Pigment Organization in Genetically Modified Reaction Centers of *Rhodobacter capsulatus*†

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ABSTRACT: Linear dichroism (LD) spectroscopy and absorption (A) spectroscopy on oriented samples of genetically modified reaction centers (RCs) have been used to investigate whether site-specific mutations change the spatial organization of the RC pigments relative to the protein. Wild-type and mutant RCs from *Rhodobacter capsulatus* were spatially oriented by compression within polyacrylamide gels and then cooled to 10K for spectroscopy. RCs are known to align with their pseudo-*C2* crystallographic axis parallel to the compressed dimension. In order to trap the primary donor (P) in the reduced or in the oxidized state, the RCs were cooled in the dark or under continuous illumination, respectively. Mutations at glutamic acid-L104, which perturb the energy of the Q_X transition of the bacteriopheophytin acceptor (H_L), do not significantly affect the orientation of this pigment relative to the *C2* axis. Similarly, the orientation of the major Q_Y transition of P in the $His^{M200} \rightarrow$ Leu or $His^{M200} \rightarrow$ Phe mutants, which result in the replacement of the special-pair bacteriochlorophyll dimer with a bacteriochlorophyll–bacteriopheophytin heterodimer, is indistinguishable from that of the wild-type. However, the LD and circular dichroism (CD) data do suggest that the electronic coupling is reduced between the two pigments constituting P in this heterodimer. While X-ray crystallographic data are not currently available for *Rb. capsulatus* RCs, comparison of these LD data with data obtained for *Rhodobacter sphaeroides* RCs demonstrates that the pigment organization is essentially the same in both species. Until X-ray structures are determined, LD spectroscopy on oriented RCs will remain an ideal method for assessing possible changes of orientation of the chromophores and/or pleiotropic structural changes after site-specific mutagenesis. Such structural data are essential for interpreting other spectroscopic results for modified RCs, including Stark effect measurements and electron-transfer kinetics.

The high-resolution X-ray structures for the reaction center (RC) from *Rhodopseudomonas viridis* and *Rhodobacter sphaeroides* (Deisenhofer et al., 1984; Michel et al., 1986b; Allen et al., 1987, 1988; Tiede et al., 1988) clearly define amino acid residues in the L and M subunits which interact with the RC chromophores. Many of these residues are conserved between these two species (Michel et al., 1986a; Williams et al., 1983, 1984, 1986) and *Rhodobacter capsulatus* (Youvan et al., 1984). The histidines at L173 and M200, which are positioned within 4 Å of the central Mg atom of each of the bacteriochlorophyll molecules (denoted P_L and P_M) forming the dimeric primary electron donor P, and glutamic acid-L104, located at a suitable distance to form a hydrogen bond with the keto carbonyl of the bacteriopheophytin associated with the L subunit (H_L), are among these conserved residues. Although the crystal structure of RCs from *Rb. capsulatus* has not been determined, the similarities between these three species facilitate mutagenesis experiments in *Rb. capsulatus*, which possesses a well-characterized genetic system (Scolnick & Marrs, 1987).

RCs bearing mutations at three different residues (His^{L173}, His^{M200}, and Glu^{L104}) positioned near pigments have been constructed and characterized for a variety of physical–chemical phenotypes, including pigment composition, absorption spectra, and primary electron-transfer kinetics (Bylina & Youvan, 1988; Bylina et al., 1988a,b; Kirmaier et al., 1988). Histidine-M200, which binds the Mg atom of the P_M bacteriochlorophyll, has been replaced with leucine ($His^{M200} \rightarrow$ Leu) and phenylalanine ($His^{M200} \rightarrow$ Phe). These mutations result in a primary electron donor where P_M is apparently replaced by a bacteriopheophytin, thus converting P into a heterodimer of bacteriochlorophyll and bacteriopheophytin (Bylina & Youvan, 1988). Glutamic acid-L104 has been replaced with glutamine ($Glu^{L104} \rightarrow$ Gln) or leucine ($Glu^{L104} \rightarrow$ Leu). Absorption studies at 77 K suggest that this glutamic acid residue is primarily responsible for the red-shift of the Q_X absorption band of H_L compared to that of the symmetric H_M pigment (Bylina et al., 1988b).

In structure/function studies, it is important to confirm that site-specific mutations do not cause global structural changes, such as unfolding of the protein. This information is best obtained through crystallography or 2D NMR of the genetically modified protein. However, in chromophoric proteins such as RCs, a variety of spectroscopic techniques are available for assessing structural aspects of the protein, such as the relative orientation of the optical transition moments of prosthetic pigments relative to the *C2* axis of the protein. In fact, the bacteriochlorophyll and bacteriopheophytin molecules can be used as "built-in" reporters for protein folding. In this work, we have used optical techniques which are sensitive to the angles between each pigment and the protein to rule out gross structural perturbations of genetically modified proteins. In addition, spectra of mutants have been obtained at lower temperatures (10 K) than previously reported. Since most absorption bands become sharper at cryogenic temperatures, these studies have led to a clearer interpretation of the effects of mutagenesis, particularly in the interpretation of the red-

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shift of the Q_X band for the photoactive bacteriopheophytin.

Materials and Methods

RCs from *Rb. capsulatus* were purified with a previously described DEAE chromatography method (Prince & Youvan, 1987; Bylina & Youvan, 1988). RC-containing chromatophores were diluted to an A_{875} of 7–8 and were solubilized in 0.3–0.5% lauryldimethylamine oxide (LDAO). After elution from the column, RCs were dialyzed against 10 mM potassium phosphate (pH 7.4)/0.05% LDAO and frozen in liquid N_2 before storage at −80 °C. Wild-type RCs were isolated from a pU2922-containing U43 derivative, which is a plasmid-complemented deletion background (Bylina & Youvan, 1987; Bylina et al., 1988c).

RCs were polymerized in polyacrylamide gels and oriented by uniaxial squeezing of the gels (Abdourakhmanov et al., 1979). RCs were added to a gel-forming mixture, with final concentrations of 60% glycerol, 10% acrylamide, 0.3% bis-(acrylamide), 0.03% tetramethylethylenediamine, 0.05% ammonium persulfate, and 5 mM Tris (pH 7.8), which was polymerized at 4 °C. The gel samples were cooled in a cryostat operating with a temperature-regulated flow of helium gas. The cooling from 290 to 10 K, which takes about 30 min, was achieved either in the dark or under continuous illumination (about 30 mW cm^{-2} with a 2-mm RG715 Schott glass filter). The recording of the absorption (A) and LD spectra, always performed in the absence of actinic light, was as described (Breton, 1985). For a given piece of oriented gel, A and LD spectra were recorded alternatively following cooling either in the light or in the dark. The fluctuations observed in the magnitudes of the signals measured under identical cooling conditions were typically 5 and 10% for the A and LD spectra, respectively. Thus, the calculated ΔA and ΔLD spectra are affected by this uncertainty. For presentation, the A and LD spectra are normalized to unity at their maximum around 800 nm, and the ΔA and ΔLD spectra are normalized around 870 nm.

