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3-(Trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine, a Hydrophobic, Photoreactive Probe, Labels Calmodulin and Calmodulin Fragments in a Ca^{2+}-Dependent Way†

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ABSTRACT: 3-(Trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID), a highly hydrophobic, carbene-generating photoreactive probe, labels calmodulin and some of its proteolytic fragments in the Ca^{2+}-bound conformation only. It is assumed that [^{125}I]TID labels hydrophobic sites exposed by the binding of Ca^{2+}. The finding offers a new and powerful means to characterize calmodulin sites that play a role in the interaction with targets.

Calmodulin belongs to a family of highly homologous proteins that contain one to four Ca^{2+}-binding domains of similar properties (Klee & Vanaman, 1982; Teo & Wang, 1973). Kretsinger (1975) developed the general "EF-hand" model for this class of proteins on the basis of the crystal structure of parvalbumin. He predicted that the Ca^{2+}-binding domains of homologous proteins will always be composed of two α-helical regions flanking a calcium-binding loop, as is the case for parvalbumin. This view has gained general acceptance and has recently been corroborated by the determination of the crystal structure of the bovine intestinal calcium-binding protein (Szebenyi et al., 1981).

Ca^{2+} binding to calmodulin induces large conformational changes in the protein [for reviews see Klee & Vanaman (1982) and Krebs (1981)], exposing hydrophobic sites (La Porte et al., 1980) that are probably responsible for the binding of phenothiazines and other drugs as originally reported by Levin & Weiss (1977). These sites are often thought to be involved in the interaction of calmodulin with its target proteins (Klee & Vanaman, 1982). In this report a new method to identify these sites will be described. The method is based on the use of a radioactively labeled photoreactive probe, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID),[1] which so far has been used exclusively to label the intramembrane segments of transmembrane proteins (Brunner & Semenza, 1981). It has also been possible to label with this probe various proteolytic fragments of calmodulin in a Ca^{2+}-dependent way. The method is useful in identifying hydrophobic sites of the calmodulin molecule (or of other Ca^{2+}-binding proteins) and also, due to its high sensitivity, in

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[1] Abbreviations: [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; TFP, trifluoperazine.

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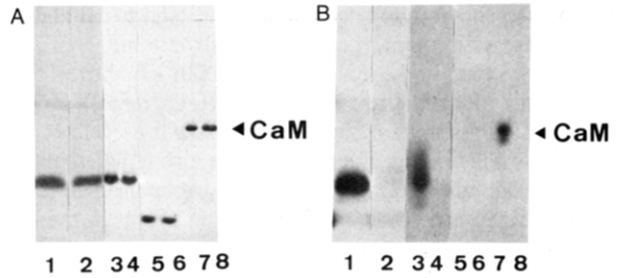

FIGURE 1: $NaDodSO_4$–15% polyacrylamide gel electrophoresis of calmodulin and its tryptic fragments. The gel was run on a Laemmli buffer system (Laemmli, 1970), stained by Coomassie Brilliant Blue (1A), and exposed for 12 h to an X-ray film for autoradiography (1B). Labeling by [^{125}I]TID has been performed either in the presence of Ca^{2+} (uneven-numbered panels) or in the presence of EGTA (even-numbered panels), respectively. Panels 1 and 2 = fragment TR_1C (=1–77); panels 3 and 4 = fragment TR_2C (=78–148); panels 5 and 6 = fragment TR_2E_1 (=107–148); panels 7 and 8 = calmodulin (CaM).

investigating the molecular details of the Ca^{2+}-induced conformational change of the protein(s).

