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Intrinsic Fluorescence of a Truncated *Bordetella pertussis* Adenylate Cyclase Expressed in *Escherichia coli*†

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ABSTRACT: A truncated, 432 residue long, *Bordetella pertussis* adenylate cyclase expressed in *Escherichia coli* was analyzed for intrinsic fluorescence properties. The two tryptophans (Trp69 and Trp242) of adenylate cyclase, each situated in close proximity to residues important for catalysis or binding of calmodulin (CaM), produced overlapping fluorescence emission bands upon excitation at 295 nm. CaM, alone or in association with low concentrations of urea, induced important modifications in the spectra of adenylate cyclase such as shifts of the maxima and change in the shape of the bands. From these changes and from the fluorescence spectrum of a modified form of adenylate cyclase, in which a valine residue was substituted for Trp242, it was deduced that, upon binding of CaM to the wild-type adenylate cyclase, only the environment of Trp242 was affected. The fluorescence maximum of this residue, which is more exposed to the solvent than Trp69 in the absence of CaM, is shifted by 13 nm to shorter wavelength upon interaction of protein with its activator. Trypsin cleaved adenylate cyclase into two fragments, one carrying the catalytic domain, and the second carrying the CaM-binding domain (Ladant et al., 1989). The isolated peptides conserved most of the environment around their single tryptophan residues, as in the intact adenylate cyclase, which suggests that the two domains of truncated *B. pertussis* adenylate cyclase also conserved most of their three-dimensional structure in the isolated forms.

Formation of adenosine 3′,5′-cyclic monophosphate (cAMP)[1] from ATP through the reaction catalyzed by adenylate cyclase is part of an important metabolic regulatory mechanism in living cells. While the chain of events leading to activation of adenylate cyclase, and the consequences of the increased cAMP in various prokaryotes or eukaryotes, is fairly well-known (Ullmann & Danchin, 1983; Gilman, 1984; Gancedo et al., 1985), much less is known about adenylate cyclase itself. In the absence of any structural data on the protein, it is very difficult to understand the catalytic and regulatory mechanisms governing this particular group of ATP-dependent enzymes.

Bordetella pertussis adenylate cyclase, a calmodulin (CaM) activated enzyme, is the object of intensive investigations due to its possible involvement in the virulence of this bacteria (Weiss & Hewlett, 1986). Synthesized as a large precursor of 1706 amino acid residues (Glaser et al., 1988), the protein of apparent molecular mass on SDS–PAGE of 200–215 kDa was shown to enter eukaryotic cells provoking unregulated synthesis of cAMP and ultimately cell death (Rogel et al., 1988; Gilboa-Ron et al., 1989; Hewlett et al., 1989; Masure & Storm, 1989; Bellalou et al., 1990). Tryptic digestion converted adenylate cyclase to a fully active form of 43 kDa. Further cleavage of this low molecular mass form of adenylate cyclase gives two complementary fragments corresponding to the catalytic and CaM-binding domains of protein. Isolated fragments have very little or no activity; however, upon reassociation, the CaM-dependent adenylate cyclase activity is restored to a significant level (Ladant et al., 1989). In the present paper we examined the fluorescence properties of a truncated *B. pertussis* adenylate cyclase expressed in *Escherichia coli* by taking advantage of the fact that the low molecular mass form of enzyme has two tryptophan residues, each situated in close proximity to residues important for catalysis or binding of CaM (Glaser et al., 1989).

Experimental Procedures

Chemicals. Adenine nucleotides, substrates, restriction enzymes and T_4 DNA ligase were from Boehringer Mannheim. CaM–agarose, bovine brain CaM, TPCK–trypsin, and soybean trypsin inhibitor were from Sigma. Urea (fluorimetrically pure) was a product of Schwarz/Mann. [α-^{32}P]-

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[1] Abbreviations: 3′dATP, 3′-deoxyadenosine 5′-triphosphate; cAMP, adenosine 3′,5′-cyclic monophosphate; CaM, calmodulin; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; IPTG, isopropyl β-D-thiogalactoside; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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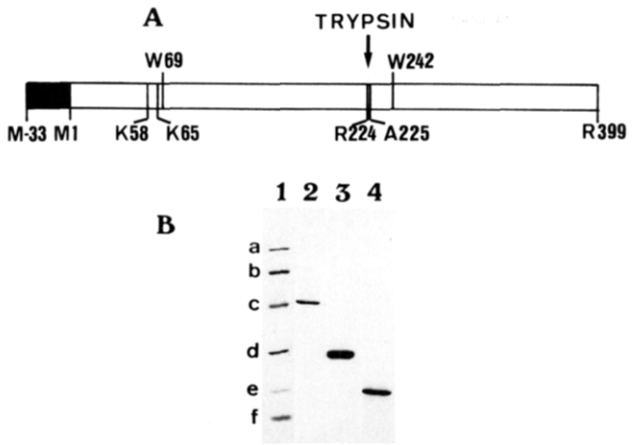

