$ coordinating positions (their analogue labeled N, corresponding to the native loop III of this troponin C). In contrast, the volume increase for La^{3+} to the corresponding peptide, also with an aspartyl residue at the $+X$ and $+Z$ positions (our peptide III), was the smallest. The peptide containing aspartyl residues at the $+X$ and the $+Y$ coordinating positions showed the smallest affinity with La^{3+} in the experiments of Marsden et al. (1988) (their peptide III). The analogous peptide in our study (peptide I) generated a volume increase only slightly larger with La^{3+} than with our peptide III. Finally, the affinity found by Marsden et al. (1988) for the peptide containing five acidic groups (aspartyl residues at $+X$, $+Y$, and $+Z$ positions—their peptide I) was somewhat smaller upon complexation with La^{3+} than for their native analogue, N (acidic residues at $+X$ and $+Z$ only). The corresponding peptide containing five acidic groups in our study (peptide II) produced by far the largest expansion upon complexation with La^{3+}.

The smaller affinity found by Marsden et al. (1988) for the peptide containing five acidic groups (their peptide I) and the much smaller affinity obtained for their peptide II compared to that for the native analogue accord with an earlier prediction by Reid and Hodges (1980) that the presence of aspartyl residues at coordinating positions $+X$ and $+Y$ or at $+Y$ and $+Z$ should result in dentate–dentate repulsion. Such repulsions would tend to contribute to a smaller association constant with metal ion than would the native analogue containing these residues only at the $+X$ and $+Z$ positions. The volume studies, on the other hand, suggest that peptides with potentially greater dentate–dentate repulsion contribute to larger expansions upon complexation with metal ion. This is most easily seen by comparing our peptide I and peptide III systems. The latter peptide, containing acidic residues at the $+X$ and $+Z$ positions, yielded smaller volume increases with all lanthanides studied than did the former peptide in which aspartyl residues occupied the $+X$ and $+Y$ rather than the $+X$ and $+Z$ positions. A rationale for expansions arising from closely spaced carboxylate ions is discussed in the next section. Our peptide II, containing aspartyl residues at all three of these nonconserved coordinating positions ($+X$, $+Y$, and $+Z$) produced much larger expansions with all lanthanides tested; the additional charged donor oxygen, however, complicates a dentate–dentate repulsion comparison because of the expected volume increase arising from decompression of water dipoles about this oxygen upon metal–ion coordination. These larger expansions for peptide II are moderately larger than what has been reported for an additional carboxylate coordinating to divalent metal ions (Katz et al., 1975, 1978).

ΔV and EF Loop Configuration. The above differences in the direction of change for metal-ion affinity and for the volume invite some further remarks, although these can be of no more than a preliminary nature at this time. Reid et al. (1981) were the first to show that longer fragments than that of an EF binding loop were required for Ca^{2+} to complex with an affinity approaching that in the native rabbit skeletal troponin C protein. This finding indicated that the binding cavities for strong complexation with Ca^{2+} are frozen in by the superstructure of the native proteins. It is currently plausible to consider these preformed cavities in the proteins

somewhat as a charged sphere in a dielectric medium. In the original approximation for electrostriction (Drude & Nernst, 1894), the volume contraction was predicted to be proportional to the *square* of the charge when charging up a test sphere of constant radius in a medium of dipoles. Although this square law has not been sustained experimentally beyond that for divalent ions, the interesting volume experiments by Kauzmann et al. (1962) are instructive in this context. These authors showed that carboxylate ions constrained to produce overlap of their electric fields generated a much larger expansion upon protonation than did carboxylates not so constrained (e.g., the dianion of maleic acid versus the dianion of fumaric acid). Some of the EF type of binding cavities in proteins may contain constrained acidic groups to also provide a charged environment for generating greater electrostriction. Upon coordination with metal ion, the expansion from decompression of the nearby water dipoles would be larger than if the carboxylate ionic fields were acting independently. This circumstance may occur particularly in those EF loops containing convergent pairs of carboxylate groups. In rabbit skeletal troponin C, the two high-affinity loops (designated loops III and IV in the C-terminal half of the molecule) contain two approximately converging pairs at the $\pm X$ and $\pm Z$ coordinating positions of the assumed nonliganded cavity.[2] In previous ΔV experiments, the first 2 equiv of Ca^{2+} added to rabbit skeletal troponin C enlarged the volume ~70 mL/mol of Ca^{2+} equivalent, or twice as much as did the second pair of Ca^{2+} equivalents; the latter apparently went to the two low-affinity sites, loops I and II (Kupke, 1986). The sequence in binding loop II allows for one possible convergent pair of carboxylates and that for loop I allows for none (Collins et al., 1977). It is noted, however, that both oxygens from the acidic group at the $-Z$ coordinating position (glutamyl) in loops III and IV are donors to Ca^{2+} (Satyshur et al., 1988). The resulting 7-fold coordination would conceivably allow for more dipole decompression around the Ca^{2+} if sites I and II are only 6-coordinate. Since the expansion found for the high-affinity pair of sites in this protein was about double that found for the low-affinity sites, one additional oxygen donor, even if charged, should not produce this 2-fold difference. Barring an improbably large expansion resulting from the creation of void spaces from conformation changes attending the complexation of the troponin C with Ca^{2+}, we suggest that the electrostatic nature of the binding cavities in loops III and IV may provide an explanation for their much larger volume increases observed during Ca^{2+} complexation than can be rationalized from these experiments on isolated binding-loop peptides complexed with lanthanide ions. Indeed, our peptide III, which was designed to contain two potentially convergent pairs of carboxylate groups, exhibited somewhat smaller expansions with the lanthanides overall than did our peptide I having only one such possible pair. Obviously, a constrained convergence of carboxylates with overlapping fields prior to the complexation with metal ions is not indicated. Nonetheless, the small but clear differences in the expansions between peptides I and III do suggest that some time-averaged overlap exists in the electric fields of the carboxylate groups in the $+X$ and $+Y$ coordinating positions of peptide I.

