Functional Regions of the Inhibitory Subunit of Retinal Rod cGMP Phosphodiesterase Identified by Site-Specific Mutagenesis and Fluorescence Spectroscopy†

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ABSTRACT: In the dark, the activity of the cGMP phosphodiesterase (PDE) of retinal rod outer segments is held in check by its two inhibitory γ subunits. Following illumination, γ is rapidly removed from its inhibitory site by transducin, the G-protein of the visual system. In order to probe the functional roles of specific regions in the PDE_γ primary sequence, 10 variants of PDE_γ have been produced by site-specific mutagenesis and expression in bacteria and their properties compared to those of protein containing the wild-type bovine PDE_γ amino acid sequence. Three questions were asked about each mutant: What is its affinity for the $\alpha\beta$ catalytic subunit of PDE? Does it inhibit catalytic activity? If so, can transducin relieve this inhibition? Binding to $PDE_{\alpha\beta}$ was determined directly using fluorescein-labeled γ by measuring the increase in emission anisotropy that occurs when γ binds to $\alpha\beta$. Inhibition of $PDE_{\alpha\beta}$ was measured by reconstitution of the γ variants with γ-free PDE generated by limited digestion with trypsin or endoproteinase Arg-C. Unlike trypsin, the latter enzyme did not remove PDE's ability to bind membranes and be activated by transducin, so that transducin activation of PDE containing specific γ variants could be assayed directly. The results indicate that mutations in many regions of γ affect its binding to $\alpha\beta$. A mutant missing the last five carboxy-terminal residues (83–87) was totally lacking in inhibitory activity. However, it still bound to $PDE_{\alpha\beta}$ tightly, although with a 100-fold lower dissociation constant ($\sim$5 nM) than that of wild-type γ ($\sim$50 pM). Thus, when added in sufficient excess over endogenous γ, this mutant was capable of activating holo-PDE. A mutant in which lysines 41, 44, and 45 were replaced with glutamines bound to $PDE_{\alpha\beta}$ and inhibited catalytic activity with similar affinity to that of wild-type γ. However, inhibition by this mutant was poorly relieved by activated transducin; PDE reconstituted with this mutant was stimulated 10-fold less by 1.5 μM transducin than was PDE reconstituted with wild-type γ. These results suggest a critical role for Lys41, Lys44, and Lys45 in activation by transducin and for the carboxy terminus in $PDE_{\alpha\beta}$ inhibition, while identifying other residues that make relatively small but measurable contributions to binding of PDE_γ to $PDE_{\alpha\beta}$.

Visual excitation in vertebrates is mediated by a light-activated enzymatic cascade (Liebman et al., 1987; Chabre & Deterre, 1989; McNaughton, 1990; Stryer, 1991). In the dark, the retinal rod is held in a relatively depolarized state by a steady influx of sodium ions through a cation channel held open by cGMP (Fesenko et al., 1985; Yau & Baylor, 1989). The light response in the rod is initiated by the photoisomerization of the visual pigment, rhodopsin, and photoisomerized rhodopsin, R*, catalyzes the exchange of GTP for bound GDP in the rod outer segment G-protein, transducin. The α subunit of transducin with its bound GTP (T_α–GTP) in turn activates the cGMP-specific phosphodiesterase (PDE), which rapidly hydrolyzes the local supply of cGMP. The cGMP-gated channels close in response to the cGMP depletion, and the rod hyperpolarizes.

The retinal rod phosphodiesterase is a peripheral membrane protein composed of three polypeptides, α (99.2 kDa), β (98.3 kDa), and γ (9.7 kDa), in a stoichiometry of $\alpha\beta\gamma_2$ (Baehr et al., 1979; Ovchinnikov et al., 1986, 1987; Lipkin et al., 1990a; Deterre et al., 1988). Chemical cross-linking data indicate that each large catalytic subunit binds a single γ (Fung et al., 1990). The catalytic activity of PDE is reciprocally regulated by its two inhibitory γ subunits and activated transducin (Wensel & Stryer, 1986). PDE can also be activated by the proteolytic removal of its γ subunits (Miki et al., 1975; Hurley & Stryer, 1982).

Despite intensive investigation, the detailed mechanism by which activated transducin (specifically T_α–GTP) activates PDE is still unclear. Results from Wensel and Stryer indicate that PDE_γ is displaced from its high-affinity inhibitory site on $PDE_{\alpha\beta}$ during activation; it is rapidly replaced by fluorescein-labeled PDE_γ when bleached rod outer segments (ROS) are incubated with GTP. Dissociation of the endogenous PDE_γ subunit occurs with a $t_{1/2} > 500$ s in the absence of activated transducin but very rapidly in the presence of T_α–GTP[γS] (Wensel & Stryer, 1990). Coimmunoprecipitation experiments have revealed a GTP-specific interaction between T_α and PDE_γ in a one-to-one stoichiometry (Fung & Griswold-Prenner, 1989). Biochemical analysis has indicated that T_α–GTP[γS] specifically binds to the PDE_γ subunit to form a complex that can be chromatographically isolated (Deterre et al., 1986). Yamazaki and co-workers (1990) have isolated a stable T_α–PDE_γ complex from activated frog outer segments that remains associated even in the T_α–GDP form in the absence of $T_{\beta\gamma}$. These results support the idea that when T_α–GTP binds to the PDE holoenzyme, it specifically binds to PDE_γ and removes it from its inhibitory site, thereby activating PDE. However, the structural elements of PDE_γ involved in interactions either with the PDE catalytic subunits or with T_α–GTP have not been clearly identified. The primary goal

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of the present study was to identify those regions of PDE_γ that are important for these interactions.

The elucidation of the amino acid sequence of the 87-residue γ subunit of bovine PDE and cloning of its cDNA by Ovchinnikov et al. (1986) made it possible to use site-specific mutagenesis to study these critical interactions in visual transduction. A cursory examination of the primary sequence of PDE_γ reveals a number of intriguing patterns. γ has a striking charge distribution: the N-terminal half, especially residues 24–45, is filled with basic residues, almost to the exclusion of acidic residues. The C-terminal half is quite acidic, with the exclusion of basic residues after lysine 45, except for two histidines. The protein is very hydrophilic except for the C-terminal tail, which contains a cluster of hydrophobic residues. A previous report described the functional expression of PDE_γ in *Escherichia coli* as a fusion protein with a portion of the cII protein from phage λ (Brown & Stryer, 1989), using the expression system of Nagai and Thøgersen (1987). This work revealed that the N-terminus of the γ subunit is relatively unimportant to its regulatory role in visual transduction but that the C-terminus is required for its inhibitory function (Brown & Stryer, 1989). Here I report site-specific mutagenesis, coupled with biochemical and biophysical analysis of 10 mutants, that has identified regions important for binding to $\alpha\beta$, further defined a region of γ necessary for inhibition of PDE catalytic activity, and revealed an area necessary for relief of this inhibition by activated transducin.

