Structural studies of calmodulin and related calcium-binding proteins by hydrophobic labeling

Jacqueline Buerkler and Joachim Krebs*

Laboratory of Biochemistry, Swiss Federal Institute of Technology (ETH), CH-8092 Zurich, Switzerland

Received 17 January 1985

The highly hydrophobic, photoreactive probe 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID) labels calmodulin in a Ca^{2+}-dependent way [(1984) Biochemistry 23, 400–403]. Similar results are obtained in the presence of different cations depending on whether their properties are closely related to those of Ca^{2+}. Labeling by [^{125}I]TID is also observed in the presence of different denaturing agents, and the extent of labeling is reduced by the phenothiazine trifluoperazine. The Ca^{2+}-dependent exposure of hydrophobic sites was studied for different Ca^{2+}-binding proteins and evidence is presented that troponin C can be labeled by [^{125}I]TID in a Ca^{2+}-dependent way whereas parvalbumin is not.

Calmodulin *Hydrophobic labeling* *Calcium-binding protein* *Ion specificity* *Denaturing agent*

1. INTRODUCTION

Calmodulin plays a pivotal role in mediating Ca^{2+}-dependent processes (review [1]). It has 4 Ca^{2+}-binding sites [2] and belongs to a family of highly homologous proteins containing Ca^{2+}-binding domains in a helix-loop-helix arrangement [3]. In 1977 Levin and Weiss [4] reported the Ca^{2+}-dependent binding of phenothiazines to calmodulin which is now known to be due to the exposure of hydrophobic sites induced by the binding of Ca^{2+} to calmodulin [5]. On the other hand, these sites are thought to be important for the interaction with the target proteins [5,6] and have recently been affinity labeled by phenothiazine derivatives [7,8].

We have recently described a new method to identify these hydrophobic sites [9]. Here, the photoactivatable hydrophobic reagent [^{125}I]TID, which labels calmodulin and proteolytic fragments of calmodulin in a Ca^{2+}-dependent way [9], will be used to label homologous Ca^{2+}-binding proteins to identify similar hydrophobic surfaces. In addition, calmodulin is labeled either in the presence of different denaturating agents or under conditions in which Ca^{2+} is substituted by other cations to investigate their ability to induce similar conformational changes of calmodulin. A preliminary account of this work has been presented elsewhere [10].

2. EXPERIMENTAL

2.1. *Materials*

The hydrophobic label [^{125}I]TID was prepared according to Brunner and Semenza [11]. Calmodulin was isolated from bovine brain as in [12]. Troponin C and crayfish calcium-binding protein were generously supplied by Dr J. Cox, Geneva, and parvalbumin was a gift from Dr C. Heizmann, Zurich. Trifluoperazine was kindly provided by Smith, Kline and French, USA.

* To whom correspondence should be addressed

Abbreviations: [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine; PAGE, polyacrylamide gel electrophoresis; TFP, trifluoperazine; TN C, troponin C

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

2.2. *Methods*

Calmodulin and related Ca^{2+}-binding proteins were labeled with [^{125}I]TID as described [9]. The labeled samples were analysed by SDS-PAGE applying a Laemmli buffer system [13]. The gels were stained with Coomassie brilliant blue, dried and exposed to X-ray film (Eastman Kodak, X-Omat AR film) for autoradiography. Some autoradiograms were analyzed with a densitometer (Shimadzu, Japan, as modified by Desaga, Frankfurt, FRG).

Protein determination was carried out by the procedure of Lowry et al. [14], using bovine serum albumin as standard.

