Structural and Ligand-Binding Properties of a Truncated Form of *Bacillus anthracis* Adenylate Cyclase and of a Catalytically Inactive Variant in Which Glutamine Substitutes for Lysine-346†

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ABSTRACT: A truncated, 541-residue-long, *Bacillus anthracis* adenylate cyclase was expressed in *Escherichia coli*. The purified protein (CYA 62) exhibited catalytic and CaM-binding properties identical with those of the wild-type enzyme secreted by *B. anthracis*. The analysis of the secondary structure of the CYA 62 protein by Fourier transform infrared spectroscopy and circular dichroism revealed the dominance of β-type structure. The protein shows a relatively low thermal stability with the midpoint denaturation temperature at 45 °C. A catalytically inactive variant of CYA 62 in which Gln substituted for Lys-346 (CYA 62 K346Q) was comparatively analyzed for its secondary structure and thermal stability, as well as ligand-binding properties with fluorescent derivatives of ATP and calmodulin. The K346Q variant of CYA 62 has a similar secondary structure and comparable calmodulin binding properties to those of the parent protein and exhibits only slightly reduced thermal stability (the apparent midpoint denaturation temperature is at 43 °C). Despite these similarities, the binding of 3′-anthraniloyl-2′-deoxy-ATP (a fluorescent ATP analogue) to the modified protein is severely impaired, from which we conclude that the prime function of Lys-346 in the wild-type enzyme from *B. anthracis* is to ensure tight binding of the nucleotide substrate to the active site.

Bacillus anthracis, a gram-positive pathogen, produces two toxins composed of three distinct proteins: the protective antigen (PA, 85 kDa), the edema factor (EF, 89 kDa), and the lethal factor (LF, 83 kDa) (Stanley & Smith, 1961; Leppla, 1984). PA and LF form the lethal toxin, while the edema toxin consists of a combination of PA and EF (Fish et al., 1968a,b). PA, which is a receptor-binding component common to both toxins, mediates the entry of LF or EF into target cells. In contrast to LF, whose biochemical target is still unknown, EF has been identified as a CaM[1]-activated adenylate cyclase (Leppla, 1982, 1984).

Cloning and expression in *Escherichia coli* of the *cya* gene encoding EF represented the first step in the investigation of the structure–function relationship of this protein (Escuyer et al., 1988; Mock et al., 1988; Tippets & Robertson, 1988). A deletion derivative of *B. anthracis* adenylate cyclase lacking 261 amino acid residues at its N-terminal end (CYA 62) exhibits CaM-binding properties and maximal catalytic activity similar to that of the wild-type adenylate cyclase secreted by *B. anthracis* (Labruyère et al., 1990).

Comparison of the primary structure of *B. anthracis* adenylate cyclase with another CaM-dependent enzyme, the adenylate cyclase from *Bordetella pertussis*, indicates little similarity, except for three short stretches (Escuyer et al., 1988; Glaser et al., 1988). The first region contains amino acids that resemble the consensus sequence GXXXXGKT(S) present in many nucleotide-binding proteins (Moller & Amons, 1985; Fry et al., 1986). Site-directed mutagenesis of Lys-65 in *B. pertussis* adenylate cyclase yields an almost inactive protein (Glaser et al., 1989). As expected, Lys-353 in *B. anthracis* adenylate cyclase (equivalent to Lys-65 in the *B. pertussis* enzyme) plays the same role; the modified variant of *B. anthracis* adenylate cyclase in which Lys-353 was replaced by Met lost its activity completely (Xia & Storm, 1990). A second basic amino acid, situated seven residues upstream from Lys-65 in *B. pertussis* or Lys-353 in *B. anthracis* adenylate cyclase, is much less conserved in other ATP-binding proteins (Figure 1). Therefore, it is of interest to recognize the importance of these vicinal Lys residues in relation to the function of bacterial cyclases. Site-directed mutagenesis of Lys-58 in *B. pertussis*, and of Lys-346 in *B. anthracis* adenylate cyclase, also yields inactive proteins (Au et al., 1989; Glaser et al., 1989; Xia & Storm, 1990). Since no structural or ligand-binding data are presently available on these bacterial enzymes, we undertook systematic studies aimed at elucidating the role played by such key residues in catalysis, in binding of nucleotide substrate or CaM, and in maintaining thermodynamically stable protein structures.

