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Circular Dichroism of the 1300-nm Band of Oxidized Reaction Centers from *Rhodopseudomonas viridis*†

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ABSTRACT: Reaction centers were prepared from the purple nonsulfur bacterium *Rhodopseudomonas viridis*, which contains bacteriochlorophyll *b*. Absorption and circular dichroism (CD) spectra were recorded from 900 to 1360 nm for reaction centers in three states: (I) untreated (cytochromes and P960 reduced), (II) ferricyanide partially oxidized plus light (cytochromes oxidized and P960 80–90% oxidized), and (III) ferricyanide oxidized in the dark (cytochromes oxidized and P960 80–90% oxidized). The absorption and circular dichroism bands of oxidized P960 at ca. 1300 nm were the same in position, shape, and sign (+) for both the chemically oxidized and photochemically oxidized samples. The shape of the 1300-nm CD band is similar to that of the absorption spectrum; there is no indication of "exciton splitting". The relatively high anisotropy ratio of ca. 10^{-3} should be useful in assigning the electronic states responsible for this transition.

When the primary electron donor of a bacterial reaction center is oxidized by the loss of a single electron, a new absorption band appears at 1160 (green sulfur bacteria), 1240–1270 [purple bacteria containing bacteriochlorophyll *a* (BChl *a*)], or 1300 nm (purple bacteria containing BChl *b*) (Olson & Thornber, 1979). Presumably, this new band is associated specifically with the cation radical of the two BChl *a* or *b* molecules forming the primary donor in bacterial reaction centers, i.e., the "special pair" (Fajer et al., 1975; Wasielewski et al., 1982).

In a recent X-ray diffraction study of crystallized reaction centers from *Rhodopseudomonas viridis* Deisenhofer et al. (1984) have shown that the two BChl *b* molecules forming the primary donor form a closely associated, noncovalently linked dimer in which the ring planes are 15° apart. They interact most closely with their pyrrole rings, which are stacked on top of each other about 3 Å apart. The acetyl group of ring I of one BChl *b* is in direct contact with the Mg atom of the other BChl *b*, and the distance between Mg atoms is about 7 Å.

With the exact configuration of the reaction-center primary donor established for *Rps. viridis*, CD may play a critical role in evaluating models of the ground- and excited-state electronic structures. This is particularly important in photosynthetic systems where electronic properties are intimately linked to biological function. Given the atomic structure of a reaction center from X-ray crystallography and the well-characterized electronic states of the chlorophyll molecules contained therein, models of the electronic states of the centers can be constructed. A critical test of such models is comparison of the CD spectra they predict with those observed experimentally. The 1300-nm (or equivalent) band is of special importance in such comparisons because it is associated specifically with that portion of the reaction center that is oxidized in the process of charge separation. Also, the 1300-nm band is the only band in the visible and near-infrared spectrum that is completely free of overlaps from bands of other reaction-center components.

Materials and Methods

Rps. viridis was grown photoheterotrophically as described by Eimhjellen et al. (1963).

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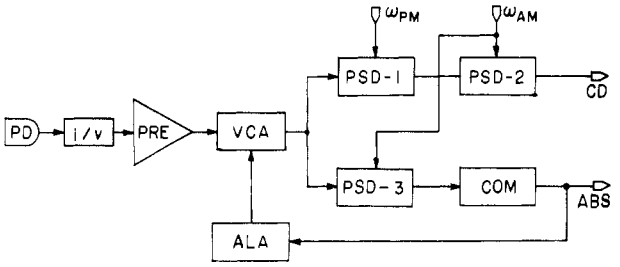

FIGURE 1: Schematic diagram of the analog electronic circuit that is used to measure CD and absorption (ABS). Components: PD, photodiode; i/v, current to voltage converter; PRE, preamplifier; VCA, voltage controlled attenuator; ALA, antilog amplifier; PSD, phase-sensitive detectors; ω_{AM} and ω_{PM}, reference signals from the amplitude and phase modulators, respectively; COM, comparator. The VCA and ALA were constructed from integrated circuits from Analog Devices (Norwood, MA), Models AD534LO and 759N, respectively.

