Poile, T. W., & Watts, A. (1989) *J. Magn. Reson.* (submitted for publication).
Rouser, G., Fleisher, S., & Yamamoto, A. (1970) *Lipids 5*, 494-496.
Seelig, J., & Seeling, A. (1980) *Q. Rev. Biophys. 13*, 19-61.
Seelig, J., Borle, F., & Cross, T. A. (1985) *Biochim. Biophys. Acta 814*, 195-198.
Spruijt, R. B., Wolfs, C. J. A., & Hemminga, M. A. (1989) *Biochemistry 28*, 9158-9165.
Sternberg, B., Gale, P., & Watts, A. (1989) *Biochim. Biophys. Acta 980*, 117-126.
Stockton, G. W., Polnaszek, C. F., Tullock, A. P., Hasan, F., & Smith, I. C. P. (1976) *Biochemistry 15*, 954-966.
Van Wezenbeek, P. M. G. F., Hulsebos, T. J. M., & Schoenmakers, J. G. G. (1980) *Gene 11*, 129-148.
Watts, A. (1987) *J. Bioenerg. Biomembr. 19*, 625-653.
Watts, A. (1989) *Curr. Opinions Cell Biol. 1*, 691-700.
Wickner, W. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1159-1163.
Woessner, D. (1961) *J. Chem. Phys. 35*, 41-48.
Wolfs, C. J. A. M., Horváth, L. I., Marsh, D., Watts, A., & Hemminga, M. A. (1989) *Biochemistry 28*, 9995-10001.

Photosynthetic Reaction Center of Green Sulfur Bacteria Studied by EPR†

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ABSTRACT: Membrane preparations of two species of the green sulfur bacteria *Chlorobium* have been studied by EPR. Three signals were detected which were attributed to iron–sulfur centers acting as electron acceptors in the photosynthetic reaction center. (1) A signal from a center designated F_B, (g_z = 2.07, g_y = 1.91, g_x = 1.86) was photoinduced at 4 K. (2) A similar signal, F_A (g_z = 2.05, g_y = 1.94, g_x = 1.88), was photoinduced in addition to the F_B signal upon a short period of illumination at 200 K. (3) Further illumination at 200 K resulted in the appearance of a broad feature at g = 1.78. This is attributed to the g_x component of an iron–sulfur center designated F_X. The designations of these signals as F_B, F_A, and F_X are based on their spectroscopic similarities to signals in photosystem I (PS I). The orientation dependence of these EPR signals in ordered *Chlorobium* membrane multilayers is remarkably similar to that of their PS I homologues. A magnetic interaction between the reduced forms of F_B and F_A occurs, which is also very similar to that seen in PS I. However, in contrast to the situation in PS I, F_A and F_B *cannot* be chemically reduced by sodium dithionite at pH 11. This indicates redox potentials for F_A and F_B which are lower by at least 150 mV than their PS I counterparts. The triplet state of P_{840}, the primary electron donor, could be photoinduced at 4 K in samples which had been preincubated with sodium dithionite and methyl viologen and then preilluminated at 200 K. The preillumination reduces the iron–sulfur centers while the preincubation is thought to result in the inactivation of an earlier electron acceptor, possibly the double reduction of a quinone which could occur at potentials higher than those associated with its functional one-electron couple. Orientation studies of the triplet signal in ordered multilayers indicate that the bacteriochlorophylls which act as the primary electron donor in *Chlorobium* are arranged with a structural geometry almost identical with that of the special pair in purple bacteria. The *Chlorobium* reaction center appears to be similar in some respects to both PS I and to the purple bacterial reaction center. This is discussed with regard to the evolution of the different types of reaction centers from a common ancestor. This has significance to the current understanding of the structure of the PS I reaction center.

Green photosynthetic bacteria (Chlorobiaceae and Chloroflexaceae) are morphologically quite distinct from other photosynthetic bacteria. They contain mainly bacteriochlorophyll (BChl)[1] *a* and *c* and have a unique antenna system called the chlorosome. Recent work, however, has demonstrated that this morphological classification is somewhat misleading. On the basis of 16 S-RNA sequences, it can be concluded that the green *non*-sulfur bacteria (Chloroflexaceae) are actually not very closely related to the green sulfur bacteria (Chlorobiaceae) (Woese, 1987).