Materials and Methods

E. coli strain AR68 was the gift of David Goldfarb (University of Rochester, Rochester, NY). A monoclonal antibody (IgG1, C2–75) specific for the N-terminus of the cII proteins from phage λ was the gift of Martin Zweig (National Cancer Institute–Frederick Cancer Research Facility) (Zweig et al., 1987). Rod outer segments (ROS), PDE, and transducin were purified as previously described (Wensel & Stryer, 1986), except that the transducin was further purified on a Superose 6 gel filtration column (Pharmacia) to remove residual PDE. Standard ROS buffer contained 20 mM MOPS, pH 7.4, 120 mM NaCl, 30 mM KCl, 2 mM $MgCl_2$, and 1 mM DTT. DNA manipulations were performed using standard methods (Maniatis et al., 1982) or per manufacturer's instructions.

Expression and Purification of Fusion Protein. The expression and purification of the cIIγ fusion protein was performed as previously described (Brown & Stryer, 1989) with the following modifications: first, expression of fusion protein was optimal when the culture media was innoculated with freshly transformed cells; cultures derived from glycerol stocks expressed the fusion protein poorly, if at all. Second, expression of the fusion protein was induced by transiently elevating the culture temperature from 30 to 42 °C. The temperature was maintained at 42 °C for 15 min and then reduced to 37 °C for 2 h. Holding the culture at 42 °C for an extended period of time substantially reduced the yield of fusion protein. Finally, the inclusion bodies were dissolved in buffer containing 6 M urea, 50 mM sodium HEPES, pH 7.5, 50 mM sodium chloride, 1 mM EDTA, 1 mM DTT, and 20 μg/mL of aprotinin, leupeptin, pepstatin, and PMSF. This extract was centrifuged and applied to the carboxymethyl-Sephadex column as rapidly as possible due to the susceptibility of the solubilized fusion protein to proteolysis in this crude mixture. Following purification, cIIγ was stable for at least 6 months frozen at −20 °C in aqueous buffers. Purification of mutant fusion proteins was done in a similar manner. Fractions

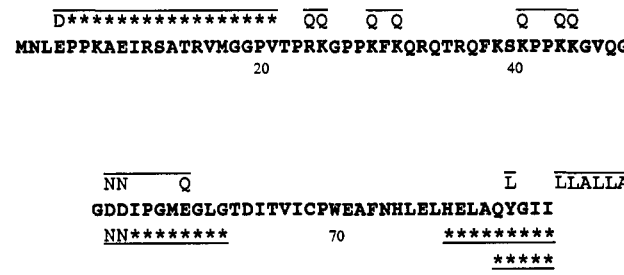

FIGURE 1: Mutants of the inhibitory subunit of the retinal cGMP phosphodiesterase. The sequence of wild-type PDE_γ is listed in bold using the one-letter amino acid code. The numbers below the sequence indicate amino acid position. Mutations are listed above or below the wild-type sequence, and a bar indicates changes in a single variant. An asterisk (*) represents a residue that has been deleted. Variants depicted from left-to-right and from top-to-bottom are Δ5–21, R24Q/K25Q, K29Q/K31Q, K41Q/K44Q/K45Q, D52N/D53N/E58Q, Y84L, α-tail, Δ54–61, T78, and T82.

containing the mutant were identified on a Western blot using the anti-cII monoclonal antibody. The integrity and purity of expressed subunits were verified by SDS–PAGE analysis, as shown previously for the wild-type cIIγ (Brown & Stryer, 1989).

Construction of Mutant Inhibitory Subunits. A previous report detailed the construction of a synthetic gene encoding the inhibitory γ subunit of the retinal rod cGMP phosphodiesterase and the construction of the pTZ19-derived vector, pTZ9-FXSG, containing this gene (Brown & Stryer, 1989). Figure 1 depicts the new mutants that are discussed in this paper. Most mutants were constructed by replacement of the appropriate restriction fragment in pTZ9-FXSG with a double-stranded oligonucleotide. Some mutant oligonucleotides were designed with a synonymous codon at one end, maintaining the amino acid coding sequence while altering the recognition site for the restriction enzyme that had been used to excise the wild-type fragment. Such a design allowed the ligation mix to be cut with this restriction enzyme to eliminate wild-type background. In this manner, the transformants were enriched in mutant colonies. Putative mutant colonies were selected and screened by restriction mapping of miniprep DNA. The mutant Δ54–61 was the result of a spontaneous mutation formed in *E. coli* during the construction of D52N/D53N/E58Q. All sequences were confirmed by dideoxy sequence analysis using Sequenase (United States Biochemicals). Additionally, Δ54–61 was confirmed by peptide sequence analysis of the C-terminal tryptic fragment of the expressed protein.

In the mutant Δ5–21, glutamate 4 has been changed to aspartate, and a peptide segment containing proline 5 through valine 21 has been deleted. Δ5–21 was constructed by digestion of pTZ9-FXSG with *Bst*EII, followed by limited treatment with mung bean nuclease (New England Biolabs), which degraded the single-stranded overhang. This was followed by digestion with *Xho*I and fill-in with the Klenow fragment of DNA polymerase. The blunt-ended linear vector was recircularized with T4 DNA ligase and transformed into *E. coli* HB101. Miniprep DNA from 24 colonies was screened for a shorter *Bam*HI–*Hin*dIII fragment. Putative mutants were then confirmed by dideoxy sequence analysis.

The α-tail mutant was constructed using the polymerase chain reaction. An oligonucleotide was synthesized coding for the last six amino acids of the wild-type gene and the amino acids LLALLA. This was followed by the termination codon, the *Hin*dIII restriction site, and four extra bases to assure efficient cleavage by the restriction enzyme. In conjunction with a 5′ oligonucleotide, this was used to form the α-tail

mutant gene by PCR using pTZ9-FXSG as a starting template. After amplification, the PCR mixture was extracted with phenol, ethanol precipitated, and restricted with *Xho*I and *Hin*dIII. The mutant DNA fragment was gel purified and cloned into *Xho*I–*Hin*dIII cut pTZ9-FXSG, and the mutation was confirmed by dideoxy sequence analysis.

