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Laser Raman Scattering from an Enzyme of Well-Documented Structure, Human Carbonic Anhydrase B

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Abstract: Raman spectra are reported for human carbonic anhydrase B and their interpretation compared with the structure determined by x-ray diffraction. The Raman spectra provide quite satisfactory quantitative agreement with x-ray data. For example, Raman reveals 19% helix, 39% β structure, and 42% "disordered", compared with x-ray values of 17, 40, and 43%, respectively. X-ray-derived models show four tyrosines "buried" and four "exposed", while quantitative treatment of the Raman data provides values of 3.7 "buried" and 4.3 "exposed". The Phe/Tyr ratio in human carbonic anhydrase B is determined as 1.41 by Raman and 1.38 by x ray. The Raman spectra indicate a conformation for human carbonic anhydrase B's two methionine residues in which C_α is trans to S and C_β gauche to the methionyl methyl. This information is not available from x-ray diffraction. Conversion of the native protein to the apo form is accompanied by little or no spectroscopically observable conformational change.

Early development of assignments of laser Raman spectra of proteins derived largely from pioneering studies by Lord and Yu[2] of proteins of well-documented structure. It is now apparent that protein Raman spectra are rich in structural information.[3,4] As the technique is now being widely applied to an increasing number of proteins, it is worthwhile to again examine a protein of well-documented structure as a test of the validity of recent premises underlying the interpretation of protein structure by Raman scattering.

As our model, we have chosen human carbonic anhydrase B (HCAB) (E.C. 4.2.1., carbonate hydrolyase), a zinc metalloenzyme which catalyses the reversible hydration of CO_2.[5] In humans, the enzyme has been found to exist as two major isoenzymes, designated as HCAB and HCAC.[6,7] The high-resolution x-ray crystallographic data are available for both isoenzymes[8,9] and indicate considerable structural homology, with some subtle differences in the vicinity of the active site. Extensive reviews[10,11] are available which detail the physical, chemical, and enzymatic properties of the carbonic anhydrases.

Presented here are the Raman spectra of HCAB and apo-HCAB in H_2O and D_2O. Structural information deduced from the Raman data is found to provide a quite satisfactory agreement with that determined by x-ray diffraction.

Experimental Section

Carbonic anhydrase B was prepared by a modification[12] of the chloroform–ethanol method.[13] Samples of the native enzyme were prepared by dissolving lyophilized protein in either H_2O or D_2O containing 0.01 M Na_2SO_4 at a final pH (pD) of 6.9. The samples were equilibrated in solvent for several hours. Protein concentrations were about 15 mM. The enzyme was assayed for esterase activity as described by Armstrong et al.[15] using *p*-nitrophenyl acetate as substrate. The activity of the samples was the same both before and after spectra were taken.

The apoenzyme was prepared by dialysis against the chelating agent 1,10-phenanthroline as described by Lindskog and Nyman.[14] After

Table I. Raman Frequencies and Assignments for Native and Apo Human Carbonic Anhydrase B

