Protein-Assisted Pericyclic Reactions: An Alternate Hypothesis for the Action of Quantal Receptors

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ABSTRACT The rules for allowable pericyclic reactions indicate that the photoisomerizations of retinals in rhodopsins can be formally analogous to thermally promoted Diels-Alder condensations of monoenes with retinols. With little change in the seven-transmembrane helical environment these latter reactions could mimic the retinal isomerization while providing highly sensitive chemical reception. In this way archaic progenitors of G-protein-coupled chemical quantal receptors such as those for pheromones might have been evolutionarily plagiarized from the photon quantal receptor, rhodopsin, or vice versa. We investigated whether the known structure of bacteriorhodopsin exhibited any similarity in its active site with those of the two known antibody catalysts of Diels-Alder reactions and that of the photoactive yellow protein. A remarkable three-dimensional motif of aromatic side chains emerged in all four proteins despite the drastic differences in backbone structure. Molecular orbital calculations supported the possibility of transient pericyclic reactions as part of the isomerization-signal transduction mechanisms in both bacteriorhodopsin and the photoactive yellow protein. It appears that reactions in all four of the proteins investigated may be biological analogs of the organic chemists' chiral auxiliary-aided Diels-Alder reactions. Thus the light receptor and the chemical receptor subfamilies of the heptahelical receptor family may have been unified at one time by underlying pericyclic chemistry.

INTRODUCTION

In the eyes of toads, rhodopsin converts light to neural signal with a quantum efficiency of ~0.5 (Baylor et al., 1979). A similar heptahelical prokaryotic molecule, bacteriorhodopsin, converts one photon into translocation of 0.25–0.7 protons across a membrane, depending on the medium (Govindjee et al., 1980). The chromophore of unilluminated rhodopsin is 11-*cis* retinal, while that of bacteriorhodopsin is all-*trans* retinal. Upon illumination these adducts undergo isomerization, from 11-*cis* to 11-*trans* in the case of rhodopsin, and from all-*trans* to 13-*cis* in the case of bacteriorhodopsin. The quantum efficiencies of these isomerizations are ~0.67 (Birge et al., 1988) and 0.64 (Rohr et al., 1992; Song et al., 1996), respectively. Although a variety of theoretical studies have been performed on these systems (Birge, 1990; Tajkhorshid et al., 1997; Pollard and Mathies, 1990; Nina et al., 1995; Yao et al., 1997), and the premises of these studies have been more plausible since a structure for bacteriorhodopsin has become available (Henderson et al., 1990), the details of the nature of the isomerizations and how they couple the chromophore isomerization to the protein activity remain elusive. Experimentation is difficult: the primary photoreaction takes only ~500 fs (Logunov et al., 1998; Hasson et al., 1996). Even so, it is clear from fluorescence decay data and from subpicosecond absorption data that at least two species with different half-lives exist in that time.

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The opsins, the heptahelical apoproteins that form rhodopsins on linking with retinal, are related to a large family of non-light receptors by homology (Attwood and Findlay, 1994; Baldwin et al., 1997) and to an immense family by heptahelical form (Troemel et al., 1995; Kuipers et al., 1997; Sullivan et al., 1995; Nef, 1993). Most pheromone receptors, including pheromones of yeast, lepidoptera, and mammals, fall in this class (Roelofs, 1995; Dulac and Axel, 1995; Prestwich, 1996; Kitamura and Shimoda, 1991). Muscarinic acetylcholine receptors, dopaminergic receptors, adrenergic receptors, and serotoninergic receptors are of this class (Trumpp-Kallmeyer et al., 1992). Gonadotropin release hormone (Kakar et al., 1993), oxytocin (Bathgate et al., 1995), cannabinoid (Thomas et al., 1991), and opioid receptors (Pogozheva et al., 1998) belong to this group. Most receptors for prostaglandins and leukotrienes also conform to the heptahelix configuration (Hirata et al., 1994; Yokomizo et al., 1997). Heptahelical receptors are frequently coupled via the molecular amplification system of G-proteins to the enhancement or suppression of intracellular cAMP (Roper et al., 1995), and they consequently relate a major second messenger of the eukaryotic cell to the primary extracellular messenger of the slime mold (Muller et al., 1998).

Some pheromone receptors can respond to their volatile agonists in a quantal way. The moth, *Bombyx mori*, responds to single molecules, or at most very few molecules of the pheromone bombykol, by increasing its wingbeat frequency (Schneider, 1969). Other examples of quantal or very low concentration response exist throughout the world of pheromones (Menini et al., 1995). The elephant shares a pheromone with the cabbage looper moth (Rasmussen et al., 1997; Green et al., 1967) that is very similar to pheromones of many other moths (Arn et al., 1992). Uncertain dilution

of these signal chemicals in large volumes of air poses a very difficult signal-to-noise situation, and it can be imagined that quantal chemical response is an evolutionary advantage for species that must communicate mating signals via wind, whether the species is mammal or moth.

One apparently efficient way to evolve quantal response is to borrow from another quantal system. The common heptahelical architecture of rhodopsins and pheromone receptors allows one to consider that such borrowing might have occurred. However, the likelihood that the heptahelical structure was purloined in the course of evolution would be greatly increased if similar chemical principles underlie the action of both light receptors and pheromones. Many pheromones, and almost all with receptors that exhibit high sensitivity, are monounsaturates. Frequently, as with bombykol and the elephant-related pheromones mentioned above, they are monoenes. A simple way in which evolution could substitute chemical for light would be to replace the quantum of light with a monoene, if the monoene could react with a retinoid. In fact, retinoids undergo both thermal- and photo-promoted Diels-Alder reactions, and are capable of behaving as 2-π or as 4-π electron donating species in both cases (Shealy et al., 1996; Burger and Garbers, 1973; Vogt et al., 1991; Pfoertner et al., 1987, 1988). Pericyclic reactions such as the Diels-Alder reaction are easily generalized. In gross outline, reactions that occur with thermal input and reactions promoted by photon absorption can share the same intermediate structure if the number of conjugated double bonds differs by one (McMurray, 1984). A retinol-monoene reaction that resulted in a cyclohexene product in the dark would be analogous to a retinal (Schiff's base) monoene reaction promoted by 550 nm light that resulted in a like product.