Experimental Procedures

Materials. [^{125}I]TID was prepared as previously described (Brunner & Semenza, 1981), resulting in a specific radioactivity of 10 Ci/mmol. Calmodulin was isolated from bovine brain by a modification of the procedure of Gopalakrishna & Anderson (1982) as will be described in detail elsewhere (D. Guerini, J. Krebs, and E. Carafoli, unpublished results). Calmodulin fragments were prepared by controlled trypsin cleavage in the presence of Ca^{2+} or EGTA, respectively, according to a modification of the procedure originally communicated by Walsh et al. (1977), and the fragments were purified by a combination of reverse-phase and ion-exchange high-pressure liquid chromatography as detailed elsewhere (D. Guerini, J. Krebs, and E. Carafoli, unpublished results). The purity of the samples was routinely checked by $NaDodSO_4$–polyacrylamide gel electrophoresis using a Laemmli buffer system (Laemmli, 1970) or on 6 M urea gels as described elsewhere (Head & Perry, 1974).

Labeling of Calmodulin and of Its Fragments by [^{125}I]TID. Freshly prepared [^{125}I]TID (2 μL) (dissolved in ethanol, 30 μCi/μL) was added to 0.5 mL of a 1.0×10^{-5} M solution of calmodulin or calmodulin fragments in buffer A (50 mM Hepes, pH 7.4, 0.5 mM $CaCl_2$) or buffer B (50 mM Hepes, pH 7.4, 2 mM EGTA) in a Pyrex glass, vortexed for 10 s, and photolyzed for 1 min in a photolysis system as described in detail elsewhere (Brunner & Semenza, 1981; Katzenellenbogen et al., 1974). An excess of free radiolabel was removed from the sample by use of diethyl ether (2 × 2 mL). Adequate amounts of labeled samples were analyzed by using the appropriate slab gel electrophoresis system (for details see the legends to the figures). After being stained with Coomassie Brilliant Blue, the gels were dried and exposed to X-ray films (Eastman Kodak, X-Omat AR film) for autoradiography. Protein determination was performed by the method of Lowry et al. (1951), using calmodulin as standard. Amino acid analysis of the calmodulin fragments was performed under standard conditions (hydrolysis with 6 N HCl at 110 °C for 20 h; the amino acid composition was determined by using an amino acid analyzer from Biotronik, Model LC 6000).

Results

The high-affinity Ca^{2+}-dependent binding of different classes of drugs to calmodulin is probably due to the Ca^{2+}-dependent exposure of hydrophobic sites (La Porte et al., 1980). Attempts have repeatedly been made to locate these hydrophobic sites by biochemical or biophysical techniques (La Porte et al., 1980; Tanaka & Hidaka, 1980; Forsén et al., 1980; Klevit et al., 1981; Krebs & Carafoli, 1982; Johnson & Wittenauer, 1983; Andersson et al., 1983). To obtain more detailed information on that matter, the carbene-generating, photoreactive radioactive probe [^{125}I]TID, which has highly hydrophobic properties (the partition coefficient of [^{125}I]TID between egg lecithin liposomes and aqueous buffer at 25 °C is 4×10^4), was used. Experiments, in which calmodulin was exposed to [^{125}I]TID in the presence of Ca^{2+} (Figure 1, panel 7) or of EGTA (Figure 1, panel 8), have shown that [^{125}I]TID labels calmodulin exclusively in the presence of Ca^{2+}. Whether saturation of all four Ca^{2+}-binding sites of calmodulin is necessary is currently being investigated. Evidently, TID recognizes hydrophobic sites that become exposed as the result of the interaction of the molecule with Ca^{2+}. Competition experiments in which increasing amounts of TFP have been added, and presumably bound to calmodulin, have shown reduced labeling by [^{125}I]TID. The exact amount of TFP required for complete inhibition of TID labeling has not yet been determined. Precise information on this point may prove difficult due to complicating factors, among them the relative hydrophobicity of TID vs. TFP and the fact that the type of interaction of the two molecules with calmodulin is different. Nevertheless, a permissible interpretation of this result is that TFP and TID interact with the same site(s) in the calmodulin molecule. Alternative explanations are also possible, for example, the relative inaccessibility of the TID site(s) caused by a conformational change induced by TFP. Indirect support for the proposal that the TFP and TID site(s) may be identical comes from preliminary results which indicate that up to two molecules of TID can be bound to calmodulin in the Ca^{2+}-induced form. Binding studies by Levin & Weiss (1977), which have been confirmed by others using different methods (Forsén et al., 1980; Klevit et al., 1981; Krebs & Carafoli, 1982; Johnson & Wittenauer, 1983; Andersson et al., 1983), have shown that 2 mol of TFP is bound to Ca^{2+}-saturated calmodulin with high affinity. The determination of the precise stoichiometry of the interaction between [^{125}I]TID and calmodulin, however, was not the aim of the present study. This matter is currently under investigation.

