Influence of temperature and denaturing agents on the structural stability of calmodulin

A ^{1}H-nuclear magnetic resonance study

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The structural stability of calmodulin was studied by ^{1}H-nuclear magnetic resonance spectroscopy under different denaturing conditions. The presence of Ca^{2+} stabilizes the structural properties of the native protein. In the absence of calcium the structural integrity of calmodulin can easily be affected by elevated temperatures or by high concentrations of denaturing agents. The unfolding process under various denaturing conditions is reversible underlining the high degree of structural flexibility of this protein.

Calmodulin *Structural stability* *Calmodulin denaturation* *Nuclear magnetic resonance*

1. INTRODUCTION

Calmodulin is a ubiquitous, Ca^{2+}-binding modulator protein, which regulates many different Ca^{2+}-dependent processes inside the cell [1]. Together with troponin-C and parvalbumin, calmodulin belongs to a family of homologous Ca^{2+}-binding proteins. They all undergo profound rearrangements of their secondary and tertiary structure upon binding of Ca^{2+} or similar divalent cations as is documented by a variety of different spectroscopic techniques [2,3]. Ca^{2+} also confers structural stability towards these proteins, since they are less susceptible to tryptic cleavage in the Ca^{2+}-bound than in the Ca^{2+}-free form [4]. Recently, the authors in [5] demonstrated that the native proteins calmodulin and troponin-C as well as some of their tryptic fragments, conserved structural integrity in the Ca^{2+}-bound form at elevated temperatures.

In order to obtain detailed information about the influence of Ca^{2+} on retaining structural stability of calmodulin, we used ^{1}H-nuclear magnetic resonance (NMR) to study thermal unfolding of calmodulin in the presence and absence of Ca^{2+}. Furthermore, we investigated the influence of denaturing agents on the structural properties of calmodulin. Several reports describe the Ca^{2+}-dependent complex formation of calmodulin with a variety of proteins and the stability of these ternary complexes in the presence of denaturing agents [6–10]. We here provide evidence that in the presence of 2 M urea, calmodulin retains its native conformation in the Ca^{2+}-bound form, whereas in the Ca^{2+}-free form calmodulin demonstrates a typical random coil ^{1}H-NMR spectrum in the presence of urea. Preliminary accounts of part of this work have been presented [11,12].

2. MATERIALS AND METHODS

Calmodulin was isolated from bovine brain by a modification of the procedure in [13] as described in detail elsewhere (Guerini, D., Krebs, J. and Carafoli, E. in preparation). Briefly, the tissue extract of 1 kg bovine brain was concentrated by a

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DEAE-cellulose batch step followed by a differential $(NH_4)_2SO_4$ precipitation before passing the calmodulin-containing solution over a Phenyl-Sepharose column (Pharmacia, Uppsala) equilibrated in 0.05 M Hepes (pH 7.5), 1 mM 2-mercaptoethanol, 0.1 mM $CaCl_2$ (buffer A). After eluting calmodulin from the column by buffer A containing 1 mM EDTA instead of $CaCl_2$, the protein was finally purified by passing it over a Sephadex G-100 column (Pharmacia) equilibrated in 100 mM NH_4HCO_3. The calmodulin-containing fractions were pooled, lyophilized, dialyzed extensively against water and freeze-dried. The purity of calmodulin was checked by sodium dodecyl sulfate/12.5% polyacrylamide slab gel electrophoresis as in [14] or by 8 M urea gel electrophoresis as in [15]. Protein concentrations were determined as in [16] using bovine serum albumin as standard. Ca^{2+}-free calmodulin was prepared as in [17]. The ^{1}H-NMR spectra were recorded on a Bruker HXS-360 spectrometer equipped with an Aspect 2000 data system and a standard Bruker temperature control unit as in [17].