Reaction centers were prepared with lauryldimethylamine oxide according to the method of Thornber et al. (1981). In the final step of chromatography on DEAE-cellulose, the reaction centers were eluted with D_2O buffer plus 0.1% Nonidet P-40 and 150 mM NaCl. Samples were quick-frozen in liquid nitrogen and stored 5–6 weeks at −70 °C before use.

After thawing, 10 mL of sample was concentrated to 4 mL with an Amicon Diaflo ultrafilter XM50 and then dialyzed against 40 mL of D_2O buffer to give a final A_{960} of 1.7, corresponding to [P960] = 17 μM (Thornber & Thornber, 1980). To oxidize the cytochromes in an 0.8-mL sample without oxidizing the P960, one to three crystals of ferricyanide were added. With the cytochromes chemically oxidized in the dark, it was possible to photooxidize the P960 about 80–90% with a blue-green light intensity of about 40 mW cm^{-2} provided by a 500-W Leitz Prado projector with a 50-mm projection lens and the following filters: Corning 1-75 (heat), Corning 4-76 (blue-green), and 2 cm of H_2O.

Absorption spectra were recorded on a Cary 14R or Cary 17 spectrophotometer (Varian Associates, Palo Alto, CA) or on the CD instrument described below. Light-induced cytochrome reactions were followed at 558 nm and light-induced P960 reactions at 603, 960–970, and 1310 nm. Absorption spectra recorded on the Cary 14R and Cary 17 spectrophotometers were digitized with the aid of a Tektronix 4662 plotter at wavelength increments of 5 nm to facilitate comparison with the CD and absorption spectra that were recorded directly in digital form in the CD spectrometer.

Infrared CD spectra were measured with a laboratory-built spectrometer that receives light from port U9B at the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory. A 2-mm diameter germanium photodiode cooled to 80 K was used as the detector. A tuning fork chopper (Bulova Watch Co., Woodside, NY, Model L2C-233) was mounted at the entrance slit of the monochromator to amplitude modulate (i.e., "chop") the light beam at 200 Hz. As in UV CD (Sutherland et al., 1980), a Rochon polarizer is placed just outside the exit slit of the monochromator and a photoelastic modulator is positioned between the toroidal focusing mirror and the sample.

The system for processing of the analog signal from the Ge photodiode is shown schematically in Figure 1. This arrangement is a modification of the design of Nafie et al. (1976). Our design permits simultaneous measurement of CD and absorption similar to the design described by Sutherland et al. (1982), using a photomultiplier. The output labeled ABS in Figure 1 is more properly called pseudoabsorbance. A true absorbance reading is obtained by taking the difference between two such readings and correcting for fluctuations in source intensity. For example, an absorption spectrum is obtained by subtracting the source-corrected pseudoabsorption of a "blank" from the corresponding spectrum of a "sample". Thus, we obtain absorption spectra of labile samples at the same time that we measure the CD. These spectra are compared with those recorded in a standard spectrophotometer (vide supra) during sample preparation. The pseudoabsorbance signal was also used to measure the absorbance changes induced by cross illumination with actinic light. The light-induced absorption difference is just the difference between the pseudoabsorption readings in the presence and absence of cross illumination. Scattered actinic radiation reaching the photodiode (PD) has no direct effect on the absorbance signal since the phase-sensitive detector PSD-3 responds only to light modulated at the frequency and phase of the amplitude modulator (ω_{AM}). The CD is also insensitive to scattered actinic light; the CD circuit detects only a signal that is the product of the 50-kHz photoelastic phase modulator and the 200-Hz amplitude modulator.

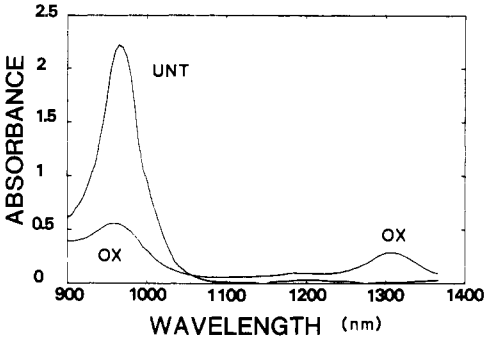

FIGURE 2: Absorption spectra of reaction centers in D_2O buffer before (UNT) and after (OX) the addition of $K_3Fe(CN)_6$ (2–5 mg/mL) sufficient to oxidize about 80% of the P960. The absorptivity of $P960^+$ at 1300 nm is estimated to be about 16 mM^{-1} cm^{-1} from the decrease in absorption at 960 nm and the known molar extinction of P960 at that wavelength (Thornber & Thornber, 1980).