The reaction center of *Chloroflexus aurantiacus* has been shown to possess pronounced similarities to the photosynthetic reaction center of purple bacteria (Shuvalov et al., 1986; Shiozawa et al., 1987; Blankenship et al., 1988; Ovchinnikov et al., 1988a,b). The structure of the purple bacterial reaction center which has been determined by X-ray crystallography

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[1] Abbreviations: RC, reaction center complex; BChl, bacteriochlorophyll; FeS center, iron–sulfur center; P_{840}, primary electron donor bacteriochlorophyll(s) of green sulfur bacteria; P_{700}, primary electron donor chlorophyll(s) of PS I; PS I, photosystem I; ZFS, zero-field splitting parameters of the spin-polarized spectrum of the triplet state of the primary donor.

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(Deisenhofer et al., 1985; Allen et al., 1986; Chang et al., 1986) can thus be used as a detailed structural model for the *Chloroflexus* reaction center. In addition, the reaction center from purple bacteria has many similarities with that of photosystem II from cyanobacteria and plants. In contrast, the poorly understood reaction center of green sulfur bacteria is usually compared with PS I.

There are four lines of evidence which suggest that PS I and the green sulfur bacterial reaction center should be classed together.

(1) The secondary acceptor quinones in purple bacterial reaction centers are not sufficiently reducing to directly reduce NAD^+. In these organisms, light-induced NAD^+ reduction is driven by ATP which is generated by a protonmotive force. By contrast, the terminal acceptors in PS I (E_m = −550 mV) are capable of direct $NADP^+$ (E_m = −320 mV) reduction without a membrane potential being involved. This was also shown to be the case for green sulfur bacterial reaction centers. Light-induced NAD^+ reduction was demonstrated in cells from *Chlorobium* even in the presence of uncoupling chemicals added in order to collapse the membrane potential (Buchanan & Evans, 1966; Knaff, 1978).

(2) EPR studies suggested that at least one FeS center is involved in the primary electron-transfer processes as a reaction center bound acceptor (Jennings & Evans, 1977; Swarthoff et al., 1981a). The presence of FeS centers again is a common feature of PS I and green sulfur bacterial reaction centers, distinguishing them from both the purple bacterial reaction center and PS II.

(3) A chemically induced dynamic electron polarization (CIDEP) spectrum has been observed in whole cells of *Chlorobium* (Heathcote & Warden, 1982). Such a spectrum can be obtained in chloroplasts and PS I, but is not observable in intact purple bacterial reaction centers and PS II.

(4) All pigments involved in primary photochemical events in purple bacteria, *Chloroflexus*, and PS II are bound by two polypeptides with a molecular mass of ≃30 kDa [reviewed in Komiya et al. (1988)]. In PS I, however, the primary donor and some early electron acceptors are most probably located on a heterodimer of proteins showing an apparent molecular mass of ≃65 kDa on SDS–PAGE [for a recent review, see Golbeck (1987)]. A purified reaction center preparation from *Chlorobium limicola* f. *thiosulfatophilum* also shows the most prominent band on an SDS gel at 65 kDa (Hurt & Hauska, 1984), and this band divides into a doublet under certain conditions (Feiler and Hauska, unpublished results).

Two groups demonstrated that a light-induced reduction of iron–sulfur centers occurs in green sulfur bacteria [Jennings & Evans, 1977; Swarthoff et al., 1981a; for a review, see Blankenship (1985)]. These results, however, were somewhat controversial: Jennings and Evans (1977) observed a signal at g = 1.90 which could be photoinduced at 4 K and estimated a midpoint potential of −550 mV. Prior to this work, a signal at g = 1.9 was reported in redox titrations performed on *Chlorobium* membranes, and it was attributed to a Rieske FeS center (Knaff & Malkin, 1976). Jennings and Evans identified their light-induced signal with this component, suggesting that the E_m of +160 mV determined by Knaff and Malkin (1976) was artifactual. This led to a reexamination of the Rieske hypothesis, confirming the original assignment and challenging the existence of the g = 1.91 photoreducible signal (Knaff et al., 1979).

In 1981, Swarthoff et al. reported the spectrum of a g = 1.94 FeS center, obtained by calculating a difference between spectra taken at −420 and +70 mV. These authors estimated a midpoint potential for this component of higher than −420 mV, and the component was attributed to the terminal acceptor in the reaction center. When a sample poised at −420 mV was frozen under illumination, another FeS center at g = 1.94 was reduced. From this observation and earlier redox data (Knaff & Malkin, 1976), it was suggested that this FeS center was an earlier electron acceptor with an E_m of ≃−560 mV functioning prior to the terminal acceptor of the reaction center (Swarthoff et al., 1981a). Recently it was shown that a spectrum comparable to that described by Swarthoff et al. (1981a) could be photoaccumulated at 200 K in a sample containing 20 mM dithionite at pH 10.5 (Nitschke et al., 1987), suggesting an E_m of <−650 mV.