Circular dichroism spectra were obtained at room temperature on a JASCO J-500C spectropolarimeter using 1-cm cells. Wavelength calibration was done with neodynium glass at 586 nm. Sodium ascorbate (1 mM) was added to the RC samples to prevent photooxidation by the measuring light.

Results

The absorption (A), linear dichroism (LD), and anisotropy (LD/A) spectra for RCs of *Rb. capsulatus* WT cooled to 10 K either in the dark (Figure 1a) or under illumination (Figure 1b) are very similar to the corresponding spectra obtained for reduced or oxidized RCs from *Rb. sphaeroides* (Breton, 1985, 1988). This observation strongly suggests that both the orientation within the gel and the orientation of the pigments within each RC is the same in the two organisms. In the case of *Rb. sphaeroides* and *Rps. viridis*, the X-ray studies have demonstrated the presence of a pseudo-*C2* symmetry axis running from the center of P through the ferrous iron (Deisenhofer et al., 1984; Allen et al., 1987, 1988; Tiede et al., 1988). It has been further shown in vivo that this *C2* axis is normal to the plane of the photosynthetic membrane (Breton, 1985; Breton & Nabedryk, 1987; Breton et al., 1987; Yeates et al., 1987). For RCs from *Rps. viridis* oriented in uniaxially compressed polyacrylamide gels, the *C2* axis is oriented along the compression axis (Breton, 1985), and thus the LD/A can give a measure of the angle ϕ of each transition moment with respect to the *C2* axis. The same conclusion can also be derived for the RCs of *Rb. sphaeroides* upon noting the close similarity between the room temperature LD spectra

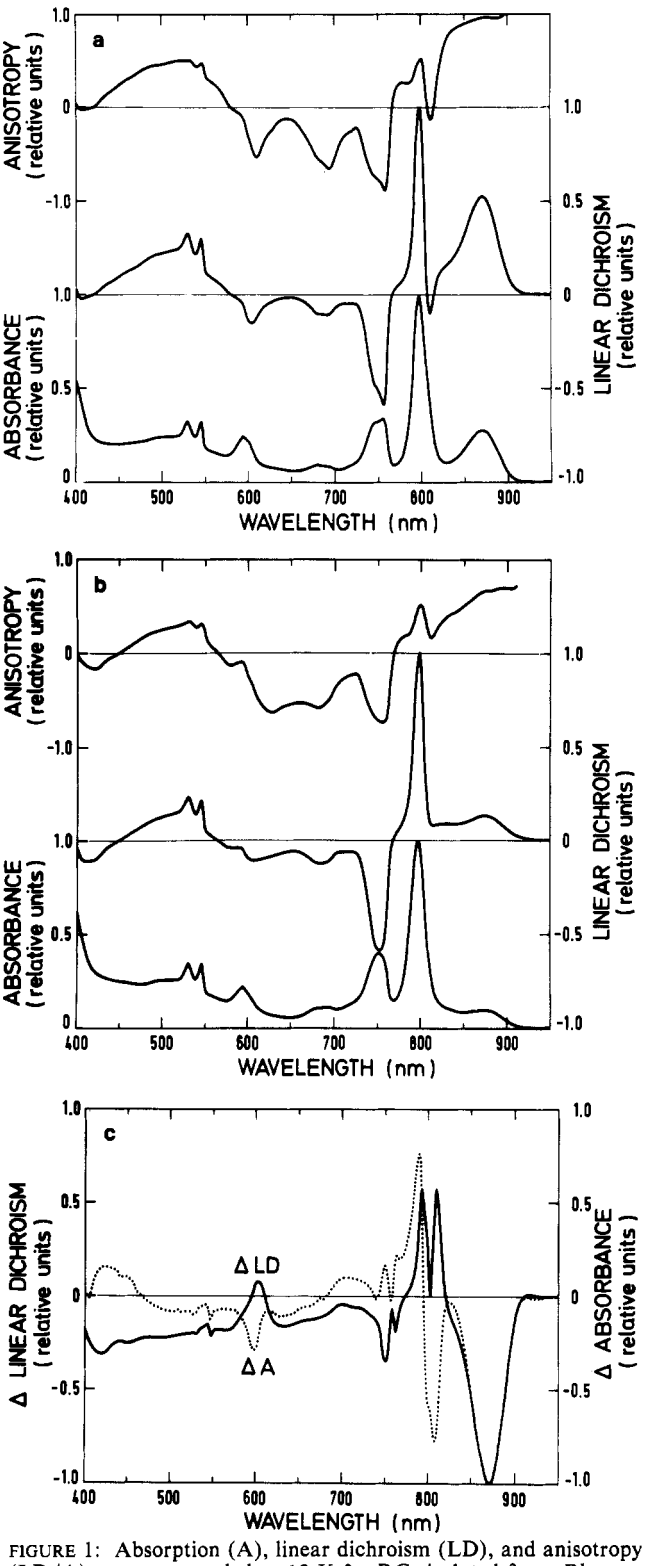

FIGURE 1: Absorption (A), linear dichroism (LD), and anisotropy (LD/A) spectra recorded at 10 K for RCs isolated from *Rb. capsulatus* wild-type that are embedded in a squeezed polyacrylamide gel. The RCs were cooled either in the dark (panel a) or in the light (panel b) in order to achieve a reduced or oxidized state of the primary donor, respectively. The absorbance at 800 nm was about 0.5 at 290 K. (Panel c) Oxidized-minus-reduced difference absorption spectrum (···) and linear dichroism spectrum (—).

for air-dried membranes containing RCs (Verméglio & Clayton, 1976; Rafferty & Clayton, 1979; Nabedryk et al., 1982) and of RCs oriented in polyacrylamide gels (Abdourakhmanov et al., 1979). The observation that the low-temperature LD and LD/A spectra of RCs from *Rb. sphaeroides* (Breton, 1985, 1988) and *Rb. capsulatus* (Figure 1) are very

similar is thus supporting evidence for a close analogy of the RCs in these two species and suggests the existence of a *C2* symmetry axis oriented along the gel compression axis in the RC of the latter organism. It will be shown in the following experiments that the shape of the LD bands in several spectral regions, notably around 755 and 800 nm and in the absorption bands of the carotenoid, is the same for RCs isolated from the WT or from the mutant strains of *Rb. capsulatus*. Furthermore, for a given compression of the gel, the LD/A magnitudes are also quite comparable (data not shown). These observations can thus be taken to indicate that a uniaxial orientation of the *C2* axis along the compression axis is also present for the RCs of the *Rb. capsulatus* mutants.

At 10 K, the main Q_Y transition of P is located at 870–875 nm in reduced RCs from *Rb. capsulatus* (Figure 1a), while it is close to 895 nm in *Rb. sphaeroides* (Breton, 1988). Furthermore, the amplitude of this band relative to the 800-nm band is slightly reduced compared to the situation in *Rb. sphaeroides* RCs. These differences appear to be related to some minor alterations of the geometry and/or environment of the chromophores during purification of RCs from *Rb. capsulatus*. This is demonstrated by measuring A and LD spectra as well as ΔA and ΔLD spectra at 10 K for chromatophores isolated from the $His^{LHI\alpha32} \rightarrow Asn$ mutant of *Rb. capsulatus* (data not shown), which contains WT RCs but lacks both the B800–850 and B875 antennae (Bylina et al., 1988c). These spectra indicate that upon isolation of the RC, the main band of P shifts from 895 to 870–875 nm, while the 800-nm band shifts from 802–803 to 797–798 nm, and the band around 750 nm shifts to the red by about 3 nm. Apart from these shifts, the orientation of all the chromophores, estimated by comparing the ΔA and ΔLD spectra, is essentially unperturbed by the isolation procedure.