After confirmation of the mutant sequences in pTZ9-FXSG-derived vectors, the *Bam*HI–*Hin*dIII fragment containing the factor Xa recognition site and the mutant γ gene was purified from a preparative agarose gel using the Gene Clean II system (Bio 101) and subcloned into pLcII-based vectors. *E. coli* strain MM294 cI^+ was used for stable maintenance and plasmid purification of the expression construct; *E. coli* strain AR68 was used for expression of the protein product.

Determination of PDE Activity. Trypsin-activated PDE (tPDE) was prepared by limited tryptic digestion of purified PDE. TPCK-treated trypsin was added to 1 μM PDE to a concentration of 50 μg/mL and incubated at 23 °C for 5 min. This treatment destroys γ, leaving a modified $\alpha\beta$ subunit that is catalytically active (Hurley & Stryer, 1982) but incapable of binding to membranes (Wensel & Stryer, 1986).

PDE activity was measured fluorimetrically by either of two methods using an SLM 8000C spectrofluorimeter. In one method, activity was determined by monitoring the decrease in fluorescence at 442 nm (using an interference filter) accompanying the hydrolysis of 2′-(*N*-methyl)anthraniloylguanosine 3′,5′-cyclic monophosphate (Molecular Probes, Pitchford, OR) as described previously (Johnson et al., 1987; Brown & Stryer, 1989). In the other method, PDE activity was measured by monitoring the proton release accompanying cGMP hydrolysis in a weakly buffered solution using the pH-sensitive fluorescent dye SNAFL-1. This dye was excited at 480 nm, and emission was measured from 510–550 nm using a Corion colored-glass 510-nm high-pass filter and a 550-nm cut-off filter. The buffer used for this assay (pH assay buffer) was 20 mM MOPS, pH 8.0, 150 mM KCl, 10 mM $MgCl_2$, and 1 mM DTT, containing 20 μM SNAFL-1. cGMP was added to this buffer to 1 mM, and the reaction was initiated by the addition of 1–5 nM PDE.

Inhibitory Activities of cIIγ and Mutants. Mutants were studied as cII fusion proteins because the wild-type fusion protein, cIIγ, was shown previously to be indistinguishable from the native PDE_γ subunit in its binding affinity for tPDE and its ability to inhibit both trypsin- and transducin-activated PDE (Brown & Stryer, 1989). The concentration of mutant subunit was estimated using a Bradford protein assay (Bio-Rad) standardized with a cIIγ stock whose concentration was determined by inhibitory activity. It was assumed that the catalytic activity of 5 nM tPDE (with two catalytic sites) was fully inhibited by 10 nM cIIγ [K_D < 100 pM (Brown & Stryer, 1989)]. tPDE concentration was estimated assuming a turnover number of 4000 cGMP s^{-1}. The inhibitory ability of a mutant subunit was determined by monitoring the decrease in hydrolytic activity of tPDE after the addition of a known amount.

Fluorescence Emission Anisotropy Measurements of On- and Off-Rate Constants for cIIγ and Mutant Subunits. Because I anticipated obtaining mutants that would bind to tPDE but not inhibit its catalytic activity, I designed an assay that measured binding affinity directly and did not depend on measurement of inhibitory activity. This assay, which employs steady-state fluorescence emission anisotropy, takes advantage of the change in molecular size when γ subunits complex with $PDE_{\alpha\beta}$ (Wensel & Stryer, 1990). As expected, the emission anisotropy of the fluorescein-labeled cIIγ (14 kDa) increased markedly as it complexed with tPDE (180 kDa). The on-rate for the binding of fluorescent cIIγ (cIIγ^F) to an excess of tPDE could be determined by monitoring the time course of anisotropy increase. Subsequently, the off-rate could be determined by monitoring the decrease in anisotropy following the addition of a 100-fold excess of unlabeled cIIγ as the cIIγ^F dissociated and was replaced by its unlabeled counterpart. This method, in addition to providing information on the kinetic constants, allows an accurate determination of the equilibrium dissociation constants at concentrations (e.g., nanomolar) well above the measured K_D values (tens of picomolar).

For fluorescence anisotropy measurements, the cIIγ subunit was specifically labeled on its sole sulfhydryl, cysteine 68, using the sulfhydryl-specific reagent iodoacetamidofluorescein (Molecular Probes, Pitchford, OR). It was shown previously that chemical modification at cysteine 68 does not measurably affect inhibition of PDE catalytic activity or relief of this inhibition by transducin (Wensel & Stryer, 1990). Wild-type or mutant cII fusion proteins (5–25 μM) were dialyzed against 50 mM sodium phosphate buffer, pH 7.2, overnight at 4 °C. Iodoacetamidofluorescein from a 20 mM stock in DMF was added under dim red light to 200 μM, and the reaction mixture was incubated in the dark at 23 °C for 1 h. The reaction was quenched by the addition of β-mercaptoethanol to 5 mM, any protein aggregates were removed by centrifugation, and the mixture was concentrated to ~1 mL in a Centricon 10 (Amicon). This mixture was then applied to a PD-10 desalting column (Pharmacia) equilibrated with sodium phosphate buffer. The cIIγ^F was eluted in the excluded volume (4–5 mL) and was well separated from unbound dye.

Fluorescence emission anisotropy was measured on an SLM 8000C spectrofluorimeter in a T-format. The solution to be measured was placed in an 4-mL acrylic cuvette which had been coated previously with BSA and was maintained at 23 °C. Excitation was from a xenon lamp passed through a monochromator set at 480 nm (16-nm slit width) and a Glans-Thompson polarizer. Orthogonal emission in the horizontal and vertical orientation was measured simultaneously using Corion 520-nm high-pass colored glass filters and Glans-Thompson polarizers. The *g* factor, which is a measure of the differing sensitivity of the instrument to vertical and horizontally polarized light, was set to 1.0 by adjusting the photomultiplier gain while the excitation light was polarized in the horizontal orientation.

For the measurement of on-rates, cIIγ^F was added to a final concentration of 5 nM in a 2-mL volume of ROS buffer with stirring at 23 °C. After a baseline was recorded, tPDE was added to a final concentration of 5 nM (Figure 2). Because binding occurs in less than 10 s at this concentration, data were collected at 250-ms intervals. For the measurement of off-rates, 500 nM of unlabeled cIIγ was added to the above solution, and the time course of anisotropy decrease was measured over a longer time scale (50 s for the Δ5–21 mutant to 3000 s for wild-type cIIγ^F) (Figure 3). Data collection increments were varied accordingly. Apparent dissociation constants were defined as k_{off}/k_{on}.