In conclusion, the volume-change results with binding-loop peptide analogues support the hypothesis that the similar, but nonidentical, sequences of these loops in the intracellular calcium-binding proteins can generate significantly distinct volume increases upon complexation with metal ions. Thus, the changes in volume observed previously upon sequential addition of Ca^{2+} to two proteins of this class may reflect principally the coordination event to specific binding loops. In addition, these results have shown that the closely related metal ions of the lanthanide series produce distinctive volume expansions upon coordination with the same peptide or with the same model sequestrant. Finally, a comparison of published results reflecting solute properties by NMR experiments with those reflecting properties of both solvent and solute by these volume measurements presents complementary insights into the nature of protein–metal interactions.

[2] Recent refinements on the crystal structure of avian skeletal troponin C (Satyshur et al., 1988) show an intervening water molecule between the converging carboxylates at $X,-X$ coordinating positions in the metal-complexed, high-affinity loops.

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Use of Site-Directed Mutagenesis To Destabilize Native Apomyoglobin Relative to Folding Intermediates†

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ABSTRACT: Site-directed mutagenesis has been used to study the effect on the stability of human apomyoglobin (apoMb) of modifying the size, hydrophobicity, and charge of a central residue in the G·B helix–helix packing interface. Some stability measurements have also been made on the corresponding holomyoglobins (heme present). Cys-110, a central helix pairing residue in the G helix, has been changed to Ala, Ser, Asp, and Leu. Stability to low-pH-induced unfolding has been measured for both native apoMb and the compact folding intermediate discovered by Griko et al. [Griko, Y. V., Privalov, P. L., Venyaminov, S. Y., & Kutyshenko, V. P. (1988) *J. Mol. Biol. 202*, 127–138]. As judged by its circular dichroism spectrum, this intermediate has a substantial helix content (about 35%). Whether or not this inferred helical structure is closely related to the myoglobin structure is not yet known. The mutational evidence shows that integrity of G·B helix pairing is important for the stability of apoMb as well as of myoglobin and that this helix pairing site is very sensitive to both steric and electrostatic disruption. Our results also suggest that G·B helix pairing does not stabilize the compact intermediate; hence, disrupting this site destabilizes the native protein relative to the compact intermediate. Such selective destabilization of the native state relative to equilibrium folding intermediates is not restricted to acid denaturation: urea denaturation of the Leu mutant appears to display at least one stable intermediate, while wild-type and the remaining mutant apoMbs undergo two-state urea unfolding transitions.

Apomyoglobin (apoMb)[1] is the classic example of a protein whose folding pathway has been inferred from its structure. In particular, the framework model of folding is based in part on the postulated folding behavior of apoMb (Ptitsyn & Rashin, 1975; Richmond & Richards, 1978; Cohen et al., 1979), in which individual α-helices form independently of each other but are stabilized by helix pairing reactions, so that a stable framework of helical structure is formed which acts as a hydrophobic container for the heme group. Recently, Griko et al. (1988) have shown, especially by calorimetric measurements, that apoMb has a well-organized hydrophobic core and may be considered as a well-defined globular protein. In view of its significance for the framework model of folding,

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[1] Abbreviations: apoMb, apomyoglobin; CD, circular dichroism; GuHCl, guanidine hydrochloride; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; Mb, myoglobin; N, I, and U, native, acid intermediate, and unfolded states of apomyoglobin, respectively.

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