In this paper we show that the inactive K346Q variant of *B. anthracis* adenylate cyclase has normal CaM-binding

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[1] Abbreviations: CaM, calmodulin; Ant-dATP, 3′-anthraniloyl-2′-deoxyadenosine 5′-triphosphate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Blue–Sepharose, Cibacron blue 3G-A Sepharose CL-6B; $P_{342-358}$, synthetic peptide corresponding to residues 342–358 of *Bacillus anthracis* adenylate cyclase having the sequence GVATKGLNVHGKSSDWG; CYA 62, deletion derivative of wild-type adenylate cyclase of *B. anthracis* lacking the first 261 residues; CYA 62 K346Q, modified form of CYA 62 in which a glutamine residue substitutes for Lys-346; FT-IR, Fourier transform infrared spectroscopy; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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Cell Source	Protein	Residues	Homologous Sequence														Reference
B. anthracis	Adenylate cyclase	342-354	G	V	A	-	T	K	G	L	N	V	H	G	K	S	(a)
E. coli	Adenylate kinase	2-14	R	I	I	-	L	L	G	A	P	G	A	G	K	G	(b)
Muscle	Adenylate kinase	9-22	K	I	I	F	V	V	G	G	P	G	S	G	K	G	(c)
E. coli	ATPase (β-subunit)	144-156	K	V	G	-	L	F	G	G	A	G	V	G	K	T	(d)
E. coli	ATPase (α-subunit)	164-176	R	E	L	-	I	I	G	D	R	G	T	G	K	T	(e)
E. coli	Rho protein	173-185	R	G	L	-	I	V	A	P	P	K	A	G	K	T	(f)
S. cerevisiae	RAD3 gene product	37-49	N	S	I	-	L	E	M	P	S	G	T	G	K	T	(g)

FIGURE 1: Alignment of homologous "G-rich" sequences in six ATP-dependent enzymes. Identical residues shared by different proteins are boxed. The basic residues are written in bold letters. References: (a) Escuyer et al. (1988); (b) Reinstein et al. (1988); (c) Kuby et al. (1984); (d) Parsonage et al. (1987); (e) Rao et al. (1988); (f) Dombrovski et al. (1988); (g) Sung et al. (1988).

properties; however, binding of Ant-dATP (a fluorescent analogue of ATP) to the modified protein was severely impaired. Since the K346Q substitution does not affect the secondary structure and has only a slight effect on the thermostability of the protein, it seems that the main function of Lys-346 in the wild-type *B. anthracis* enzyme is to ensure tight binding of nucleotide substrate to the active site.

Experimental Procedures

Chemicals. Adenine nucleotides, restriction enzymes, and T4 DNA ligase were from Boehringer Mannheim. Oligonucleotides were synthesized according to the phosphoamidinate method by using a commercial DNA synthesizer (Applied Biosystems). Bovine brain CaM and dansyl-CaM were from Sigma. Blue–Sepharose, Ultrogel AcA44, polybuffer exchanger 94, and polybuffer 96 were from Pharmacia LKB Biotechnologies. Ant-dATP and [α^{32}P]-Ant-dATP were prepared from 2′-dATP or [α-^{32}P]-2′-dATP and isatoic anhydride essentially by the same procedure as described by Hiratsuka (1983) for the synthesis of Ant-ATP. The identity and purity of the compounds were checked by UV spectrophotometry and proton NMR. [α-^{32}P]ATP (3000 Ci/mmol), [α-^{32}P]-2′-dATP (1000 Ci/mmol), [^{3}H]cAMP (40 Ci/mmol), and [^{35}S]-2′-dATPαS (1000 Ci/mmol) were obtained from the Radiochemical Centre, Amersham (U.K.). Rabbit antibodies against synthetic peptide $P_{342-358}$ were obtained as previously described (Goyard et al., 1989).