In the present study, we have resolved these apparent inconsistencies and have gained further insights into the structure and function of the reaction center of green sulfur bacteria using EPR on improved membrane preparations from two different species.

Experimental Procedures

Cultures of *Chlorobium limicola* f. *thiosulfatophilum* strain tassajara were obtained from the Deutsche Sammlung von Mikroorganismen (DSM), Braunschweig; *Chlorobium phaeobacteroides* strain 4930 was a kind gift of Prof. N. Pfennig, Konstanz. Cells were grown anaerobically in a medium as described by Biebl and Pfennig (1978) in 1-, 2-, and 10-L bottles with the modification that the trace element solution SL 10 (Widdel et al., 1983) was used. After 3 days of growth, cells were harvested by centrifugation and stored at −80 °C until use. After being thawed, cells were resuspended in 50 mM MOPS, pH 7.0, and broken by passing 3 times through a French pressure cell (20000 psi) in the presence of DNase.

Chlorosome-depleted membranes were prepared as described by Schmidt (1980). Chlorosome-depleted membranes contained typically 0.5–3 BChl *c*/BChl *a*.

Redox titrations of membranes (in 100 mM MOPS, pH 7.0) were carried out essentially as described by Dutton (1971). The following redox mediators were used: benzoquinone, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine, diaminodurol, 2,6-dichlorophenolindophenol, 2,5-dimethylbenzoquinone, methylene blue, pyocyanine, indigodisulfonate, indigotetrasulfonate, 1,4-dihydroxynaphthoquinone, 2,5-dihydroxy-*p*-benzoquinone, anthraquinone-2,6-disulfonate, anthraquinone-2-sulfonate, acridinium orange, safranine T, neutral red, benzyl viologen and methyl viologen at 100 μM, and phenazine methosulfate and phenazine ethosulfate at 25 μM.

Reductive titrations were performed with sodium dithionite, and oxidative titrations were carried out with porphyrexide.

Oriented membrane multilayers were obtained by using the technique of Blasie et al. (1978): chlorosome-depleted membranes in H_2O were dried in a 90% humidity atmosphere for approximately 24 h in darkness at 4 °C. Mildly reducing conditions in these membranes were achieved by applying solutions of sodium ascorbate or sodium dithionite to the dried membranes followed by drying under a stream of argon gas in darkness. Strongly reducing conditions were achieved by injecting a solution of reductant (20 mM sodium dithionite, 200 mM glycine, pH 11, ±50 μM benzyl viologen and methyl viologen) into the EPR tube containing the dried membranes followed by rapid freezing in an ethanol/dry ice mixture. Samples treated in this way showed no marked loss of orientation.

EPR spectra were obtained by using a Bruker 300 X-band spectrometer fitted with an Oxford Instruments cryostat and temperature control system. Illumination in the EPR cavity

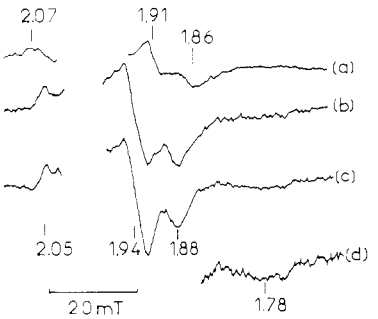

FIGURE 1: Light-induced EPR spectra of *Chlorobium limicola* f. *thiosulfatophilum* membranes poised at an ambient potential of −417 mV as described under Experimental Procedures. The spectra shown are differences of spectra taken under three different conditions: (1) sample frozen after 5-min dark adaptation; (2) after 10-min illumination at 4 K within the cavity; (3) after 20-min illumination at 200 K in a quartz Dewar containing a mixture of ethanol and dry ice. The following differences are shown: (a) spectrum 2 minus spectrum 1; (b) spectrum 3 minus spectrum 1; (c) spectrum 3 minus spectrum 2; (d) spectrum c expanded 4-fold. Instrument settings: temperature, 15 K; microwave power, 6.3 mW; frequency, 9.45 GHz; modulation amplitude, 1.6 mT; gain, 1×10^5.

was carried out by using an 800-W tungsten projector providing 16000 μE m^{-2} s^{-1} of white light at the EPR cavity window after being filtered through 2 cm of water and two Calflex filters (Balzers) to remove infrared radiation.