Frequency, cm^{-1} (intensity)				
Native		Apo		
H_2O	D_2O	H_2O	D_2O	Assignment
624 (0.7)	624 (0.7)	624 (0.9)	622 (0.8)	Phe
645 (0.6)	645 (0.6)	645 (0.8)	645 (0.17)	Tyr; O-deuterated Tyr
700 (0.5)	700 (0.4)	700 (0.5)	700 (0.6)	ν(C–S) methionine
760 (3.6)	758 (2.8)	760 (3)	758 (2.8)	Trp; N-deuterated Trp
830 (1.7)	830 (1.2)	830 (2.2)	830 (1.3)	Tyr; O-deuterated Tyr
859 (1.5)	858 (1.3)	859 (2)	858 (1.4)	Tyr; O-deuterated Tyr
882 (1.5)	886 (0.6)	885 (1.4)	885 (0.8)	ν(C–C), Trp
938 (0.6)	938 (1.5)	939 (0.8)	940 (1.5)	ν(C–C)
963 (0.8)		962 (0.9)		ν(C–C), Pro (cis)
	977 (1.6)		975 (1.9)	ν(C–C), deuterated amide III
992 (sh)			994 (sh)	ν(C–C)
1004 (10)	1004 (10)	1004 (10)	1004 (10)	Phe, Pro
1011 (sh)	1001 (sh)	1011 (sh)	1011 (sh)	Phe, Trp
1033 (2.3)	1034 (1.8)	1034 (2.4)	1033 (1.9)	Phe
1080 (1.5)	1080 (0.4)	1080 (1.6)	1080 (0.8)	ν(C–N)
1090–1115		1090–1115		ν(C–N)
1126 (1.9)	1126 (0.9)	1125 (2.5)	1124 (2.2)	ν(C–N)
1156 (0.4)	1157 (0.3)	1158 (0.6)	1157 (0.7)	ν(C–N)
1175 (0.4)	1178 (0.9)	1173 (0.5)		Try
1194 (0.2)		1197 (0)		Try
1207 (1.3)	1207 (2)	1208 (1.5)	1208 (2)	Try, Phe; O-deuterated Tyr
1240 (3.4)	1239 (1.2)	1240 (3.5)	1238 (0.8)	Amide III β sheet and "disordered"
1258 (2.4)	1253 (1.2)	1259 (2.5)	1253 (0.8)	Amide III 3_{10} helix
	1268 (1.3)		1265 (0.6)	
1276 (sh)		1276 (sh)		Amide III α helix
	1291 (0.1)		1290 (0)	
1305 (0.2)		1308 (0.5)		
1325 (0.8)	1319 (1.8)	1325 (1.5)	1323 (2.2)	C–H defo
1341 (2.3)	1335 (3.3)	1340 (2.7)	1335 (4)	C–H defo
			1347 (sh)	C–H defo
1363 (1.3)	13	1360 (1.4)	1358 (sh)	Trp; N-deuterated Trp
	1386 (1.3)		1386 (1.8)	
1401 (0.3)		1402 (0.3)		ν(COO–) symmetric
			1410 (1.3)	N-Deuterated imidazolium ion
1431 (0.2)		1427 (0.4)		N–H bend indole ring
1451 (5.3)	1453 (6.5)	1451 (4.5)	1454 (6)	$C-H_2$, $C-H_3$ defo
1461 (sh)	1460 (sh)	1463 (sh)	1463 (sh)	$C-H_2$, $C-H_3$ defo, Pro
1554 (3.4)	1553 (2.8)	1554 (3.5)	1553 (3)	Trp
	1575 (0.7)		1575 (1.2)	
1586 (0.6)	1587 (0.7)	1585 (0.9)	1587 (1)	Tyr
1607 (2)	1608 (1.2)	1606 (2.7)	1608 (2.2)	Phe, Tyr
1621 (1.1)	1616 (0.7)	1618 (2)	1615 (2.2)	Phe, Tyr, Trp
1650 (4.5)	1650 (4.5)	1651 (5.7)	1654 (5.7)	Amide I helix
1666 (8.5)	1665 (7.2)	1666 (6.5)	1665 (6.8)	Amide I β sheet and "disordered"
1672 (sh)	1671 (sh)	1673 (sh)	1670 (sh)	Amide I β sheet

removal of the zinc ion the sample was exhaustively dialyzed against deionized H_2O. The sample, which showed less than 5% esterase activity, was then lyophilized and redissolved in 0.01 M phosphate buffer at a pH (pD) of 6.0.

Spectra were recorded with a Spex 1401 double monochromator, utilizing a cooled RCA 31034 photomultiplier. A Coherent Radiation CR-5 argon laser provided approximately 200 mW of power at the sample using the 5145-Å exciting line. Samples of typically 10 μL were held in sealed Kimax capillary tubes with scattered radiation collected at a 90° angle from the direction of the incident light. Spectra were recorded with a spectral slit width of 4 cm^{-1} and are accurate to ±2 cm^{-1}.

Results

The characteristic Raman spectra of the native and the metal-free forms of the enzyme are reproduced in Figure 1. The spectra share many features in common with spectra of other proteins,[3,4] a fact which aided greatly in the assignments. Specific frequencies and assignments for native and apoenzyme in both H_2O and D_2O are tabulated in Table I. Spectra are not shown below 600 cm^{-1} due to the absence in that region of any Raman bands significant to this study.

Discussion

Native Enzyme. Although specific assignments have varied somewhat, it is generally assumed that both the amide I (peptide C=O stretch) and amide III (peptide C–N stretch and N–H bend) bands are indicative of distinct components of a protein's secondary structure. Model studies with both homopolypeptides[16-18] and proteins of well-documented structure[19-21] have established the following assignments: α-helix, ~1655 cm^{-1}; antiparallel β-pleated sheet, ~1670 cm^{-1}; disordered structure, ~1665 cm^{-1}.