Bacterial Strains, Plasmids, and Growth Conditions. E. coli strain CAG 1139, a protease-deficient strain (*lon*$^-$) (Grossman et al., 1983), was transformed with the plasmid pMMA 861.19N, which harbors a partial deletion of the *cya* gene lacking 261 codons at the 5′-end (Labruyère et al., 1990). Cultures were performed in LB medium (Miller, 1972) supplemented with 200 μg/mL ampicillin.

Site-Directed Mutagenesis and Sequence Analysis. Oligonucleotide-directed mutagenesis was performed by using the Amersham kit and following the supplier's instructions. The Lys (AAG) codon at position 346 was modified to a Gln (CAG) codon by using the nucleotide 3′-TAAGTTAGG*GA*-CACATCGGTG-5′. The modification was performed on the *Eco*R1–*Bam*H1 pMMA 861.19N DNA fragment cloned in phage M13mp19 and controlled by DNA sequencing using a modified dideoxynucleotide chain terminator with sequenase as DNA polymerase (Tabor & Richardson, 1987).

Purification and Assay of Adenylate Cyclase. Bacteria from 8 L of culture were harvested by centrifugation for 30 min at 9000*g*, resuspended in 200 mL of 50 mM Tris-HCl (pH 8), and disrupted by sonication at 20 kHz and 100 W (3 × 3 min). Cell debris was removed by centrifugation at 12000*g* for 20 min at 4 °C. Both forms of truncated adenylate cyclase were purified with chromatography on Blue–Sepharose, Ultrogel AcA44, and chromatofocusing (Labruyère et al., 1990). In the case of the K346Q variant, progress in the purification was monitored by assaying proteins (by using either the procedure of Bradford or the absorbance at 280 nm), SDS–PAGE, and immunoblot analysis. Purified proteins were stored as frozen solutions at −20 °C in 50 mM Tris-HCl (pH 8) or lyophilized after extensive dialysis against 50 mM ammonium bicarbonate. Enzyme activity was measured according to the procedure of White (1974) as described previously (Ladant et al., 1989). One unit of adenylate cyclase activity corresponds to 1 μmol of cAMP formed in 1 min at 30 °C and pH 8.

Fluorescence Measurements. Binding of dansyl-CaM or of Ant-dATP to adenylate cyclase was analyzed with a Perkin-Elmer LS-5B luminescence spectrometer thermostated at 25 °C using 1 × 1 cm or 1 × 0.2 cm UV-grade quartz cuvettes (sample volume of 2 or 0.3 mL). The dansyl moiety of CaM was excited at 340 nm, and adenylate cyclase was added to the medium containing 50 mM Tris-HCl (pH 8), 100 mM NaCl, 0.1 mM $CaCl_2$, and 150 nM dansyl-CaM. Spectra were recorded from 400 to 550 nm. Ant-dATP was excited at 330 nm, and adenylate cyclase was added to the medium containing 50 mM Tris-HCl (pH 8), 100 mM NaCl, 0.5 mM $CaCl_2$, 40 μM CaM, and 10 μM Ant-dATP. Spectra were recorded from 350 to 500 nm. The titration of fluorescence enhancement or quenching was performed by recording the fluorescence emission at 480 nm (dansyl-CaM) or 420 nm (Ant-dATP). One data point corresponds to the fluorescence intensity integrated over a total time of 8 s.

Fourier Transform Infrared Spectroscopy. Infrared spectra were recorded at 22 °C with a Digilab FTS-60 spectrometer using a high-sensitivity deuterated triglycine sulfate detector. Samples (0.3 mg of protein dissolved in 15 μL of buffer) were prepared by using 50 mM HEPES and 100 mM NaCl buffer in D_2O (pH 7.4) and were placed between two CaF_2 windows separated by a 50-μm Teflon spacer. For each spectrum, 100 interferograms were accumulated and Fourier transformed to yield infrared spectra with a nominal resolution of 2 cm^{-1}. Overlapping infrared bands were resolved according to Fourier self-deconvolution procedures as described previously (Kauppinen et al., 1981).