3. RESULTS

3.1. *Thermal unfolding*

The temperature dependence of the ^{1}H-NMR spectrum was followed in the range 25–90°C for Ca^{2+}-saturated and Ca^{2+}-free calmodulin. Fig.1 shows the aromatic region of the spectrum of Ca^{2+}-saturated calmodulin at various temperatures. As can be seen from the figure only

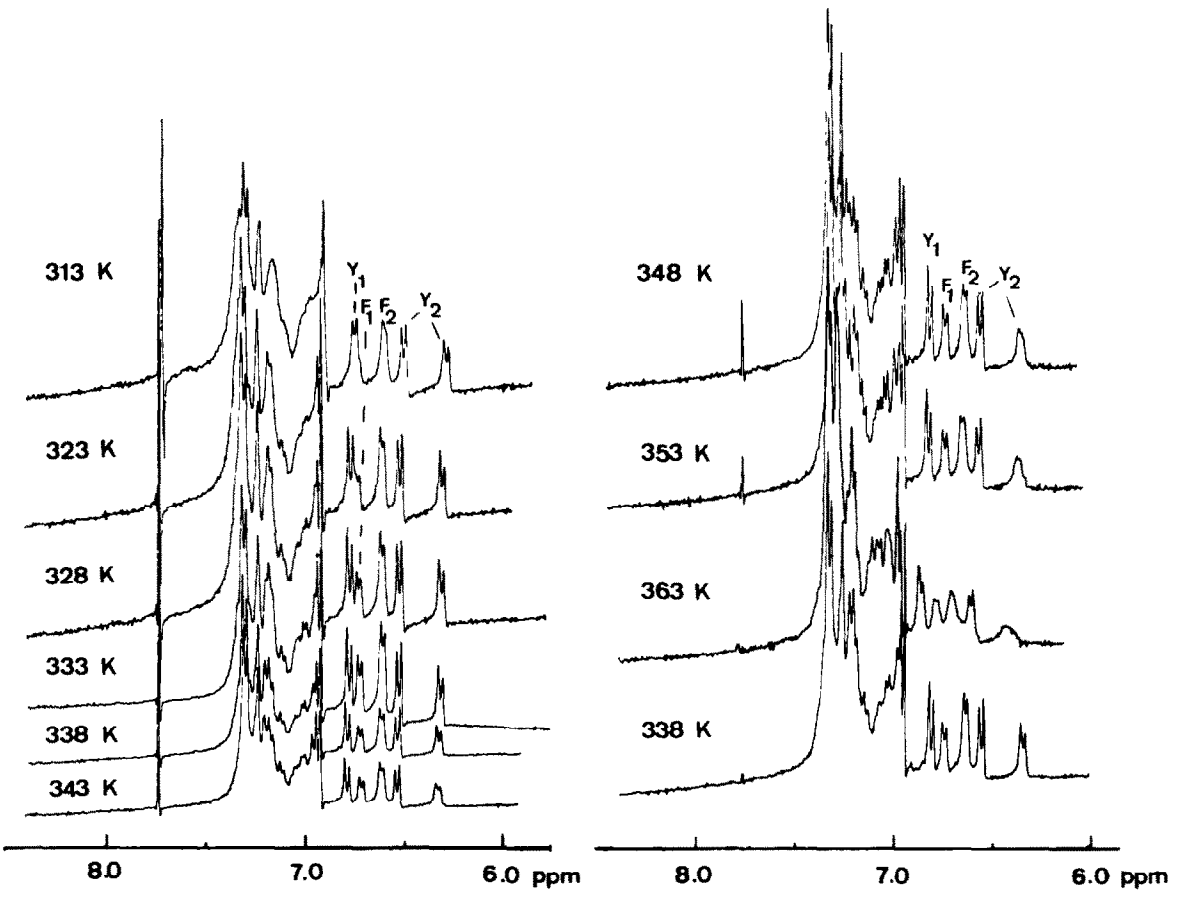

Fig.1. Aromatic regions of ^{1}H-NMR spectra of Ca^{2+}-saturated calmodulin at different temperatures. 360 MHz ^{1}H-NMR spectra of 2 mM Ca^{2+}-saturated calmodulin in ^2H_2O (pH 7.5) were recorded at the given temperatures. (Y_1) Tyr_{99}, (Y_2) Tyr_{138}, (F_1 and F_2) high field shifted phenylalanines.

gradual changes can be observed for various resonances which mainly narrow with increasing temperature. This is especially prominent for the broad resonance at 7.2 ppm and the broad shoulder at about 7.0 ppm, which reveal several well resolved resonances at elevated temperatures. Above 70°C chemical shift changes of most resonances become more marked. But even at 90°C the spectrum indicates that in the Ca^{2+}-saturated form calmodulin retains most of its native conformation. In addition, by lowering the temperature the spectral changes are fully reversible. Similar observations have been made for skeletal troponin-C [18] resembling the close relationship between the two proteins.