Although the intramembrane environments surrounding sensory rhodopsins contain high percentages of docosahexaenoic acid, which is essentially a multiple monoene (Yuan et al., 1998), there is no known monoene that inhabits the active site of rhodopsin or bacteriorhodopsin. However, tryptophan (or the indole ring) can participate as a monoene in pericyclic reactions (Kraus et al., 1988; Benson et al., 1996). Furthermore, aromatic residues are routinely used for chiral auxiliaries in Diels-Alder reactions, improving reaction rates and specificities in ways that are not understood (Westwell and Williams, 1997). The retinal in bacteriorhodopsin lies in a bath of aromatic residues, three of which are tryptophans implicated in the binding site of the retinal (Rothschild et al., 1989). However, the structure of two antibodies that promote Diels-Alder reactions are known (Heine et al., 1998; Romesberg et al., 1998). We reasoned that, especially in view of the fact that the protein backbone of bacteriorhodopsin is primarily α-helix, while that of the antibodies is primarily β-sheet, similarities in the local environment of the retinal in bacteriorhodopsin and of the mock reaction intermediates in the Diels-Alder antibodies might indicate that pericyclic chemistry plays a role in responses of rhodopsins, and that it may have been essential in the evolutionary path of heptahelical receptors. Comparisons shown below do demonstrate an unusual similarity in the active sites. This similarity is extended to include the active site of the photoactive yellow protein. Furthermore, quantum chemical calculations of molecular orbitals indicate that the isomerizing retinal of bacteriorhodopsin can participate in an apparently transient pericyclic reaction with tryptophan 86. Finally, we show that the photoactive yellow protein 4-hydroxycinnamoyl chromophore can also participate in a pericyclic reaction as it isomerizes, and that this pericyclic reaction is analogous to that of bacteriorhodopsin in its capability for coupling the adduct isomerization to the protein conformational change.

METHODS

The coordinates of bacteriorhodopsin were obtained from the Protein Data Bank, accession number 1BRX (Luecke et al., 1998). These were obtained from x-ray crystallographic refinement of bacteriorhodopsin. They were chosen rather than coordinates 2BRD from the electron crystallographic studies (Grigorieff et al., 1996), because the data were intrinsically of better resolution. The Protein Data Bank coordinates of the Diels-Alder antibody, 39A11, with a bicyclic representation of the reaction intermediate, a bicyclo[2.2.2]octene, in the active site, has accession number 1a4k (Romesberg et al., 1998). The Protein Data Bank coordinates of the structure of its germline precursor without included mock intermediate are designated 1a4j (Romesberg et al., 1998). The antibody 39A11 was obtained by affinity maturation of its germline precursor against the bicyclic surrogate intermediate. Coordinates of the second Diels-Alder antibody (13G5), which was complexed with 1-carboxyl-1′-[(dimethylamino)carbonyl]ferrocene instead of a surrogate intermediate, were obtained directly from the Wilson laboratory (Heine et al., 1998). This antibody was raised against a slightly different ferrocene derivative coupled to keyhole limpet hemocyanin. The coordinates of the unbleached photoactive yellow protein have Protein Data Bank accession number 2PHY, while those of the steady state illuminated form have number 2PYP (Genick et al., 1997). In the latter ~50% of the 4-hydroxycinnamoyl adduct is in the *cis* (post light exposure) form and 50% is in the *trans* (unbleached) form.

Comparisons of the active sites of bacteriorhodopsin and the Diels-Alder antibodies were performed with the program Molmol. Quanta was used for visualization of allowable directions of isomerization in bacteriorhodopsin and in the photoactive yellow protein. In both cases, using as criterion non-intersection of the adduct with the protein backbone, there was a preferred direction of twist about the isomerizing double bond. Quanta was also used for production of new coordinates for the retinal and 4-hydroxycinnamoyl adducts in partially isomerized positions derived from twisting in the allowed direction. Molecular orbitals of the adducts and of the presumed critical aromatic residues were calculated separately at the 3-21G level of ab initio calculation using MacSpartan. The resultant orbital calculations were then arrayed on the appropriate pdb-derived scaffold in order to display the juxtaposition of orbitals. Both the retinal (in Schiff's base form) and the 4-hydroxycinnamoyl moiety were modeled as neutral species. The results were visualized with MacSpartan.

RESULTS

The distribution of aromatic side chains in the active sites of bacteriorhodopsin and the Diels-Alder antibody 39A11 was strikingly similar despite the fact that the backbone structure of the important region of bacteriorhodopsin is all helical, and that of the antibody mostly β-sheet. Three aromatic residues are distributed at roughly three of the vertices of a rectangle in each case (Fig. 1 *A*). These surround the central body of the retinal in bacteriorhodopsin and the bicyclo ring