Reconstitution of Membrane-Bound PDE with Mutant cIIγ Subunits. An ROS suspension was digested with endoproteinase Arg-C (Boehringer Mannheim) at a concentration of 1:100 (Arg-C to rhodopsin; w/w) at room temperature. Membrane-bound PDE was separated from soluble PDE by pelleting the ROS membranes in a Beckman Airfuge (5 min, 30 psi). The supernatant and pellet were then assayed for PDE activity separately. Total PDE (active plus inactive) in each

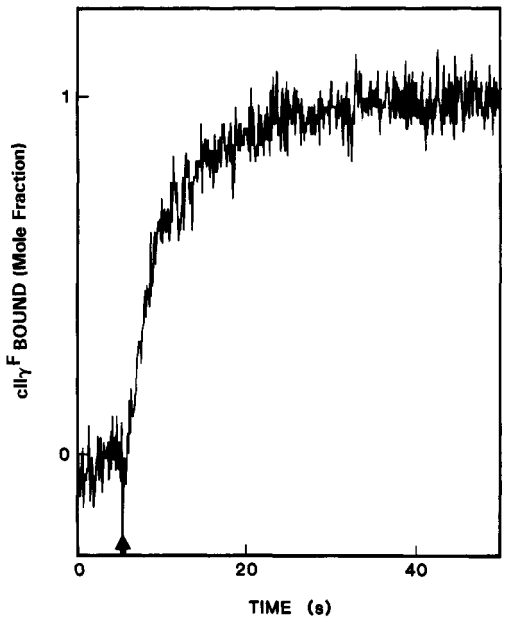

FIGURE 2: Time course of cIIγ binding to tPDE measured by fluorescence emission anisotropy. The cuvette initially contained 5 nM cIIγ^F; at the arrow, tPDE was added to 5 nM. The steady-state anisotropy value increased from 0.14 for the free cIIγ^F to 0.24 for the complex. All mutants showed a time course of binding similar to that of wild-type cIIγ shown here.

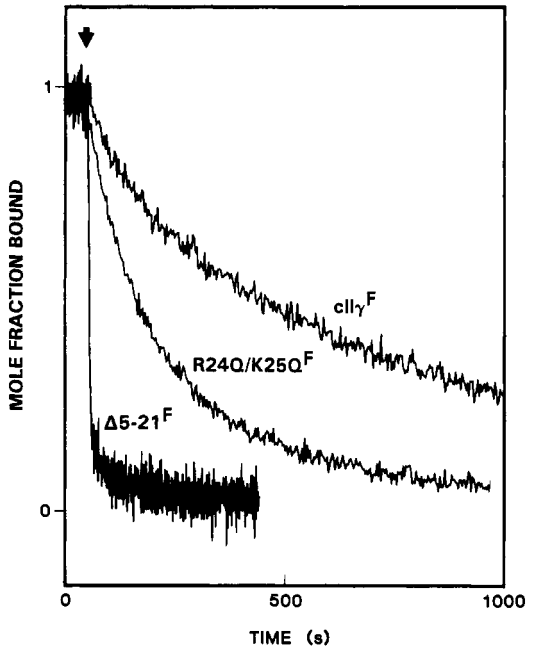

FIGURE 3: Time course of cIIγ dissociation from tPDE measured by fluorescence emission anisotropy. The cuvette initially contained 5 nM cIIγ^F, or labeled mutant, bound to 5 nM tPDE. At the arrow, unlabeled cIIγ was added to 0.5 μM. This figure displays results for cIIγ^F and two mutants, R24Q/K24Q^F and Δ5–21^F, which have τ_{off}'s of 830, 295, and 9 s, respectively.

fraction was determined by subsequent trypsin treatment.

After activation of greater than 95% of membrane-bound PDE by Arg-C, a 5-fold excess of wild-type or mutant cIIγ was added and allowed to incubate at room temperature for 5 min. The ROS were washed with ROS buffer three times by centrifugation at 30 psi for 5 min in a Beckman Airfuge to remove excess protease, inhibitor, and soluble PDE. The membranes were then resuspended in ROS buffer at about 200 μM R*.

To test the reconstituted PDE in a ROS suspension for transducin activation, it was used at a concentration of 2–5 nM (approximately 0.5 μM R*) in either assay system. PDE-free transducin was typically added to a concentration of 2 μM and activated by the subsequent addition of 5 μM GTP[γS]. Alternatively, purified T_α–GDP could be activated by the addition of aluminum chloride and sodium fluoride (Bigay et al., 1987). Maximal PDE activity was determined by in situ activation with 50 μg/mL trypsin.

Results

Assay of Binding Affinity by Fluorescence Emission Anisotropy. Previous work had shown that the fluorescence emission anisotropy of fluorescently labeled γ increased dramatically upon binding to tPDE (Wensel & Stryer, 1990). Free cIIγ^F had an anisotropy of 0.14; upon addition of tPDE, the anisotropy increased linearly with tPDE added until saturating at 0.23. The anistropy increase and decrease could be measured in a time-dependent manner. Addition of a saturating amount of tPDE (5 nM; 10 nM cIIγ binding sites) to 5 nM cIIγ^F resulted in a time-dependent increase in anisotropy with a $1/e$ time of approximately 5 s, indicating that the on-rate constant is greater than 2×10^7 M^{-1} s^{-1}. Addition of 10 nM tPDE did not speed the binding reaction, suggesting that the observed rate is limited by the finite mixing time in the cuvette (about 2–4 s). The off-rate was measured by observing the time course of the decrease in emission anisotropy following the addition of a 100-fold excess of unlabeled cIIγ as the cIIγ^F dissociated and was replaced by its unlabeled counterpart. The dissociation curve was well fit by a single exponential with a τ_{off} = 830 s. All values listed in Table I were representative of 3–4 determinations with a standard deviation of less than 5%. The dissociation constant defined by these values (50 pM) is consistent with that determined earlier for native PDE$_\gamma$ using alternative methods (Hurley & Stryer, 1982; Wensel & Stryer, 1986; Lipkin et al., 1990b).

Reconstitution of PDE with Mutant Inhibitory Subunits. To reproducibly reconstitute PDE with mutant subunits, some of which possessed decreased binding affinity compared to wild-type, it was necessary to proteolytically remove >98% of the endogenous PDE$_\gamma$ in a ROS suspension. Because only membrane-bound PDE is efficiently activated by transducin (Bennett & Clerc, 1989), trypsin was not suitable for this purpose because it caused the release of PDE from the membrane prior to degradation of the γ subunit (Wensel & Stryer, 1986). I therefore decided to use an arginine-specific protease, endoproteinase Arg-C, to activate PDE. Because this enzyme only cleaves after arginine residues, I hoped it would activate PDE prior to releasing the catalytic moiety from the membrane. In fact, as shown in Figure 4, membrane-bound PDE was fully activated by Arg-C while almost 50% of the total activity remained associated with the membrane. After this treatment, the activated PDE was readily reconstituted with wild-type or mutant cIIγ. Because the reconstituted PDE remained associated with the membranes, it was readily purified away from the protease and excess inhibitor, which were soluble, by three centrifugal wash steps.