It is of interest to note here that the 2,6-proton doublet resonance of a phenylalanine residue (F_1) which is usually masked underneath the 3,5-proton doublet of Tyr_{99} (Y_1) at 6.82 ppm [17] becomes visible due to a temperature-induced upfield shift initiated at a relatively low temperature. This resonance begins to appear upfield of Tyr_{99} at 40°C. It becomes clearly visible at 55°C and is finally located at 6.74 ppm at 70°C. Another pronounced temperature effect is the marked line broadening of the 2,6-proton doublet of Tyr_{138} (Y_2) at 6.35 ppm starting gradually above 60°C

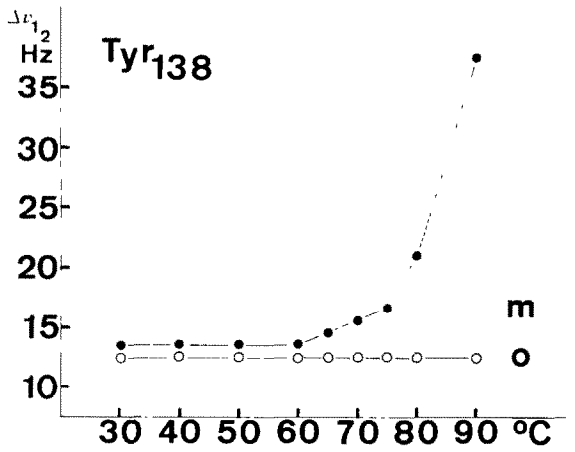

Fig.2. Temperature-dependent line broadening of the 2,6-proton doublet of Tyr_{138}. The linewidth of the resonances were measured (in Hz) at half height and plotted *vs* temperature. ('m') doublet of the protons *meta* to the hydroxyl group = 2,6-doublet; ('o') doublet of the protons *ortho* to the hydroxyl group = 3,5-doublet.

with a dramatic increase above 75°C as can be seen from fig.2 ('m'). This is in contrast to the 3,5-proton doublet of Tyr_{138} (fig.2, 'o') which does not show an appreciable change of linewidth over the whole temperature range. The reason for this unusual line broadening of the 2,6-proton doublet of Tyr_{138} at elevated temperature might be due to an exchange process of the *m*-protons of Tyr_{138} between their native (6.35 ppm) and the corresponding random coil position (~7.2 ppm). In Ca^{2+}-saturated calmodulin the resonance of the 2,6-proton doublet of Tyr_{138} is located at an unusual high field shift position (6.35 ppm; e.g., [17,19]), which is upfield of the 3,5-proton doublet, probably due to the unique structural environment of this tyrosine. This situation might change at a certain temperature resulting in a defined local conformational transition indicated by the above exchange process.

In contrast to the Ca^{2+}-saturated molecule, the Ca^{2+}-free form of calmodulin is much more susceptible to temperature-induced conformational changes. As can be seen from fig.3, some of the resonances of the aromatic region of calmodulin shift to their random coil positions already at relatively low temperature. So already at 40°C (fig.3b) the resonance of the two doublets of Tyr_{138} at 6.70 ppm is strongly reduced in intensity, and they tend to shift to their random coil positions (6.8 and 7.2 ppm, respectively), a process which is completed at about 50°C (fig.3d). Similar findings can also be made for several resonances of phenylalanine residues (e.g., at 6.47, 6.95 and 7.15 ppm). These observations are corroborated by the ring-current shifted resonances of various high field shifted methyl residues and the methyl resonances of the various methionines which all shift continuously to their unperturbed positions (not shown). Therefore, already at 60°C the spectrum of Ca^{2+}-free calmodulin closely resembles that of a random coil protein (fig.3e). But it is remarkable that even after heating the sample up to 70°C (fig.3f), calmodulin can still regain almost completely its native conformation after reducing the temperature to 30°C (see fig.3g, T = 30°C). Only if the temperature has been increased up to 90°C can some irreversible shift changes be observed for the Ca^{2+}-free form, indicating again that the Ca^{2+}-free form is much less heat stable in comparison to the Ca^{2+}-saturated form.