Circular Dichroism Measurements. The CD spectra were recorded with a Jobin-Yvon CD6 dichrograph interfaced to

a microcomputer. The protein was solubilized in 10 mM potassium phosphate buffer (pH 7.4) at 0.8 mg/mL (about 13 μM). Measurements were performed in quartz optical cylindrical cells (Hellma) with a 0.02-cm path length for the spectral range from 180 to 260 nm, the temperature being maintained constant at 20 °C with a water bath ministat (Bioblock). Results are given as the mean residue molar ellipticity [θ] expressed in deg·cm^2·dmol^{-1}. For the estimation of secondary structure, CD curves in the 190-260 nm range were analyzed by the method of Chen et al. (1974) by using the program of Yang et al. (1986).

Analytical Procedures. Protein concentration was determined according to Bradford (1976) with a Bio-Rad kit. SDS-PAGE was performed as described by Laemmli (1970). Gels were either stained with Coomassie Blue or subjected to immunoblot analysis (Towbin et al., 1979). Western blots were probed with an immunoserum obtained from rabbits immunized with the synthetic peptide $P_{342-358}$ (Goyard et al., 1989) and used at a 1/1000 dilution. Immunodetected proteins were visualized by using ^{125}I-labeled protein A and autoradiography on X-ray films. Binding of [α-^{32}P]-Ant-dATP to adenylate cyclase by equilibrium dialysis was determined at 6 °C in chambers of 0.5-mL capacity. Aliquots of 0.25 mL of solution having a final concentration of 0.01–0.05 mM adenylate cyclase plus an equimolar concentration of CaM, 50 mM Tris-HCl (pH 8), 100 mM NaCl, 0.5 mM $CaCl_2$, and 2 mM $MgCl_2$, were equilibrated for 12 h with an equal volume of the same solution in which the enzyme was replaced by radiolabeled nucleotide (from 5 to 200 μM). Equilibrium data were analyzed by Scatchard plots.

Results

Expression and Purification of the K346Q Variant of the Truncated B. anthracis Adenylate Cyclase. The plasmid pMMA 861.19N, in which the first 261 codons of the *B. anthracis* adenylate cyclase gene were deleted, was transformed into a protease-deficient strain (CAG 1139) of *E. coli*. The transformed strain expressed a protein with an apparent molecular mass on SDS–PAGE of 62 kDa. The truncated *B. anthracis* adenylate cyclase (CYA 62), representing approximately 2% of the total *E. coli* proteins, was purified to apparent homogeneity in three steps that involve chromatography on Blue–Sepharose, Ultrogel AcA44, and chromatofocusing (Labruyère et al., 1990). The modified variant of the truncated adenylate cyclase (CYA 62 K346Q) was found catalytically inactive, although CYA 62 and the mutated variant were both expressed in similar amounts in recombinant *E. coli* strains, as detected by immunoblot experiments using antibodies directed against peptide $P_{342-358}$ (Figure 2). In some bacterial extracts, immunoblot analysis evidenced a lower molecular mass peptide, which probably represents a degradation product of CYA 62 K346Q. The modified enzyme was purified by the same protocol as that used for the purification of CYA 62 (Labruyère et al., 1990).

Structural Analysis of the CYA 62 Protein and of Its Inactive Variant, CYA 62 K346Q. No structural data are presently available on adenylate cyclases, irrespective of their origin or cellular localization (i.e., soluble or membrane-bound forms). Hence, we have performed a comparative investigation of the secondary structure and the thermal stability of CYA 62 and CYA 62 K346Q by two spectroscopic techniques, infrared spectroscopy and circular dichroism.

The infrared spectrum of CYA 62 dissolved in D_2O as illustrated in Figure 3 for the conformationally sensitive amide I region. The original spectrum (A) shows a single broad band with the maximum at 1646 cm^{-1}, which results from the overlapping of component bands representing different secondary structure elements (Byler & Susi, 1986; Surewicz & Mantsch, 1988). The spectrum obtained after band narrowing by Fourier self-deconvolution (B) allows the identification of at least seven component bands in the amide I region of CYA 62. On the basis of established spectra–structure correlation (Byler & Susi, 1986), the bands at 1623 and 1634 cm^{-1} are assigned to β-sheet structures, the band at 1658 cm^{-1} is in the spectral region characteristic of α-helical structures, and the strong band at 1647 cm^{-1} represents mainly unordered polypeptide segments but may also contain contributions from α-helices. The band at 1668 cm^{-1} originates from peptide segments engaged in turns, while the weaker bands at 1675 and 1682 cm^{-1} are in the wavenumber range characteristic of turns and/or β-strands.