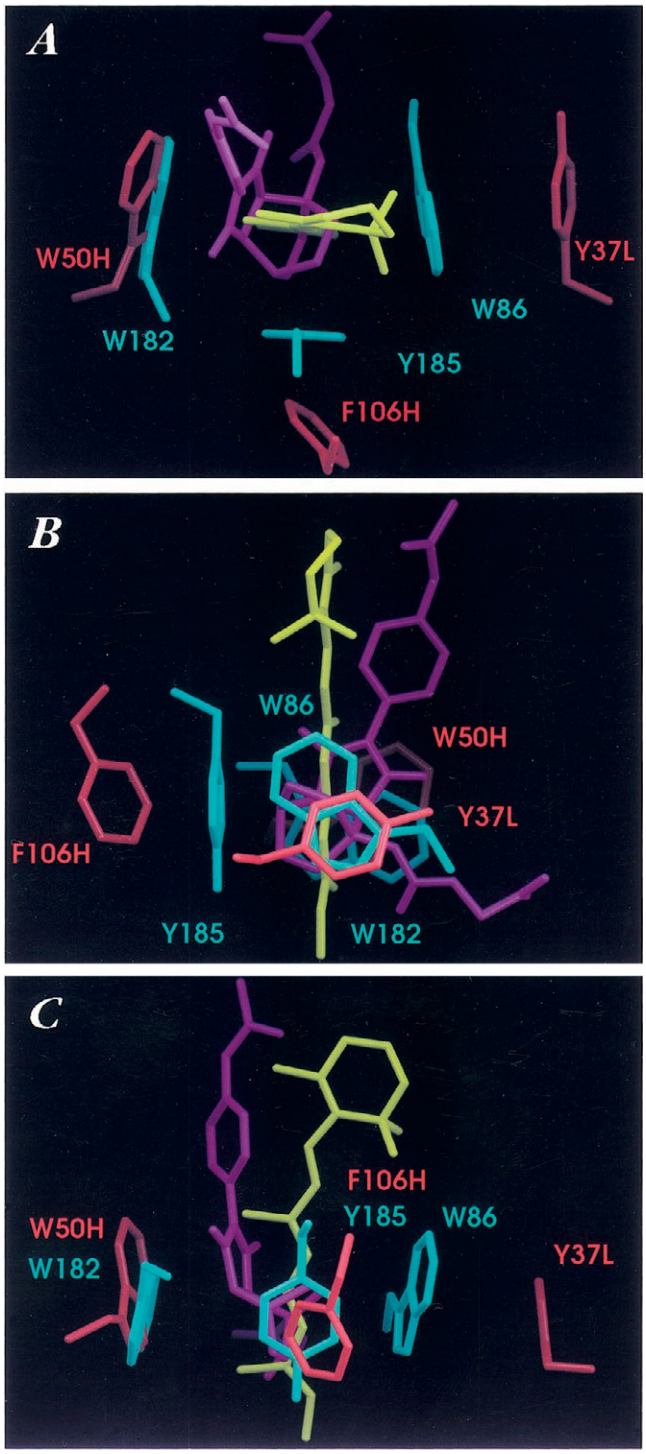

FIGURE 1 Comparison of the reaction pockets of bacteriorhodopsin and one of the Diels-Alder antibodies. Retinal, *yellow*; bicyclo intermediate, *purple*; bacteriorhodopsin residues, *blue*; Diels-Alder residues, *orange*. (*A*) Top view (looking along the axis of the retinal) of bacteriorhodopsin overlaid on a Diels-Alder antibody (1a4k) raised against a bicyclo surrogate Diels-Alder intermediate. In this overlay the retinal pierces the bicyclo ring of the mock intermediate. The vertices of two incomplete squares are easily identifiable. (*B*) Side view of the overlaid reaction pockets. A straight line can be drawn through bacteriorhodopsin residues, antibody residues and regions of the inclusions implicated in the reactions. In this view one looks directly along this line. From the viewer in sequence the residues are 39A11 Tyr-37L, bacteriorhodopsin Trp-86, overlapped retinal and bicyclo mock intermediate, bacteriorhodopsin Trp-182 and, nearly coincident, the antibody Trp-50H. Antibody Phe-106H and bacteriorhodopsin Tyr-185 appear to the left. (*C*) Alternate side view of the two molecules rotated ~90° about the vertical axis in view *B*, so that the viewer is closest to Phe-106H. The residues to the right, antibody Tyr-37L (*orange*) and bacteriorhodopsin Trp-86 (*blue*), show especially clearly how the bacteriorhodopsin residues in the plane of the reaction pocket are disposed like the Diels-Alder antibody residues, but closer to the reactant.

of the pseudointermediate in the Diels-Alder antibody. In bacteriorhodopsin, Tyr-185 is parallel to and ~3.5 Å from the retinal, while in the Diels-Alder antibody Trp-50H is ~3.5 Å from the pseudo-intermediate. With the retinal of bacteriorhodopsin threaded through the suicide substrate analog of the Diels-Alder active state, the rectangle of bacteriorhodopsin aromatic residues fits within the rectangle of antibody active site residues. In fact, one can draw a straight line corresponding to the overlapping diagonals of the rectangles: antibody Trp-50H-bacteriorhodopsin Trp-182-Diels-Alder intermediate and retinal-bacteriorhodopsin Trp-86-antibody Tyr-37L (Fig. 1 *B*). The vertex of the 39A11 rectangle not on this line is inhabited by Phe-106H, while that of bacteriorhodopsin displays bacteriorhodopsin Tyr-185. If the superimposed reactive pockets are viewed from a vantage point where Phe-106H appears nearest to the observer, it is clear that all the aromatic residues lie in a plane that includes the reactive center of the bicyclo Diels-Alder mock intermediate and the C13-C12 bond of the bacteriorhodopsin retinal (Fig. 1 *C*). The symmetry is such that the residues and retinal of bacteriorhodopsin can be rotated 180° about an axis through Tyr-185 and the retinal, and with little adjustment the square within square relationship holds. In either combined configuration the retinal is nearly colinear with one of the extended chains of the mock intermediate and the rectangles are nearly perpendicular to the direction defined by the retinal.

The ferrocene derivative complexed Diels-Alder antibody (13G5) also exhibited the motif of three aromatic residues, Trp-103H, Tyr-96L, and Tyr-36L. A fourth residue, Trp-47H, almost resides in the plane of residues surrounding the mock intermediates, but it is far enough out of plane to ignore for this analysis. Although the ferrocene derivative appears nearly symmetric, these residues partially ring one pentagonal end of the intermediate analog. Overlay of these residues with those of the Diels-Alder antibody raised against the bicyclo intermediate and with those of the photoactive yellow protein shows the similarity in their distributions (Fig. 2). Two 13G5 residues, Trp-103H and Tyr-96L, bracket their intermediate precisely, so that a straight line runs through the three moieties. Similar lines can be drawn for Trp-50H, Tyr-37L, and the bicyclo-octene moiety of 39A11; and for Phe-96, Tyr-98, and the 4-hydroxycinnamoyl adduct of the photoactive yellow protein. The result in the overlay is that two groups of amino acids and the superimposed faux intermediates plus the 4-hydroxycinnamoyl group form essentially a straight line,