Powderlike spectra were generated by summing up spectra obtained on oriented membrane multilayers. The appropriate weighing factors for orientations of the magnetic field with respect to the membrane within 0° and 90° were calculated essentially following the routine proposed by Blum et al. (1978).

Results

Low-Temperature Photoreduction of FeS Centers. In *Chlorobium* membranes frozen in the dark over a range of different redox conditions, a series of EPR signals from iron–sulfur centers were observed. Our redox titrations on these "dark" signals essentially confirm the results published by Knaff and Malkin (1976). These centers appear not to be associated with the photochemical reaction center.

At redox potentials, where P_{840} is reduced, illumination at 4 K resulted in the appearance of a spectrum with g values at $g_x = 1.86$, $g_y = 1.91$, and $g_z = 2.07$, which can be attributed to an FeS center (Figure 1a). The spectrum was fully developed after 5-min illumination and was stable in the dark at temperatures up to 100 K. At 200 K, the spectrum decayed within hours and was completely restored by renewed illumination at 4 K. Formation and loss of the spectrum of this FeS center were always correlated to appearance and disappearance of a free radical attributable to P_{840}^+. The reversibility persisted throughout the redox range and is attributed to a back-reaction under most conditions.

Illumination at 200 K in the presence of dithionite resulted in a considerably larger light minus dark spectrum (Figure 1b) in the spectral region of the FeS centers (characterized by $g_x = 1.88$, $g_y = 1.94$, and $g_z = 2.05$) and changes in the $g = 2$ region as already described (Nitschke et al., 1987).

A close examination of the spectrum shows that in addition to the signals mentioned above there is another feature at relatively high field (Figure 1d). This spectral component appears as a trough at $g_x = 1.78$ with a half-width of $\simeq$45 G.

After a short period of illumination at 200 K (a few minutes), the line intensities of the three dominant lines reached a constant level with respect to the 4 K photoinduced spectrum

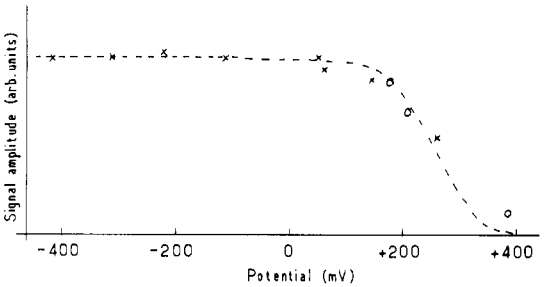

FIGURE 2: Redox titration at pH 7.0 of the 4-K light-induced spectrum shown in Figure 1a. The amplitude of the $g = 1.91$ absorption is plotted vs the ambient potential. EPR spectra were obtained as described in the legend to Figure 1. Crosses and circles denote data points collected while titrating in the negative and positive directions, respectively. The dashed line represents the theoretical Nernst curve for an $n = 1$ component with an E_m of +250 mV.

whereas the trough at $g = 1.78$ was hardly discernible. After 20-min illumination at 200 K, the $g = 1.78$ spectrum was completely formed, and prolonged illumination did not induce further changes. All of the signals photoaccumulated at 200 K were stable at 200 K in the dark. Thawing the sample and refreezing it in the dark essentially abolished all the light-induced changes and allowed for a new illumination cycle.

In all samples examined, the double integral of the 200 K photoaccumulated spectrum was 2.0–2.2 times greater compared to the spectrum induced at 4 K. This indicates that at 200 K a second signal (disregarding the component with $g = 1.78$) is additionally produced in a 1:1 stoichiometry to the 4 K photoreducible center. However, the two centers photoreduced at 200 K probably interact magnetically (see Discussion). This could result in some inaccuracy in the comparison of the double integrals and thus in the estimates of the stoichiometry of the two centers. A 200 K − 4 K difference spectrum representing the second species is shown in Figure 1c.

It should be mentioned that these illumination protocols applied to PS I samples yield comparable results both with regard to the number of photoreduced centers and with regard to their stability at various temperatures.

By comparison to the g values of the EPR signals reported in PS I (see Discussion), the center photoreduced at 4 K will be called "center F_B", the additional center photoreduced at 200 K and having g values of 2.05, 1.94, and 1.88 will be called "center F_A", and the species giving rise to the g_x trough at 1.78 will be called "center F_X".

Virtually identical spectra were obtained for both species of green sulfur bacteria examined, i.e., *Chlorobium limicola* f. *thiosulfatophilum* and *Chlorobium phaeobacteroides*.