The estimate of the secondary structure obtained from the curve fitting of the infrared spectrum of CYA 62, following the procedure of Byler and Susi (1986), provides the following numbers: ~34% β-structures, ≥10% α-helices, ~14% turns, and the remainder of ≤42% corresponds to unordered protein segments. Analysis of the CD spectrum of the truncated

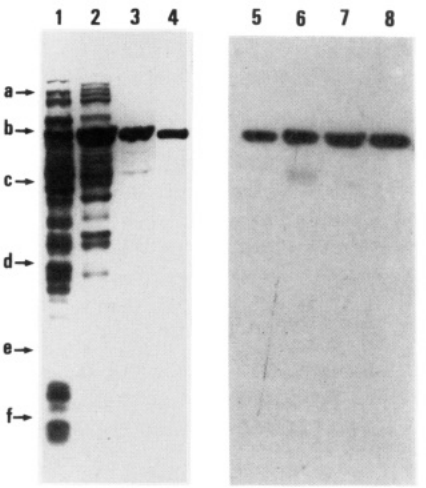

FIGURE 2: SDS–PAGE (lanes 1–4) and immunoblot analysis (lanes 5–8) of *B. anthracis* CYA 62 K346Q protein in different purification steps. Lanes 1 (50 μg of protein) and 5 (10 μg of protein), crude bacterial extract; lanes 2 (28 μg of protein) and 6 (4 μg of protein), after Blue–Sepharose chromatography; lanes 3 (3 μg of protein) and 7 (0.7 μg of protein), after chromatofocusing; lanes 4 (1.5 μg of protein) and 8 (0.6 μg of protein), after Ultragel AcA 44 chromatography. Arrows on the left side indicate the molecular mass markers: (a) phosphorylase *b* (94 000); (b) bovine serum albumin (67 000); (c) ovalbumin (43 000); (d) carbonic anhydrase (30 000); (e) soybean trypsin inhibitor (20 300); (f) lysozyme (14 400).

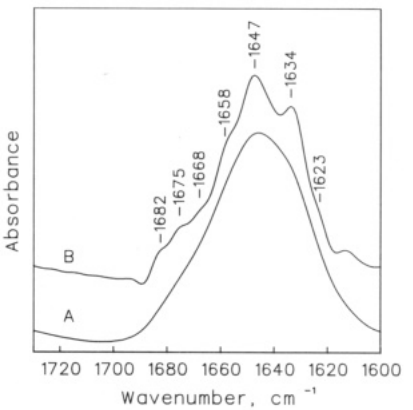

FIGURE 3: Infrared spectrum in the amide I region of CYA 62 protein (A) and the same spectrum (B) after band narrowing by Fourier self-deconvolution using a Lorentzian band shape of 15-cm^{-1} half-width and a resolution enhancement factor of 2.5 (Kauppinen et al., 1981).

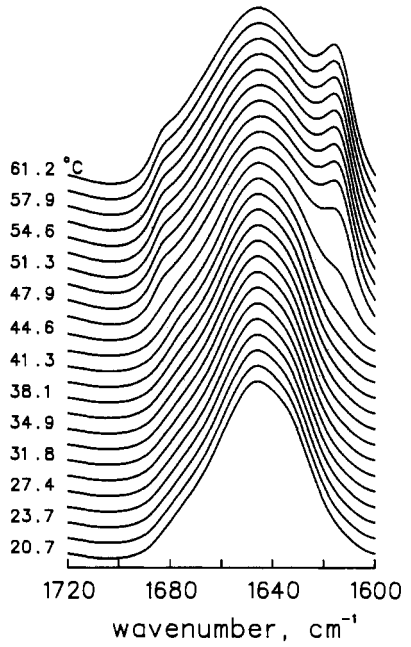

FIGURE 4: Temperature dependence of the infrared spectrum of CYA 62. Spectra were recorded during the heating cycle at a heating rate of approximately 10 °C/h.

adenylate cyclase of *B. anthracis* yields ~36% β-structures and ~12% α-helical structures. Thus, two independent spectroscopic techniques indicate that the secondary structure of CYA 62 consists of β-sheets and contains relatively little α-helix.