FIGURE 1: Structural organization of the truncated *B. pertussis* adenylate cyclase expressed in *E. coli* as deduced from the nucleotide sequence of the corresponding gene (A) and SDS–PAGE control for purity of adenylate cyclase and complementary tryptic peptides (B). The black box situated within M-33 and M1 corresponds to the vector coding sequence. The position of some key residues in the 47-kDa truncated form of *B. pertussis* adenylate cyclase, as well as the site of cleavage by trypsin, is indicated in (A). Samples of pure enzyme (lane 2) or tryptic fragments (T28, lane 3; T19, lane 4) were run onto a 10% SDS–PAGE and then stained with Coomassie Blue. The molecular weight markers (lane 1) were as follows: (a) phosphorylase *a* (94000); (b) bovine serum albumin (67000); (c) ovalbumin (43000); (d) carbonic anhydrase (30000); (e) soybean trypsin inhibitor (20300); (f) lysozyme (14400).

ATP (3000 Ci/mmol), [α-^{35}S]ATP (>1000 Ci/mmol), and [^{3}H]cAMP (40 Ci/mmol) were obtained from the Radiochemical Center, Amersham, U.K. Oligonucleotides were synthesized according to the phosphamidinate method using a commercial DNA synthesizer (Applied Biosystems).

Bacterial Strains and Growth Conditions. The production of recombinant protein was performed by using the protease-deficient strain Y1083 BNN 103 (Young & Davis, 1983) which harbors the plasmid pDIA5227, a derivative of pDIA5202 (Glaser et al., 1989). Plasmid pDIA5227 encodes a 432 residue long truncated adenylate cyclase of *B. pertussis*. The 33 N-terminal amino acids of this protein (MTMITPSSNTTHYRESLHACTSTLEDLTAVEIH) numbered from −33 to −1 are encoded by vector sequences. They are followed by the first 399 amino acid residues of the adenylate cyclase (Figure 1A). Cultures were grown in LB medium (Miller, 1972) supplemented with 100 μg/mL ampicillin and 20 μg/mL chloramphenicol. Synthesis of truncated adenylate cyclase was induced by IPTG (1 mM) when cultures reached an optical density of 0.5. Bacteria were harvested by centrifugation 4 h after induction.

Site-Directed Mutagenesis and Sequence Analysis. Oligonucleotide-directed mutagenesis was performed on the single-stranded form of pDIA5222 using the Amersham system based on the method of Taylor et al. (1985). The glutamine (CAG) codon at position 400 was modified to a stop (UAG) codon using the oligonucleotides $^{5'}$GTC GAA CGC TAG GAT TCC G$^{3'}$. Tryptophan codon (at position 242 in the corresponding protein) was replaced by a Val codon by exchanging the *Eco*RV–*Bcl*I restriction fragment by the equivalent DNA fragment from pDIA5202 derivative in which a TTG Trp codon was modified to a GTC Val codon by site-directed mutagenesis (Glaser et al., 1989).

Purification and Assay of Adenylate Cyclase. Truncated *B. pertussis* adenylate cyclase expressed in *E. coli* was purified by affinity chromatography on CaM–agarose (Ladant, 1988; Haiech et al., 1988) essentially as described by Glaser et al. (1989). The enzyme, adsorbed onto CaM–agarose, was cleaved with TPCK–trypsin (25 μg for each 1000 units of enzyme activity) for 10 min at 4 °C, and then fragments were separated from uncleaved protein by gel permeation chromatography under denaturing conditions (Figure 1B). Details concerning preparation and analysis of isolated peptide, T28 and T19 (corresponding to the N- and C-terminal domains, respectively), of bacterial enzyme will be published elsewhere.