3. RESULTS AND DISCUSSION

Teo and Wang [2] first described the Ca^{2+}-binding properties of calmodulin, but only recent results demonstrated the existence of two high- and two low-affinity Ca^{2+}-binding sites of calmodulin [15,16]. Tb^{3+}, Nd^{3+} and Pr^{3+} can also form biologically active calmodulin complexes with K_{diss} values even smaller than that of Ca^{2+} [17,18], but other cations like Sr^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+} bind to calmodulin with lower affinity than does Ca^{2+} [2,18]. Therefore, it was of interest to determine whether cations other than Ca^{2+} are able to induce exposure of hydrophobic sites in calmodulin which can subsequently be labeled by [^{125}I]TID as described before [9]. As can be seen from fig.1, Ca^{2+}-free calmodulin can be labeled by [^{125}I]TID in the presence of different cations. It is evident from this figure that calmodulin becomes strongly labeled by [^{125}I]TID only in the presence of Sr^{2+}, Cd^{2+} and Tb^{3+} (even if the latter was present at 3-fold lower concentrations, fig.1, panels 3,6,7). On the other hand, labeling of calmodulin by [^{125}I]TID in the presence of Mn^{2+} (panel 2), Zn^{2+} (panel 4) or Co^{2+} (panel 5) was either only marginal or, in the presence of Mg^{2+} (panel 8), even non-existent. These findings are in excellent agreement with the recent observation of Chao et al. [18]. These authors provided evidence that there is a strong correlation between the similarity of the ionic radii of the different cations and their efficiency to substitute for Ca^{2+} in the function of calmodulin.

As demonstrated by NMR, calmodulin retains its structural integrity in the presence of Ca^{2+} even

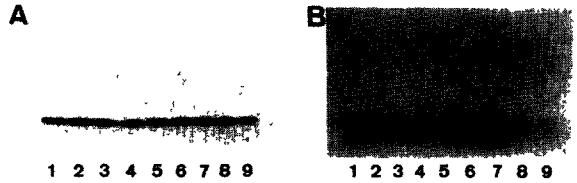

Fig.1. SDS-PAGE of [^{125}I]TID-labeled calmodulin saturated with different cations. The 12% polyacrylamide gel was run using a Laemmli buffer system [13], stained with Coomassie brilliant blue (A) and exposed for 24 h to an X-ray film for autoradiography (B). Ca^{2+} was removed from calmodulin and the buffer by passing the solutions through a Chelex 100 column. The solutions of the metal ions were tested for Ca^{2+} contamination by atomic absorption spectroscopy. The following total cation concentrations were present in the [^{125}I]TID labeling experiment: (1) 600 μM Ca^{2+}, (2) 600 μM Mn^{2+}, (3) 500 μM Sr^{2+}, (4) 600 μM Zn^{2+}, (5) 600 μM Co^{2+}, (6) 600 μM Cd^{2+}, (7) 200 μM Tb^{3+}, (8) 5 mM Mg^{2+}, (9) 2 mM EGTA.

under highly denaturing conditions [19]. These findings are corroborated by the results presented in fig.2. Ca^{2+}-saturated calmodulin is labeled by [^{125}I]TID either in the presence of 6 M urea (fig.2, panel 3) or in the presence of 0.1% SDS (fig.2, panel 5), as much as in the absence of the denaturing agents (fig.2, panel 1). However, in contrast to urea, Ca^{2+}-free calmodulin is also labeled by [^{125}I]TID, at least partly (it should be noted here that the second band of labeled calmodulin which can be observed only on the autoradiograms most probably represents doubly labeled calmodulin molecules thereby gaining a higher mobility on SDS-gels), in the presence of 0.1% SDS (see fig.2, panels 4 and 6) indicating that the presence of SDS is sufficient to make hydrophobic sites of calmodulin accessible to [^{125}I]TID, even in the absence of Ca^{2+} (fig.2, panels 5 and 6).

Two molecules of phenothiazine per molecule of calmodulin are bound in a Ca^{2+}-dependent way with high affinity [4]. Recently, evidence has been provided that hydrophobic binding sites exist in each half of the calmodulin molecule [9,20]. Since the phenothiazines may bind to similar sites of calmodulin as [^{125}I]TID we undertook competition experiments to see whether TFP reduces the binding of [^{125}I]TID to calmodulin. As shown in fig.3, this is indeed the case, but complete inhibition of