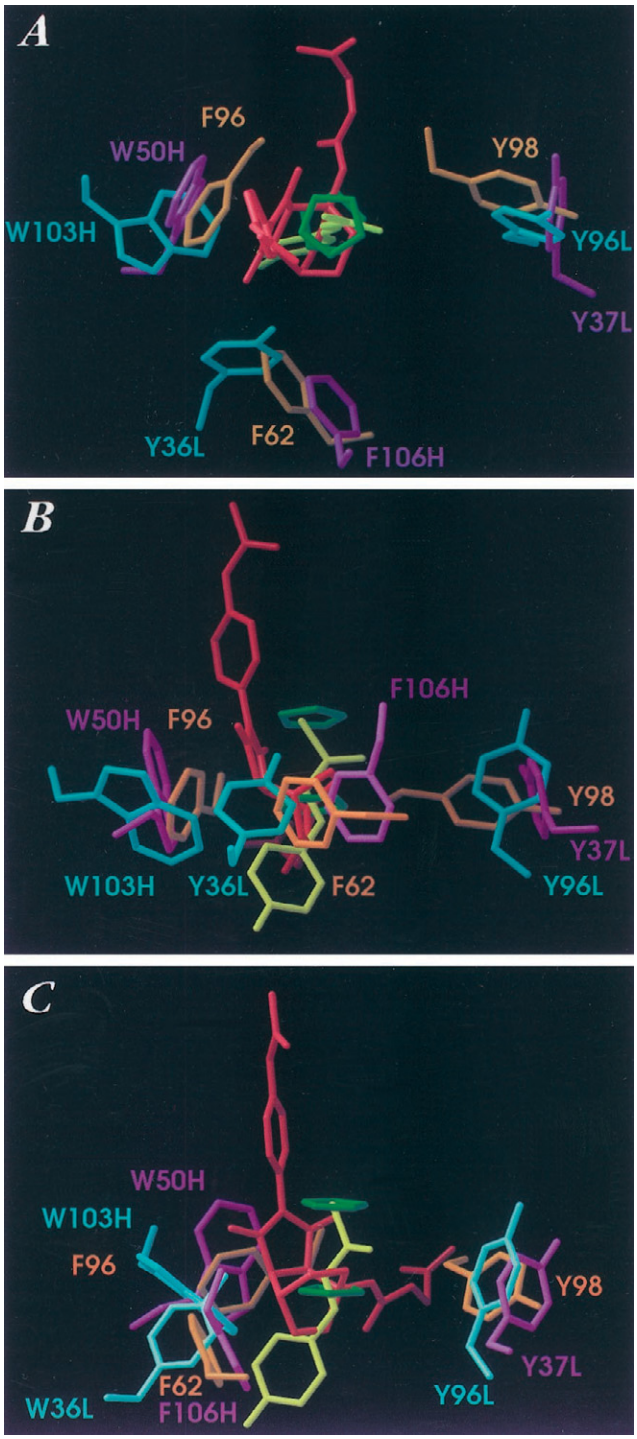

FIGURE 2 Comparison of the two Diels-Alder antibody reaction pockets and that of the photoactive yellow protein. Ferrocene inclusion, *green*; residues from the ferrocene including antibody, *blue*; bicyclo inclusion, *dark orange*; bicyclo including antibody residues, *purple*; 4-hydroxycinnamoyl adduct, *yellow*; residues from the photoactive yellow protein, *light orange*. (*A*) Top view corresponding to Fig. 1 *A*. The aromatic triples are all nearly in the same plane. They clearly segregate into three regions at roughly three vertices of a rectangle, leaving one avenue of approach or exit. To the left are grouped 39A11 Trp-50H, 113G5 Trp-103H, and photoactive yellow protein Phe-96. At the bottom, strung in a connected line, are 39A11 Phe-106H, 13G5 Tyr-36L, and photoactive yellow protein Phe-62. To the right are 39A11 Tyr-37L, 13G5 Tyr-96L, and photoactive yellow protein Tyr-98. (*B*) Side view corresponding to Fig. 1 *C*. Phe-106H from 39A11, 13G5 Tyr-36L, and photoactive yellow protein Tyr-36 are closest to the viewer. This diagram demonstrates how all the residues can be arranged in near coplanarity. (*C*) A view from the non-obstructed side, i.e., the region where there are no aromatics in the overlay. In this overlay of three reaction pockets the top pentacarbon ring of the ferrocene mock intermediate inclusion resides within the bicyclo octene mock intermediate of the other antibody, as do the C2-C3 and C3-C1′ bonds of the photoactive yellow protein.

while Tyr-36L of 13G5, Phe-106H of 39A11, and Phe-62 of the photoactive yellow protein form a sort of extended glob of residues at a third corner of the imaginary rectangle (Fig. 2 *A*). All these side chains reside in a plane whose depth is little more than one aromatic residue's width (Fig. 2 *B*). This plane also incorporates the bicyclic ring, the top of the ferrocene and the C2-C3, and C3-C1′ bonds of the 4-hydroxycinnamoyl adduct (Fig. 2 *C*). For clarity the overlay of bacteriorhodopsin's active site aromatic triplet with those of both antibodies at once has not been shown in this figure, but clearly a single line can be drawn through two aromatic residues and the mock intermediate, retinal, or 4-hydroxycinnamoyl adduct of all four simultaneously. When such a line is drawn, the mock intermediates, the retinal carbon chain, and the 4-hydroxycinnamoyl moiety are oriented in a remarkably similar manner.

The above results are consistent with the possibility that there is some pericyclic character in the retinal isomerization of bacteriorhodopsin and the photoactive yellow protein, and that the likely pericyclic reactions involve aromatic residues. To evaluate this possibility ab initio calculations of molecular orbitals were run at the 3-21G level for the individual amino acids and the retinal. Retinal orbitals were calculated with the 13-14 double bond at *trans*, and at *cis* at −135°, −120°, and −90° (viewed from carbon 14 to carbon 13). The choice of turning toward −90° rather than +90 was determined by evaluating, using Quanta, whether the polyene would intersect a protein backbone chain upon rotation about the 13-14 bond. In the case of rotation from −180° to −90° it does not.