Analogous to the situation in RCs from *Rb. sphaeroides* (strains 241 and R26) (Breton, 1988), the absorption spectrum in Figure 1a shows a nonsymmetrical 800-nm band, indicating the presence of a component absorbing around 810 nm to which is associated a prominent negative LD band. The bacteriopheophytin Q_Y transitions show peaks at 757 and 747 nm for the molecules assigned to H_L and H_M, respectively. The dichroism, negative for both, is slightly larger for H_L than for H_M, leading to angles of 30° ± 5° (H_L) and 35° ± 5° (H_M), when one assumes that the angle of the main Q_Y transition of P with the *C2* axis is 90°, as reported for the RCs of *Rps. viridis* (Paillotin et al., 1979) and of *Rb. sphaeroides* (Verméglio & Clayton, 1976; Rafferty & Clayton, 1979). In the Q_X regions, the H_L and H_M transitions absorb at 545.5 and 529.5 nm, respectively, and are both oriented at 60–75° from the *C2* axis. The broad carotenoid band around 500 nm exhibits positive LD corresponding to an orientation rather perpendicular ($\phi > 70°$) to the *C2* axis, as previously reported for *Rb. sphaeroides* 241 and *Rps. viridis* RCs (Breton, 1985). The ΔA and ΔLD spectra (Figure 1c) clearly show the bleaching of the main components of P at about 870 and 600 nm, with an orientation essentially perpendicular and parallel to the *C2* axis, respectively. The blue-shift of the 800-nm band is associated with a positive LD, while at least a large fraction of the bleaching observed around 810 nm corresponds to a negative LD signal, which is thus assigned to the high-energy exciton component of P (P_{Y+}). In the LD spectrum of the RCs cooled under illumination (Figure 1b), the components at 595 and 600 nm are assigned to the Q_X transitions of the two B molecules. As shown by examination of the LD/A spectrum (Figure 1b), the 595-nm transition exhibits a larger LD (positive) than the 600-nm component. All these components,

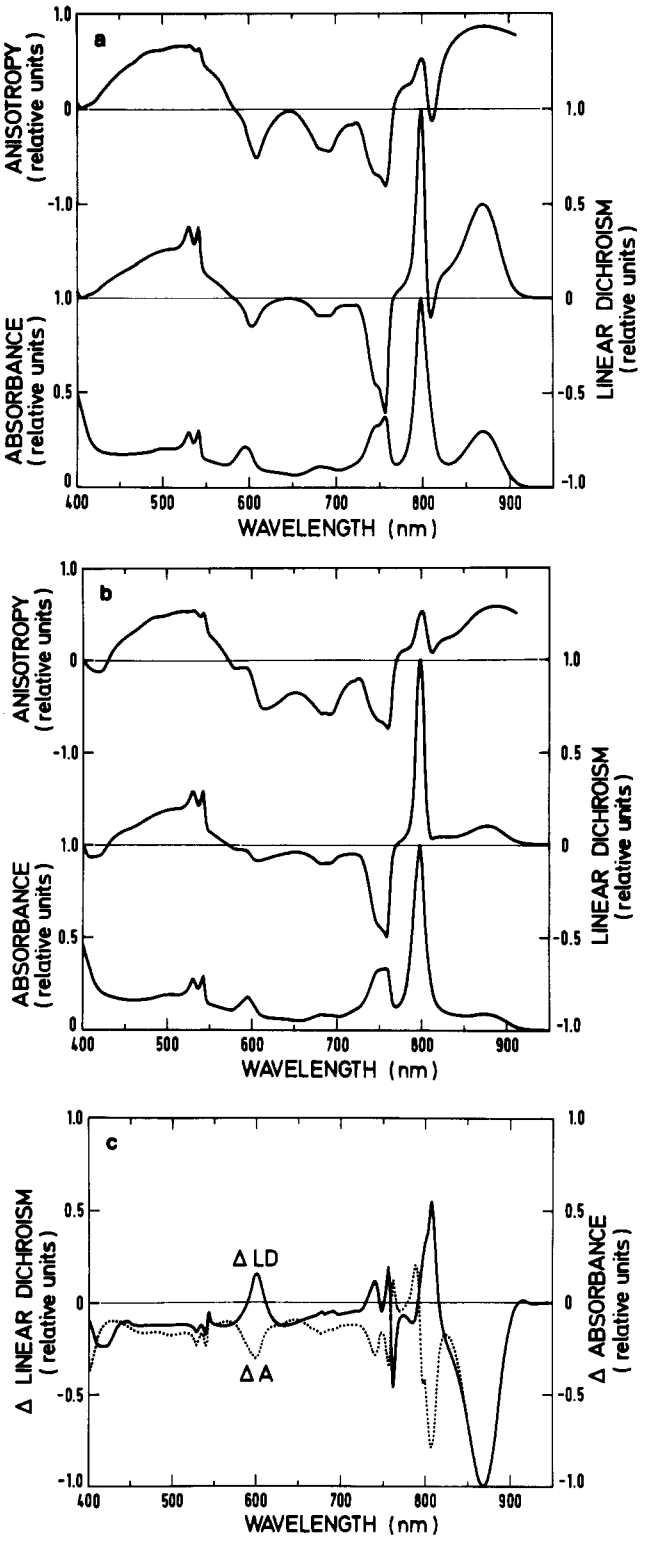

FIGURE 2: Same conditions as Figure 1, but for RCs isolated from a mutant of *Rb. capsulatus* in which the glutamic acid residue L104 has been replaced by glutamine ($Glu^{L104} \rightarrow Gln$).

with identical associated dichroisms, have been previously described and assigned in RCs from *Rb. sphaeroides* (Breton, 1988).