The amide I band contour in the infrared spectrum of the K346Q variant is virtually identical with that of the active protein CYA62. Furthermore, no significant differences between the two proteins could be detected in the positions or fractional areas of the component bands resolved by Fourier self-deconvolution and band-fitting analysis. Thus, within the resolution limit of this method, there are no detectable differences between the secondary structure of the active protein CYA 62 and that of the modified inactive protein CYA 62 K346Q.

Examination of the temperature dependence of infrared spectra in the amide I region shows that the band contour characteristic of a "native" secondary structure of adenylate cyclase does not change in the temperature range up to approximately 42 °C. A further increase in temperature leads to a broadening of the central portion of the amide I band contour, which is accompanied by the appearance of a strong, well-defined band at 1616 cm^{-1} and a weaker band at 1683 cm^{-1} (Figure 4). These spectral changes occur over a relatively narrow temperature range and reflect the cooperative unfolding of adenylate cyclase. The low-wavenumber band at 1616 cm^{-1}, characteristic of infrared spectra of many other thermally denatured proteins (Surewicz et al., 1990), is believed to represent a special kind of extended structures that are formed upon aggregation of the thermally unfolded species. The apparent midpoint denaturation temperature of CYA 62 obtained by using an empirical parameter, $I_{1616}/(I_{1616} + I_{1646})$ (where I_{1616} and I_{1646} are the intensities of the amide I band contour at 1616 and 1646 cm^{-1}, respectively), is at 45 °C (Figure 5). This value is very close to the unfolding temperature determined by CD and differential scanning calorimetry (unpublished observation). The midpoint denaturation temperature of the modified protein is shifted to 43 °C (Figure 5). This relatively small but fully reproducible shift indicates that the K346 → Q346 mutation results in slightly diminished

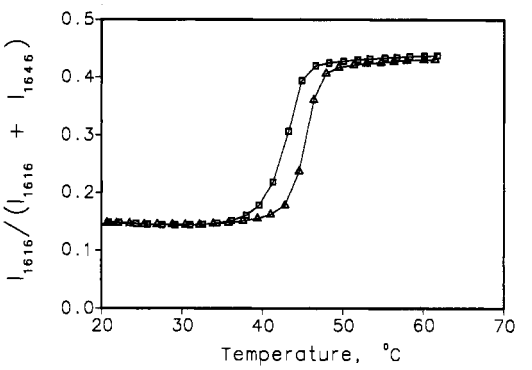

FIGURE 5: Temperature dependence of the band intensity ratio $I_{1616}/(I_{1616} + I_{1646})$ in the CYA 62 protein (△) and in the K346Q variant (□). I_{1616} and I_{1646} represent the peak height intensities of the amide I band contour at 1616 and 1646 cm^{-1}, respectively.

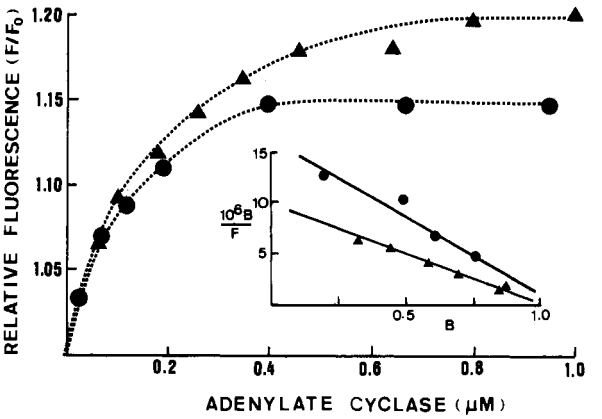

FIGURE 6: Binding of dansyl-CaM to CYA 62 (▲) and to its K346Q modified form (●). The experimental conditions are described under Experimental Procedures.

overall thermodynamic stability of adenylate cyclase.

