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VOLUME CHANGES UPON ADDITION OF Ca^{2+} TO CALMODULIN: Ca^{2+}-CALMODULIN CONFORMATIONAL STATES

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SUMMARY: Measurement of the volume change by a rapid density method upon sequential addition of calcium ion to calmodulin showed relatively large, nonuniform increases for the first 4 moles Ca^{2+} per mole calmodulin. Substantially larger volume increases ($\sim$ 15 ml/mol protein) were observed upon addition of the second and fourth moles Ca^{2+} relative to the first and third moles added per mole calmodulin. A total volume increase of $\sim$ 170 ml/mol protein attended the addition of 4 moles Ca^{2+}, as expected for multidentate carboxylate coordination to metal ion. Marginal changes in volume were observed upon further additions, the data showing a remarkably sharp transition after $[Ca^{2+}]/[\text{calmodulin}] = 4$. The results are consistent with an ordered binding of Ca^{2+} in which pair-wise additions produce similar volume changes; the volume change behavior, however, does not indicate an absence of distinct conformational states for a Ca_1^{2+}-calmodulin and a Ca_3^{2+}-calmodulin complex as has been proposed on the basis of ^{1}H-NMR evidences.

The change in volume, ΔV, upon sequential addition of Ca^{2+} to calmodulin provides an independent thermodynamic measure of the interaction of this metal ion with the 4 evenly distributed calcium-binding loops in this single-chain polypeptide. It is through such interaction that calmodulin can modulate a variety of structurally-unrelated enzymes in all eukaryotic cells [for a thorough review, see (1)]. Recently, via ^{1}H-NMR evidence, it has been firmly determined that the 2 highest affinity calcium-binding loops are located in the carboxy terminal half of the bovine calmodulin molecule and that the 2 lower affinity loops are located in the amino terminal half (2,3). It has been further deduced, however, that the binding of Ca^{2+} to bovine calmodulin occurs in a pair-wise manner without producing a distinct Ca_1^{2+}-calmodulin or a Ca_3^{2+}-calmodulin conformational state upon addition of the first or third equivalent of Ca^{2+}, respectively (3, cf.4). This conclusion emerged from the

Abbreviation: Hepes = 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid.

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observation that the spectral changes via ^{1}H-NMR upon adding the first or third equivalent of Ca^{2+} are precisely half that seen as when the second or fourth Ca^{2+} equivalent is added, respectively; the spectral changes observed during the sequential addition of Ca^{2+} occurring in a continuous, linear manner over each pair of equivalents added (3). On the other hand, the change in volume, as reported herein, using similar pH and concentrations of bovine calmodulin, are clearly different between the addition of the first and second Ca^{2+} equivalents and also between the third and fourth equivalents added to this calmodulin.

Theoretical Notes: The putative dehydration of metal ion and of the charged residues in the binding loops of the calcium-binding proteins during the coordination process can be tested in a reasonably direct manner by measuring the volume changes. The principal volume change attending the binding of metal ion to protein results from the release of electrostricted water molecules clustering about the charged metal ion and the charged groups of the protein forming a coordination site. The released water molecules then occupy a substantially larger volume, thereby increasing the total volume of the solution as has been observed (5). Relatively small volume changes, positive or negative, may attend the shifting of some amino acid residues during the coordination process, but these effects cannot yet be assigned. Hence, in the absence of extensive exposure of hydrophobic groups (e.g., denaturation) or of disruption of internal salt bridges (both effects leading to decreases in volume), the binding of Ca^{2+} in the binding loops of calmodulin containing multi-carboxylate residues, should display relatively large volume increases - the volume increment increasing nearly linearly for each charged carboxylate group coordinating to a metal ion in the binding loop (viz., 11-13 ml/mol $-COO^{-}$) (6,7). If calmodulin takes up Ca^{2+} into the 4 tight binding loops in an ordered and/or cooperative manner, the volume increments will not necessarily be uniform during the sequential addition of this metal ion. That is, the extent of dehydration at equilibrium upon each mole Ca^{2+} added per mole calmodulin need not be similar unless a random-type uptake occurs wherein a

distribution of populated states obtains upon each addition; a rather monotonic increase in volume should then accompany the sequential addition of Ca^{2+} because the dissociation constants for the 4 tightly-bound calcium ions are approximately within the same order of magnitude [viz., 10^{-5} to 10^{-6} M for the low and high affinity pairs of sites, respectively (1)]. On the other hand, the condition of a pairwise uptake of Ca^{2+}, in which each equivalent of metal ion of the pair to be added produces an identical volume response, would lead to at most 2 unique volume increases corresponding to the conformers, Ca_2^{2+}-calmodulin and Ca_4^{2+}-calmodulin, for the high and low affinity pairs, respectively. An uneven, stepwise volume increase for each equivalent of metal ion added, however, would allow for a metal-ion uptake mode which is still consistent with each half of the calmodulin molecule containing a higher affinity and a lower affinity pair of binding sites - each pair of equivalents may produce a similar stepwise pattern. In this case, distinct conformers, representing complexes for each equivalent of Ca^{2+} added, may not be ruled out; this case conforms to the observations in this report.