RESULTS

The near-infrared absorption spectra of untreated and ferricyanide-oxidized reaction centers are shown in Figure 2. Progressive oxidation results in a decrease in the band at 960 nm and a concomitant increase in the band at about 1300 nm. (The exact maximum of this band is between 1300 and 1310 nm.) Attempts to titrate to complete disappearance of the 960-nm band always resulted in precipitation of the reaction centers. Thus, in all of our studies, 10–20% of the P960, as judged by absorption, was in the reduced state. The total oscillator strength of the 1300-nm band of the oxidized species was only about one-fifth of that lost at 960 nm.

The near-infrared CD spectra of untreated and ferricyanide-treated reaction centers are shown in the top panel of Figure 3. The preparations were the same as those used in Figure 2. The oxidized-minus-untreated absorption difference spectrum measured in the CD spectrometer, shown in the bottom panel of Figure 3, shows an absorption difference at 1300 nm, only slightly less than that recorded in the conventional spectrometer. This result reflected minor deterioration of the sample as a result of light exposure and the handling necessary to transport it to the NSLS. The large positive CD peak at 960 nm, off scale for the untreated sample, was similar to the CD peak reported previously (Philipson & Sauer, 1973). The residual CD peak at 960 nm supported the conclusion that the residual absorption at that wavelength was indeed reduced P960. The oxidized-minus-reduced CD spectrum, shown as the center panel in Figure 3, has a shape

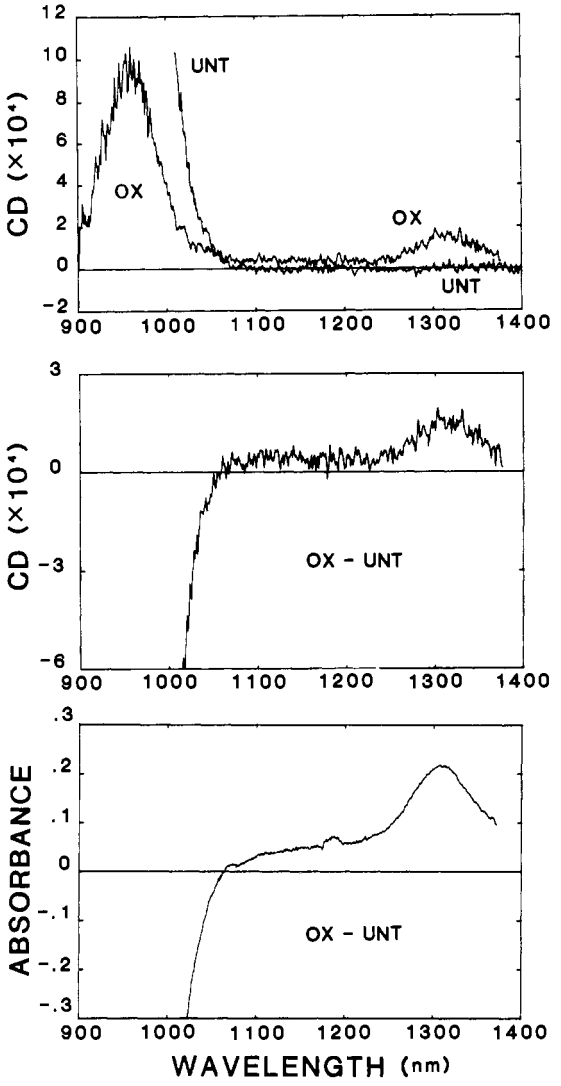

FIGURE 3: CD spectra of untreated (UNT) and chemically oxidized (OX) reaction centers (top) and the oxidized-minus-untreated CD difference spectrum (center) and absorption difference spectrum (bottom). The CD spectrum of the oxidized sample is noisier for wavelengths less than 1000 nm because of reduced sensitivity of the Ge photodiode.

similar to that of the absorption difference spectrum; there is no indication of derivative-like components in the CD spectrum, frequently referred to as "exciton splittings", but the amplitude of the CD is much greater than that of reduced BChl *a* and BChl *b* monomers (see Table I).