Almost 40% of the secondary structure of carbonic anhydrase is comprised of ten segments of twisting β-pleated sheet.[9] Two pairs of chain segments are parallel; the remainder are antiparallel. In the HCAB Raman spectra, we may confidently assign the main amide I peak at 1666 cm^{-1} to these regions of twisted β structure. Intensity also is contributed from a broad, underlying band centered at approximately 1660 cm^{-1} due to regions of disordered structure.

Band shape is an informative qualitative feature of the amide I. A sharp intense band indicates a narrow distribution of ϕ/ψ angles[22] and uniform H bonding[23] while a broad band is suggestive of a wider range of peptide conformations. Thus the

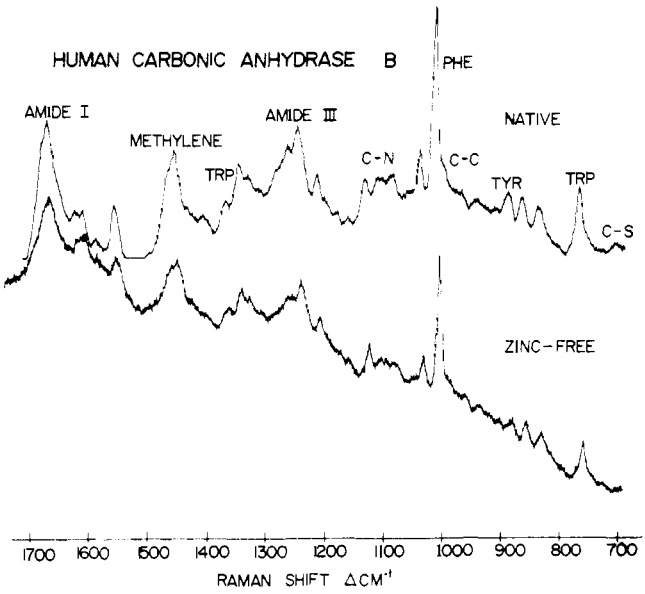

Figure 1. Raman spectra of native carbonic anhydrase B (upper trace) and the zinc-free enzyme (lower trace).

Table II. Comparison of Structural Information by Raman Scattering and X-Ray Diffraction for Human Carbonic Anhydrase B

	Raman	X-ray[a]
Secondary structure		
Helix	19%	17%
β	39% (33%)	40%
"Disordered"	42%	43%
Tyrosine environment		
"Buried"	3.7	4
"Exposed"	4.3	4
Phenylalanine/tyrosine ratio	1.41	1.38
Tyrptophan environment		
Some residues buried		3 buried 2 partially exposed
Methionine conformation		
C_α trans to S C_β gauche to $-CH_3$		Not determined
Conformational change (native → apo)		
None to very slight		Not determined for HCAB; none for HCAC

[a] Data from ref 9.

disordered domain is characterized by a low degree of uniformity for hydrogen bonding to the amide carbonyls and a spread of torsional dihedral angles of the polypeptide backbone. The slight shoulder on the high frequency side of amide I near 1672 cm^{-1} is at the frequency generally accepted as characteristic of β structure and probably represents regions of more uniform conformation.

Helices make up 17% of the HCAB secondary structure. Of these, only one segment (seven residues) is close to an α-helical form, the rest being short distorted helices approaching more nearly the 3_{10} conformation.[9] The weak shoulder in amide I at 1651 cm^{-1} reports these helical regions. Additionally, the two *parallel* pairs of β structure may add intensity near 1650 cm^{-1}, for as Krimm and Abe[24] have noted, the amide I frequency of the *parallel* β sheet is predicted to fall quite near that of the α helix. (However, the prediction has not yet been verified in the Raman effect.)

Amide III. Like the amide I, there exist characteristic amide III frequencies indicative of secondary structure. The presence of helix in protein[19] or polypeptide[16] is generally signaled by weak amide III structure below ~1275 cm^{-1}. A sharp feature around 1230-1240 cm^{-1} is typical of a β-pleated sheet. The frequency is usually somewhat higher in proteins with substantial β structure than it is in the corresponding homopolypeptides.[25] Disordered structure is revealed by a broad band near 1240-1245 cm^{-1}, and it is often difficult to discern disordered from β structure except on the basis of line shape and intensity. We assign the sharp, intense amide III peak at 1240 cm^{-1} to the same β structure responsible for the 1666-cm^{-1} amide I peak. There is a very weak shoulder on the low-frequency side of the 1240-cm^{-1} peak at 1231 cm^{-1} which may arise from that same portion of very regular β structure which gives rise to the shoulder at 1672 cm^{-1} in the amide I. As a consequence of the N-H bending component in amide III, it has been suggested that the mode is sensitive to H bonding, the frequency decreasing with weaker bonding.[18] Thus a lower amide III frequency might be anticipated for the more weakly hydrogen-bonded 3_{10} helix than for the α helix. On this basis, the peak at 1258 cm^{-1} is tentatively assigned to the distorted 3_{10} helix and the shoulder at 1276 cm^{-1} to the small amount which is nearly α helical.