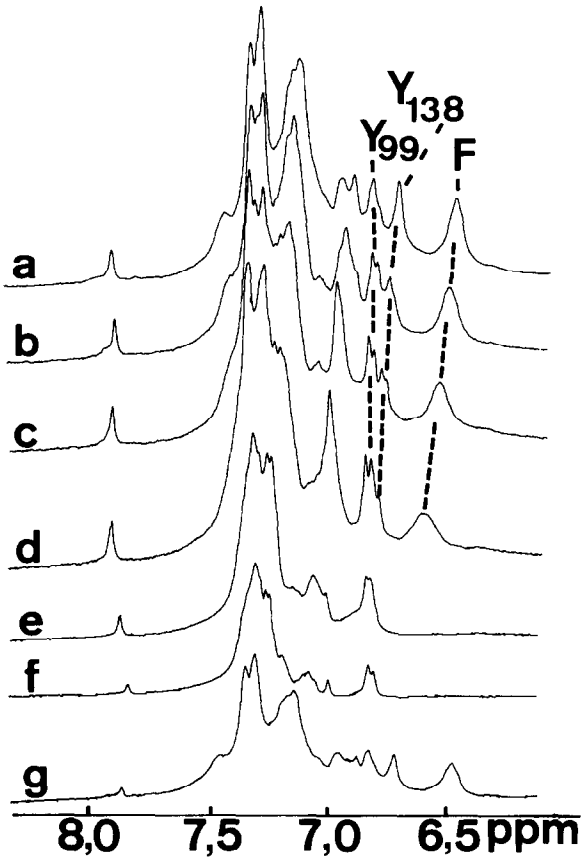

Fig.3. Aromatic regions of ^{1}H-NMR spectra of Ca^{2+}-free calmodulin. Calmodulin, after passing through a chelex-100 column (see [17]), was lyophilized and a 2 mM solution in ^2H_2O (pH 7.5) was prepared. 360 MHz ^{1}H-NMR spectra were recorded at different temperatures. (a) 30°C, (b) 40°C, (c) 45°C, (d) 50°C, (e) 60°C, (f) 70°C, (g) 30°C.

3.2. *Unfolding by urea*

The influence of denaturing agents such as urea on the unfolding process of calmodulin has been studied in the presence and absence of Ca^{2+}. Fig.4 shows the ^{1}H-NMR spectra of Ca^{2+}-free calmodulin in the presence (a) and absence (b) of 2 M urea. It is clearly demonstrated that in the presence of 2 M urea, calmodulin reveals a typical random coil spectrum (fig.4a). On the other hand, fig.5 shows that Ca^{2+} can confer structural stability on calmodulin even in the presence of 2 M urea. Fig.5a shows the same random coil spectrum of Ca^{2+}-free calmodulin in the presence of 2 M urea as presented in fig.4a. Fig.5b–d are spectra of

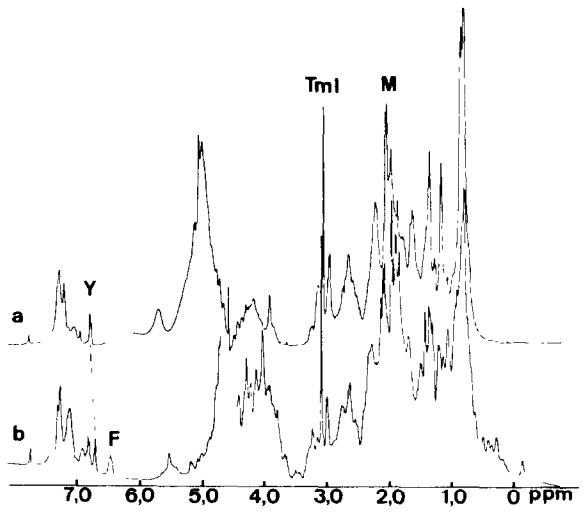

Fig.4. ^{1}H-NMR spectra of Ca^{2+}-free calmodulin in the presence and absence of 2 M urea. Calmodulin, prepared in the Ca^{2+}-free form as in [17], was lyophilized and dissolved either in ^2H_2O/2 M urea (a) or in ^2H_2O (b) (pH 7.5 and T = 30°C). (F) phenylalanine, (M) methionine, (Tml) trimethyllysine, (Y) tyrosine.