Recent studies with tryptic and CNBr fragments of calmodulin have indicated that the two TFP-binding sites are probably located in each of the two halves of the protein (Head et al., 1982; Vogel et al., 1983; Brzeska et al., 1983; Thulin et al., 1983; Newton et al., 1983; see below). It thus was of interest to perform labeling experiments with TID on tryptic fragments of calmodulin. Tryptic cleavage has been performed in the presence of Ca^{2+} or of EGTA (Walsh et al., 1977), obtaining the fragments indicated in the schematic representation of Figure 2. Details of the conditions of the enzymatic digestion and on the purification of the fragments will appear elsewhere (D. Guerini, J. Krebs, and E. Carafoli, unpublished results).[2] The purity of the fragments has been

[2] A detailed analysis of the proteolytic fragmentation pattern indicates that the original observations by Walsh et al. (1977) that calmodulin in the presence of Ca^{2+} is split by trypsin in two halves corresponding to the sequences 1–77 and 78–148 may have been oversimplified (D. Guerini and J. Krebs, unpublished results). Studies under way in this laboratory indicate that besides cleavage at Lys_{77}, additional cleavage points at Arg_{74} and Lys_{75} also occur. Therefore, fragments TR_1C and TR_2C (see scheme of Figure 2) are probably mixtures of products differing by one or two amino acids. These products have been purified by ion-exchange high-performance liquid chromatography and identified by partial amino acid sequence analysis (D. Guerini and J. Krebs, unpublished results). Similar observations have been made in the laboratories of Klee and Forsén, respectively (Newton et al., 1983; Thulin et al., 1983).

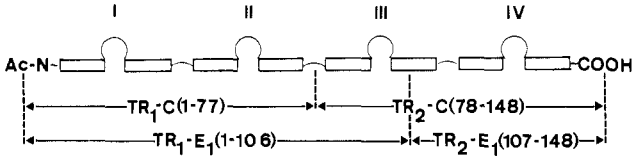

FIGURE 2: Schematic representation of the four Ca^{2+}-binding domains (I–IV) of calmodulin as proposed according to the amino acid sequence [e.g., Klee & Vanaman (1982)]. The different tryptic fragments as obtained in the presence of Ca^{2+} (TR_1C and TR_2C) or in the presence of EGTA (TR_1E and TR_2E) according to Walsh et al. (1977) are indicated in the figure.

Table I: Amino Acid Composition of the Tryptic Fragments of Calmodulin[a]

	TR_1C[d] (1–77)	TR_2C[d] (78–148)	TR_2E (107–148)
Asx	12.2 (11)[b]	11.6 (12)	6.4 (7)
Thr	7.1 (8)	3.9 (4)	2.5 (3)
Ser	1.8 (2)	2.0 (2)	0.3 (0)
Glx	13.7 (13)	14.2 (14)	7.9 (9)
Gly	6.6 (6)	4.9 (5)	2.9 (3)
Ala	6.2 (6)	4.8 (5)	2.1 (2)
Val	2.5 (2)	4.8 (5)	3.2 (4)
Met	3.9 (5)	3.8 (4)	3.8 (4)
Ile	4.5 (4)	3.7 (4)	2.0 (2)
Leu	5.9 (6)	3.0 (3)	1.9 (2)
Tyr	0.2 (0)	2.0 (2)	0.8 (1)
Phe	5.1 (5)	3.0 (3)	1.1 (1)
Lys	4.5 (5)	3.7 (3)[c]	2.1 (2)[c]
His	0.0 (0)	0.9 (1)	0.7 (1)
Arg	2.0 (2)	3.7 (4)	1.2 (1)
Pro	2.5 (2)	0.0 (0)	0.0 (0)

