Volume 298, number 2,3, 211–214 FEBS 10744 February 1992

Dissection of Calbindin D_{9k} into two Ca^{2+}-binding subdomains by a combination of mutagenesis and chemical cleavage*

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Received 23 December 1991

Calbindin D_{9k} is a 75-residue globular protein made up of two Ca^{2+} binding subdomains of the EF-hand type. In order to examine the subdomains independently, a method was devised to selectively cleave the loop between them. Using site-directed mutagenesis, a unique methionine was substituted for Pro^{43} in the loop, thus allowing cleavage using cyanogen bromide. Agarose gel electrophoresis shows that the fragments have a high affinity for one another, although less so in the absence of calcium. ^{1}H-NMR spectra of the fragments indicate that the structures of the heterodimers are changed little from that of the intact protein. However, the Ca^{2+} binding constants of the individual subdomains are several orders of magnitude lower than for the corresponding sites in the uncleaved protein.

NMR; Site-directed mutagenesis, Cyanogen bromide, Calbindin D_{9k}

1. INTRODUCTION

Calbindin D_{9k} is one of the smallest members of the calmodulin superfamily of calcium binding proteins. It is composed of two helix–loop–helix calcium binding subdomains, also called EF-hands [1]. These two EF-hands subdomains associate to form a very stable four-helix bundle. It has been observed for the vast majority of this class of proteins that these calcium binding units occur in pairs; calbindin D_{9k} has one pair; calmodulin has two pairs; etc.. We wished to examine the properties of the individual EF-hand subdomains of calbindin D_{9k} in order to gain an understanding of their individual contributions to the structure and function of the intact protein. Studies on fragments of proteins have been shown to be useful in determining their contribution to the structure, folding and function of the intact proteins.

In order to obtain sufficient quantities of the desired fragments, we have devised a method for cleaving the protein specifically at a chosen point between the two subdomains. Since the protein contains no internal methionine residues, one can be introduced and used as a target for cleavage by CNBr. By substituting Met for Pro at position 43 and cleaving, this procedure yields two fragments designated F1, residues 1–43, and F2, residues 44–75. In this way, the fragments can be prepared in large quantities without resorting to expression and purification of the individual subdomains in *E. coli* where the yield could be quite low, or to solid-phase peptide synthesis which could prove a difficult task in the case of large peptides. This method could potentially be applied to the production of fragments from many small proteins with few or no methionine residues.

* Originally presented at the Seventh International Symposium on Calcium-Binding Proteins in Health and Disease, March 11, 1990 at Banff, Alberta, Canada.

Abbreviations: NMR, nuclear magnetic resonance; CNBr, cyanogen bromide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Met, methionine; Pro, proline.

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2. MATERIALS AND METHODS

2.1. *Mutagenesis and protein production*

Mutagenesis and expression of calbindin D_{9k} were performed as described previously [2,3] using an oligonucleotide cassette having the desired CCG to ATG codon change. The protein was purified according to the modified procedure described by Chazin et al. [4] in order to avoid deamidation of the protein.

2.2. *CNBr cleavage*

250 mg of the P43M protein was dissolved in 30 ml 80% trifluoroacetic acid. 3 g CNBr was added and the solution was cooled on ice while N_2 was bubbled through it. The solution was left at room temperature for 20 h after which it was evaporated using a Buchi Rotavapor. The sample was redissolved in 150 ml of 5 mM EDTA and the pH was adjusted to 7.5 using 1 M Tris-base (final concentration of Tris was 24 mM). The solution was then pumped onto a 2.2 × 27 cm DEAE-Sephacel column pre-equilibrated in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5. The applied sample was eluted with a linear NaCl gradient from 0.05 M to 0.30 M. Fragment F1 will elute in the loading wash under these conditions while fragment F2 will be retarded more than intact calbindin. Fractions were pooled, lyophilized, redissolved in 1 ml H_2O and desalted on a 4.5 × 20 cm superfine Sephadex G-25 column using water as the eluant. Just before the sample application, 15 ml of saturated NaCl, that had been treated with Chelex 100 to

remove any Ca^{2+} contamination, was applied to the column. The NaCl saturation of the G-25 column eliminates non-specific EDTA binding to the protein. The amounts of lyophilized product obtained were: fragment F1, 108 mg; fragment F2, 58 mg; and 34 mg of uncleaved protein.

2.3. *^1H NMR studies*

All ^1H NMR experiments were performed using a GE Omega 500 spectrometer at 300 K. Samples were dissolved to a concentration of 4.2–4.6 mM in 10% D_2O/90% H_2O, pH 6.0. For Ca^{2+} binding studies the samples were titrated with 0.1 M $CaCl_2$.