Upon deuteration of the native enzyme most of the amide III intensity shifts to below 1000 cm^{-1} where unambiguous assignment becomes difficult. For proteins in which the β sheet is abundant, deuterated amide III has been assigned at 980-897 cm^{-1}.[16,26] As would be anticipated, a strong Raman band occurs at 977 cm^{-1} and is readily assigned to the β structure. A small amount of intensity remains in the amide III D_2O region. While this could represent a portion of the 20% of amide nitrogens termed "hard to exchange" in infrared studies,[13] it should be noted that side groups do contribute some intensity in this region as well.[27] Surprisingly, deuteration does not produce the small shift (3-8 cm^{-1}) to lower frequency usually observed for the amide I.

To this point we see that Raman spectra, coupled with currently accepted assignments, provide a reliable qualitative view of the secondary structure of HCAB. We now show that the data yield quantitative information as well.

A method of quantitating secondary structure by comparison of the relative intensities of amide I and amide III components in D_2O and H_2O has been suggested recently by Lippert et al.[25] The Lippert technique employs a set of four simultaneous equations, three of the form, $C^{\text{protein}} I_\nu^{\text{protein}} = f_\alpha I_\nu^\alpha + f_\beta I_\nu^\beta + f_R I_\nu^R$, and the fourth, $f_\alpha + f_\beta + f_R = 1.0$. A relationship is thereby established between the fractional secondary structural content (f_α, f_β, f_R) and protein Raman intensity (I_ν^{protein}) relative to the intensities ($I_\nu^\alpha, I_\nu^\beta, I_\nu^R$) determined experimentally from poly(L-lysine) in its helical, β sheet and "disordered" forms. The scaling constant C^{protein} represents the relative methylene band intensity in the protein. Using this basic procedure with the spectra presented here, we obtain rather good agreement with the x-ray data (Table II). Note, however, that the scaling constant (0.85) was chosen empirically so as to best fit the known amount of β structure as taken from the x-ray data. Moreover, Lippert's original procedure was developed with the assumption that the only structural components were α helix, *antiparallel* β sheet, and disordered structure, whereas the helix in HCAB is mostly 3_{10} and there is a substantial amount of *parallel* β sheet present. We believe that while the *relative portions* of secondary structure determined by Raman are valid, the unusually good congruence with the x-ray data is, to some extent, fortuitous. A quantitation procedure which does not require an arbitrary scaling factor, but yields only the fraction of β structure, has been proposed by Pezolet et al.[28] This method assumes that a linear relationship holds between the fraction β structure and

the intensity ratio I_{1240}/I_{1450} normalized to the average number of CH_2 per amino acid residue. We estimate ~33 ± 4% β structure by this method. Thus Raman data do provide realistic quantitative estimates of the elements of tertiary structure.

Side Group Environment. There is only one half-cystine in HCAB and hence no peak attributable to an S-S stretching frequency is found in the region 500–550 cm^{-1} where it would normally appear. The peak at 700 cm^{-1} is assigned to a C-S stretch contributed primarily by the two methionine residues. In simple alkyl sulfides the C-S stretching frequency is correlated with the conformations about the C-C and CH_2-S bonds of the $-CCH_2SCH_3$ residue.[29] For example, if hydrogen occupies the position trans to the sulfur ν_{C-S} falls between 630 and 670 cm^{-1}, while for carbon in the trans position ν_{C-S} is in the range 700–745 cm^{-1}. From the frequency of the C-S stretch in HCAB we infer a conformation in which the C_α is trans to the sulfur and C_β is gauche to the methionyl methyl group. This information is not available from the x-ray structure.