[a] Calculation of the number of residues per mole of peptide fragment was normalized to both the expected values of leucine and phenylalanine on the basis of the sequence of calmodulin from bovine brain as reported by Watterson et al. (1980). [b] Numbers in parentheses represent the expected values as based on the sequence. [c] These values of lysine also include the value for trimethyllysine. [d] See footnote 2.

checked by polyacrylamide gel electrophoresis as shown in Figure 1A. Their identity was established by analysis of the amino acid composition, as shown in Table I. The results of the [^{125}I]TID labeling of the various proteolytic fragments are given in Figure 1B. The autoradiograph shows that also the fragments are labeled only in the presence of Ca^{2+}, demonstrating the fact that hydrophobic sites are exposed by the interaction with the cation as well. In line with the observation that the hydrophobic sites are located in each of the two halves of the calmodulin molecule (Vogel et al., 1983; Brzeska et al., 1983; Newton et al., 1983) are the findings that fragments TR_1C (=1–77)[2] and TR_2C (=78–148)[2] are labeled by [^{125}I]TID in the presence of Ca^{2+}, whereas fragment 107–148 (TR_2E) is not (Figure 1).

Discussion

The observation by Levin & Weiss (1977) that TFP and other antipsychotic drugs bind to calmodulin with high affinity in a Ca^{2+}-dependent way and inhibit the stimulatory effect of calmodulin on cyclic nucleotide phosphodiesterase was related to the pharmacological activity of these drugs. This view was later disproven by a number of observations, among them the similar inactivation of calmodulin by pharmacologically active and inactive enantiomers (Norman et al., 1979). La Porte et al. (1980) provided compelling evidence that high-affinity binding of these drugs is due to the Ca^{2+}-induced exposure of calmodulin hydrophobic sites. This view is strongly supported by the findings presented here since TID is structurally different from the other drugs that bind to calmodulin but shares with them the highly hydrophobic character (water/lipid partition coefficient 1/40 000; see above). The high specific radioactivity of [^{125}I]TID (10 Ci/mmol), its high reactivity, and its small molecular size make it useful not only in mapping the binding site in detail but also in assessing the degree of contamination of the preparations used by other (TID-binding) proteins. This has proven useful in the case of the calmodulin fragments in which no contamination by intact calmodulin was detected (Figure 1B).

As indicated above, two high-affinity binding sites for TFP and other drugs are present in calmodulin (Levin & Weiss, 1977; Forsén et al., 1980; Klevit et al., 1981; Krebs & Carafoli, 1982; Johnson & Wittenauer, 1983; Andersson et al., 1983). It has been reported that fragments from both halves of calmodulin bind to a phenothiazine- or phenyl-Sepharose affinity column in a calcium-dependent manner (Vogel et al., 1983; Brzeska et al., 1983; Newton et al., 1983), suggesting that each half of the protein contains one high-affinity hydrophobic binding site for these drugs. The existence of such hydrophobic sites in each of the two halves of calmodulin is supported by the present results since both fragments TR_1C and TR_2C are labeled by [^{125}I]TID in the presence of Ca^{2+} (Figure 1). It has become apparent from other studies [reviewed by Seamon & Kretsinger (1983)] that only the fragments that contain at least two adjacent Ca^{2+}-binding domains, conserving the cooperative properties of these sites, repeat the properties of the native protein, including the ability to undergo conformational change (i.e., exposure of the hydrophobic sites) upon binding of Ca^{2+}. Fragment 107–148 (TR_2E) is therefore not labeled because it only contains one Ca^{2+}-binding site and/or it may not contain the hydrophobic site to be exposed in the presence of Ca^{2+}. This would exclude the location of one of the hydrophobic sites at the carboxy-terminal part of Ca^{2+}-binding site IV of calmodulin as was suggested by Klevit et al. (1981).