Redox Properties of the Photoinduced Signals. The ability to photoinduce a signal at 4 K was examined at several ambient potentials. The results are shown in Figure 2. The ability to photoinduce the signal at 5 K is fully developed at potentials lower than $\simeq$200 mV. The potential dependence of the signal amplitude is compatible with that measured earlier for the P_{840}/P_{840}^+ couple by optical techniques (Fowler et al., 1971; Prince & Olson, 1976; Knaff et al., 1979). In figure 1, a theoretical curve with this E_m of +250 mV is drawn through the data points. The photoinducible signal can still be generated even at the lowest potentials achieved in the titration. The same observation holds for the 200-K photoaccumulated spectrum (data not shown). We were unable to detect any decrease of signal amplitude at the lowest potentials. This puts the midpoint potential of both photoreduced centers at lower than −500 mV. An even lower limit is imposed by the observation that in the presence of 20 mM dithionite, pH

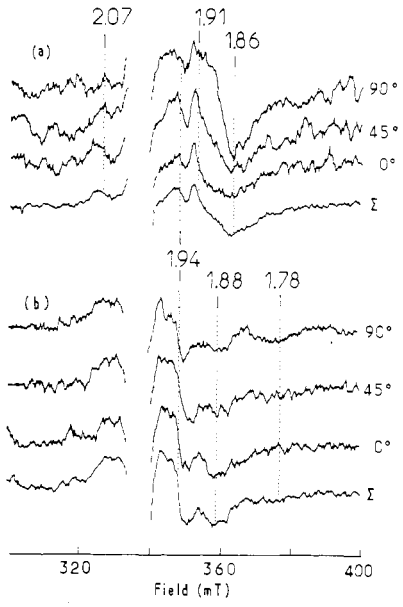

FIGURE 3: Orientation dependence of the light-induced difference spectra obtained on oriented membrane multilayers from *Chlorobium limicola* f. *thiosulfatophilum* treated with (a) sodium ascorbate, as described under Experimental Procedures (after 10-min illumination at 4 K minus dark), and (b) sodium dithionite (after 20-min photoaccumulation at 200 K minus dark). Angles are given between the plane of the multilayer and the direction of the magnetic field. The spectrum labeled "Σ" is a powderlike spectrum calculated as described under Experimental Procedures. EPR conditions are as in Figure 1 except for the gain being 2×10^5 for (a) and 4×10^4 for (b).

11, with or without redox mediators (benzyl viologen, methyl viologen), the signal at $g = 1.91$ is still the dominant photoreduced spectral component at 4 K. In photosystem I, this treatment *even without mediators* results in the complete reduction of the iron–sulfur centers F_A/F_B and partial reduction of center F_X. Unfortunately, due to the number of FeS centers already reduced in the dark, a direct estimation of the chemical reduction of both acceptors under these conditions is precluded.

The results obtained on oriented membrane multilayers also demonstrate that neither center F_B nor center F_A is measurably chemically reduced by 20 mM dithionite, pH 11, in the presence or absence of mediators. Conclusions with respect to the midpoint potential of F_A and F_B in this case are less stringent, because access of dithionite to the centers in dry membranes is probably less than optimal.

Orientation Dependence of Centers F_A and F_B. Partially ordered membrane multilayers were used to examine the orientation of the observed signals. After illumination at 4 K, the spectra showed orientation-dependent signals at the g values of center F_B. However, additional signals at the field positions of center F_A were also clearly visible (Figure 3a). Compared to the spectrum in Figure 1a, a line at $g = 1.94$ is superimposed on the spectrum of center F_B. This is most clearly seen when all orientations are summed up (using appropriate weighing factors) to represent a powderlike spectrum (spectrum labeled "Σ"). Thus, in the oriented sample, the specificity of photoreducing center F_B at 4 K is diminished. Treating the sample with 20 mM dithionite, pH 11, in the presence of mediators did not change these results (not shown).

Major spectral changes were induced by photoaccumulation at 200 K. Signals at $g = 1.94$ and $g = 1.88$ became predominant, and the "powder-sum" very much resembles the spectrum obtained for the unoriented sample under the same conditions. Polar plots for the respective signals are depicted in Figure 4.