Adenylate cyclase activity was measured according to the procedure of White (1974) as described previously (Ladant, 1988; Ladant et al., 1989), in 100 μL of medium containing 50 mM Tris-HCl (pH 8), 6 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 μM bovine brain CaM, 0.5 mg/mL bovine serum albumin, 0.1 mM cAMP (10^4 cpm/assay), and 2 mM ATP (2500 cpm/mmol). One unit (1 U) of adenylate cyclase activity corresponds to 1 μM cAMP formed in 1 min at 30 °C and pH 8.

Analytical Procedure. Proteins were measured according to Bradford (1976) or by amino acid analysis after hydrolysis for 24 h at 110 °C with 6 N HCl. SDS–PAGE was performed as described by Laemmli (1970). Fluorescence measurements were done with a Perkin-Elmer LS-5B luminescence spectrometer thermostated at 25 °C using 1 × 1 cm UV-grade quartz cuvettes (sample volume of 2 mL). Emission spectra of adenylate cyclase and corresponding fragments (λ_{exc} = 295 nm) were recorded from 305 to 400 nm. Circular dichroism measurements were done at 22 °C with a Jobin-Yvon CD6 dichrograph connected to a computer. Ellipticities at 222 nm were expressed in deg·cm^2·dmol^{-1}.

Results

Upon excitation at 295 nm, truncated adenylate cyclase exhibits a fluorescence emission spectrum with a maximum at 342 nm. Addition of equimolar or excess CaM in the presence of Ca^{2+} shifted this maximum to 334 nm with no or slight (up to 5%) increase in the peak height (Figure 2A). A more careful analysis of the spectra indicates narrowing upon addition of CaM to adenylate cyclase (the bandwidth at half-height decreases from 55 to 51 nm). Excess EGTA almost completely reversed the effect of CaM on the fluorescence spectrum of enzyme (not shown).

Urea also produced important changes in the fluorescence spectra of protein which were most visible at a 3 M concentration. Thus, in the absence of CaM, the fluorescence maximum of adenylate cyclase was shifted from 342 to 352 nm with no apparent changes of the bandwidth at half-height. CaM broadened the fluorescence spectrum of enzyme (bandwidth at half-height of 60 nm), and the maximum at 335 nm was accompanied by a shoulder at 346 nm (Figure 2B). Gradual changes induced by different concentrations of urea on the fluorescence properties of adenylate cyclase, complexed or not with CaM, are shown in Figure 3.

These results can be rationalized as follows as a first approximation: the two tryptophan residues of *B. pertussis* adenylate cyclase (W69 and W242) produce overlapping fluorescence bands upon excitation at 295 nm. However, when ligand alone or in association with denaturing agent interacts with the protein, the microscopic environments of these amino acid residues are markedly different, which results in shifts of the maxima and band narrowing or broadening. The shift to higher frequencies upon addition of CaM to adenylate cyclase, both in the presence and in the absence of urea, can be attributed to W242. This residue belongs to a helical segment of adenylate cyclase, as predicted from the primary structure of the protein using an algorithm of Gibrat et al. (1987). A synthetic peptide corresponding to residues 235–254 of *B. pertussis* adenylate cyclase ($P_{235-254}$) possessing residual α-helix structures, as indicated by ^{1}H NMR analysis (Craescu