Calmodulin-Binding Properties of CYA 62 K346Q. The K_d of CYA 62 for Ca^{2+}-complexed bovine brain CaM (23 nM as determined from the enzyme activation curve) is very close to that found for the wild-type protein secreted by *B. anthracis* in culture supernatants (Labruyère et al., 1990). As the modified CYA 62 K346Q protein is inactive, its affinity for bovine brain CaM was determined indirectly from competitive binding experiments (Erickson-Viitanen & DeGrado, 1987). The shift in the activation curve of CYA 62 by CaM in the presence of a constant concentration of catalytically inactive enzyme was used to measure the affinity of the latter for CaM. We obtained a K_d value of 20 nM for the K346Q mutant for CaM from three separate experiments (data not shown). In another set of experiments, a fluorescent derivative of CaM, dansyl-CaM, was used to monitor the interaction of cyclase with activator protein. CYA 62 was activated by dansyl-CaM and excess calcium ions at the same maximal level as the CaM; yet, the half-maximal activation was reached at 130 nM dansyl-CaM. The Scatchard plot analysis of the titration curve of dansyl-CaM with CYA 62 yielded a K_d value of 100 nM, a result in good agreement with that obtained from kinetic experiments (Figure 6). The modified K346Q enzyme enhanced the fluorescence of dansyl-CaM to a level that was 25% lower than that of CYA 62; nevertheless, the K_d (70 nM) was close to that found for CYA 62. Thus, two independent approaches show that binding of CaM to *B. anthracis* adenylate cyclase is not affected by substitution of Gln for Lys-346.

Nucleotide-Binding Properties of CYA 62 K346Q. Equilibrium dialysis experiments with radiolabeled 3′-dATP, a noncyclizable analogue of ATP acting as a competitive in-

Table I: Binding of Ant-dATP to CYA 62 and to Its Inactive Variant CYA 62 K346Q As Determined by Fluorescence Analysis[a]

enzyme (40 μM)	CaM (40 μM)	relative fluorescence (%)
	+	107
CYA 62	-	114
CYA 62	+	335
CYA 62 K346Q	-	108
CYA 62 K346	+	116

[a] The "basal" medium (final volume of 0.3 mL) contained 50 mM Tris-HCl (pH 8), 100 mM NaCl, 0.5 mM $CaCl_2$, and 10 μM Ant-dATP. The fluorescence at 420 nm (λ_{exc} = 330 nm) relative to the "basal" medium is expressed as percentage.

hibitor of CYA 62, showed that optimum binding of nucleotide to the bacterial enzyme requires the presence of both CaM and divalent ions. The apparent K_d of CYA 62 for 3′-dATP in the presence of excess Ca^{2+} and CaM was 30 μM (Labruyère et al., 1990). In the absence of Ca^{2+}–CaM, binding of 3′-dATP to the bacterial enzyme was almost undetectable. Ant-dATP, a fluorescent analogue of ATP acting also as a strong competitive inhibitor of CYA 62 (K_i = 9 μM), was used to monitor binding of nucleotide to the active site of the truncated *B. anthracis* adenylate cyclase and its K346Q inactive form. As shown in Table I, the fluorescence of Ant-dATP was only slightly increased by CaM or CYA 62 alone. When Ca^{2+}-complexed CaM and CYA 62 were simultaneously present in the test cuvette, the fluorescence of Ant-dATP increased more than 3 times. Excess ATP or 3′-dATP quenched the fluorescence of the Ant-dATP–cyclase–CaM complex, indicating that the interaction of the analogue with the catalytic site of CYA 62 is specific (Sarfati et al., 1990). Under identical experimental conditions, CYA 62 K346Q was ineffective in increasing the fluorescence yield of Ant-dATP. This could be explained by the fact that the modified protein has a very low affinity for the fluorescent derivative of ATP, irrespective of the presence or absence of CaM and divalent ions. Another possible explanation could be that a subtle conformational change occurs at the active site of CYA 62 as the result of the K346Q substitution, which results in a serious decrease in the quantum yield of enzyme-bound Ant-dATP. In order to address this question, the binding of Ant-dATP to adenylate cyclase was determined by equilibrium dialysis with a radioactive ligand. Ant-dATP interacted with CaM-complexed CYA 62 in a stoichiometric ratio close to 1:1 and with an apparent K_d of 8 μM. Binding of the ATP analogue to the CYA 62 K346Q protein was indeed strongly affected, and experimental points were too scattered for a precise determination of K_d. With the assumption of a binding stoichiometry identical with that of the active truncated adenylate cyclase, the K_d of the inactive K346Q form of CYA for Ant-dATP was estimated to be around 300 μM (data not shown). It may be thus concluded that the substitution of Lys-346 by Gln (and likely by other natural amino acids) affects primarily the binding of the nucleotide to the active site.