calmodulin taken during a Ca^{2+} titration in the presence of 2 M urea representing molar ratios of Ca^{2+} : calmodulin of 1, 2 and 4, respectively. It can be clearly seen that even at subsaturating condi-

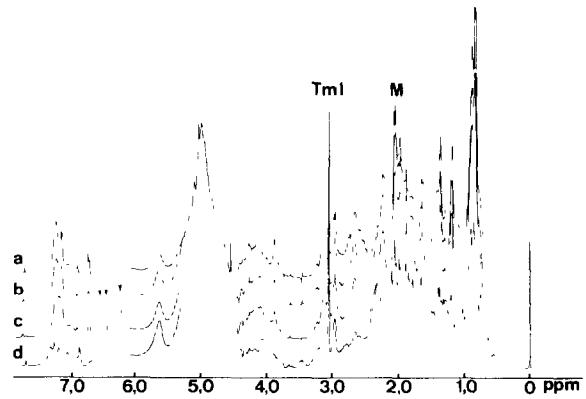

Fig.5. ^{1}H-NMR spectra of a Ca^{2+} titration of calmodulin in the presence of 2 M urea. Ca^{2+}-free calmodulin, dissolved in ^2H_2O/2 M urea ([calmodulin] = 2 mM) (pH 7.5), was recorded at different Ca^{2+} : calmodulin molar ratios: (a) 0, (b) 1, (c) 2, (d) 4. Spectra were recorded at T = 30°C. The arrows indicate the positions of resonances of Tyr_{138} and high field shifted phenylalanines (probably $F_{89,92}$; see [17]).

tions of Ca^{2+}, resonances appear in the spectra which are typical for the spectrum of the native Ca^{2+}-saturated calmodulin [17]. These are marked in the aromatic region by arrows indicating resonances at positions of Tyr_{138} and probably phenylalanine 89 or 92 [17]. This becomes also obvious in the region of the high field shifted methyl resonances (0–0.8 ppm). The spectrum of fig.5d is almost identical with a spectrum of Ca^{2+}-saturated calmodulin in the absence of urea [17] indicating the stabilizing effect of Ca^{2+} in conserving the native conformation even in the presence of high concentrations of denaturing agents.

4. DISCUSSION

The results presented here clearly show that Ca^{2+} confers structural stability on calmodulin at elevated temperatures or in the presence of denaturing agents. Changes in the ^{1}H-NMR spectra are only marginal for Ca^{2+}-saturated calmodulin even at 90°C indicating the strong thermostability of this protein. Furthermore, all the observed changes are fully reversible after decreasing the temperature. On the other hand, in the absence of Ca^{2+} gross spectral changes can be observed, even at relatively low temperatures. These changes tend towards a spectrum of a random coil protein with increasing temperature, a process which is fully reversible only if the temperature has not been increased above 70°C. This finding is important in the view that often a heating step is included (usually 95°C, 5 min) during the isolation of calmodulin. Since calmodulin is usually extracted from tissues in the presence of EDTA, Ca^{2+} should be added prior to heating to stabilize calmodulin, if losses of the protein are to be avoided.

Similar observations of the influence of Ca^{2+} on the thermostability of calmodulin have recently been reported in [5] using circular dichroism. The authors further provided evidence that even calmodulin-derived fragments which encompass at least 2 of the 4 Ca^{2+}-binding sites show similar thermostability properties in the presence of Ca^{2+}. In fact, advantage was taken of the Ca^{2+}-stabilizing effect towards calmodulin to prepare fragments by controlled proteolysis [20].