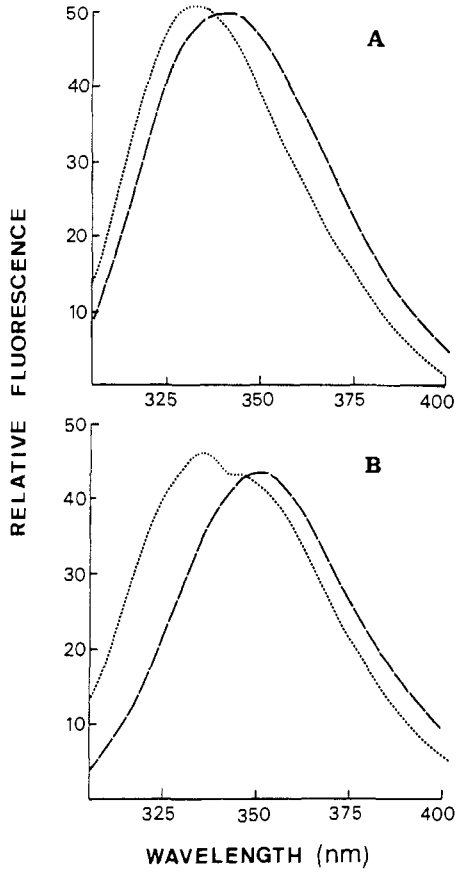

FIGURE 2: Fluorescence emission spectra of truncated *B. pertussis* adenylate cyclase in the absence (A) or presence of 3 M urea (B). The intrinsic fluorescence spectra of adenylate cyclase (1 μM) in 50 mM Tris-HCl (pH 8), 100 mM NaCl, and 0.1 mM $CaCl_2$ corrected for the buffer and urea were recorded in the absence (--) or presence (···) of 1.2 μM CaM. Protein solutions in 3 M urea were prepared 2 h before measurements; occasionally, spectra were recorded after overnight incubation of samples in urea at 4 °C with essentially identical results.

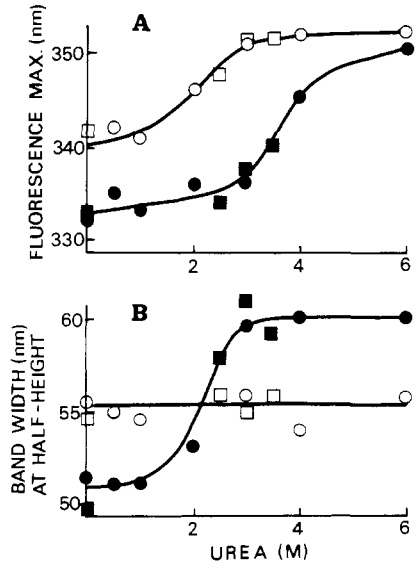

FIGURE 3: Effect of different concentrations of urea on the fluorescence emission maximum (A) and bandwidth at half-height (B) of *B. pertussis* adenylate cyclase alone (□, ○) or in the presence of CaM (■, ●). The experimental conditions are identical with those described in Figure 2. Squares and circles represent different preparations of adenylate cyclase.

et al., unpublished results), showed similar changes in its fluorescence properties upon binding of CaM (Ladant et al.,

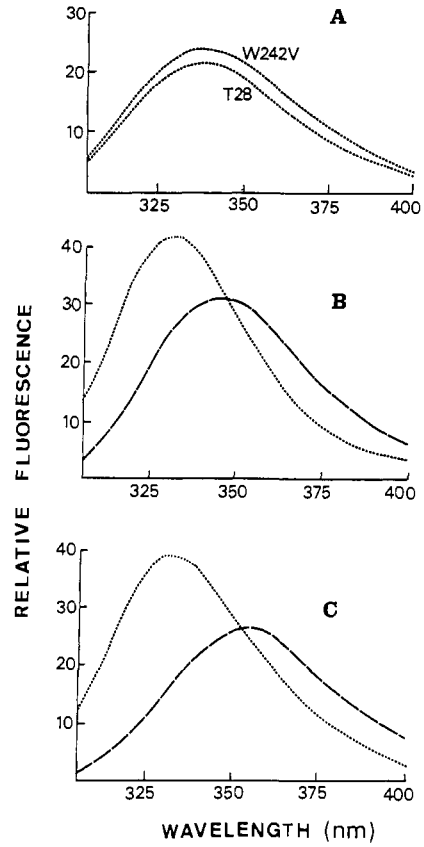

FIGURE 4: Fluorescence spectra of W242V-modified adenylate cyclase, T28 peptide (A), and T19 peptide (B and C) in the presence (···) or absence (--) of CaM. The experimental conditions in (A) and (B) are identical with those described in Figure 2A. Spectra in (C) were obtained in the presence of 3 M urea.

1989). The shoulder observable at 346 nm in the presence of urea and CaM is mostly due to W69.

To validate this interpretation, two experimental approaches were considered. First, W242 was substituted with a valine residue by site-directed mutagenesis. The W242V-modified enzyme essentially showed the same catalytic activity and CaM-binding properties as the wild-type adenylate cyclase (Glaser et al., 1989). The fluorescence spectrum of W242V-modified adenylate cyclase was different from that of wild-type protein in many respects. It exhibited a maximum at 338 nm, independent of the presence or absence of CaM, and the peak height at identical concentration of enzyme represented less than 50% of that of wild-type protein (Figure 4A). A 3 M concentration of urea displaced the maximum from 338 to 352 nm. In a second approach adenylate cyclase was cleaved at Arg224 by trypsin into two fragments, one carrying the catalytic site (T28) and the other carrying the CaM-binding domain (T19). Each fragment possesses a single tryptophan and exhibits a characteristic fluorescence spectrum upon excitation at 295 nm. Like W242V-modified protein, T28 exhibited a peak at 338 nm which was shifted by 3 M urea to 355 nm. CaM did not affect the fluorescence properties of T28 fragment for which the peak height was approximately 40% of wild-type protein (Figure 4A). The T19 fragment of adenylate cyclase behaved differently. The maximum in fluorescence at 345 nm in the absence of CaM was shifted to 332 nm when equimolar or excess CaM was added to the peptide (Figure 4B). As for the wild-type protein, excess EGTA almost completely reversed the effect of CaM on the fluorescence spectrum of T19 (not shown). Two other properties of T19 peptide fluorescence should also be mentioned. The peak height at 345 nm in the absence of CaM represented