The absorption and LD spectra of RCs from the $Glu^{L104} \rightarrow Gln$ and $Glu^{L104} \rightarrow Leu$ mutants are shown in Figures 2 and 3, respectively, for samples cooled either in the dark (Figures 2a and 3a) or under illumination (Figures 2b and 3b). In the region of absorption of the four bacteriochlorophylls, these spectra closely correspond to the respective ones of the WT RC, with the exception of a Q_X band in the $Glu^{L104} \rightarrow$

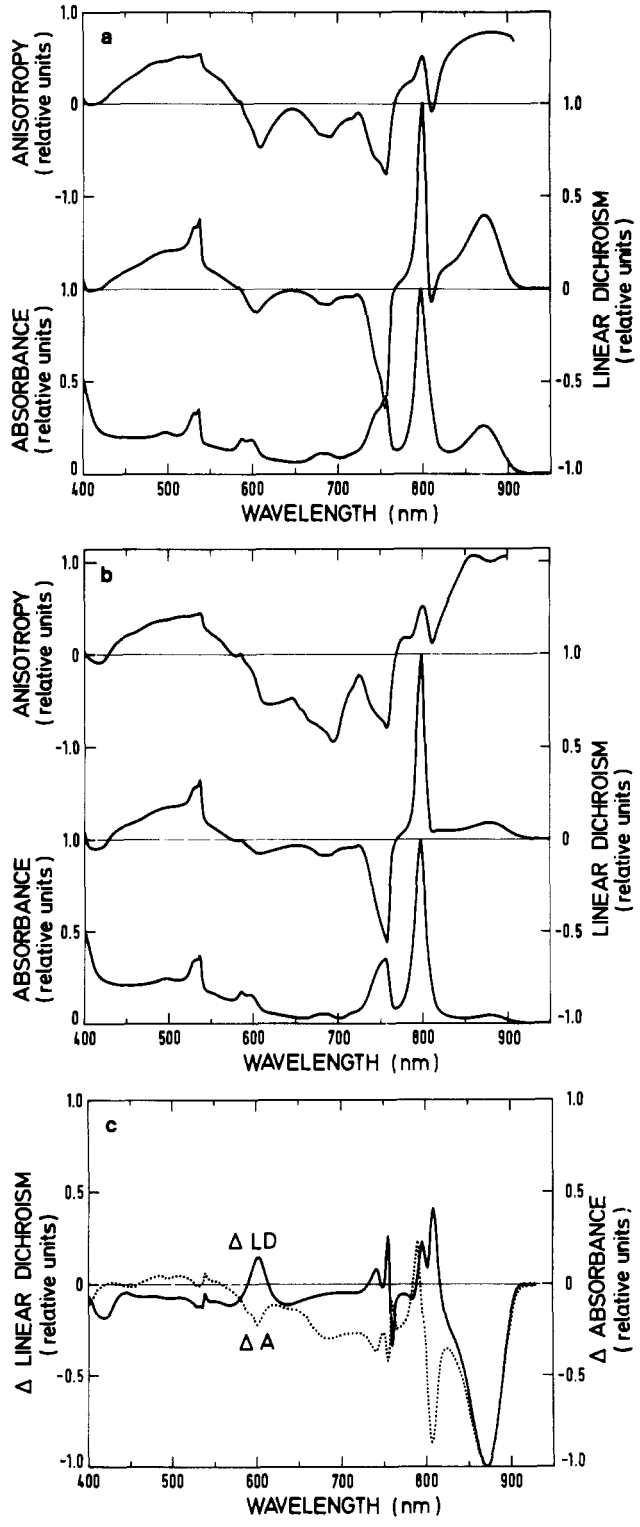

FIGURE 3: Same conditions as in Figure 2, but the residue L104 has been replaced by leucine ($Glu^{L104} \rightarrow Leu$).

Leu mutant which appears at 587 nm. This band is actually seen as a minor shoulder in the spectra of RCs from both $Glu^{L104} \rightarrow Gln$ (Figure 2) and WT (Figure 1). Although changes in the bacteriochlorophyll Q_X bands have been observed in a number of other RC mutations (Bylina and Youvan, unpublished observations), the cause of these changes remains unclear. In addition, we have consistently observed that the magnitude of the ΔA signal resulting from the blue-shift of the 800-nm band is smaller in the Glu^{L104} mutants compared to that in the WT. This might be due to a slight modification of the conformation of the more polarizable

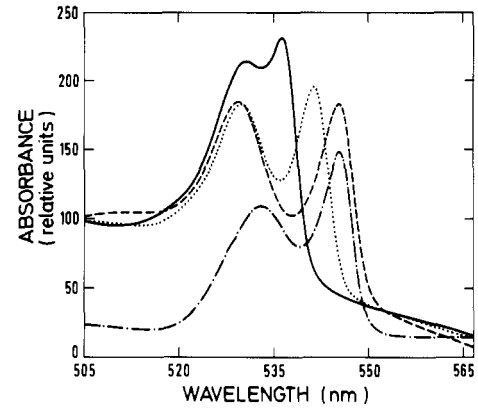

FIGURE 4: Absorption spectra at 10 K in the Q_X regions of the two bacteriopheophytins for RCs isolated from *Rb. capsulatus* wild-type (---), $Glu^{L104} \rightarrow Gln$ (···), and $Glu^{L104} \rightarrow Leu$ (—). The spectrum (–·–) of RCs from *Rb. sphaeroides* R-26 is redrawn from Breton (1988).

residues located in the vicinity of the P and/or B molecules.

As expected, the main differences related to the site-directed mutagenesis of Glu^{L104} are observed in the region of absorption of the H_L molecule. In the Q_Y region, an increase in the asymmetry of the 757-nm peak is visible, with the long-wavelength peak increasing relative to the 747-nm shoulder when going from WT to $Glu^{L104} \rightarrow Gln$ to $Glu^{L104} \rightarrow Leu$. In the Q_X region (Figure 4), the peak position of the H_M band stays constant at 529.5 nm while that of the H_L band shifts from 545.5 nm in WT to 541.5 nm in $Glu^{L104} \rightarrow Gln$ and to 536.5 nm in $Glu^{L104} \rightarrow Leu$, in agreement with a previous report for spectra at 77 K (Bylina et al., 1988b). However, at 10 K, the splitting of the two Q_X bands in the RC of $Glu^{L104} \rightarrow Leu$ is clearly resolved. Furthermore, the difference in shape of these two bands (i.e., H_M is significantly broader than H_L), which is present in RCs from *Rb. sphaeroides* and *Rb. capsulatus* WT (Figure 4), is also maintained after the mutations. In addition, the shape of the LD/A spectra in the range 520–560 nm (Figures 1–3) indicates that the mutations induce no detectable variation of the tilt of the Q_X transition of H_L with respect to the *C2* axis.

The A, LD, and LD/A spectra for RCs from the $His^{M200} \rightarrow Leu$ mutant of *Rb. capsulatus* cooled either in the dark or under illumination are shown in Figure 5a and Figure 5b, respectively. Around 755 nm, in the Q_Y region of the bacteriopheophytins, the spectra of the reduced sample (Figure 5a) are almost identical with those of the RCs from WT. A strikingly different situation is observed at longer wavelengths, as previously reported for room temperature spectra (Bylina & Youvan, 1988). At 10 K, the broad band assigned to the heterodimer extends from 820 nm to about 1000 nm and acquires some structure notably around 850 nm. The large positive LD in this region leads to an LD/A value (relative to that near 755 nm) close to the LD/A measured for the 870-nm band in RCs from WT (Figure 1a). The main absorption band peaks at 794 nm and exhibits a small shoulder at 802 nm, with both of these features exhibiting positive LD. In this region, the LD/A spectrum shows very little structure and increases smoothly from its negative minimum at 756 nm to its maximum at wavelengths larger than about 850 nm, with no indication of the pronounced trough observed around 810 nm in the spectra of RCs from either WT (Figure 1a) or the Glu^{L104} mutants (Figures 2a and 3a).