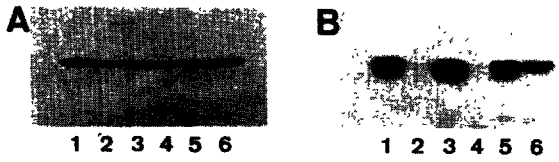

Fig.2. SDS-15% polyacrylamide gel electrophoresis of calmodulin labeled with [^{125}I]TID under denaturing conditions. Labeling of calmodulin by [^{125}I]TID was performed either in the presence of Ca^{2+} (nos 1,3,5) or in the presence of EGTA (nos 2,4,6), respectively. The Laemmli gel [13] was stained with Coomassie brilliant blue (A) and subjected to autoradiography for 2 days (B). Special conditions for the labeling procedure were as follows: (1 + 2) none, control; (3 + 4) 6 M urea; (5 + 6) 0.1% SDS.

[^{125}I]TID labeling by TFP has not been observed, probably due to the different types of binding of the two molecules to calmodulin. Similar observations have recently been reported by Jarret [8], demonstrating the competition between TFP, W7 or fluphenazine and a phenothiazine derivative used as affinity label.

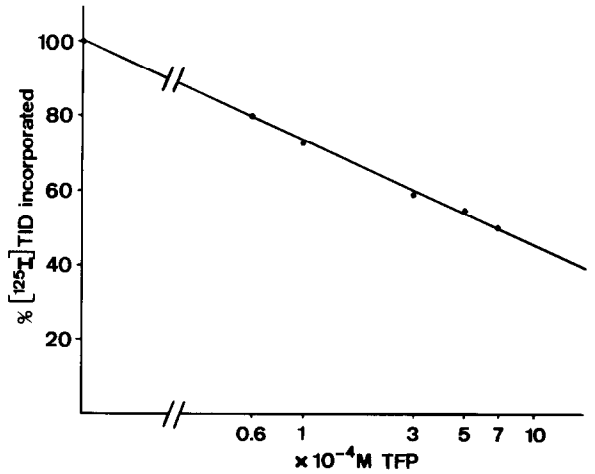

Fig.3. Reduced [^{125}I]TID-labeling of calmodulin by TFP. SDS-PAGE according to Laemmli [13] was carried out with calmodulin samples labeled by [^{125}I]TID in the presence of increasing amounts of TFP. The 12%-polyacrylamide gel was stained with Coomassie brilliant blue and exposed to an X-ray film for 15 h. The autoradiogram was analyzed with a densitometer. The computed areas under the peaks were calculated relative to the control, taking Ca^{2+}-calmodulin labeled in the absence of TFP as 100%.

Since calmodulin belongs to a family of different Ca^{2+}-binding proteins we were interested in whether some of these proteins expose similar hydrophobic sites due to the binding of Ca^{2+}. As can be noted from fig.4, TN C was labeled by [^{125}I]TID in a similar Ca^{2+}-dependent way (panels 3 and 4). These findings are corroborated by recent observations reported by Marshak et al. [21] who provided evidence for the Ca^{2+}-dependent interaction of calmodulin, troponin C and S 100b-protein with immobilized phenothiazines. Parvalbumin and crayfish calcium-binding protein show a different behaviour if exposed to [^{125}I]TID. Parvalbumin is not labeled by [^{125}I]TID in the presence of Ca^{2+}, on the contrary, if labeled at all, then only in the presence of EGTA (fig.4, panels 5 and 6). These properties are further corroborated by the fact that parvalbumin is not retained by a phenothiazine affinity column in a Ca^{2+}-dependent way [21]. On the other hand, the crayfish calcium-binding protein is labeled by [^{125}I]TID, in both the presence or absence of Ca^{2+} (fig.4, panels 7 and 8).