Several studies provided convincing evidence that Ca^{2+}-loaded calmodulin retains its interaction with target molecules even in the presence of denaturing agents [6–9]. Therefore, it was interesting to see whether Ca^{2+} can also stabilize the native conformation of calmodulin in the presence of a denaturing agent such as urea. From the results presented in fig.4,5 it became evident that in the absence of Ca^{2+} calmodulin reveals a typical random coil ^{1}H-NMR spectrum in the presence of 2 M urea, whereas under the same denaturing conditions in the presence of saturating amounts of Ca^{2+} the spectrum is almost indistinguishable from that of the native protein in the absence of urea. These findings suggest that in the presence of calcium, calmodulin retains its native conformation even in the presence of 2 M urea. Similar results have also been obtained in the presence of 6 M urea (not shown). In this respect, it is of interest that the authors in [9] reported on the interaction between phosphorylase *b* kinase and calmodulin δ-subunit in the presence of 8 M urea, provided Ca^{2+} was present. These results suggest that Ca^{2+}-loaded calmodulin retained its native conformation which enabled it to interact with the other subunits of phosphorylase *b* kinase even in the presence of 8 M urea. A similar situation occurs in the interaction between troponin-C and troponin-I, which also form a complex that is resistant to dissociation by 8 M urea in the presence of Ca^{2+} [21]. From the results presented here, the following conclusions can be made:

(i) Ca^{2+} confers a high degree of structural stability on the protein. Therefore, increasing temperature or the presence of denaturing agents have only marginal effects on the structural properties of calmodulin.
(ii) In the absence of Ca^{2+} calmodulin is highly susceptible to denaturing conditions.
(iii) The high degree of reversibility of the unfolding process, also in the absence of the stabilizing effect of Ca^{2+}, underlines the flexible nature of calmodulin, which facilitates its structural adaptation to interact with many different targets.

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REFERENCES

[1] Klee, C.B. and Vanaman, T.C. (1982) Adv. Protein Chem. 35, 213–321.
[2] Seamon, K.B. and Kretsinger, R.H. (1983) in: Metal Ions in Biology (Spiro, T.G. ed) vol.4, Wiley, New York, in press.
[3] Krebs, J. (1981) Cell Calcium 2, 295–311.
[4] Ho, H.C., Deshi, R. and Wang, J.H. (1975) FEBS Lett. 50, 374–377.
[5] Brzeska, H., Venyaminoo, S.V., Grabarek, Z. and Drabikowski, W. (1983) FEBS Lett. 153, 169–173.
[6] Comte, M., Maulet, Y. and Cox, J.A. (1983) Biochem. J. 209, 269–272.
[7] Head, J.F., Weeks, R.A. and Perry, S.V. (1977) Biochem. J. 161, 465–471.
[8] Grand, R.J.A. and Perry, S.V. (1980) Biochem. J. 189, 227–240.
[9] Shenolikar, S., Cohen, P.T.W., Cohen, P., Nairn, A.C. and Perry, S.V. (1979) Eur. J. Biochem. 100, 329–337.
[10] Burgess, W.H., Jevriolo, D.K. and Kretsinger, R.H. (1980) Biochim. Biophys. Acta 623, 257–270.
[11] Krebs, J., Schäfer, B. and Guerini, D. (1983) Hoppe Seyler's Z. Physiol. Chem. 364, 346.
[12] Guerini, D., Schäfer, B. and Krebs, J. (1983) in: Calcium-Binding Proteins in Health and Disease (Carafoli, E. et al. eds) Elsevier, Amsterdam, New York, in press.
[13] Gopalkrishna, R. and Anderson, W.B. (1982) Biochem. Biophys. Res. Commun. 104, 830–836.
[14] Laemmli, U.K. (1970) Nature 227, 680–685.
[15] Swank, R.T. and Munkres, K.D. (1971) Anal. Biochem. 39, 462–477.
[16] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[17] Krebs, J. and Carafoli, E. (1982) Eur. J. Biochem. 124, 619–627.
[18] Levine, B.A., Mercola, D., Coffman, D. and Thornton, J.M. (1977) J. Mol. Biol. 115, 743–760.
[19] Seamon, K.B. (1980) Biochemistry 19, 207–215.
[20] Walsh, M., Stevens, F.C., Kuznicki, J. and Drabikowski, W. (1977) J. Biol. Chem. 252, 7440–7443.
[21] Head, J.F. and Perry, S.V. (1974) Biochem. J. 137, 145–154.