The intermediates might be expected to conform to Woodward-Hoffman rules (Hoffmann and Woodward, 1968; Schipper, 1988). Under this supposition the excited state of retinal, corresponding to LUMO in the MacSpartan calculations, would be expected to either form a six-carbon intermediate with LUMO density of a neighboring active site aromatic side chain, or a four-carbon intermediate from interaction with the HOMO density of the nearby aromatic group. Nothing in the *trans* or *cis* calculations led directly to the clear indication that some pericyclic mechanism might occur upon excitation of the polyene by light. In the −120° calculation, however, it is clear that a twisted cyclobutyl ring can form as a result of the apposition of LUMO orbital electron density over the 12-13 single bond of the retinal and HOMO electron density of Trp-86 (Fig. 3 *A*). The twisted nature of the possible cyclobutyl ring makes it likely that it would be transient. There is ~0.5 Å clear space

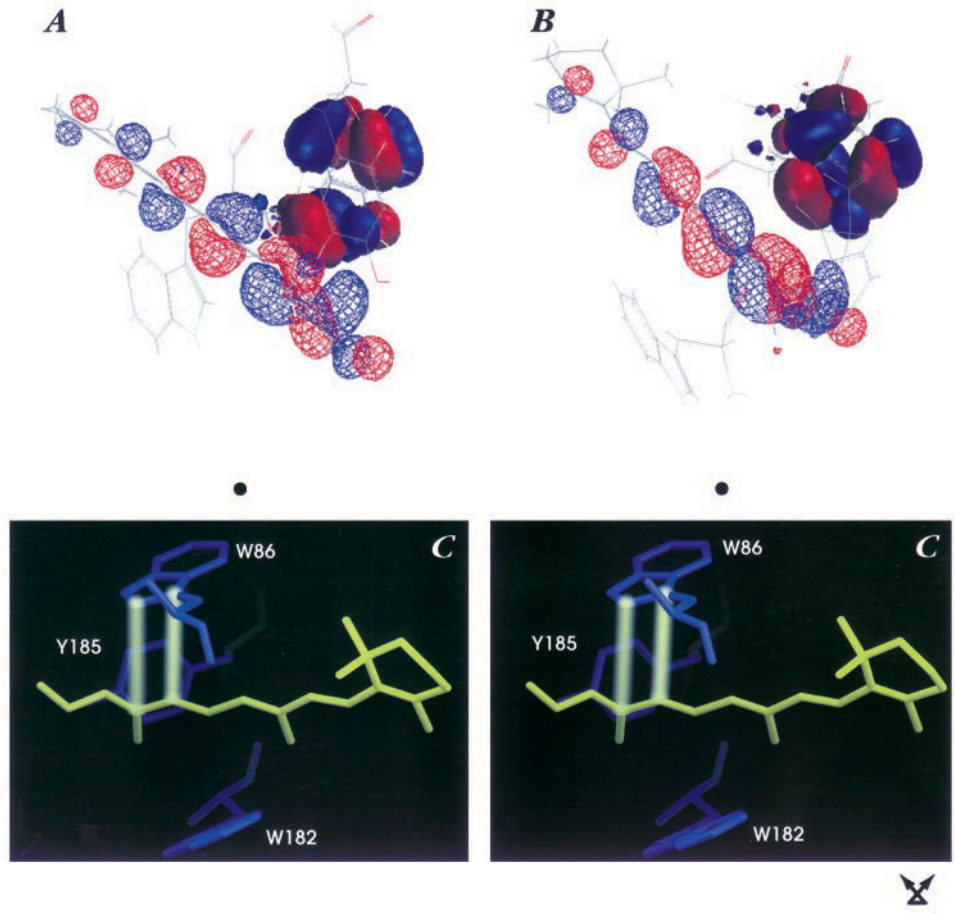

FIGURE 3 Ab initio molecular orbital calculation with retinal C14-C13 double bond at −120°. (*A*) Oblique view with retinal in front of Trp-86. For clarity the LUMO orbitals of the retinal are shown in mesh outline. The blue electron density lobe of the retinal C13-C12 bond is apposed to the red lobe of the Trp-86 Cδ1-Cτ bond. The two were calculated independently, so the apparent sign difference would have no effect on the reaction possibility. (*B*) View demonstrating separation of retinal LUMO and Trp HOMO electron densities. The 99.8% occupancy electron densities show ~0.5 Å empty space between the retinal C13-C12 bond and the Trp-86 Cτ-Cδ1 bond. However, because the calculations of the aromatic residues were done separately, the distortion of retinal orbitals due to Tyr-185 and Trp-182 is not represented. It would be expected to close the gap. (*C*) Diagram of pericyclic reaction with C14-C13 at −120°. The nascent bonds are shown as glowing *light yellow* lines.

between the 99.8% occupancy level density of the closest point of the orbitals (Fig. 3 *B*). This distance is very likely overestimated, and the densities in a more thorough calculation would be expected to nearly touch, because the nonbonding bacteriorhodopsin Tyr-185 and Trp-182 should distort the retinal orbitals so that more electron density shows on the Trp-86 side. These interactions were not calculated in this approximation. At −120° the carbon-carbon distances are 4.01 for the retinal C13 to tryptophan Cδ1 apposition and 3.81 Å for the retinal C12 to Trp-86 Cτ distance (Fig. 3 *C*). This cyclobutyl ring can form as early as −155°, where the nascent bond lengths are 4.01 and 3.67 Å. Most interesting is the fact that formation of a transient intermediate specifically involving carbons 12 and 13 (rather than 13 and 14, the carbons of the isomerizing double bond) is calibrated to pull the retinal toward isomerization while simultaneously transmitting the initial effects of isomerization to the protein matrix. In fact in this approximation, where the protein is not allowed to relax, at −120° the retinoid is beginning to pull away from Trp-86.

These principles were tested on the photoactive yellow protein, where a considerably different polyene chromophore, the 4-hydroxycinnamoyl thioadduct, also undergoes *trans-cis* isomerization upon absorption of light. Two structures of the protein are available, one in which it is dark-adapted (see methods) and one in which the chromophore inhabits both its initial and final positions, *trans* and *cis*, respectively. Again a trial rotation about the C2-C3 bond indicated that the intermediate at +90° (viewing the bond from C2 to C3, and noting that C1 is attached via a thio group to the protein) would be difficult to reach, while the one at −90° would be sterically possible, if the aromatic portion of the polyene were allowed to rotate, i.e., if the C3-C1′ bond were allowed freedom of rotation. In fact, with the C2-C3 bond at −90°, there is little freedom of motion left for the aromatic ring. In order to avoid close contacts with Phe-96 the C3-C1′ bond must be rotated to ~+36°, where the aromatic portion of the 4-hydroxycinnamoyl adduct is very nearly parallel with the Phe-96 side chain. In this position, using all coordinates from the *cis* form of the chromophore and the protein coordinates associated with the *cis* form, a possible Diels-Alder intermediate becomes exceptionally clear (Fig. 4). It is, however, a six-membered ring and appears as an apposition of the LUMO orbital of the 4-hydroxycinnamoyl adduct with the LUMO orbital of Phe-96 (Fig. 4 *A*). The distance from Cδ2 of Phe-96 to C6′ of the 4-hydroxycinnamoyl group is 3.23 Å, while that from Cδ1 of Phe-96 to C3 of the 4-hydroxycinnamoyl group is 3.56 Å. In this case the 99.8% occupancy orbitals are osculating or nearly so (Fig. 4 *B*). Again the central bond of the chromophore involved in the reaction, C3-C1′, would be just distal from the isomerizing double bond, C2-C3 (Fig. 4