In the region of the Q_X transitions, the absorption spectrum at 10 K (Figure 5a) confirms the one previously reported at 77 K (Bylina & Youvan, 1988) with notably the presence of two bands at 582 and 599 nm. Also, the 545.5-nm band has

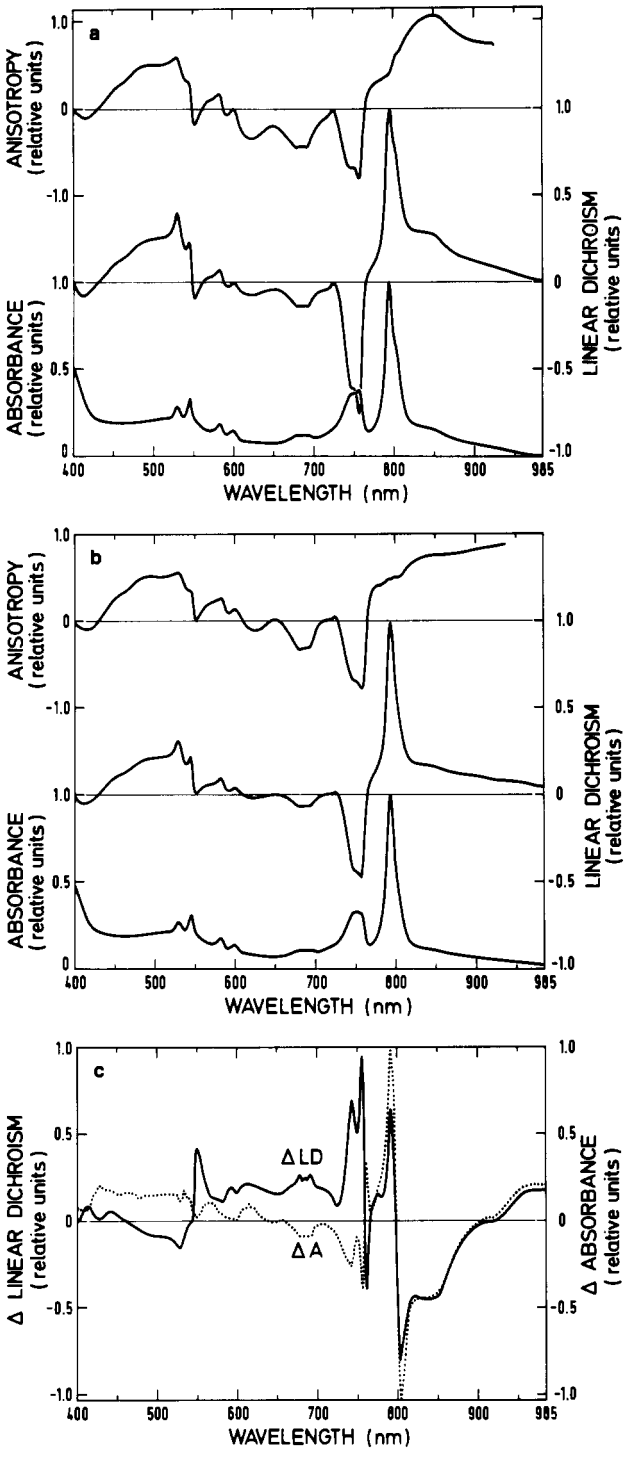

FIGURE 5: Same conditions as in Figure 1, but for RCs isolated from a mutant of *Rb. capsulatus* in which the histidine residue M200 has been replaced by a leucine ($His^{M200} \rightarrow Leu$).

more amplitude than the 530-nm band, while in the spectra shown in Figures 1–3 these two bands have approximately equal amplitude. These four bands all exhibit positive LD (Figure 5a). The main difference between the LD spectra of WT and of $His^{M200} \rightarrow Leu$ is the absence in the latter of the large negative LD signal at 600 nm. Instead, a large negative signal is observed at 551 nm. In the region 520–570 nm, the LD and LD/A spectra (Figure 5a) suggest the overlap of two bands peaking at about 545 and 550 nm with the latter exhibiting a negative LD, while the former has a positive LD.

Upon illumination during cooling, a bleaching of about 50% of the long-wavelength ramp and an apparent blue-shift of the 802-nm band to about 794 nm are observed (Figure 5b). The

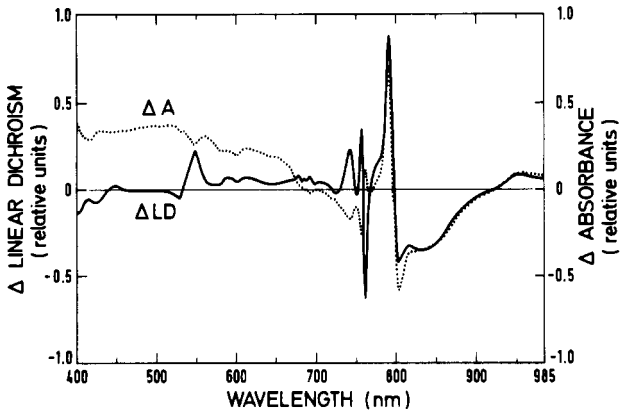

FIGURE 6: Same conditions as in Figure 5c, but the residue M200 has been replaced by a phenylalanine ($His^{M200} \rightarrow Phe$).

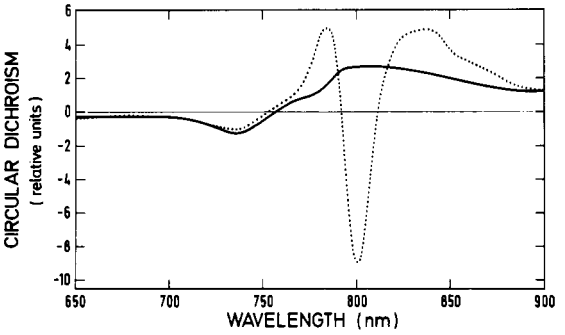

FIGURE 7: Circular dichroism spectra recorded at 290 K for a suspension of RCs isolated from *Rb. capsulatus* wild-type (···) and its $His^{M200} \rightarrow Leu$ mutant (—) with the primary donor reduced. Each of the RC samples (in a 1-cm cell) had an absorbance at 800 nm of 0.9.

remaining 802-nm band in the absorption spectrum in Figure 5b seems to correspond to the 50% of RCs in which P has not bleached. The main band at 794 nm appears little affected by the charge separation. The ΔA and ΔLD spectra (Figure 5c) show in addition the red-shift of the H_L molecule around 760 nm and the bleaching of a small band at 548 nm to which is associated a pronounced negative ΔLD. Although our measurement is not optimized for wavelengths >950 nm, we have consistently observed that ΔA and ΔLD were null around 900–920 nm and increased significantly at longer wavelengths (Figure 5c). Such an absorbance increase, which we assign to P^+, is not observed in this spectral range for the other RCs. For $His^{M200} \rightarrow Phe$, the absorption, LD, and LD/A spectra for reaction centers cooled in the dark or under illumination are almost indistinguishable (data not shown) from the corresponding spectra of the $His^{M200} \rightarrow Leu$ mutant. This is also demonstrated by comparison of the ΔA and ΔLD spectra (Figure 6) with those shown in Figure 5c.