PDE Reconstituted with Fusion Protein Is Efficiently Activated by Transducin. The reconstituted PDE, like native PDE, showed a low basal activity but was efficiently activated by transducin and GTP[γS] in the presence of bleached membranes. Because transducin was added to an almost 500-fold excess over PDE, it was verified for each transducin preparation that contaminating PDE was present at less than 1 part in 10000 by assaying cGMP hydrolysis following trypsinization. The extent of PDE activation was variable among transducin preparations; therefore, activation for each

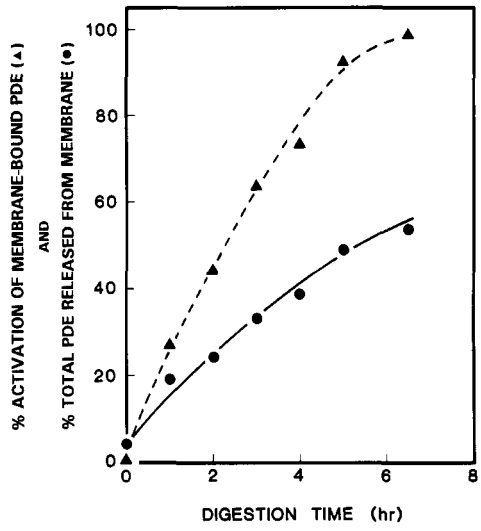

FIGURE 4: Activation of PDE by endoproteinase Arg-C occurs faster than release of PDE from the membrane. The reaction was initiated at time 0 by the addition of 100 μg of endoproteinase Arg-C to 1 mL of an ROS suspension containing 10 mg of bleached rhodopsin. The sample was incubated at 23 °C, and 100-μL aliquots were removed at the times indicated. Membrane-bound PDE was centrifugally separated from soluble PDE by spinning the ROS in a Beckman Airfuge. Both the supernatant and the pellet were assayed for PDE activity resulting from Arg-C treatment. Total PDE (active plus inactive) in each fraction was determined by trypsin treatment. Percent activation by Arg-C proteinase of PDE remaining on the membrane (▲) and percent of total PDE released from the membrane (●) are plotted as a function of incubation time.

mutant was compared to activation of PDE reconstituted with cIIγ, measured in parallel using the same transducin preparation. PDE reconstituted with all inhibitory mutants except K41Q/K44Q/K45Q was activated as well as or better than PDE reconstituted with wild-type cIIγ by 1.5–2.0 μM transducin.

PDE could also be activated by purified T_α–GDP with the addition of NaF and $AlCl_3$. These salts recombine in solution to form AlF_3, which mimics the γ-phosphoryl group of GTP and induces the active conformation of T_α when bound (Bigay et al., 1987).

Mutational Analysis of the Functional Regions of the Inhibitory γ Subunit of the Retinal Rod cGMP Phosphodiesterase. A number of mutations were designed to investigate the role of the charged regions of the PDE_γ subunit. Double and triple point mutations were made in which charged residues were replaced with polar but uncharged residues, such as glutamine or asparagine. A large deletion at the N-terminus (Δ5–21) was designed to test the functional role of the N-terminal region; this is the only region of the rod PDE_γ subunit that is not highly conserved in the PDE_γ subunit found in blue cones (Hamilton & Hurley, 1990). Finally, a series of mutations at the C-terminus were designed to further define this region's role in inhibition and to determine whether this effect was primarily due to a loss of binding affinity or to a loss of inhibitory ability even when bound to $PDE_{\alpha\beta}$.

Each of the cIIγ mutants characterized in this study fell into one of three broad classes on the basis of three criteria: binding affinity, inhibitory activity, and ability to functionally interact with transducin (summarized in Table I). Mutants in one class fully retained their ability to inhibit tPDE and to interact with transducin. However, as shown in Figure 3, they varied in their dissociation rates (τ_{off}) from the catalytic subunits from 9 s for Δ5–21, to 295 s for R24Q/K25Q, to 830 s for wild-type cIIγ. This class also included cIIγ variants with the mutations K29Q/K31Q, D52N/D53N/E58Q, and

Table I: Mutants of the PDE_γ Subunit and Their Functional Attributes[a]

	τ_{off} (s)	inhibits tPDE?	activated by T?
cIIγ	830	yes	yes
Δ5–21	9	yes	yes
R24Q/K25Q	295	yes	yes
K29Q/K31Q	285	yes	yes
D52N/D53N/E38Q	265	yes	yes
Y84L	530	yes	yes
α-tail		yes	
Δ54–61	8	no	
T78		no	
T82	9	no	
K41Q/K44Q/K45Q	820	yes	no

[a] τ_{off} values were measured by fluorescence emission anisotropy as described in the text. Inhibition was measured as dimunition of the catalytic activity of 5 nM tPDE. All inhibitory mutants with the exception of α-tail were effective at nearly stoichiometric concentrations. The α-tail mutant was effective only at concentrations approaching 1 μM. Noninhibitory mutants were defined as those mutants which showed no inhibition of tPDE catalytic activity up to 1 μM. Mutants which were functional inhibitors were tested for transducin activation using the reconstitution assay described in the text.

Y84L. The estimated dissociation constants ranged from 75 pM to 5 nM, and these affinity differences were primarily due to increased off-rates for the mutant subunits. The on-rate for each mutant was indistinguishable from that of wild-type cIIγ; however, this may be due in part to limited time resolution. The α-tail mutant, which had an additional six hydrophobic residues on the C-terminus, also inhibited tPDE but with a very low affinity. In fact, its affinity was only measurable by inhibitory activity. Anisotropy measurements using nanomolar concentrations were insensitive to the low binding level (<1% bound) of this mutant.

The second class of mutants included those which still bound tPDE with nanomolar affinity but had lost their inhibitory capability. This class consisted of the C-terminal truncation mutants T78 and T82 and the deletion mutant Δ54–61. These mutants bound competitively with wild-type cIIγ; in the presence of one of these mutants, a much higher concentration of wild-type inhibitor was required to completely suppress tPDE catalytic activity than was required with tPDE alone. As shown in Figure 5 for T82, these mutants actually activate holo-PDE when added in excess, presumably by displacement of the native inhibitory subunit.