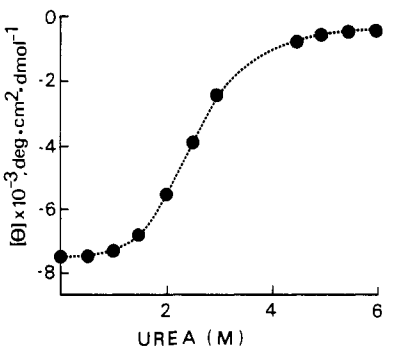

FIGURE 5: Dependence of mean residue molar ellipticity at 222 nm of truncated adenylate cyclase on urea concentration. Measurements were made at 20 °C in 0.02-cm circular cells at 3.8 μM protein concentration in buffer containing 100 mM Tris-HCl (pH 8), 100 mM NaCl, and various concentrations of urea.

60% of that of wild-type protein. On the other hand, CaM increased 1.4-fold the maximum at 332 nm in addition to shifting the spectrum to higher frequencies. A 3 M concentration of urea in the absence of CaM provoked a shift in the fluorescence maximum of T19 from 345 to 352 nm, whereas the maximum at 332 nm in the presence of CaM was practically unchanged (Figure 4C).

Several conclusions should be retained from these last two experiments: (i) the two tryptophans in *B. pertussis* adenylate cyclase have different environments and behave differently upon excitation at 295 nm in both their fluorescence intensities and wavelengths at maximum fluorescence; (ii) conformational changes induced by CaM upon binding to the protein affected a single tryptophan residue, namely, W242; (iii) the two isolated peptides, T28 and T19, appear to conserve most of, if not the same, environment around their tryptophan residues as they have in the intact adenylate cyclase; (iv) the two domains in intact adenylate cyclase probably unfold differently in urea, the domain corresponding to the catalytic site being more sensitive to this chaotropic agent. An indirect indication in this sense is the fact that adenylate cyclase activity declines rapidly in the presence of urea (40% of maximum activity in 1 M urea, 8% of maximum activity in 2 M urea), whereas the CaM-binding properties of wild-type protein or of T19 fragment are much less affected. Determination of the ellipticity of adenylate cyclase at 222 nm as a function of urea concentration indicated, however, a sigmoidal profile with a single point of inflection at 2.4 M urea (Figure 5).

In previous experiments we showed that two lysine residues (K65 and K58) in *B. pertussis* adenylate cyclase, close to W69, are essential for catalysis, since their replacement by glutamine reduced activity of the bacterial enzyme by more than 3 orders of magnitude (Glaser et al., 1989). K65, as part of a sequence resembling the well-known motif GXXXXGKT(S) in ATP-binding proteins (Moller & Amons, 1985; Fry et al, 1986), belongs to a β-turn segment as predicted from the primary structure of protein. We expected, therefore, that binding of nucleotides (or pyrophosphate) would provoke conformational changes in this region of the protein sufficient to affect the fluorescence of the single tryptophan residue in W242V-modified enzyme. Neither 3′dATP (a noncyclizable ATP analogue acting as a competitive inhibitor) nor pyrophosphate affected the fluorescence properties of W242V adenylate cyclase, irrespective of the presence or absence of divalent ions or CaM.