In order to assess the coupling of the pigments within the heterodimer, the CD spectrum at room temperature of $His^{M200} \rightarrow Leu$ RCs has been compared to that of the WT (Figure 7). In the Q_Y absorption region, the CD spectrum of RCs from *Rb. capsulatus* WT exhibits bands at 735 nm (−), 784 nm (+), 801 nm (−), and 835 nm (+). These bands closely correspond in shape and relative intensity to those observed in RCs from *Rb. sphaeroides* R26 at 735, 788, 804, and 850 nm, respectively (Sauer et al., 1968; Reed & Ke, 1973; Shuvalov et al., 1986). The shifts observed in the position of these bands between the two types of RCs are consistent with those found in the room temperature absorption spectra (data not shown). Although the small negative CD band at 735 nm is well preserved in the CD spectrum of RCs from the His^{M200}

→ Leu mutant, the three other main bands present in the spectrum of WT RCs are drastically altered, being essentially replaced by a broad and quasifeatureless band with positive CD extending from 780 nm to wavelengths >900 nm (Figure 7). This observation is taken to indicate a reduced coupling of the pigments in the heterodimer–containing RCs.

Discussion

Rb. capsulatus WT. Recently, the orientation of the chromophores in RCs isolated from *Rb. sphaeroides* WT (strain 241) and its carotenoidless derivative R26 as well as in borohydride–treated RCs from the latter strain has been investigated by using the same experimental approach as described here (Breton, 1988). We have shown that the orientation of the two main transitions of P at 600 nm (P_{X+}) and 895 nm (P_{Y-}), as well as the orientation of the X and Y directions for each of the four monomeric pigments B_L, B_M, H_L, and H_M, could be determined by analyzing the A and LD spectra at 10 K of the RCs cooled either in the dark (primary donor in the state P) or in the light (state P^+). Furthermore, from the shape of the difference absorption (ΔA) and the difference linear dichroism (ΔLD) spectra, it was concluded that three major overlapping spectral features contribute to the absorption changes in the range 795–815 nm: a bleaching around 810 nm assigned to the high-energy (P_{Y+}) exciton component of P and the blue-shift of the Q_Y transitions of B_L and B_M absorbing around 800 and 810 nm, respectively. Calculations by Parson and Warshel (1988) have shown that slight alterations of the geometry of P in *Rps. viridis* RCs could induce a large shift of the long-wavelength band of P. For example, a 22-nm shift of the 960-nm band could be generated either by a variation of 0.1 Å of the distance between the two bacteriochlorophylls in P or by a rotation of 10–20° of the acetyl group on these molecules. Thus, the shift of the long-wavelength absorption band observed upon isolation of the RC from *Rb. capsulatus* could be induced by very small changes in the geometrical organization of the P_L and P_M chromophores during the purification process. Such geometrically induced shifts are also likely to be responsible for the differences in the shape of the ΔA and ΔLD spectra, notably in the congested region extending from 795 to 815 nm, which are observed when comparing the spectra of isolated RCs from *Rb. capsulatus* WT with either those of chromatophores from *Rb. capsulatus* $His^{LH1\alpha32}$ → Asn or those of RCs from *Rb. sphaeroides* (Breton, 1988).

The close similarity between the orientation of the transition moments of all the chromophores in the RCs of *Rb. capsulatus* WT and of *Rb. sphaeroides* 241 demonstrates that the organization of the bacteriochlorophyll, bacteriopheophytin, and carotenoid pigments is essentially the same in these two systems. This conclusion is further strengthened by the resemblance of the CD spectra of RCs from these two species. Together with the homology of amino acid sequences of the L and M polypeptides for the two organisms (Youvan et al., 1984; Williams et al., 1986), this is strong evidence that the X-ray structure of the RC from *Rb. sphaeroides* is an appropriate model for the *Rb. capsulatus* RC. Thus, the LD spectra reported here for *Rb. capsulatus* can be interpreted in terms of the tilt angle ϕ of each detectable transition with respect to the *C2* axis.

Glutamic Acid-L104 Mutations. Mutations at this residue essentially perturb the energy of the Q_X transition of H_L but not its orientation. The band shifts from 545.5 nm in WT to 541.5 nm in Glu^{L104} → Gln and to 536.5 nm in Glu^{L104} → Leu. Considering that the M branch residue homologous to Glu^{L104} is a nonpolar valine at position M131 (Tiede et al., 1988), the observation that the Q_X transition of H_M for RCs from *Rbs. capsulatus* WT and from all the presently investigated mutants absorbs at 529.5 nm indicates that in vivo a large fraction of the red-shift of the Q_X transition of H_L is indeed due to the bonding interaction between H_L and Glu^{L104} (Bylina et al., 1988b). However, the remaining 7-nm splitting between the Q_X transitions of H_L and H_M in the Glu^{L104} → Leu mutant shows that other interactions also participated in the red-shift of the Q_X transition of H_L in vivo. Furthermore, the difference in width of the two Q_X bands (i.e., H_L sharp and H_M broad) is maintained in the Glu^{L104} → Leu mutant and thus cannot be ascribed to the difference in the bonding interaction at the keto carbonyls. This difference of width of the two Q_X transitions is conserved in RCs from *Rb. sphaeroides* and *Rps. viridis* (Breton, 1985). The report of electron transfer along the L branch in Glu^{L104} → Leu RCs (Bylina et al., 1988b) suggests that this difference in width of the two Q_X transitions could reflect some intrinsic contribution to unidirectionality of electron transfer. One can further speculate that the difference in width is to be ascribed at least in part to the looser structure (dynamic freedom and/or static disorder) of the chromophores in the M branch compared to the L branch, which is demonstrated by analyzing the R factors of the pigments in the two branches (J. Deisenhofer, personal communication). Finally, it is worth noting that the shift of the Q_X of H_M from 529.5 nm in *Rb. capsulatus* RCs to 533 nm in *Rb. sphaeroides* (Figure 4) is consistent with the presence in the latter of a more polar threonine residue at the appropriate distance for hydrogen bonding to the keto carbonyl of H_M (Tiede et al., 1988).