Yu et al.[30] first proposed that the relative intensities of the ring modes at 828 and 853 cm^{-1} might indicate whether tyrosine residues were "buried" or "exposed". In a joint study from the laboratories of Professors Lord and Shimanouchi[31] it is shown that the doublet near 850 and 830 cm^{-1} results from Fermi resonance between a ring-bending overtone and a ring-breathing fundamental. In HCAB this doublet is observed at 859 and 830 cm^{-1}. The nature and extent of hydrogen bonding to the phenolic hydroxyl determine the degree of negative charge on the oxygen atom which, in turn, influences the respective intensities of the doublet. Basically two major classes of hydrogen bonding patterns affect the doublet intensities. In one, the phenolic oxygen acts as a strong proton donor, with a resulting intensity enhancement of the ratio, $I_{859}/I_{830} \simeq 0.5$. This has been suggested to be the case for residues buried within the protein.[29] If, on the other hand, the phenolic oxygen acts as a much weaker proton donor, or as an acceptor to an external acidic proton, I_{859}/I_{830} lies near 1.25.

The value of the doublet ratio for the eight tyrosines in HCAB is 0.9. With the simplified assumption that only two tyrosine environments exist and labeling them (somewhat arbitrarily) "buried" or "exposed", we may use the coefficients above to calculate the distribution of tyrosines from the Raman data. Thus

$$\text{tyrosines "buried"} + \text{tyrosines "exposed"} = 8$$

$$(0.5) \times (\text{tyrosines "buried"}) + (1.25) \times (\text{tyrosines "exposed"}) = 0.9$$

Again, excellent agreement (Table I) is achieved between the estimate based on Raman parameters and the x-ray data. Small deviations in the Raman estimate reflect the assumptions made and suggest that very careful correlation with proteins of well-documented structure could permit a valuable refinement of this technique both in terms of the coefficients chosen and the number of "classes" of tyrosines assumed. (For example, as Riddiford[32] has shown the tyrosines of HCAB may actually be classified into three groups: two with "normal" pK_a values (~9.8), one or two with somewhat higher pK_a values (~10.5), and four masked residues with $pK_a \simeq 11.5$.)

Yu et al.[33] obtained a reliable estimate of the phenylalanine to tyrosine ratio in lens protein by comparison of intensities at 624 (phenylalanine) and 644 cm^{-1} (tyrosine). When applied to HCAB, the procedure yields Phe/Tyr = 1.41; the ratio determined from the amino acid composition is 1.38.

A sharp intense Raman line near 1360 cm^{-1} is diagnostic for "buried" tryptophane.[34] Four of the tryptophan residues of HCAB are buried in a highly hydrophobic domain;[9] they are observed in the Raman spectrum at 1363 cm^{-1}.

Apoenzyme. When the zinc ion is removed from HCAB there is no discernible change in the spectra in either of the amide regions. The only hint of a conformational change is the band at 1126 cm^{-1}, assigned to C-N stretching, which sharpens and increases in intensity in the apoprotein spectra. Broadening and a loss of intensity in this band have been seen upon protein denaturation.[35] Thus the observed sharpening may reflect a small local increase in rigidity or ordering of portions of the protein backbone.

X-ray crystallographic studies of HCAB have revealed a network of hydrogen bonds involving several buried residues, including tyrosine and histidine, near the active site. The network's structural and functional significance is not known, although its destruction and the unmasking of the residues to solvent upon denaturation dislodge zinc ion and result in irreversible loss of enzymatic activity.[36] We believe that the apoenzyme shows no radical breakdown of the network. As evidence we cite the tyrosine Fermi doublet ratio in the apoenzyme which is identical with that of the native enzyme, thus indicating no change in tyrosyl hydrogen bonding. Similarly, the tryptophan environment appears to be the same as that of native protein.

It appears, as has been shown to be the case for HCAC by electron difference mapping,[36] that almost no change in the conformation of HCAB accompanies transformation to the apoprotein. This is the first such observation for the B isoenzyme.

Acknowledgments. Our sincere thanks to Professor W. L. Peticolas for his critical reading of the manuscript and for support (through NIH GM-15547) during its preparation. We gratefully acknowledge the cooperation of Professors R. C. Taylor and M. D. Morris, Department of Chemistry, The University of Michigan, for making their laser Raman spectrometer available to us. Primary support for this work was provided by the Research Corp. B.P.G. is a research fellow of the National Cancer Institute (CA 5488-01).