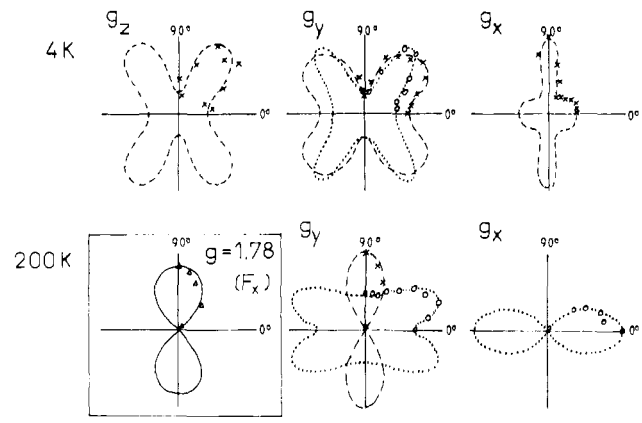

FIGURE 4: Polar plots for the line intensities of the signals described in Figure 3. The upper three plots represent signals photoinduced at 4 K (Figure 3a); the lower three plots are signals photoaccumulated at 200 K (Figure 3b). Data points obtained on signals attributed to center F_B ($g_z = 2.07$, $g_y = 1.91$, $g_x = 1.86$) are denoted by crosses, and the fitted orientation dependence is drawn as a dashed line; data points on F_A ($g_z = 2.05$, $g_y = 1.94$, $g_x = 1.88$) are marked as open circles, and the corresponding curve is drawn as a dotted line. Data points on the g_z and g_x lines of center F_A (at 4 K) and on the g_z peak of F_A and F_B (at 200 K) and the g_x trough of F_B (at 200 K) could not be obtained. The lower left plot (boxed) represents the orientation dependence of the signal at $g = 1.78$ (attributed to center F_X).

Spin-Polarized Triplet Signal. As the occurrence of a spin-polarized triplet signal in partially purified reaction centers from *Prosthecochloris aestuarii* has been demonstrated (Swarthoff et al., 1981b), we tried to find the equivalent signal in our membrane preparation of *Chlorobium limicola* f. *thiosulfatophilum*. In the presence of dithionite at pH 11, no triplet signal could be detected, either before or after illumination at 200 K. A two-step pretreatment was required before the photoinduced triplet state could be detected at 5 K: first a dark incubation in the presence of dithionite *and* redox mediators (benzyl viologen, methyl viologen); second, a preillumination at 200 K to reduce the FeS centers (monitored by EPR). In the first step, a component other than the iron–sulfur centers is thought to be reduced (see Discussion).

The triplet spectrum is similar to that reported by Swarthoff et al. (1981b), having splitting parameters of $|D| = 209 \times 10^{-4}$ cm^{-1} and $|E| = 40 \times 10^{-4}$ cm^{-1}.

Using the same pretreatment, we were able to detect the triplet signal in the oriented multilayers. Figure 5a shows spectra taken at a range of orientations of the membrane with respect to the magnetic field. Polar plots of the signal amplitudes (Figure 5b) reveal that the z- and x-axis directions are both parallel to the membrane plane, whereas the y axis is perpendicular.

Discussion

Terminal FeS Acceptors. Three different photoreduced electron acceptors have been demonstrated in this work. Two of them can be confidently assigned to iron–sulfur centers on the basis of the range of their g values. Only the iron–sulfur center with g values of 2.07, 1.91, and 1.86 can be stably photoreduced at 4 K in aqueous samples. It is probably identical with that giving rise to the signal at $g = 1.91$ reported by Jennings and Evans (1977). The 200-K photoaccumulated spectrum (g values of 2.05, 1.94, and 1.88) resembles the spectrum published by Swarthoff et al. (1981a) when reduced samples were frozen under illumination. Illumination of a sample while freezing or at 200 K can result in the photoreduction of more than one electron acceptor, since P_{840} can be rereduced by exogenous electron donors (dithionite) in both

Table I: Comparison of *g*-Tensor Orientations of the FeS Centers F_A and F_B in *Chlorobium limicola* f. *thiosulfatophilum* and PS I[a]

	after illumination at 4 K				after illumination at 200 K			
	F_A		F_B		F_A		F_B	
Chlorobium g_z	no[c]	no	2.07	50–60°	2.05	no	2.05	no
PS I g_z	2.05	0–20°	2.07	50°	2.05	90°	2.05	0°
Chlorobium g_y	1.94	55°	1.91	40°	1.94	25°	1.91	90°
PS I g_y	1.94	35–60°	1.92	35°	1.94	30–50°	1.92	90°
Chlorobium g_x	no	no	1.86	0/90°[b]	1.88	0°	1.88	no
PS I g_x	1.86	40–60°	1.89	0/90°	1.88	0°	1.88	0°

[a] Data taken from Dismukes and Sauer (1978), Prince et al. (1980), Aasa et al. (1981), and Hootkins and Bearden (1983). [b] Prince et al. (1980) and Hootkins and Bearden (1983) attributed only the 0° component to the FeS center F_B, although the 90° component was present. [c] no, not observable.