Finally, a single mutant was found that exhibited wild-type character in its inhibitory and binding properties but was severely impaired in its ability to functionally interact with activated transducin to relieve PDE inhibition. This mutant was formed by replacing the last three basic residues, lysines 41, 44, and 45, with glutamines. When PDE reconstituted with this mutant was assayed for activation by T_α–GDP and aluminum fluoride (Figure 6), it was stimulated only to 4% of maximal under conditions where PDE reconstituted with cIIγ was activated to 24%. This result was obtained as well with $T_{\alpha\beta\gamma}$ and GTP[γS] as the activating species. Because the affinity of this mutant for tPDE as determined by anisotropy measurements was virtually identical to that of wild-type cIIγ, this impaired activation cannot be attributed to an increased binding affinity for PDE.

Discussion

The results obtained by coupling site-specific mutagenesis with biochemical and biophysical analysis make it possible to identify several functional regions on the PDE_γ subunit. The C-terminus of the γ subunit is critical for its inhibitory activity.

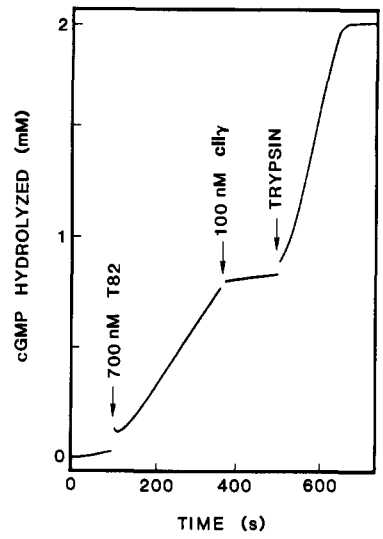

FIGURE 5: The C-terminal truncation mutant, T82, activated holo-PDE. PDE activity was monitored by SNAFL-1 which reports proton release due to cGMP hydrolysis. The cuvette initially contained 2 mM cGMP in pH assay buffer. At time zero, holo-PDE was added to a concentration of 5 nM. The addition of 700 nM T82 activated the PDE to approximately 20% of its full activity. The effect was reversed by the addition of 100 nM cIIγ, which returned the PDE to its initial activity level. Finally, the PDE was fully activated by the addition of trypsin to 50 μg/mL.

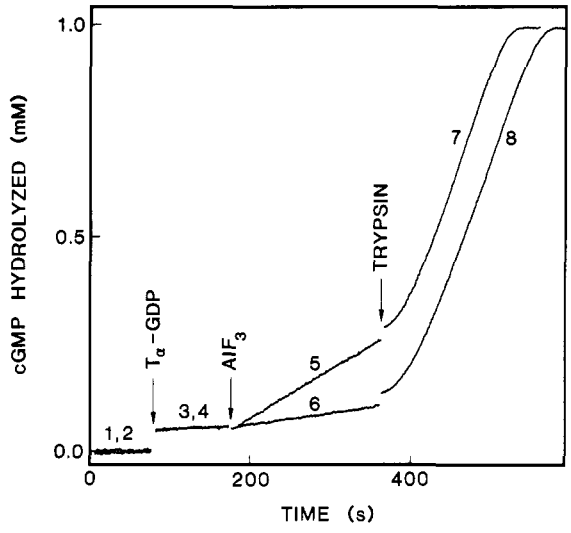

FIGURE 6: Activation by transducin of PDE reconstituted with the mutant K41Q/K44Q/K45Q was highly impaired. Shown here is a comparison of the activation by T_α–GDP–AlF_3 of PDE reconstituted with either cIIγ or the mutant K41Q/K44Q/K45Q. The arrows indicate the addition of 1.5 μM T_α–GDP, then of 10 mM NaF and 30 μM $AlCl_3$, and finally of 50 μg/mL trypsin to a cuvette originally containing 5 nM PDE. Trace segments 1, 3, 5, and 7 depict the activation of PDE reconstituted with wild-type cIIγ. The basal level was about 2% of the tryptic level and was stimulated to 24% following transducin activation. Trace segments 2, 4, 6, and 8 depict the degree of activation of PDE reconstituted with K41Q/K44Q/K45Q. Once again, the basal level was about 2% of the tryptic level; however, stimulation reached only 4% following transducin activation. The total PDE contained in each sample was nearly identical, as shown by final activation with trypsin.

I have previously identified a C-terminal truncation mutant, T74, missing amino acids 75–87 of the native subunit, that did not inhibit the catalytic activity of tPDE. In the present study, I have further localized a region necessary for inhibition to the last five amino acids. The C-terminal truncation mutant, T82, missing residues 83–87, did not inhibit catalytic activity. However, as shown by fluorescence emission anisotropy, T82 binds to the catalytic subunits with nanomolar affinity as compared with 50 pM for the wild-type subunit. This loss in binding affinity is not sufficient to account for a loss of inhibitory activity. In fact, as shown in Figure 5, T82 actually activated holo-PDE when added in excess, presumably by displacing the endogenous subunit. However, the activation appears too rapid to be strictly rate-limited by the dissociation of the endogenous inhibitor. One possible explanation for this puzzle is that T82 displaces the functional inhibitory region of endogenous γ before the entire subunit dissociates from PDE. These data strongly suggest that the truncation mutants have a tertiary structure similar to the native subunit and bind to the same site on the catalytic subunits. The deletion mutant, Δ54–61, exhibited similar characteristics to the C-terminal truncation mutants described above. This result suggests that a second region of the PDE_γ subunit may be required for inhibition of catalytic activity. A second possibility is that there are spatially distinct binding sites on $PDE_{\alpha\beta}$ for the C-terminal region of PDE_γ and a region prior to Ile54, and that a certain length of peptide chain is required to span this distance allowing the C-terminus to inhibit catalytic activity.

Peptide studies reported by Cunnick et al. (1990) also implicated the C-terminal region of PDE_γ as the region of the peptide primarily responsible for inhibition of PDE catalytic activity. When peptides derived from different regions of PDE_γ were tested for their ability to inhibit catalytic activity, the peptide derived from residues 80–87 was the most effective. However, even this most potent peptide was only effective at millimolar concentrations.