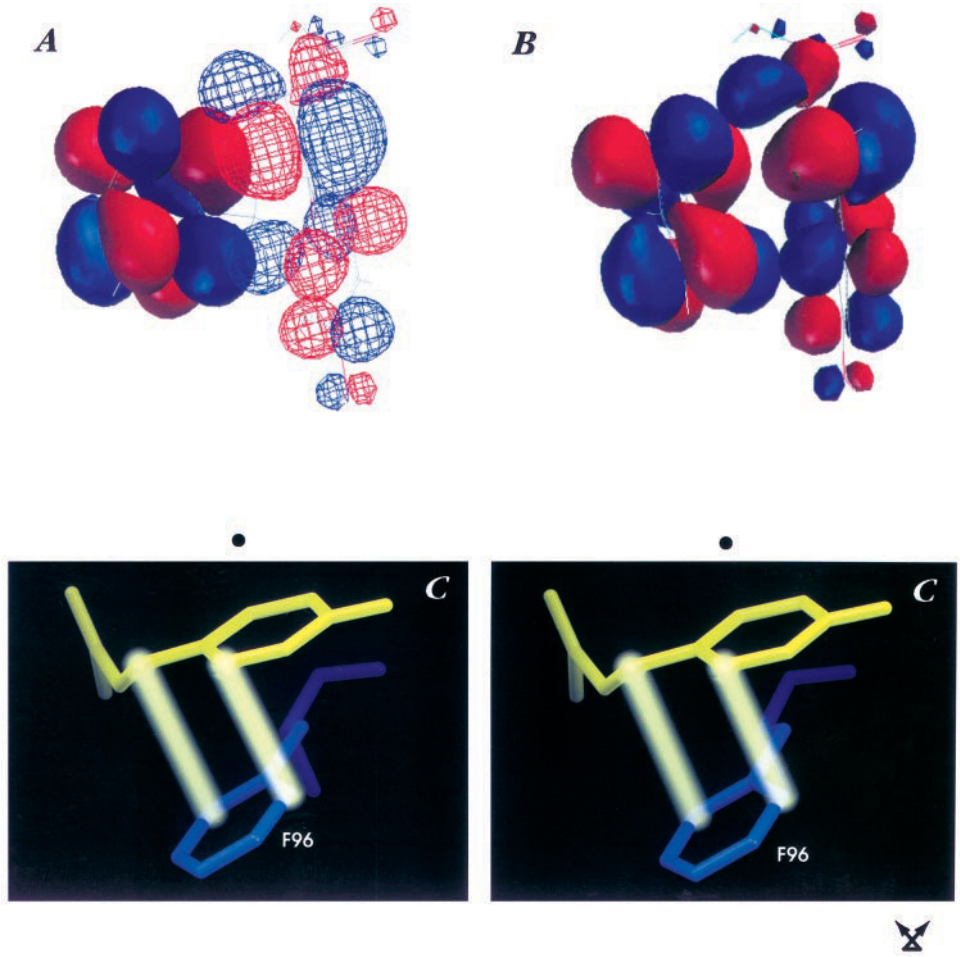

FIGURE 4 The results of ab initio molecular orbital calculations for the 4-hydroxycinnamoyl adduct and reaction pocket aromatic residues of the photoactive yellow protein. (*A*) View of 4-hydroxycinnamoyl–Phe-62 apposition from the adduct toward the phenylalanine. The 4-hydroxycinnamoyl adduct is represented in mesh. The first two sets of LUMO electron density lobes belong to the 4-hydroxycinnamoyl, the next set to the phenyl of the phenylalanine. The C2-C3 bond of the 4-hydroxycinnamoyl adduct has been rotated to the −90° position, but with the protein atomic coordinates and C1 and C2 coordinates of the adduct taken from the *cis* conformation. The C3-C1′ bond has been allowed to twist to +36°. This implies that the protein has had time to relax somewhat so that the thiol bond positioning the adduct reaches the correct angle. (*B*) View demonstrating closeness of Phe-62 and 4-hydroxycinnamoyl LUMO-LUMO electron densities. The 99.8% occupancy electron densities are osculating, or nearly so. (*C*) Diagram of the six-carbon transient condensate of the photoactive yellow protein. The nascent bonds are shown as glowing *light yellow* lines.

C; note the numbering convention on this chain differs from that of retinal). However, this time the involvement of the pericyclic reaction would occur later in the reaction process.

DISCUSSION

The aromatic residues clustered closely around the reactive centers in bacteriorhodopsin, the Diels-Alder antibodies, and the photoactive yellow protein exhibit a spatial pattern that is similar for all four proteins. In all four the reaction cavity has three aromatic residues disposed in a plane that intersects the chromophore or mock substrate. In all four cases the plane of these residues seems to be roughly perpendicular to an important axis of the adducts. In all cases two aromatic residues frame the inclusion so that one can draw a straight line that intersects some part of the aromatic rings and a part of the adduct near the reaction site. Thus, the reaction pockets of these four dissimilar proteins exhibit unexpected similarity, especially in view of the fact that one of these pockets, that of bacteriorhodopsin, is formed in an almost totally helical polypeptide environment, while the antibodies are essentially β-sheet structures, and the photoactive yellow protein reaction pocket is bordered by both α-helix and β-sheet. Given that the two antibodies promote Diels-Alder reactions, and that photopericyclic reactions can occur within picoseconds, as would be required (Reid et al., 1993), it does seem reasonable to consider that bacteriorhodopsin and the photoactive yellow protein may undergo their isomerizations in a manner involving pericyclic intermediates.