2.4. *Electrophoresis*

Agarose gel electrophoresis was carried out in sodium barbitone buffer, pH 8.6, using a 1% agarose gel. Either 2 mM EDTA or 2 mM $CaCl_2$ was added to the buffer. After running, the protein was visualized by staining with Coomassie blue.

3. RESULTS

In order to examine the individual calcium binding subdomains of calbindin D_{9k}, it was necessary to produce the protein fragments in high yield. A cleavage point in the loop connecting the two subdomains was desired so that the perturbation to the structure of the individual helix–loop–helix subdomains would be minimal. Trypsin digestion was attempted first but gave too many products. CNBr was then selected since a unique cleavage point could be designed by inserting a methionine to serve as the target for the CNBr. Pro^{43} was chosen as the residue to replace with Met because it is one of the residues in the loop (residues 36 to 43) between the two subdomains and because by removing this proline, the observed conformational heterogeneity associated with the *cis-trans* isomerization of Pro^{43} [5] would be avoided.

However, the choice of this residue introduced a problem for the cleavage reaction. In case where residues having hydroxyl side-chains (serine and threonine) lie immediately after the bond to be cleaved, the reaction efficiency is reduced under the conditions normally used, 70% formic acid and a 100:1 ratio of CNBr to methionine [6]. In the case of calbindin D_{9k}, the serine at position 44 presented such a problem. In order to improve the reaction efficiency, 80% trifluoroacetic acid and a ratio of 1000:1 CNBr/methionine was used. Under these conditions only approximately 17% of the protein remained uncleaved.

Table I

Properties of fragments of calbindin D_{9k}

	Fragment F1	Fragment F2
Residues[1]	1–43	44–75
Molecular weight (Da)	4850	3650
Net charge[2]	–1	–6
EF-hand charge[2]	–2	–4
K_{Ca} (M^{-1})	8×10^5	2×10^5
k_{off} (s^{-1})	<3	38

[1] The amino-terminal methionine added to the recombinant calbindin D_{9k} for purpose of expression on *E. coli* is designated residue 0.
[2] Without calcium.

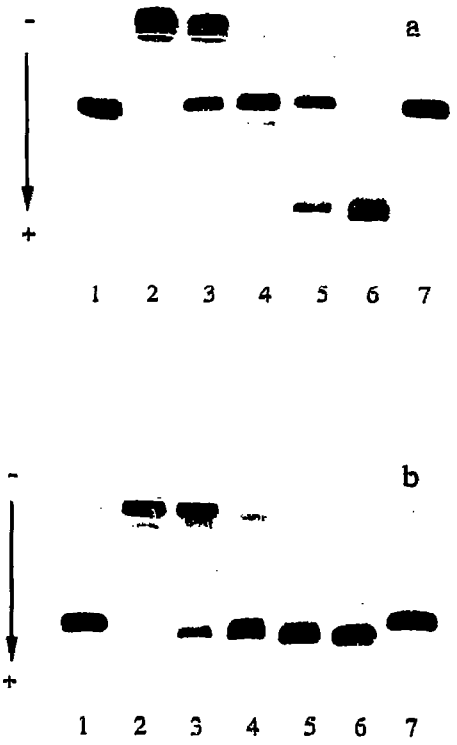

Fig. 1. Agarose gel electrophoresis of P43M calbindin D_{9k} and fragments F1 and F2. (a) In the presence of Ca^{2+}: (1,7) intact P43M calbindin; (2) F2 only; (3) 1:3, F1/F2; (4) 1:1, F1/F2; (5) 3:1, F1/F2; (6) F1 only. (b) In the absence of Ca^{2+}: lane assignments are the same as for (a).

The ability of the purified fragments to associate was examined by agarose gel electrophoresis. As shown in Fig. 1a, with calcium present, a third band not corresponding to the individual F1 or F2 fragments is observed. This is most likely a heterodimer of the F1 and F2 fragments since it migrates at the same position as the intact protein. This result demonstrates the formation of a dimer similar to the intact protein. In the absence of calcium (Fig. 1b), any dimerization of the fragments is too weak to be observed with this method. In both cases, the remainder of the fragments not involved in heterodimer formation migrate as expected based on their charges (Table I).