Histidine-M200 Heterodimer Mutations. The pigment composition (three bacteriochlorophylls and three bacteriopheophytins) together with the altered absorption spectrum and the position of the mutation has led to the conclusion that the primary donor in RCs from both His^{M200} → Leu and His^{M200} → Phe is no longer a dimer of bacteriochlorophylls but is replaced by a heterodimer of bacteriochlorophyll and bacteriopheophytin (Bylina & Youvan, 1988). Although the quantum yield of initial charge separation is reduced by a factor of about 2 compared to the WT, the RC from this mutant still performs photochemistry along the L branch (Kirmaier et al., 1988). The shape of the main band of P in the heterodimer spectra is considerably altered compared to that of the WT, but the orientation of this transition (preferentially perpendicular to the *C2* axis) is remarkably conserved. Integration of the absorption band region from 820 to 985 nm indicates that the oscillator strength of P in this region is reduced by no more than 30% compared to WT, a decrease which can be assigned to the lower oscillator strength of bacteriopheophytin compared to bacteriochlorophyll (Kirmaier et al., 1988). The larger spread in energy of this band compared to the 870-nm transition of RCs from WT could be due to increased fluctuations of the distance separating the two halves of the heterodimer and/or to larger contributions from charge-transfer states which can be expected due to its intrinsic electronic asymmetry (Petke & Maggiora, 1986). In the 800-nm region, the shape of the absorption band with its long-wavelength shoulder is reminiscent of that observed in RCs from WT *Rb. capsulatus* (Figure 1a) and from *Rb. sphaeroides* (Breton, 1988). In the spectrum of the latter, this shoulder has been assigned to contributions both from the B_M molecule and from P_{Y+} (Breton, 1988). In the heterodimer RCs, the absence of a negative LD component in this region is taken to indicate that the coupling between the two pigments of P is smaller, as previously suggested (Kirmaier et al., 1988),

and as illustrated by the small magnitude of the CD signals in the region where P absorbs (Figure 7). However, a change in the geometry of the dimer, with the two Q_Y transition moments of the monomers in P more parallel than in the WT, could also lead to the disappearance of both the 810-nm P_{Y+} component and the S-shaped CD signal. An absorption band around 1240 nm has been specifically assigned to the bacteriochlorophyll dimer radical cation in the P^+-minus-P spectrum of *Rb. sphaeroides* RCs (Dutton et al., 1975). For monomeric bacteriochlorophyll *a* in vitro, the radical band is located around 870 nm (Fajer et al., 1975), and the corresponding Q_Y transition has been shown to keep the same polarization as for the neutral species (Otten, 1971). Thus, the positive ΔA and ΔLD signals observed for wavelengths >900–920 nm for RCs from the $His^{M200} \rightarrow$ Leu mutant, which we tentatively assign to the monomer-like radical cation of the red-shifted bacteriochlorophyll within the heterodimer, also point to a smaller coupling between the two partners of the special pair in this mutant RC compared to WT.

The orientation of the main transition of the dimer and the B and H molecules appear remarkably conserved in the His^{M200} mutants. In the Q_X region of the bacteriochlorophylls, the two bands at 582 and 599 nm seem to belong mainly to the two B molecules rather than to P, as they do not bleach appreciably when P^+ is generated and as they exhibit the small positive dichroism characteristic for these bands. A wavelength of 582 nm is unusually low for bacteriochlorophyll *a* in situ and might reflect an altered environment of one of the B molecules. For bacteriochlorophyll *a* in vitro, this wavelength is characteristic of a 5-coordinated state of the central magnesium atom with oxygen as a fifth ligand (Callahan & Cotton, 1987). The presence in the RC structure of *Rps. viridis* at 2.3-Å resolution of two water molecules in the vicinity of P, one bridging His^{M200} and B_L, while the other is located in between His^{L173} and B_M (Deisenhofer & Michel, 1988), makes it conceivable that the replacement of His^{M200} by a leucine or a phenylalanine modifies the bonding of B_L. We further note that no change is observed in the orientation of the carotenoid, which we putatively locate close to B_M by analogy with its situation in *Rb. sphaeroides* (Allen et al., 1988) and in *Rps. viridis* (Deisenhofer & Michel, 1988).

One of the new important observations in the present work is the finding that the transition of the heterodimer at 548 nm, which bleaches upon P^+ formation (Figures 5c and 6; Bylina & Youvan, 1988; Kirmaier et al., 1988), is oriented rather close to the *C2* axis. Although the overlap of the 548-nm transition with the 545-nm Q_X transition of H_L precludes the determination of ϕ from the spectra in Figure 5a, a comparison of ΔA and ΔLD (Figures 5c and 6) shows that the bleaching of the 548-nm transition is more dichroic than the signal assigned to the red-shift of the Q_Y transition of H_L. This is compatible with a ϕ value of $<30°$ for the 548-nm transition, which is an angle comparable to that of the P_{X+} band of P at 600 nm in WT RCs. Thus, the linear dichroism data indicate similar geometries for the angle between the *C2* axis and the main transitions of P in both normal and heterodimer-containing RCs.

Registry No. Bacteriochlorophyll *a*, 17499-98-8.

References

Abdourakhmanov, I. A., Ganago, A. O., Erokhin, Y. E., Solov'ev, A. A., & Chugunov, V. A. (1979) *Biochim. Biophys. Acta 546*, 183–186.

Allen, J. P., Feher, G., Yeates, T. O., Komiya, H., & Rees, D. C. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5730–5734.

Allen, J. P., Feher, G., Yeates, T. O., Komiya, H., & Rees, D. C. (1988) *NATO ASI Ser., Ser. A 149*, 5–11.

Breton, J. (1985) *Biochim. Biophys. Acta 810*, 235–245.

Breton, J. (1988) *NATO ASI Ser., Ser. A* 149, 59–69.

Breton, J., Deprez, J., Tavitian, B. A., & Nabedryk, E. (1987) in *Progress in Photosynthesis Research* (Biggins, J., Ed.) Vol. I, pp 387–394, Martinus Nijhoff Publishers, The Hague, The Netherlands.

Bylina, E. J., & Youvan, D. C. (1987) *Z. Naturforsch. 42C*, 769–774.

Bylina, E. J., & Youvan, D. C. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7226–7230.

Bylina, E. J., Jovine, R., & Youvan, D. C. (1988a) *NATO ASI Ser., Ser. A 149*, 113–118.

Bylina, E. J., Kirmaier, C., McDowell, L., Holten, D., & Youvan, D. C. (1988b) *Nature (London) 336*, 182–184.

Bylina, E. J., Robles, S., & Youvan, D. C. (1988c) *Isr. J. Chem. 28*, 73–78.

Callahan, P. M., & Cotton, T. M. (1987) *J. Am. Chem. Soc. 109*, 7001–7007.

Deisenhofer, J., & Michel, H. (1988) *NATO ASI Ser., Ser. A 149*, 1–3.

Deisenhofer, J., Epp, O., Miki, K., Huber, R., & Michel, H. (1985) *Nature (London) 318*, 618–624.

Dutton, P. L., Kaufmann, K. J., Chance, B., & Rentzepis, P. M. (1975) *FEBS Lett. 60*, 275–280.

Fajer, J., Brune, D. C., Davis, M. S., Forman, A., & Spaulding, L. D. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4956–4960.

Kirmaier, C., Holten, D., Bylina, E. J., & Youvan, D. C. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7562–7566.

Michel, H., Weyer, K. A., Gruenberg, H., Dunger, I., Oesterhelt, D., & Lottspeich, F. (1986a) *EMBO J. 5*, 1149–1158.

Michel, H., Epp, O., & Deisenhofer, J. (1986b) *EMBO J. 5*, 2445–2451.

Nabedryk, E., Tiede, D. M., Dutton, P. L., & Breton, J. (1982) *Biochim. Biophys. Acta 682*, 273–280.