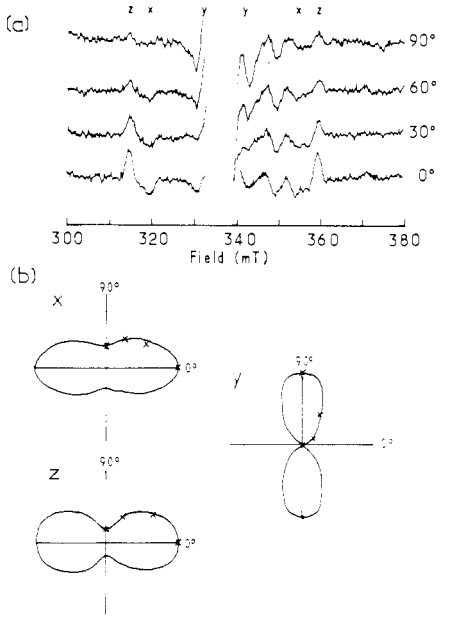

FIGURE 5: Spin-polarized triplet spectrum of P_{840} in oriented membrane multilayers from *Chlorobium limicola* f. *thiosulfatophilum* photoinduced at 4 K. Very reducing conditions were achieved as described under Experimental Procedures, and the required pretreatments crucial for the observation of the triplet state of P_{840} are described in the text. (a) Light minus dark difference spectra taken at a range of orientations. The additional signals around 350 mT are subtraction artifacts due to the fact that the sample temperature is slightly increased by the illumination. Angles are as defined in Figure 3. (b) Polar plots of the signals arising from the three canonical triplet axes. Instrument settings: temperature, 4 K; microwave power, 63 μW; frequency, 9.45 GHz; modulation amplitude, 2.4 mT; gain, 2×10^5.

of these treatments. The double integral of the 200-K photoaccumulated spectrum is about twice that of the 4-K illuminated spectrum, suggesting that there are two different acceptors present in a 1:1 stoichiometry.

This resolves the long-standing controversy on the spectral characteristics of the terminal acceptor (Jennings & Evans, 1977; Knaff et al., 1979; Swarthoff et al., 1981a). No evidence was found for a terminal acceptor showing the characteristics of an FeS center ($g = 1.94$) with an E_m of −420 mV as suggested by Swarthoff et al. (1981a) (see section on the midpoint potentials of F_A and F_B below for an explanation of this discrepency).

Comparison to PS I. In photosystem I from higher plants, an almost identical sequence of experimental results can be obtained: low-temperature illumination in PS I results in the photoreduction of center F_A (see Table I for *g* values), whereas photoaccumulation at 200 K or freezing under illumination additionally reduces center F_B (Table I). The 4-K photoreduced center F_A is stable only at very low temperatures (up to ≃100 K). At higher temperatures, a temperature-dependent back-reaction takes place, just as is the case for the signal studied in this work.

This strikingly similar behavior with respect to photoreduction and back-reaction of the two iron–sulfur centers found in green sulfur bacteria and PS I suggests an analogous function in both systems.

On the basis of the similarity of the g_y values in green sulfur bacteria compared to PS I, we designate the center photoreduced at 4 K in *Chlorobium* as center F_B and the second center (additionally photoaccumulated at 200 K) as center F_A.

Adopting this nomenclature implies a reversed pattern of iron–sulfur center photoreduction at low temperature. In green sulfur bacteria, center F_B is the 4-K photoreduced species whereas center F_A is (usually) photoreduced in PS I under comparable conditions. This is probably due to minor structural differences since, even in PS I, there are reports of center F_B being photoreduced at low temperature prior to center F_A:

(a) In the blue-green alga *Phormidium laminosum*, the sequence of FeS center reduction is partially reversed (Cammack et al., 1979).

(b) In PS I from spinach, the specificity of 4-K photoreduction is largely diminished on addition of glycerol (Evans & Heathcote, 1980).

(c) In samples of dried membrane multilayers, 4-K illumination results in reduction of center F_A in some reaction centers and center F_B in others (Prince et al., 1980; Hootkins & Bearden, 1983). The fact that the same holds true for oriented samples from *Chlorobium* obtained by this method further strengthens the similarity of both systems. It also indicates that as in PS I, the E_m values of F_B and F_A are likely to be very similar to each other since they seem to be influenced by small conformational changes, probably arising from an altered protein surface/solvent interaction.