The structures of the fragments were compared to that of the intact protein by examination of their one-dimensional ^1H NMR spectra. Fig. 2a and b show the spectra of the dimer of F1 and F2 and uncleaved P43M protein in the presence of calcium. The differences between the two spectra are very small and indicate that the structure of the protein is largely unaffected by the cleavage of the peptide bond at Met^{43}. As can be seen in Fig. 2c and d, the structural differences between the intact and cleaved protein are also minimal in the absence of calcium indicating that dimers are formed under these conditions. Preliminary data from ^1H 2D-

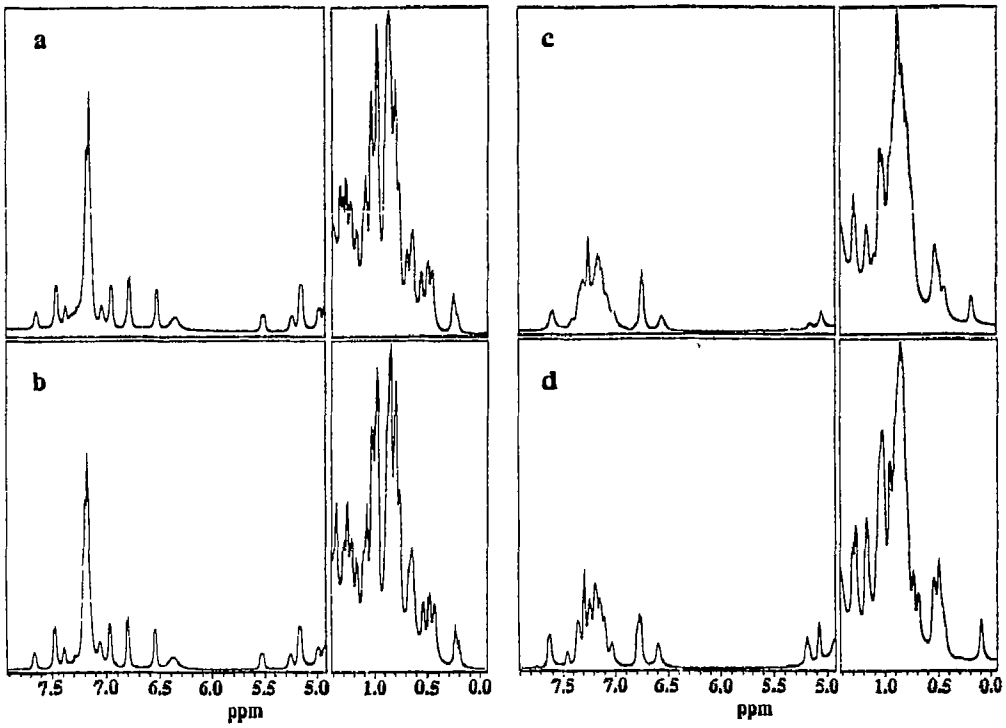

Fig. 2. ^{1}H NMR spectra of the F1–F2 heterodimer and uncleaved P43M calbindin D_{9k}. (a) F1–F2 with calcium; (b) P43M with calcium; (c) F1–F2 without calcium; (d) P43M without calcium.

NMR studies of the fragments also indicate few changes in the structure of the F1–F2 heterodimer compared to the intact protein (J. Kördel, unpublished results).

The calcium binding behavior of the fragments was determined by titration of the fragments with $CaCl_2$ and observation of the shifts and linewidths of the aromatic sidechain protons by ^{1}H NMR as used previously to characterize the intact protein [3]. As shown in Table I, the binding constants are several orders of magnitude lower than those reported for the corresponding sites in intact calbindin D_{9k}. These binding constants were confirmed by microcalorimetric studies (data not shown).

4. DISCUSSION

The results presented above indicate that the two calcium binding subdomains largely retain their structure after cleavage of the loop separating them. As shown by the lack of change in the NMR spectra, the structure of the F1–F2 dimer is essentially identical to that of the intact protein. Clearly the major force holding the two subdomains together in their specific orientation is not the covalent linkage between them but noncovalent forces at the interface of the two subdomains. As shown in Fig 3, the interior of the intact protein is made up entirely of hydrophobic residues. This large area of hydrophobic contact, which is buried upon association of the two subdomains, bestows on calbindin D_{9k} a large conformational stability as has been observed previously [8]. Electrostatic interactions, on the other hand, have been shown to have a negative overall effect on the stability of the protein due mostly to the repulsion of many negatively charged side-chains in the region of the calcium binding loops [9]. Binding of calcium stabilizes the F1–F2 dimer through a combination of neutralization of the negative charges and, possibly, by inducing a conformation more favorable to dimerization.

Electrostatic interactions also play a role in the calcium binding properties as has been demonstrated in the case of the intact protein [10,11]. This is also true for the fragments. The calcium off-rate is lower in F1 than in F2 as is the on-rate. This is consistent with the lower charge of F1 versus F2 (Table I). These rates may also be influenced by the lower stability of the individual fragments as compared to the intact protein. Studies are in progress to address this issue.

In summary, it is possible to use a combination of mutagenesis and chemical cleavage to isolate specific protein subdomains in high yield. This method should prove useful for studies related to the structure and function of the protein from which the fragments are derived.