Discussion

The fluorescence of tryptophan in proteins is heterogeneous, each residue having different maximum and quantum yield depending on its local environment and degree of exposure to solvents (Burnstein et al., 1973; Sun & Song, 1977). Thus, the fluorescence maxima of proteins containing "buried" tryptophan residues occur around 330 nm, while the maxima for proteins containing exposed tryptophan residues are shifted to lower frequencies. Since the fluorescence band shapes are broad in all cases, they overlap and little information can be gained about individual tryptophans. Conservative replacement by site-directed mutagenesis of tryptophans by other amino acids (or vice versa, insertion of tryptophans in place of other residues) proved to be a new and helpful tool in interpretation of the intrinsic fluorescence of proteins (Kilhoffer et al., 1988; Nishimura et al., 1990). Since the two tryptophan residues of truncated *B. pertussis* adenylate cyclase respond differently to CaM in the presence or absence of urea, their contribution to the overall fluorescence of intact protein can be "decomposed", at least qualitatively. Comparison of the spectra exhibited by wild-type enzyme and W242V-modified protein suggested that Trp69 is less exposed to the solvent than Trp242, and its environment is not affected by binding of CaM, nucleotides, or pyrophosphate. On the contrary, the fluorescence maximum of Trp242, which is more exposed to the solvent in the absence of CaM, is shifted by 13 nm to shorter wavelength upon interaction of protein with its activator. A likely explanation is that CaM binding shields Trp242 from water accessibility. Simulated spectra from this simplified "decomposition" accounted correctly for the shifts in maxima of fluorescence or bandwidth narrowing or broadening upon binding of CaM in the absence or presence of urea (not shown). From intrinsic fluorescence experiments it was not clear if the CaM-dependent conformational changes of enzyme are equally critical for binding of the nucleotide substrate. It should be mentioned that binding experiments by equilibrium dialysis done with another CaM-activated enzyme, namely, a truncated *Bacillus anthracis* adenylate cyclase expressed in *E. coli*, showed that, in the absence of CaM, binding of [α-^{32}P]3′dATP to the bacterial enzyme was severely impaired (Labruyère et al., 1990).

The fact that the isolated fragments, T28 and T19, conserve an environment around their tryptophan residues similar to that in the intact protein indicates that the catalytic and CaM-binding domains in adenylate cyclase possess well-defined three-dimensional structures. Such a "modular" construct of *B. pertussis* adenylate cyclase might be relevant to its remarkable property, namely, activation by a protein (CaM) present in the target eukaryotic cells, but absent in bacteria. The bringing together of independent modules might have allowed construction in a primitive organism of a functional protein in which modules coevolved toward CaM activation and virulence.

It is interesting to note that, at concentrations of urea at which both enzyme and CaM unfold to a significant degree, the proteins still interact strongly as indicated by the fluorescence shift of Trp242 in adenylate cyclase. This means that interactions between "complementary" structures in CaM and adenylate cyclase (or T19 fragment) are strong enough for mutual recognition and binding at 3 M urea. We do not know if these structures, restricted most probably to short helical segments, are particularly resistant to the chaotropic agent or if they were stabilized upon electrostatic interaction of amino acid residues of opposite charge in CaM and adenylate cyclase.

The reversal by excess EGTA of the effect of CaM on enzyme fluorescence spectrum indicates that the interaction between the helical segment around Trp242 in *B. pertussis*

adenylate cyclase and its complementary structure in the CaM molecule is considerably weakened or completely abolished. Since EGTA does not dissociate CaM from adenylate cyclase,[2] the question arises, therefore, whether other segments in adenylate cyclase contribute to the tight binding of CaM, and perhaps to activation. Analysis of C-terminal deletion derivatives of *B. pertussis* adenylate cyclase seems to indicate that a fragment situated between residues 343 and 373 is essential for both binding of CaM and activation (P. Glaser et al., unpublished results). This observation might explain at least in part why the synthetic peptide $P_{235-254}$ exhibited a much lower affinity for CaM than intact adenylate cyclase or T19 fragment.

In conclusion, under properly selected experimental conditions, the intrinsic fluorescence of adenylate cyclase offers useful information on local structural changes which occur in protein upon interaction with CaM. Such information is particularly important when enzyme modified by site-directed mutagenesis is inactive, and kinetic experiments cannot be used to discriminate between structural changes that affect binding of CaM or activation.

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Registry No. Ca, 7440-70-2; adenylate cyclase, 9012-42-4; Trp, 73-22-3.

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[2] Under our experimental conditions the two proteins exist predominantly as a complex (98.6% with excess Ca^{2+} and 85.4% with excess EGTA, respectively). These figures were based on a K_d of CaM for bacterial adenylate cyclase of 0.2 nM in excess Ca^{2+} and 25 nM in excess EGTA, respectively (Haiech et al., 1988).