Amino acid residues from many regions of the γ subunit contribute to the high binding affinity for the catalytic subunits either through direct contact or by stabilization of a high-affinity peptide conformation. The mutations Δ5–21, R24Q/K25Q, K29Q/K31Q, D52N/D53N/E58Q, T82, and Y84L,scattered widely throughout the subunit, lead to small losses in binding affinity. The α-tail mutant, which had an additional six hydrophobic residues on the C-terminus, also inhibited PDE activity, but with a very low affinity. This may indicate that there is a tight fit of the C-terminus into its inhibitory site on $PDE_{\alpha\beta}$. At room temperature, the standard free energy of binding, $\Delta G°$, for the association of γ with the catalytic subunits can be calculated to be approximately −14.8 kcal/mol [$\Delta G° = -RT \ln (1/K_D)$]. A number of double and triple point mutations scattered throughout the subunit lead to losses in binding energy of about 0.7 kcal/mol. Even the relatively major deletions of 17 amino acids at the N-terminus or five amino acids at the C-terminus lead to the loss of only 2.7 kcal/mol. Perhaps it is not surprising that contacts are dispersed throughout the molecule; crystal structures of other high-affinity protein–protein interaction sites have revealed extensive contacts. For example, the complex formed between trypsin and soybean trypsin inhibitor has a dissociation constant of 10^{-11} M. In addition to arginine 63 of trypsin inhibitor that binds to the active site aspartic acid, there is a network of hydrogen bonds and hydrophobic interactions formed between the "binding loop" of the inhibitor and the "binding pocket" on trypsin (Sweet et al., 1974). Perhaps widespread surface contact will prove to be common in high-affinity protein–protein interactions; high-affinity binding may be due to the summation of a large number of relatively weak interactions.

In this study, I have also identified amino acid residues in PDE_γ involved in the interactions with transducin that relieve PDE inhibition. When PDE reconstituted with the mutant K41Q/K44Q/K45Q was assayed for activation by transducin,

it was stimulated 10-fold less than was PDE reconstituted with cIIγ. This result suggests that these residues either are the site of interaction with transducin or are involved in an allosteric switch mechanism which facilitates the release of PDE_γ from its inhibitory site on the catalytic moiety of PDE. Biochemical and immunological data have implicated the basic region of PDE_γ (residues 24–45) in both binding to $PDE_{\alpha\beta}$ and in PDE activation by transducin (Lipkin et al., 1988). Further evidence for interaction of this region with T_α has been provided by Morrison et al. (1989), who showed that a peptide derived from PDE_γ residues 31–45 inhibited GTP uptake by transducin. Finally, it has been known since the mid-1970's that PDE can be activated by polycations such as protamine and polylysine, presumably by the disruption of electrostatic contacts (Miki et al., 1975). It is possible that the interaction between transducin and the basic region of PDE_γ may weaken or disrupt similar electrostatic interactions and dislodge the γ subunit.

Over the past five years, Lipkin et al. have accumulated a wealth of biochemical data identifying functional regions of PDE_γ. In 1988 they reported that limited carboxypeptidase treatment removing seven residues from the C-terminus resulted in a loss of inhibitory activity. More recently, data from the in vitro expression of a C-terminal truncation mutant (Δ81–87) suggested that the C-terminus was necessary for the inhibitory activity of γ. Lipkin et al. (1990b) have also expressed point mutations in the basic region of PDE_γ and, like the present study, reported that most of these mutants inhibited PDE catalytic activity with lowered affinity. However, there is one significant difference with the present study: their mutant R24G possessed virtually no inhibitory ability and only minimal binding affinity. In contrast, a mutation of a similar region in my study, R24Q/K25Q, inhibited tPDE fully with only slightly lowered affinity. This difference might be explained if the greater rotational freedom provided by glycine at position 24 destabilized the inhibitory conformation of the protein. Finally, the studies of Lipkin et al. (1990b) implicated the basic region in PDE_γ–T_α interactions; in their study they measured binding of PDE_γ to immobilized T_α–GTP[γS] on an ELISA plate. This binding was diminished by mutations at arginine 24, lysine 28, and arginine 33; however, binding was minimally affected by single point mutations at lysines 41, 44, and 45. In my study, PDE reconstituted with the triple point mutant K41Q/K44Q/K45Q was poorly activated by transducin. The reason for this apparent discrepancy is unclear; however, activation of PDE on ROS membranes may be a quite different process than the binding of immobilized transducin to free γ. It would be informative to determine if K41Q/K44Q/K45Q binds to transducin using the method of Lipkin et al.

Mounting evidence supports the idea that the C-terminal region of γ is the functional inhibitor of PDE catalytic activity; the N-terminal half of the peptide appears to anchor it in place on the catalytic α and β subunits. One site of interaction with activated transducin is near the end of the basic region of PDE_γ (residues 41–45). Binding of transducin to this site on PDE_γ may disrupt its interaction with the catalytic subunits, displacing it from its inhibitory site and causing activation of PDE. A challenge for the future will be to determine the structural basis of these interactions using techniques like nuclear magnetic resonance and X-ray crystallography.

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REFERENCES

Baehr, W., Devlin, M., & Applebury, M. (1979) *J. Biol. Chem. 254*, 11669–11677.

Bennett, N., & Clerc, A. (1989) *Biochemistry 28*, 7418–7424.

Bigay, J., Deterre, P., Pfister, C., & Chabre, M. (1987) *EMBO J. 6*, 2907–2913.

Brown, R. L., & Stryer, L. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4922–4926.

Chabre, M., & Deterre, P. (1989) *Eur. J. Biochem. 179*, 255–266.

Cunnick, J. M., Hurt, D., Oppert, B. Sakamoto, K., & Takemoto, D. J. (1990) *Biochem. J. 271*, 721–727.

Deterre, P., Bigay, J., Robert, M., Pfister, C., Kuhn, H., & Chabre, M. (1986) *Proteins: Struct., Funct., Genet. 1*, 188.

Deterre, P., Bigay, J. Forquet, F., Robert, M., & Chabre, M. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2424.

Fesenko, E., Kolesnikov, S. & Lyubarsky, A. (1985) *Nature 313*, 310–313.

Fung, B. K.-K., & Griswold-Prenner, I. (1989) *Biochemistry 28*, 3133–3137.

Fung, B. K.-K., Young, J. H., Yamane, H. K., & Griswold-Prenner, I. (1990) *Biochemistry 29*, 2657–2664.

Hamilton, S., & Hurley, J. (1990) *J. Biol. Chem. 265*, 11259–11264.

Hurley, J., & Stryer, L. (1982) *J. Biol. Chem. 257*, 11094–11099.

Johnson, J., Walters, J., & Mills, J. (1987) *Anal. Biochem. 162*, 291–295.

Liebman, P. A., Parker, K. R., & Dratz, E. A. (1987) *Annu. Rev. Physiol. 49*, 765–791.