As shown in Figure 4, photooxidation of the reaction centers gives CD and absorbance changes similar to those produced by ferricyanide oxidation. The concentration of reaction centers was the same as for the samples shown in Figures 2 and 3. The magnitude of the absorption difference (bottom panel, Figure 4) was slightly greater than that achieved chemically (bottom panel, Figure 3). The magnitude of the CD difference (center panel, Figure 4) is also slightly greater than that observed for chemical oxidation (center panel, Figure 3), but the ratio of CD difference to absorption difference is similar for chemical oxidation and photooxidation. This supports the conclusion that the chemically oxidized sample had degraded somewhat between the data recorded in Figure 2 and the data recorded in Figure 3.

Sustained exposure to actinic light resulted in a gradual decrease in the magnitude of both absorbance and CD at 1300 nm. To minimize the exposure time during actinic radiation, we recorded the CD spectrum in a shorter time when the actinic radiation was on by reducing the period of digital integration of the CD at each wavelength. This resulted in noisier spectra, as can be seen by comparing the top panels of Figures 3 and 4.

Table I: Comparison of the Anisotropy of the Q_y Band of Monomeric BChl *a*[a] and BChl *b*[b] with That of the 1300-nm Band of P960+[c]

chlorophyll	λ (nm)	$(\epsilon_L - \epsilon_R)/\epsilon \times 10^5$
BChl *a* in ether	770	6
BChl *a* in CCl_2 + pyridine	782	20
BChl *b* in ether	794	<6
P960+ (ferricyanide)	1300	100 ± 30
P960+ (light)	1300	100 ± 30

[a] Sauer et al., 1968; Philipson & Sauer, 1972. [b] K. Sauer, personal communication. [c] The peak position of BChl *b* in ether is from Steiner et al. (1983). The anisotropy is defined as the ratio of the CD, i.e., $\epsilon_L - \epsilon_R$, the difference between the molar extinction coefficient for left and right circularly polarized light, divided by ϵ, the molar extinction coefficient for unpolarized light. Since both the absorption and CD are proportional to the concentration of chromophore and the path length through the sample, the anisotropy can be calculated directly from the observed (extrinsic) absorption and CD even when the concentration of the chromophore is unknown.

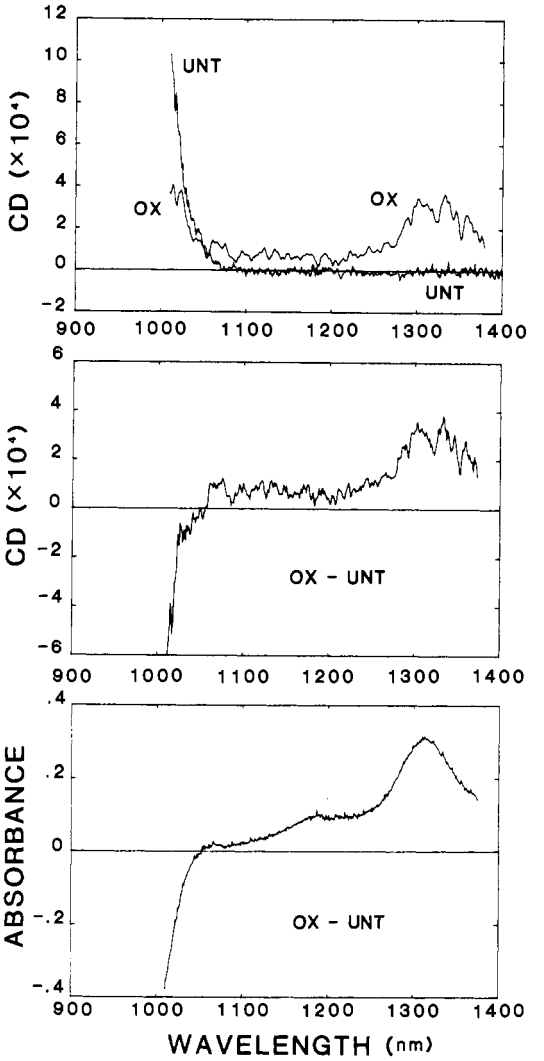

FIGURE 4: CD spectra of untreated (UNT) and light-oxidized (OX) reaction centers (top) and the light-minus-dark CD difference spectrum (center) and absorption difference spectrum (bottom). The CD spectrum of the light-oxidized preparation is noisier than for the chemically oxidized sample (Figure 2) because the spectrum was collected more rapidly to minimize light-induced degradation of the sample.