Otten, H. A. (1971) *Photochem. Photobiol. 14*, 589–596.

Paillotin, G., Verméglio, A., & Breton, J. (1979) *Biochim. Biophys. Acta 545*, 249–264.

Parson, W. W., & Warshel, A. (1987) *J. Am. Chem. Soc. 109*, 6152–6163.

Petke, J. D., & Maggiora, G. M. (1986) *J. Chem. Phys. 84*, 1640–1652.

Prince, R. C., & Youvan, D. C. (1987) *Biochim. Biophys. Acta 890*, 286–291.

Rafferty, C. N., & Clayton, R. K. (1979) *Biochim. Biophys. Acta 546*, 189–206.

Reed, D. W., & Ke, B. (1973) *J. Biol. Chem. 248*, 3041–3045.

Sauer, K., Dratz, E. A., & Coyne, L. (1968) *Proc. Natl. Acad. Sci. U.S.A. 61*, 17–24.

Scolnik, P. A., & Marrs, B. L. (1987) *Annu. Rev. Microbiol. 41*, 703–726.

Shuvalov, V. A., Shkuropatov, A. Ya., Kulakova, S. M., Ismailov, M. A., & Shkuropatova, V. A. (1986) *Biochim. Biophys. Acta 849*, 337–346.

Tiede, D. M., Budil, D. E., Tang, J., El-Kabbani, O., Norris, J. R., Chang, C. H., & Schiffer, M. (1988) *NATO ASI Ser., Ser. A 149*, 13–20.

Verméglio, A., & Clayton, R. K. (1976) *Biochim. Biophys. Acta 449*, 500–515.

Williams, J. C., Steiner, L. A., Ogden, R. C., Simon, M. I., & Feher, G. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6505–6509.

Williams, J. C., Steiner, L. A., Feher, G., & Simon, M. I. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7303-7307.
Williams, J. C., Steiner, L. A., & Feher, G. (1986) *Proteins: Struct., Funct., Genet. 1*, 312-325.
Yeates, T. O., Komiya, H., Rees, D. C., Allen, J. P., & Feher, G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6438-6442.
Youvan, D. C., Bylina, E. J., Alberti, M., Begusch, H., & Hearst, J. E. (1984) *Cell 37*, 949-957.

Characterization of Multiple Forms of the Ah Receptor: Comparison of Species and Tissues†

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ABSTRACT: Biochemical and toxic responses to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) appear to be mediated via the Ah receptor, a gene-regulatory protein that, like steroid hormone receptors, undergoes a ligand-dependent acquisition of affinity for nuclei and DNA. Since responses to TCDD are highly species- and tissue-specific, we compared DNA-binding properties of Ah receptor from several tissues of rat, C57BL/6 mouse, hamster, and guinea pig, using DNA–Sepharose chromatography. Hepatic cytosol from all species contained TCDD·receptor complexes that eluted at ~0.15 (peak 1) and ~0.33 M NaCl (peak 2). The relative proportions of these forms as well as of TCDD·receptor that did not bind to DNA (i.e., was present in flowthrough fractions) varied among species. In each case, the yield of the higher affinity form (peak 2) increased with time or temperature of incubation. Cytosol from lung, thymus, kidney, and testis contained the same two forms; peak 2 was the major DNA-binding form only in thymus. In KCl extracts of hepatic nuclei from animals treated with [^{3}H]TCDD, only the higher affinity form (peak 2) was found. Peak 1 isolated from cytosol by DNA–Sepharose and incubated with hepatic cytosol from D2 mouse (which contains no detectable receptor) transformed into peak 2, suggesting that these two forms are different conformations of the same protein. Sucrose density gradient and gel filtration analyses of peaks 1 and 2 isolated from DNA–Sepharose indicated that (i) the untransformed form (peak 1) was smaller than the unoccupied and the transformed forms, (ii) 0.4 M KCl in the density gradients had little effect on these isolated forms, and (iii) nuclear receptor sedimented like peak 2. On the basis of these results, we hypothesize that the Ah receptor exists in several forms: When occupied, it has no affinity for DNA. Ligand binding initially yields a smaller form with low DNA affinity (i.e., peak 1), as well as, in some cases, a form with no DNA affinity (flowthrough fractions); further incubation in the presence of cytosolic factor(s) induces a change conferring higher DNA affinity and faster sedimentation (i.e., peak 2). The latter form is likely the transcriptionally active form in vivo. Species and tissue differences in this scheme are quantitative rather than qualitative.

The Ah (*a*ryl *h*ydrocarbon) receptor is a soluble intracellular protein that binds numerous halogenated and nonhalogenated aromatic hydrocarbons and thereby appears to mediate many of the biochemical and toxic responses to these xenobiotics, of which 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)[1] is the most potent (Poland & Knutson, 1982; Whitlock, 1987). Both physicochemically and functionally, the Ah receptor is analogous to steroid hormone receptors, although no endogenous ligand for the Ah receptor has yet been identified. The Ah and glucocorticoid receptors are strikingly similar in their molecular size, surface charge, and sedimentation coefficient (Wilhelmson et al., 1986; Cuthill et al., 1987). Binding of their respective ligands confers on both Ah and steroid receptors greater thermostability, greater resistance to disruption by reagents such as salt and SH modifiers, and higher affinity for cell nuclei and for DNA in vitro (Okey et al., 1979; Carlstedt-Duke et al., 1981; Schmidt & Litwack, 1982; Hannah et al., 1986; Kester & Gasiewicz, 1987; Henry et al., 1988). These ligand-induced changes are presumed to result from a conformational change(s) in the receptor protein. Most importantly, the Ah receptor, like the steroid receptors, is a gene regulatory protein: the ligand·receptor complexes have high affinity for nuclear material and modify expression of a receptor- and tissue-specific battery of genes (Whitlock, 1987).

In the case of the Ah receptor, the best-studied effect at the genetic level is the activation of the gene(s) encoding specific cytochrome P-450 isozymes (e.g., the gene P450IA1 encoding cytochrome P_1-450 in mouse, P-450c in rat) [reviewed by Eisen et al. (1983) and Jones et al. (1985)]. Tukey et al. (1982) demonstrated a direct correlation between the presence of the TCDD·receptor complex in the nucleus and synthesis of P_1-450 mRNA. Upstream from the P_1-450 gene, three dioxin-responsive elements that are transcriptional enhancers have been identified, and they are functionally dependent on the TCDD·receptor complex (Durrin & Whitlock, 1987; Denison et al., 1988). Although the induction of these P-450s and associated mixed-function oxidases by TCDD is well docu-

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[1] Abbreviations: BSA, bovine serum albumin; B6, C57BL/6J; D2, DBA/2J; HAP, hydroxylapatite; HEDG, 25 mM Hepes, 1.5 mM EDTA, 1 mM dithiothreitol, and 10% (v/v) glycerol (pH 7.6); hsp90, 90-kDa heat shock protein; PMSF, phenylmethanesulfonyl fluoride; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDF, 2,3,7,8-tetrachlorodibenzofuran; TCDD ± TCDF, [^{3}H]TCDD ± 100-fold excess of TCDF.

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