METHOD AND MATERIALS

Volume changes were determined with a magnetic-suspension densimeter at 20° in which 0.2 ml of sample was used per density measurement. The construction and operation of the densimeter was as reported (8); [Recent modifications reduce the sample volume to 0.1 ml and allow for a linear relationship between signal output and density - manuscript in preparation]. The precision of measurement in terms of volume change was ± 0.5 nl. Calibration at 20° was with heat-dried "Suprapur" cesium chloride (E. Merck, Darmstadt) in water related to the sucrose tables of the U. S. National Bureau of Standards, using their standard sucrose after careful drying *in vacuo* at 50°.

For the calculation of ΔV by density (9), the sum of the pre-mixed volumes of the protein and of the calcium-containing solutions are subtracted from the final volume after mixing. Typically, weights, (to 0.01 mg) of about 0.5 ml protein solution (∿ 1 mM) and of membrane-equilibrated dialyzate (0.01 M Hepes - 0.1 M KCl, pH 7.4) containing an added amount of dry $CaCl_2$, both of known density, were mixed and the density of the mixture was determined. This final volume allowed for rinses and for 3-4 density determinations. The change in volume for this operation is:

$$\Delta V = \frac{m_{A+B}}{\rho_{A+B}} - \left[\frac{m_A}{\rho_A} + \frac{m_B}{\rho_B} \right] \qquad (1)$$

where m is the mass in grams, ρ is the density in grams per milliliter and subscripts A and B refer to the protein and the calcium-containing solutions, respectively. The pre-mixed volume is represented within the brackets. As

is evident, the sum of the weights within the brackets is identical to that outside; hence, small weighing errors have no significant effect on the value of ΔV. The 3 density values, however, are independent and each must be determined with high precision.

Control experiments were carried out similarly with $CaCl_2$-dialyzate solutions instead of protein solutions in order to determine the volume change attending the dilution of $CaCl_2$; these corrections were within the experimental error of the method. [The dilution in the same solvent of the protein produces no significant change in volume since macromolecules generally show no measurable change in partial volume over a broad concentration range (9).]

Calmodulin was prepared from bovine testes according to the method of Klee (10) except for minor modifications in the initial steps (T. H. Crouch, unpublished). Accepted safeguards were employed to maintain solvent media and utensils metal-ion free. The final calmodulin preparations were all found to contain <0.04 moles Ca^{2+} per mole calmodulin by atomic absorption analyses; the solvent media were $<10^{-7}$ M in Ca^{2+}. The UV spectra and SDS polyacrylamide gel electrophoresis analyses agreed with those published for authentic calmodulin (1). The material found most reliable for our purpose exhibited a ratio of specific absorbancies at the 277 nm to the 259 nm maxima of 1.30 or slightly higher. High-precision dry-weight determinations were carried out as reported (11). A specific absorbance of 0.197 ± 0.004 ml/mg/cm at 277 nm (21^{o}) was found for the calcium-free material, and a value of 0.179 ± 0.002 ml/mg/cm for the calcium-loaded protein in the buffer medium as employed here (cf. 12). This medium (0.01 M Hepes-0.1M KCl, pH 7.4) was chosen because in it calcium binding has been reported to reflect a cooperative behavior (12,13). Samples for a measurement series were kept frozen at -22^{o} following extended dialysis against metal-ion free buffer solution in the presence of Chelex 100 (BioRad). Both protein and dialyzate solutions were then filtered through 0.2 micron, buffer-washed filters to remove any particulates. Since calcium-free calmodulin solutions were observed to suffer some change in ΔV values if at room temperature over a few hours, the weighings and density determinations were made as quickly as possible after thawing a sample.

$CaCl_2$ of AnalaR grade was heated to dryness *in vacuo*. An amount was added to a weighed volume of solvent quickly after breaking the vacuum seal and the mixture of solvent and salt was reweighed. Correspondence with atomic absorption analyses was 2% or better. All other chemicals used were of reagent grade prior to demetalization in the solvent media employed.