It is clear that the method used in this paper can be applied to a high degree of sensitivity to identify Ca^{2+}-binding proteins with properties similar to calmodulin (i.e., EF-hand proteins). Studies with a number of these proteins currently under way in our laboratory appear very promising. Investigations in which [^{125}I]TID labeling is used as a tool to characterize calmodulin–target interactions in detail are also under way.

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Registry No. [^{125}I]TID, 79684-41-6; Ca, 7440-70-2.

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Aqueous Solution Structure of an Intercalated Actinomycin D-dATGCAT Complex by Two-Dimensional and One-Dimensional Proton NMR†

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ABSTRACT: Two-dimensional NOESY and COSY ^{1}H NMR techniques have been employed to determine the conformation of the complex formed by actinomycin D and the hexanucleoside pentaphosphate dATGCAT. One-dimensional NOE experiments in H_2O confirmed the intact nature of the oligonucleotide double helix within the complex. The drug chromophore was intercalated between the GC base pairs, with the pentapeptide lactones nestled in the minor groove. No significant conformational change of the pentapeptide lactones between bound and free drug was observed.

The actinomycins (Mauger, 1980) are a class of antitumor antibiotics that inhibit DNA-directed RNA synthesis in both eucaryotic and procaryotic cells by binding to the DNA template. Actinomycin D (Figure 1) was shown to be one of the most effective of the natural analogues, which share the same chromophore structure but differ only by amino acid substitutions in the pentapeptide lactone moieties. The inhibition of RNA synthesis was shown to correlate less directly with binding constants than with rates of association and dissociation from DNA (Muller & Crothers, 1968), which are quite slow ($\sim 10^{-3}$ s^{-1}) compared to other typical DNA binding drugs such as ethidium bromide and daunomycin (10^1-10^3 s^{-1}). The relatively slow rates have been attributed to conformational changes in the pentapeptide lactones (Muller & Crothers, 1968; Shafer et al., 1980; Brown et al., 1982; Mirau & Shafer, 1982) or in the nucleic acid (Sobell, 1974).

A crystal structure of actinomycin D (ACTD)[1] complexed with deoxyguanosine has been published (Jain & Sobell, 1972), from which a model of ACTD bound to the hexanucleoside pentaphosphate duplex dATGCAT was proposed (Sobell & Jain, 1972). NMR data obtained from binding ACTD to various dinucleotides (e.g., dCpG, dGpC, etc.), mononucleotides (Reinhardt & Krugh, 1977; Krugh & Chen, 1975; Patel, 1974a), and oligomers (Patel, 1974b; Patel et al., 1981; Reid et al., 1983) have been interpreted to be consistent with the proposed model. However, the mono- and dinucleotide systems investigated showed fast kinetics (Davanloo & Crothers, 1976) and may not be truly representative of the slowly associating/dissociating binding mode directly correlated to RNA synthesis inhibition. These previous NMR

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[1] Abbreviations: ACTD, actinomycin D; COSY, ^{1}H two-dimensional *J*-correlation NMR; NOESY, ^{1}H two-dimensional NOE correlation NMR in the pure absorption mode; TSP, sodium 3-(trimethylsilyl)-tetradeuteriopropionate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; FID, free induction decay; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; Nmv, L-*N*-methylvaline; Thr, L-threonine; Dva, D-valine; Pro, L-proline; Sar, sarcosine; TOE, truncated driven nuclear Overhauser effect; HPLC, high-pressure liquid chromatography; DP, data point.

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