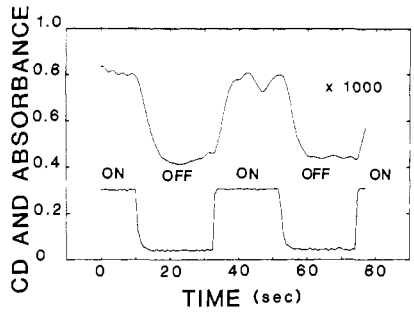

FIGURE 5: CD (upper) and absorption (lower) spectra of reaction centers at 1310 nm as a function of actinic light. The apparent lag in the CD signal relative to the absorption signal is due to the longer time constant of the CD circuit (PSD-2). The CD signal was multiplied by 1000 and displaced vertically to facilitate comparison with the absorbance.

The dependence of the absorption and CD on actinic light is demonstrated in Figure 5. The wavelength of the CD spectrometer was fixed at 1310 nm, and CD and absorbance were recorded as a function of time. Data collection started with the actinic beam on; it was turned off after 10 s. The on–off cycle was repeated twice, with each period lasting about 20 s.

The data shown in Figures 2–5 were obtained with samples prepared from a single stock solution of reaction centers and, where possible, with exactly the same samples to facilitate comparisons between the two methods of oxidation and the different instruments used to measure absorption. These results are typical, however, of spectra obtained with many different samples derived from two separate purifications of the reaction centers.

Discussion

Prior to oxidation, the longest wavelength bands of the BChl *b* molecules in reaction centers are split by exciton coupling; the resulting bands cannot be assigned to specific BChl molecules but rather are properties of the ensemble (Sauer et al., 1968; Philipson & Sauer, 1972). Is the 1300-nm band of oxidized reaction centers similarly a collective property of multiple interacting BChl *b* molecules, i.e., the two components of the special pair, or does it result from a transition localized on a single molecule?

Oxidation of isolated Chl *a*, BChl *a*, and several related molecules to form radical π cations results in disappearance of the longest wavelength absorption band and the appearance of weaker bands at longer wavelengths (Borg et al., 1970; Fajer et al., 1974), a behavior similar to that observed for reaction centers (cf. Figure 2). (To date it has not been possible to form stable radical cations of BChl *b* at room temperature; J. Fajer, personal communication.) This behavior and the lack of exciton splitting observed in the CD (Figures 2 and 3) are consistent with the notion that the 1300-nm band is the property of just one of the two BChl molecules in the special pair that had been oxidized to a π-cation radical.

Other authors have attributed the 1300-nm band to interactions between the two BChl molecules in the special pair. Shuvalov & Parson (1981) proposed that the analogous band in reaction centers from *Rhodospirillum sphaeroides* (at 1270 nm) is due to transitions involving both BChl molecules in the special pair in which one molecule is promoted to a triplet excited state. This proposal was based on the similarity in energy of the 1270-nm band and of the lowest triplet state of neutral BChl relative to its ground state.

Vermeglio & Paillotin (1982) extended this idea into a three-orbital model that predicts two excited states of the special pair: a higher energy excited state that is all singlet in character and has approximately three-fourths of the oscillator strength of the Q_y transition of a neutral BChl *b* and a lower energy transition that has both singlet and triplet character and only about one-fourth of the oscillator strength of the Q_y band. The mixing of the singlet and triplet states of the neutral BChl *b* of the special pair is attributed to the presence of the unpaired spin on the BChl b^+. The 1300-nm band is assigned to the transition with triplet character and a band at 808 nm to the higher energy component with singlet character. Philipson & Sauer (1973) reported slight changes in absorption and CD at 808 nm when *Rps. viridis* reaction centers are oxidized, but quantitative evaluation is hindered by overlapping spectra from other chromophores.