The active site cavities of the four proteins are strongly reminiscent of chiral auxiliary systems for Diels-Alder reaction promotion. Chiral auxiliaries are almost always designed to have an aromatic moiety (Westwell and Williams, 1997). In one case, where R-prolyl-S-phenylalanyl is the auxiliary moiety, the phenyl group can stack partially over the vinyl dienophilic substituent, but it is also in position to stabilize the aromatic features of the reaction intermediate, or to interact directly as a transient reaction participant (Le et al., 1997). Another example furthers the notion that bacteriorhodopsin and the photoactive yellow protein might act via a pericyclic mechanism: in Diels-Alder condensations of small dienophiles with a naphthol-anthracenophane, there is a seemingly unlikely tendency of the dienophiles to attack the anthracene moiety by insertion between the two wings of the molecular V of the anthracenophane rather than from the outside (Mataka et al., 1995). Here again it is unclear whether the promotion of the reaction is due to stacking interactions stabilizing the position of the dienophile, stacking stabilization of the reaction intermediate, or

transient reaction participation of the naphthol group. The biological reaction cavity described here in bacteriorhodopsin displays a number of similarities to the naphthol-anthracenophane when the retinal is at −120°, approaching the −90° position. The reactant resides in one cleft found between the two angled aromatic residues, Tyr-185 and Trp-86, and simultaneously in another formed by Tyr-185 and Trp-182. The narrower cleft formed by Tyr-185 and Trp-86 has an opening of ~7.3 Å, which narrows to ~4.4 Å (the opening of the naphthol-anthracenophane is ~5.0 Å, but its angle of aperture is narrower, so it narrows over the first ring's width to ~4.0 Å). By analogy, in the reaction where retinal would form a cyclobutyl intermediate with Trp-86, the retinal would be acting as a monoene. The situation is also similar in the photoactive yellow protein. There is an aromatic wedge consisting of Phe-96 and Phe-62 that brackets the isomerizing chromophore. However, it is not as restrictive as those of naphthol-anthracenophane or bacteriorhodopsin. The shortest distance between these phenylalanines is 5.22 Å and the longest is 9.44 Å, so the angle of aperture is wider than that of the Tyr-185–Trp-86 wedge of bacteriorhodopsin, and the pocket is looser.

If the aromatic wedge is involved in the course of the initial photoreaction, mutants of Trp-86 and Tyr-185 should show changes in the reaction's course. These occur even with the minimal alteration of either aromatic to phenylalanine. Both mutations cause substantial spectroscopic shifts of the dark-adapted chromophore. Mutation of Trp-86 to phenylalanine reduces the overall production of protons to ~30% of normal (Mogi et al., 1989), while producing an unusual species apparently related to bR548, a 13-*cis* retinal containing state of bacteriorhodopsin (Rothschild et al., 1989). Recent studies have shown that a significant amount of unusual isomers of retinal is produced by this mutant (Hatanaka et al., 1997). The mutation of Tyr-185 to phenylalanine results in an abnormal and unproductive photocycle at pH 6. At pH 8 the mutant bacteriorhodopsin will pump protons, with a shorter photocycle that seems to be missing the intermediate state M (Jang et al., 1990). It has been suggested that this mutant also generates or utilizes abnormal isomers of retinal (Dunach et al., 1990). Because Trp-182 is a member of the motif and also part of the second aromatic wedge, a mutation in Trp-182 is also of interest. Alteration of Trp-182 to phenylalanine reduces photocycling (Ahl et al., 1988) apparently by reducing the amount of photoisomerization (Rothschild et al., 1989).

Prior molecular orbital calculations have shown that the bacteriorhodopsin protein might contribute electron density to the retinal via HOMO-LUMO interactions (Sakai et al., 1997). However, because the charge transfer was stipulated to occur via a hydrogen bond to the Schiff's base, the possibility that a transient covalent bond might be formed was not considered. Furthermore, in a general sense the protein has been considered as a catalyst for the retinal isomerization (Hermone and Kuczera, 1998), but in these studies no specific covalent catalytic mechanism was invoked. In the work presented here ab initio molecular orbital calculations (Figs. 3 and 4) show explicitly how pericyclic reactions might take part in the isomerization reactions of the retinal in bacteriorhodopsin and the 4-hydroxycinnamoyl adduct of the photoactive yellow protein. Most interestingly, these hypothetical transient reactions provide a clear idea of how the isomerization can be coupled to protein motion and why the time courses of the two isomerizations are different (Chosrowjan et al., 1997, 1998; Changenet et al., 1998). In bacteriorhodopsin the putative reaction forms a cyclobutyl ring, involving the Cτ and Cδ1 carbons of Trp-86 and retinal C13 and C12, the second pair of which are at their closest distance of approach at −147°, before the isomerizing bond has completed one-quarter of its turn. At −105° the retinal C12 is seriously beginning to pull away from its Trp-86 partner. Given this geometry, the transient formation of the cyclobutyl ring, which would both promote isomerization and transmit initial effects of that isomerization directly to the protein, would occur very quickly after excitation, and the entire formation and dissolution of the cyclobutyl intermediate might well occur within the first 500 fs after absorption of the light quantum (Gai et al., 1998).

However, the potential photoactive yellow protein cyclohexenyl intermediate is likely to form when the protein reaction pocket residues are in positions consistent with the *cis* form of the chromophore and when the cinnamoyl adduct's 2-3 bond has fully reached −90°. Here the nascent bonds are 3.5 Å long or less in the absence of any calculation of protein reaction to bond formation. The implication is that, after photon absorption, the protein must relax and the adduct must wander a bit before the transient six-carbon intermediate is suddenly formed. In this case the transient nature of the reaction would be less likely to be due solely to twisting-pulling restrictions of the protein environment and more likely to depend on the requirements of both the interacting phenylalanine and the cinnamoyl adduct to regain aromaticity. The envisioned process is consistent with a fivefold decrease in reaction forward rate over that of bacteriorhodopsin (Chosrowjan et al., 1997). The formation of a six-membered ring transient reaction product is also consistent with the disposition of the aromatic side chains of the reaction product. The aromatic moieties of the photoactive yellow protein can be interdigitated with those of the Diels-Alder antibodies (Fig. 2), which must also promote six-membered ring intermediates, while the pocket aromatics of bacteriorhodopsin form a much smaller cavity (Fig. 1), which is consistent with the smaller size of the postulated transient cyclobutyl product (Fig. 3).