RESULTS AND DISCUSSION

Table 1 shows the increases in volume upon addition of integral moles of Ca^{2+} per mole calmodulin. The $\Delta(\Delta V)$ values reflect the increase from the addition of each successive mole of Ca^{2+}. The comparatively large positive ΔV increments upon equimolar additions of Ca^{2+} is strong support for coordination to multiple carboxylate groups to each binding loop of the protein. It is also significant that after 4 moles of Ca^{2+} were added, further large increases in volume were not observed in the 3 calmodulin preparations tested. Additional experiments (not shown) for fractional equivalents of Ca^{2+} showed

TABLE 1: Change in total volume per mole calmodulin upon Ca^{2+} addition (20°)

$\frac{[Ca^{2+}]}{[Calmodulin]}$	ΔV^* (ml/mol)	$\Delta(\Delta V)^{\#}$ (ml/mol)
1	37 (35-39)	37
2	88 (85-91)	51
3	121 (118-124)	33
4	170 (166-174)	49
5	171 (168-174)	1
6	170 (169-171)	-1
10	177 (170-184)	7

* Values in parentheses represent extremes observed from 3 or more samples each drawn from 3 or more density measurements. [Calmodulin] = 1.01-1.25mM.

\# See text.

that the change in ΔV to a nearly zero increase after the addition of 4 equivalents was sharp indeed.

These data also show that the sequential addition of Ca^{2+} is not consistent with a monotonic increase in volume over the first 4 equivalents of Ca^{2+} added. If these preparations reflect the native properties of this protein, the ΔV results suggest a symmetry in pairs of binding loops. A pairwise effect of Ca^{2+} on calmodulin has been noted from other kinds of studies (2,3,4) and may be viewed following Reid & Hodges (14) as a cooperativity of paired sites in each half molecule. This view is consistent with the recently-derived X-ray structure showing 2 globular domains (each containing 2 calcium-binding loops) separated by a length of polypeptide chain (15). We may suppose from the ΔV results that after a second Ca^{2+} has been added to one domain, some additional dehydration occurs as a result of conformation changes (cf. 14), because the addition of the second and fourth equivalents of Ca^{2+} exhibit significantly larger ΔV values than when the first and third equivalents were added. These larger ΔV values might also result in part if the putative conformational changes involve an increase in helicity as has been deduced by several laboratories (cf. 1) upon pairwise addition of Ca^{2+}. The formation

of α-helices can entail a volume increase of perhaps 1 ml per mole of amino acid residue entering the helix (16). In general, these ΔV data are consistent with the idea that each half of the calmodulin molecule folds somewhat independently - the one half containing a pair of higher affinity sites than does the other half (2,3). The data, however, does not lend support to the contention that a pair-wise uptake of Ca^{2+} provides no distinct Ca_1^{2+}-calmodulin and Ca_3^{2+}-calmodulin conformational states as deduced from ^{1}H-NMR studies (3).

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REFERENCES

1. Klee, C. B. and Vanaman, T. C. (1982) Adv. Protein Chem. 35, 213-321.
2. Thulin, E., Andersson, A., Drakenberg, T., Forsen, S., and Vogel, H. J., (1984) Biochemistry 23, 1862-1870.
3. Klevit, R. E., Dalgarno, D. C., Levine, B. A., and Williams, R. J. P., (1984) Eur. J. Biochem. 139, 109-114.
4. Seamon, K. B., (1980) Biochemistry 19, 207-215.
5. Katz, S., and Roberson, L. C., (1976) Bioinorg. Chem. 6, 143-154.
6. Katz, S., Uhrmacher, J. A., and Shinaberry, R. G., (1978) J. Coord. Chem. 7, 149-153.
7. Katz, S., and Shinaberry, R. G., (1978) Bioinorg. Chem. 8, 215-224.
8. Kupke, D. W., and Crouch, T. H., (1978) Methods Enzymol. 48, 29-68.
9. Kupke, D. W., (1973) Physical Principles and Techniques of Protein Chemistry, Part C (Leach, S. J., ed.) pp. 1-75, Academic Press, New York.
10. Klee, C. B., (1977) Biochemistry 16, 1017-1024.
11. Kupke, D. W., and Dorrier, T. E., (1978) Methods Enzymol. 48, 155-162.
12. Crouch, T. H., and Klee, C. B., (1980) Biochemistry 19, 3692-3704.
13. Haiech, J., Klee, C. B., and Demaille, J. G., (1981) Biochemistry 20, 3890-3897.
14. Reid, R. E., and Hodges, R. S., (1980) J. Theor. Biol. 84, 401-444.
15. Babu, Y. S., Sack, J. S., Greenhough, T. J., Bugg, C. E., Means, A. R., and Cook, W. J., (1985) Nature, 315, 37-40.
16. Noguchi, H., and Yang, J. T., (1963) Biopolymers 1, 359-369.