Lipkin, V., Dumler, I. L., Muradov, K. G., Artemyev, N. O., & Etingof, R. N. (1988) *FEBS Lett. 234*, 287.

Lipkin, V. M., Khramtsov, N. V., Vasilevskaya, I. A., Muradov, K. G., Gubanov, V. V., Li, T., Johnston, J. P., Volpp, K. J., & Applebury, M. L. (1990a) *J. Biol. Chem. 265*, 12955–12959.

Lipkin V., Udovichenko, I. P., Bondarenko, V. A., Yurovskaya, A. A., Tolnykh, E. V., & Skiba, N. P. (1990b) *Biomed. Sci. (London) 1*, 305–308.

Maniatis, T., Fritsch, E., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Lab, Cold Spring Harbor, NY.

McNaughton, P. A. (1990) *Physiol. Rev. 70*, 847–883.

Miki, N., Baraban, J. M., Keirns, J. J., Boyce, J. J., & Bitensky, M. W. (1975) *J. Biol. Chem. 250*, 6320–6327.

Morrison, D. F., Cunnick, J. M., Oppert, B., & Takemoto, D. J. (1989) *J. Biol. Chem. 264*, 11671–11681.

Nagai, K., & Thøgersen, H. C. (1987) *Methods Enzymol. 153*, 461–481.

Ovchinnikov, Y. A., Lipkin, V. M., Kumarev, V. P., Gubanov, V. V., Khramtsov, N. V., Akhmedov, N. B., Zagranichny, V. E., & Muradov, K. G. (1986) *FEBS Lett. 204*, 288–292.

Ovchinnikov, Y. A., Gubanov, V. V., Khramtsov, N. V., Ischenko, K. A., Zagranichny, V. E., Muradov, K. G., Shuvaeva, T. M., & Lipkin, V. M. (1987) *FEBS Lett. 223*, 169–173.

Stryer, L. (1991) *J. Biol. Chem. 266*, 10711–10714.
Sweet, R. M., Wright, H. T., Janin, J., Chothia, C. H., & Blow, D. M. (1974) *Biochemistry 13*, 4212–4228.
Wensel, T., & Stryer, L. (1986) *Proteins: Struct., Funct., Genet. 1*, 90–99.
Wensel, T., & Stryer, L. (1990) *Biochemistry 29*, 2155–2161.
Yamazaki, A., Hayashi, F., Tatsumi, M., Bitensky, M. W., & George, J. S. (1990) *J. Biol. Chem. 265*, 11539–11548.
Yau, K.-W., & Baylor, D. (1989) *Annu. Rev. Neurosci. 12*, 289.
Zweig, M., Showalter, S., DuBois, G. C., Sisk, W. P., & Court, D. L. (1987) *Gene 55*, 47–53.

Interactions between the *trp* Repressor and Its Operator Sequence As Studied by Base Analogue Substitution†

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ABSTRACT: A series of modified *trp* operator sequences has been prepared by the incorporation of seven different base analogues. Four of the analogues allow the site-specific deletion of functional groups present on the dA-dT and dT-dA base pairs at positions −4/+4 and −5/+5 in the *trp* operator. The remaining three analogues permit the incorporation of structural analogues of the native dA-dT or dG-dC base pairs. The duplex operator sequences all exhibit T_m values well above ambient temperature (48–70 °C), and these values generally correlate very well with the number of interstrand hydrogen bonds present. The affinity between the *trp* repressor and 14 modified operator sequences was examined using a recently developed alkaline phosphatase protection assay. The results from the analogue sequences used in this study suggest that the structure of the dA-dT or dT-dA base pairs at positions −4/+4 and −5/+5, respectively, has relatively little effect upon the solution binding by the *trp* repressor, but the protein is very sensitive to the orientation of the amino and carbonyl functional groups at the −4/+4 positions, which are involved in the formation of an interbase hydrogen bond present in the major groove. (The term structure in this case refers to the hydrogen bonding structure of the base pairs. We recognize that the introduction of conservative functional group deletions or reversals may affect other structural criteria such as hydration.) The deletion of individual functional groups from the operator sequence suggests that the carbonyl at dT_{+4} is critical for formation of the high-affinity sequence-specific complex. Additionally, the thymine methyl group at dT_{+4} and the N7 nitrogen of dA_{+5} appear to be critical contacts necessary for high-affinity binding by the repressor. The thymine carbonyl and the adenine N7 nitrogen are each responsible for approximately −1.5 kcal/mol of apparent free energy of binding. The thymine methyl provides a somewhat smaller contribution of −0.7 kcal/mol. Deletion of either of the adenine amino groups at dA_{-4} or dA_{+5} results in a sequence that binds to the repressor with a higher affinity than observed with the native sequence; this can be explained in that the functional groups lost are not critical for binding, and the resulting increased flexibility of the operator, or the creation of a more hydrophobic surface at these sites, enhances van der Waals contacts between the protein and the nucleic acid.

The formation of high-affinity sequence-specific recognition complexes between proteins and nucleic acids can be explained most easily in terms of multidentate ionic interactions, hydrogen bonding interactions, and van der Waals contacts between the amino acid side chains of the protein and the base, carbohydrate, and phosphate residues of the nucleic acid (Seeman et al., 1976; von Hippel & Berg, 1986). While some protein–DNA contacts contribute to the overall binding affinity between the two macromolecules, it is the direct or indirect readout of the bases which allows the protein to discriminate correct from incorrect base sequences. With direct sequence readout, the protein appears to contact specific functional groups present on the base residues, and a target sequence will ideally contain a unique set of functional groups available for such interactions. Binding at the correct or incorrect sequence will depend both on the binding energy realized from the formation of specific interactions and on the potential binding energy lost as the result of steric interactions that preclude the formation of such bonds. Reports on the structure of a number of sequence-specific protein–DNA complexes, including bacterial phage repressor proteins (Anderson et al., 1987; Aggarwal et al., 1988; Jordan & Pabo, 1988; Wolberger et al., 1988) and the *Eco*RI restriction endonuclease (McClarin et al., 1986; Kim et al., 1990; Rosenberg, 1991), using X-ray diffraction methods have generally confirmed the existence of the direct readout mechanism. Indirect readout of base sequences has been suggested as an alternative mechanism by which proteins can form high-affinity complexes (Otwinowski et al., 1988). In this case, the functional groups present on the base residues will contribute to the overall geometry of the recognition site and may be important in allowing the intimate association of the protein with the nucleic acid, but most of the direct contacts occur between the amino acid side chains of the protein and the phosphodiester residues of the DNA.

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