Such a mechanism can relate three seemingly unrelated observations. 1) The core protein structure of bacteriorhodopsin responds to light absorption at least as early as the K intermediate (Kluge et al., 1998), the first intermediate in which the isomerization is known to be complete. In fact, experiments indicate that altered protein interactions modify the transient spectrum of bacteriorhodopsin as early as 200 fs after absorption (Akiyama et al., 1996, 1997). 2) Bacte-

riorhodopsin can be active, even when the chromophore is not covalently bound to the host protein (Schweiger et al., 1994). 3) Isomerization does not seem necessary for the activity of the 4-hydroxycinnamoyl chromophore of the photoactive yellow protein (Cordfunke et al., 1998). Furthermore, bacteriorhodopsin, when reconstituted with a locked C13-C14 bond retinal, exhibits some light-dependent conformational change measurable by atomic force spectroscopy (Rousso et al., 1997), but no photocycle or proton pumping ability (Rousso et al., 1998). Non-isomerizable analogues of retinal were reported to permit photoactivation of photoaxis receptors according to a population migration assay (Foster et al., 1989), but not in cell tracking studies (Lawson et al., 1991; for discussion see Spudich et al., 1995). The results are consistent with the idea that formation of the transient intermediate or formation closely followed by breakup is sufficient for at least partial generation of the signal, without complete isomerization of the adduct. All that is necessary is for the appropriate electron densities to be in apposition. The ability of a non-isomerizable 4-hydroxycinnamoyl moiety to trigger the PYP signal might be expected, because the mechanism proposed above allows time for the protein to rearrange before the intermediate forms, and it is therefore probable that the non-isomerizable adduct can reach an appropriate position. In bacteriorhodopsin with non-isomerizable retinal the atomic force measurement data are consistent with the possibility that illumination leads to some version of the transient intermediate and a consequent conformational change. However, the intermediate would necessarily be an extremely strained one that immediately would fall apart, giving back the original geometry without leading to proton translocation. Interestingly, in these experiments reduction of one bond of the retinal eliminated even the conformational change (Rousso et al., 1998), an effect that is entirely congruent with our hypothesis, because the molecular orbital phases (and also the number of electrons in the retinal LUMO orbital) would be exactly wrong for the formation of the intermediates.

SUMMARY

The remarkable similarity of the active pocket aromatic residue environments in the Diels-Alder antibodies, bacteriorhodopsin, and the photoactive yellow protein supports the hypothesis that the latter two proteins accomplish signal reception via a process involving transient pericyclic reactions. Furthermore, similarities between the putative bacteriorhodopsin pericyclic reaction and the monoene additions of naphthol-anthracenophane support the initial concept that isomerization and chemical addition are related, and that they can both be the basis for quantal response. Explicit molecular orbital calculations of the chromophores and their local aromatic environments in bacteriorhodopsin and the photoactive yellow protein do yield possible transient cyclic reaction products. These are consistent with the known differences in reaction times and with the necessity of physically transmitting information that the signal quantum has been absorbed to the protein in a timely manner. Thus, a story emerges from these studies that indicates that there is probably a pericyclic component to the photoisomerizations of bacteriorhodopsin and the photoactive yellow protein, and that the possibility is real that quantal detection of chemicals was evolutionarily plagiarized from quantal detection of light, or vice versa.

This work also raises questions. First, no non-aromatic mutants of Trp-86, Trp-182, or Tyr-185 have been reported; although, as mentioned above, each has been mutated to phenylalanine with consequent alterations in isomerization and proton pumping. None of these alterations was absolutely incapacitating. However, the reaction pockets discussed here contain four tryptophans, five tyrosines, and three phenylalanines, indicating that, at least in this small four-protein sample, the major requirement for participation in the reaction pocket is aromaticity, rather than being a particular type of aromatic residue. If the hypothesized pericyclic intermediates are important, it would be expected that non-aromatic mutants of each of these amino acids, but particularly of Trp-86, should very seriously alter the course of retinal isomerization and the time course of formation of intermediates in the proton pumping cycle. Changing two or all three of these aromatic residues to non-aromatics would be expected to create an even greater disturbance in isomerization and bacteriorhodopsin function. Similar results could be expected with the photoactive yellow protein. Second, computational approaches to describing Diels-Alder reactions have not been brought to bear in ways that include possible transient chemical interaction with complex environments. As plausible as the photoactive yellow protein's six-carbon intermediate appears, it is not a normally considered reaction product in organic chemistry texts. More importantly, the molecular comparisons presented here indicate that the protein itself, whether bacteriorhodopsin or photoactive yellow protein, is directly involved in the adduct isomerization reactions. All of the molecules investigated here have three regions of aromatics forming the planar reaction pocket, and this work has so far only dealt with one. The roles of the other two remain to be explained. In bacteriorhodopsin there is a strained but close apposition between the C11-C10 bond LUMO electron density and Trp-182 HOMO electron density when the isomerization has reached $\sim -105°$. Thus the possibility arises that one transient intermediate leads to another in a relay fashion. Furthermore, in antibody-catalyzed Diels-Alder reactions where both substrates are exogenous to the protein, it is not obvious how the protein might be directly but transiently involved. We suspect that there is a kind of multiple concerted electron movement, which will also prove to be the case in the chiral auxiliary enhancement of organic reactions. Simulations of such transient intermediate reaction processes should be the focus of ongoing quantum chemical calculations. Finally, this work brings to the fore the interrelated questions of how underlying chemistry

channels evolutionary processes, limiting the universe of accessible proteins, and conversely how protein evolution limits available biological chemical processes. A deeper understanding of these evolutionary constraints may help integrate structural and sequence analyses of relatedness.

Discussions leading to these considerations first occurred in the laboratory of Dr. Jenny Mather, then of Rockefeller University, now of Raven Biotechnologies, Inc., San Carlos, CA.

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