In conclusion, the results presented here provide evidence that (i) cations of similar ionic radii to Ca^{2+} induce in calmodulin the exposure of hydrophobic sites; (ii) structural integrity of calmodulin is stabilized by Ca^{2+} even in the presence of high concentrations of denaturing agents; (iii) trifluoperazine can reduce the degree of labeling of calmodulin by [^{125}I]TID; and (iv)

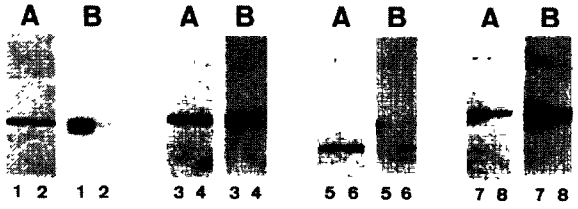

Fig.4. [^{125}I]TID labeling of calcium-binding proteins. The [^{125}I]TID-labeled Ca^{2+}-binding proteins were analysed by SDS-12% polyacrylamide gel electrophoresis. The gel was stained with Coomassie brilliant blue (A) and subjected to autoradiography for 7 days (B). The labeling experiment was carried out either in the presence of Ca^{2+} (1,3,5,7) or in the presence of EGTA (2,4,6,8). (1 + 2) Calmodulin; (3 + 4) troponin C; (5 + 6) parvalbumin; (7 + 8) crayfish calcium-binding protein.

some homologous Ca^{2+}-binding proteins expose hydrophobic sites in a Ca^{2+}-dependent way similar to calmodulin. The determination of the topology of these TID-binding sites is currently under investigation.

ACKNOWLEDGEMENTS

The authors are indebted to Drs J. Cox, Geneva, and C. Heizmann, Zurich, for providing samples of different Ca^{2+}-binding proteins. We also would like to express our gratitude to Dr E. Carafoli for his continuous interest and support for this work. This research was supported by the Swiss National Fonds (grant no. 3.189-0.82).

REFERENCES

[1] Klee, C.B. and Vanaman, T.C. (1982) Adv. Protein Chem. 35, 213–321.
[2] Teo, T.S. and Wang, J.H. (1973) J. Biol. Chem. 248, 5950–5955.
[3] Seamon, K.B. and Kretsinger, R.H. (1984) in: Metal Ions in Biology (Spiro, T.G. ed.) vol.VI, pp.1–51, Wiley, New York.
[4] Levin, R.M. and Weiss, B. (1977) Mol. Pharmacol. 13, 690–697.
[5] La Porte, D.C., Wierman, B.M. and Storm, D.R. (1980) Biochemistry 19, 3814–3819.
[6] Blumenthal, D.K. and Stull, J.T. (1982) Biochemistry 21, 2386–2391.
[7] Newton, D.L. and Klee, C.B. (1984) FEBS Lett. 165, 269–272.
[8] Jarret, H.W. (1984) J. Biol. Chem. 259, 10136–10144.
[9] Krebs, J., Buerkler, J., Guerini, D., Brunner, J. and Carafoli, E. (1984) Biochemistry 23, 400–403.
[10] Buerkler, J. and Krebs, J. (1984) Experientia 40, 604.
[11] Brunner, J. and Semenza, G. (1981) Biochemistry 20, 7174–7182.
[12] Guerini, D., Krebs, J. and Carafoli, E. (1984) J. Biol. Chem. 259, 15172–15177.
[13] Laemmli, U.K. (1970) Nature 227, 680–685.
[14] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[15] Forsen, S., Thulin, E., Drakenberg, T., Krebs, J. and Seamon, K.B. (1980) FEBS Lett. 117, 189–194.
[16] Anderson, A., Forsen, S., Thulin, E. and Vogel, H.J. (1983) Biochemistry 22, 2309–2313.
[17] Wallace, R.W., Tallant, E.A., Dockter, M.E. and Cheung, W.Y. (1982) J. Biol. Chem. 257, 1845–1854.
[18] Chao, S.H., Suzuki, Y., Zysk, J.R. and Cheung, W.Y. (1984) Mol. Pharmacol. 26, 75–82.
[19] Guerini, D. and Krebs, J. (1983) FEBS Lett. 164, 105–110.
[20] Vogel, H.J., Lindahl, L. and Thulin, E. (1983) FEBS Lett. 157, 241–246.
[21] Marshak, D.R., Watterson, D.M. and Van Eldik, L.J. (1981) Proc. Natl. Acad. Sci. USA 78, 6793–6797.