Assignment of the 1300-nm band, which is equivalent to characterization of the electronic states of the oxidized special pair, will require further experiments on the CD of radical π cations of model systems, hopefully including BChl *b*, as well as calculations of the CD expected from charge-transfer transitions such as those proposed by Vermeglio & Paillotin (1982). The CD of the 1300-nm band is important because it is free of interference from overlapping transition of other components of the reaction center and because CD–absorption anisotropy has proven useful in assigning the origin of the near-infrared bands of related molecules such as heme proteins (Eaton & Charney, 1969; Eaton et al., 1978).

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Transducin and the Inhibitory Nucleotide Regulatory Protein Inhibit the Stimulatory Nucleotide Regulatory Protein Mediated Stimulation of Adenylate Cyclase in Phospholipid Vesicle Systems†

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ABSTRACT: The adenylate cyclase coupled inhibitory nucleotide regulatory protein (N_i) and the bovine retinal nucleotide regulatory protein transducin (T) appear to share some common functional properties since their GTPase activity is stimulated to similar extents by the retinal photoreceptor rhodopsin. In the present work, we sought to assess whether these functional similarities might extend to their interaction with adenylate cyclase. This necessitated the development of reconstitution systems in which guanine nucleotide regulatory protein mediated inhibition of adenylate cyclase activity could be demonstrated and characterized in a lipid milieu. In the absence of the pure human erythrocyte stimulatory nucleotide regulatory protein (N_s), the insertion into phospholipid vesicles of either pure N_i from human erythrocytes or pure bovine T with the resolved catalytic moiety of bovine caudate adenylate cyclase (C) does not establish GppNHp inhibition of either Mg^{2+}- or forskolin-stimulated adenylate cyclase. However, the coinsertion into lipid vesicles of either N_i or T with N_s and resolved C results in an inhibition of N_s(GppNHp) stimulatable C activity. As is the case in intact membranes, the reconstituted inhibition of the N_s-stimulated C activity extends into the steady-state phase of time courses of activity. This inhibition is highly sensitive to the $MgCl_2$ concentration. At 2 mM $MgCl_2$, the inhibition is greater than 80% while at 50 mM $MgCl_2$ it is only ~20%. Overall these results suggest (1) that the inhibition of adenylate cyclase activity by N_i is due to an interference with the N_s stimulation of the C activity rather than a direct effect on the intrinsic activity of the catalytic moiety itself and (2) that the retinal nucleotide regulatory protein, transducin, substitutes for N_i in causing inhibition of N_s-stimulated C activity.

Adenylate cyclase activity is regulated through distinct stimulatory and inhibitory pathways. Stimulation is initiated by a hormone–receptor (β-adrenergic, glucagon, prostaglandin E_1, etc.) interaction which results in the promotion of GTP binding to the stimulatory nucleotide binding regulatory protein (N_s). The N_s·GTP complex then directly interacts with the catalytic moiety of adenylate cyclase (C). This protein has been purified to homogeneity from several sources (Northup et al., 1980; Sternweis et al., 1981; Codina et al., 1984; Hanski et al., 1981, 1982) and has been found to be heterotrimeric (molecular weight: α, 42000–52000; β, 35000; γ, ~5000) (Gilman, 1984; Hildebrandt et al., 1984a).

In a similar manner, the inhibition of adenylate cyclase activity is initiated by a hormone–receptor (α_2-adrenergic, muscarinic, dopamine, etc.) interaction which effects the activation of an inhibitory nucleotide binding regulatory protein (N_i). This regulatory protein has recently been purified to apparent homogeneity, and like N_s, it is heterotrimeric (molecular weight: α, 39000–41000; β, 35000; γ, ~5000) (Codina et al., 1983, 1984; Bokoch et al., 1983).

A significant amount of information regarding the activation of N_s and its resultant stimulation of adenylate cyclase activity has been obtained both from studies in intact membranes (Iyengar & Birnbaumer, 1980; Northup et al., 1983) and more recently from reconstituted systems (Asano et al., 1984; Asano & Ross, 1984; Cerione et al., 1984, 1985). Recently, attention has begun to focus on understanding the manner in which activated N_i inhibits C activity. Various mechanisms have been postulated including the $\beta\gamma$-deactivation hypothesis (Gilman, 1984; Katada et al., 1984). This model suggests that the activation of nucleotide regulatory proteins reflects subunit dissociation such that upon activating N_i, the increased levels of $\beta\gamma$ can interfere with the activation (and subunit dissoci-

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