Acknowledgements: We gratefully acknowledge Dr. Theo Hoffman for stimulating discussions and suggestions. We are grateful to Drs. Peter Brodin and Tomas Grundström for making the P43M mutant of calbindin D_{9k}. We thank the Swedish National Science Research Council for a postdoctoral fellowship (B.E.F.), graduate fellowship (J.K.), and operating grants (S.F.).

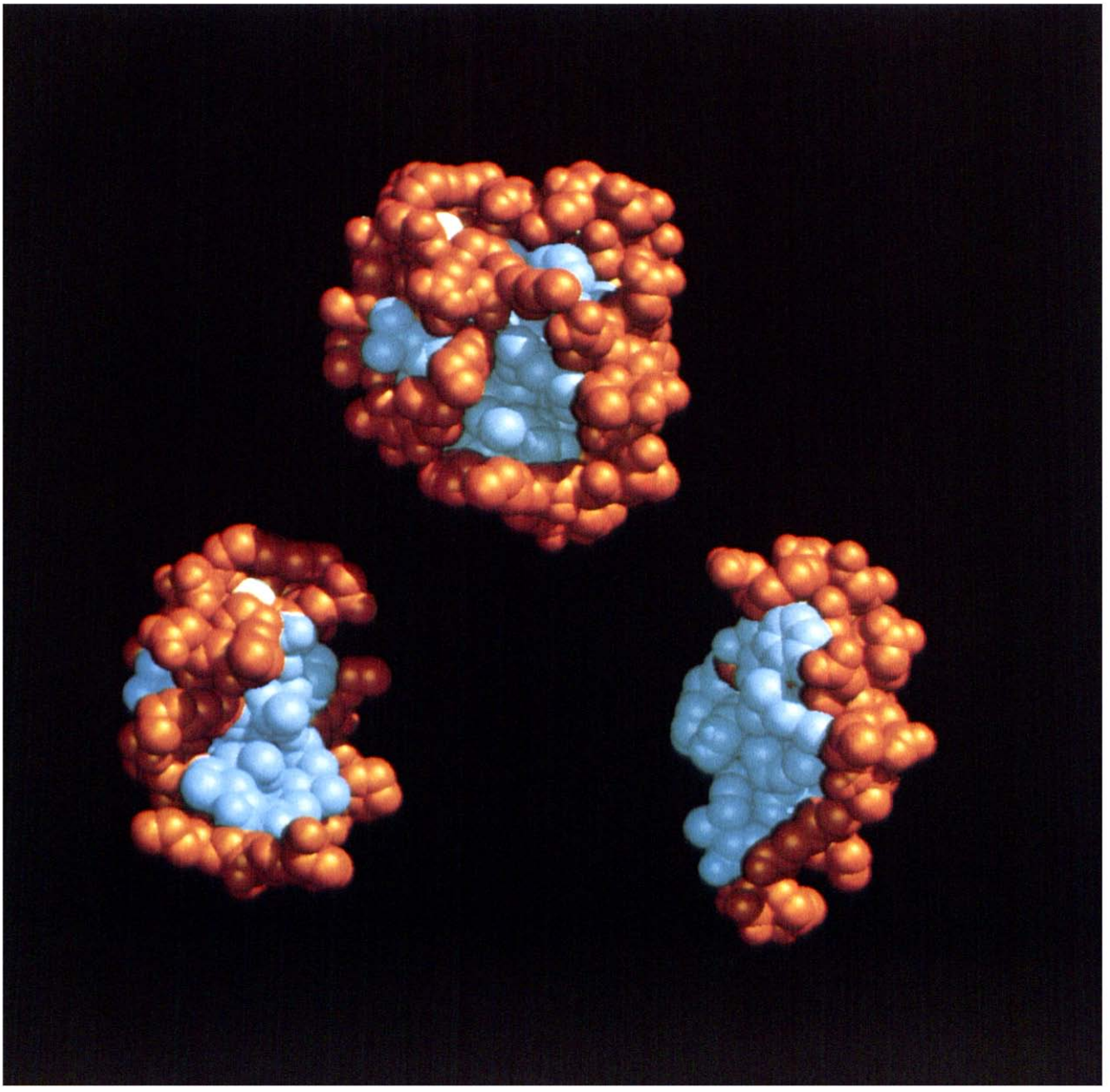

Fig. 3. Space-filling models of intact calbindin D_{9k} (upper) and the F1 and F2 fragments (lower) based on the X-ray structure coordinates [7]. The hydrophobic residues are colored blue and all others orange. The calcium ions are colored white. (Molecular graphics image produced with the MidasPlus software system from the Computer Graphics Laboratory, University of California, San Francisco.)

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