Discussion

Adenylate cyclase from *B. anthracis*, an 800 amino acid residue protein, is composed of at least three functionally distinct domains. One domain, probably the N-terminal, is required for the interaction of protein with PA and the subsequent internalization of adenylate cyclase into eukaryotic cells (Leppla, 1984; Cataldi et al., 1990). The rest of the molecule, endowed with ATP-cyclizing activity, is composed of two subdomains, one interacting with the nucleotide substrate and another interacting with CaM. Since the deletion of a large N-terminal fragment of 261 amino acid residues did not affect the binding of CaM to adenylate cyclase or its kinetic parameters, we may assume the PA-binding and the catalytic domains of adenylate cyclase act as independent entities. This particular feature of the protein offers obvious advantages for structure–function studies, since each individual domain may be independently analyzed with respect to its structural, biochemical, immunochemical, and biological properties.

Results obtained in this study enable us to put forward the hypothesis that the Lys-346 in *B. anthracis* adenylate cyclase is primarily required for the binding of the nucleotide substrate. Its substitution with Gln dramatically alters the capacity of the protein to interact with Ant-dATP, without affecting the secondary structure and the CaM-binding properties of the enzyme. As chemical modification and site-directed mutagenesis of similar Lys residues in other ATP-binding proteins (acting either as ATPase or kinases) were reported to result in a partial or total loss of catalytic activity and/or decrease in nucleotide binding properties, the present observations are not that unexpected. On the other hand, the analysis of individual enzymes cannot provide us with a single, easily interpretable picture regard to the role played by Lys-346 in *B. anthracis* adenylate cyclase. As shown in Figure 1, alignment of homologous "G-rich" sequences in six different ATP-utilizing enzymes—frequently referred to as "model" proteins—underlines the presence of a highly conserved Lys that corresponds to Lys-353 in *B. anthracis* adenylate cyclase. Furthermore, there is a general consensus in defining these Lys residues as being "essential" despite the fact that site-directed mutagenesis of an equivalent Lys in the enzymes as shown in Figure 1 affects the catalysis and ATP-binding properties in different ways. Notably, only two out of the six proteins (*B. anthracis* adenylate cyclase and *E. coli* adenylate kinase) exhibit a very drastic decrease of their activity upon site-directed mutagenesis of conserved Lys residues (Reinstein et al., 1988; Xia & Storm, 1990). In those few cases where nucleotide-binding experiments were available, the replacement of a "conserved" Lys with other amino acids decreased the affinity of the corresponding enzymes for ATP only by a factor of 2 or 3 (with a single exception, a K175I substitution in the α-subunit of *E. coli* ATPase) (Rao et al., 1988). In view of this, it is difficult to evaluate unequivocally the role of Lys-346 in *B. anthracis* adenylate cyclase, as it occupies a quite unique place in the consensus sequence. We are tempted to postulate that such a "tandem" arrangement of Lys-346 and Lys-353 in *B. anthracis* adenylate cyclase is required for a more "rigid" orientation of the polyphosphate group in ATP, as its cyclization imposes more demanding steric constraints on the molecule than hydrolysis or phosphate group transfer.

In view of the present data it would be of interest to establish the nucleotide-binding properties of the K346R modified form of *B. anthracis* adenylate cyclase, which has been shown to exhibit a measurable residual activity (Xia & Storm, 1990). An analysis of the modified protein K346R, similar to that performed here on CYA62 and CYA K346Q, should provide an answer to the critical question as to whether Lys-346 is also directly involved in catalysis or whether it is only essential for the "correct" orientation of the nucleotide substrate at the catalytic site of *B. anthracis* adenylate cyclase.

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Registry No. ATP, 56-65-5; Ant-dATP, 130996-25-7; L-Lys,

56-87-1; adenylate cyclase, 9012-42-4.

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