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Anion Radicals of Bacteriochlorophyll a and Bacteriopheophytin a. Electron Spin Resonance and Electron Nuclear Double Resonance Studies

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Abstract: Chemical, electrochemical, and photochemical reductions of bacteriopheophytin a (BPh) and deuterated BPh yielded stable anion radicals which were characterized by electron spin resonance and electron-nuclear double resonance techniques. Radicals of the model bacteriochlorin compound, 5,10,15,20-tetraphenyl-2,3,12,13-tetrahydroporphyrin, selectively deuterated, were prepared to guide the interpretation of the BPh data and the experimental results compared with self-consistent field-molecular orbital calculations. ESR and ENDOR results for bacteriochlorophyll a (BChl) and deuterated BChl are also assigned with the aid of a model, zinc tetraphenyltetrahydroporphyrin, and molecular orbital calculations. These data thus help define the electronic profile of the BPh and BChl anions, the radical species presently postulated to result, within picoseconds, from the primary charge separation of bacterial photosynthesis.

Pulsed laser spectroscopy has revealed[2-5] two short-lived intermediates involved in the primary photochemistry of the photosynthetic bacterium, *Rhodopseudomonas spheroides*. The first appears[3-5] in less than 10 ps and is postulated to consist of a dimeric bacteriochlorophyll a cation radical[5,6] and of an anion radical[6] of bacteriopheophytin, a demetallated chlorophyll. This charge separation, caused by the photooxidation, within the reaction center, of a pair of bacteriochlorophylls (BChl) that absorbs at 870 nm (P_{870}) and the concomitant reduction of a nearby bacteriopheophytin (BPh), results[6] in an estimated 1 eV of free energy. For incident light of 870 nm or 1.4 eV, this represents a 70% energy efficiency, an attractive number for a primary biological step.[7] Under physiological conditions, the BPh anion reduces, within 200 ps,[3,5] the next electron carrier (X), a ubiquinone-iron complex,[8-11] whose anion then exists for milliseconds. The electron is eventually returned to the oxidized P_{870} via an established cyclic process which generates the intermediates required for carbon dioxide fixation. If, however, normal chemistry is blocked by poising the reaction center at potentials such that X is reduced, the light-induced charge separation is annihilated in nanoseconds[2] by recombination of the radicals to yield fluorescence[12] and a triplet:[13,14]

$$P_{870}^{+}\cdot + BPh^{-}\cdot \xrightarrow{6\ ns} {}^{3}P_{870} + BPh$$

The latter exhibits anomalously polarized ESR spectra probably induced[5,6,15] by a recombination mechanism similar to that which causes chemically induced dynamic electron polarization.[16]

The putative BPh anion can be trapped[17-19] in reaction centers of *Chromatium minutissimum* and *vinosum* in the presence of $X^{-}\cdot$ and ferri- or ferrocytochrome c_{553}. The optical spectra observed are consistent with those expected[6] on reduction of BPh but also include shifts in the bands of the other bacteriochlorophylls (P_{800}) known[11] to be present in the reaction center. The BChl spectral changes may result from delocalization of the reducing electron unto BChl as well as BPh or may simply reflect electrochromic shifts due to the nearby $BPh^{-}\cdot$, $X^{-}\cdot$, and cytochrome.

Although a number of porphyrin, chlorin, and bacteriochlorin anion radicals have been prepared, little detailed information exists about their ESR characteristics (see ref 20–22 for reviews). Few compounds display hyperfine resolution and, of these, even fewer have yielded to hard core analysis of the number, amplitude, and splittings of the hyperfine structure. Among the bacteriochlorins, anions of BPh and BChl have been described.[6,23,24] We have previously assigned[23] the ESR spectrum of zinc tetraphenylbacteriochlorin (ZnTPBC) by selective deuterations. Feher et al.[25] reported a single ENDOR transition for $BChl^{-}\cdot$. Radicals of tetraphenylbacteriochlorin[26] (H_2TPBC), BChl b, and BPh b[27] (found in the bacterium *Rhodopseudomonas viridis*) have also been identified without hyperfine analysis.

We present here ESR and ENDOR data which, combined with model compound studies, isotopic substitution results, and self-consistent field-molecular orbital calculations, help characterize the electronic configuration of the BPh and BChl anions. The line widths, saturation behavior, and g values of the monomeric radicals resemble those ascribed[17,18] to the primary electron acceptor in vivo. Added to recent ESR and ENDOR observations[25,28,29] on oxidized bacteriochlorophylls, these results specify the ESR parameters and spin-density profile of the radical species presently thought to exist, within a nanosecond time domain, in the primary charge separation of bacterial photosynthesis.