Orientations and Interaction of F_A and F_B. Further evidence for an analogy between centers F_A and F_B in *Chlorobium* and PS I comes from the study of the orientations of their respective g tensors. In PS I, the orientations of the *g* tensors have been measured when either center F_A or center F_B is reduced and when both are reduced at the same time. In the fully reduced state, some orientations diverge from those found when only one center is paramagnetic (Aasa et al., 1981; Prince et al., 1980; Hootkins & Bearden, 1983). This is explained by a magnetic interaction (probably of the exchange type) between both reduced centers, an effect which had previously been evoked to explain *g*-value shifts on going from the partially to the fully reduced state (Malkin & Bearden, 1978; Cammack et al., 1979). Recently it has been shown that both center F_A and center F_B are located on an 8-kDa protein subunit [Ohyama et al., 1986; Wynn & Malkin, 1988; see also Lagoutte et al. (1984)], providing an explanation for the observed interaction.

Table I shows a comparison of *g* values and *g*-tensor orientations between green sulfur bacteria and PS I based on the

identification of FeS centers described above.

Unfortunately, due to the poorer signal-to-noise ratio in oriented samples from *Chlorobium*, not all of the signals found in frozen solution samples can be detected. However, it can be seen that, for all signals for which an evaluation was possible, no major difference between orientations of g tensors in PS I and *Chlorobium* is found. The only discrepancy is the position of the g_x peak of center F_B which has a g value of 1.86 in *Chlorobium* compared to 1.89 in PS I. The orientation of the g_y peak and the value of the g_z peak change in the *Chlorobium* sample in exactly the same manner as observed in PS I.

These results demonstrate that (a) the application of the PS I nomenclature to the FeS centers seen in the green sulfur bacteria reflects actual homology and (b) an interaction between centers F_A and F_B is found in *Chlorobium* just as in the case of PS I, suggesting that the structure of the F_A- and F_B-carrying protein is conserved in PS I and green sulfur bacteria. Therefore, we propose that a small subunit carrying the iron–sulfur clusters F_A and F_B must be an essential part of the green sulfur bacterial reaction center. According to SDS–PAGE, the partially purified reaction center reported by Hurt and Hauska (1984) apparently lacks such a subunit. This is in line with the virtual absence of EPR signals from centers F_A and F_B in this preparation (Nitschke and Hauska, unpublished results).

Midpoint Potentials of F_A and F_B. There is one major difference between the acceptors of green sulfur bacteria and PS I. Whereas in PS I iron–sulfur centers F_A and F_B can be chemically reduced by dithionite at high pH ($E_m \simeq -550$ mV), this cannot be achieved in the green sulfur bacterial reaction center. Thus, both centers F_A and F_B in green sulfur bacteria must be more negative than their PS I counterparts by at least 150 mV.

It is of note that there are several other redox centers in green sulfur bacteria, which are $\simeq$150 mV more reducing than comparable components in purple bacteria and plants:

(A) For P_{840}, a midpoint potential of +250 mV has been determined (Fowler et al., 1971; Prince & Olson, 1976; this work) compared to +450 mV for PS I (Golbeck, 1987).

(B) The Rieske center in *Chlorobium* has an E_m of +160 mV (Knaff & Malkin, 1976; our results), whereas the "normal" value is $\simeq$+300 mV (Trumpower, 1981).

(C) Cytochrome c_{555} which is the soluble donor to P_{840} and is equivalent to cytochrome c_2 ($E_m \simeq +340$ mV) in purple bacteria shows a midpoint potential of +140 mV (Meyer et al., 1968).

Swarthoff et al. (1981a,b) proposed that the terminal acceptor in their samples had a midpoint potential more positive than −420 mV. This was based on an enhancement of optically detected P_{840} triplet formation and the appearance of reversibility of the P_{840}^+ photoinduced at low temperature. This was correlated with the observation of a dark-reduced FeS center at $\simeq$−400 mV. These effects may correspond to the dark reduction step required for triplet formation which we report here and which we attribute tentatively to the two-electron reduction of a quinone-type acceptor, A_1 (see the section on the spin-polarized triplet). It is of note that methyl viologen was present in the samples used in the work of Swarthoff et al. (1981a). It seems likely that the FeS centers reduced at potentials of around −400 mV are part of a separate enzyme system not involved in photochemical processes occurring in the reaction center.

Center F_X. In addition to the two FeS centers described above, samples photoaccumulated at 200 K showed a broad (45-G half-width) trough at $g = 1.78$. A virtually identical feature is found in PS I, where the complete spectrum of this component, designated center F_X, is characterized by $g_z = 2.04$, $g_y = 1.88$, and $g_x = 1.78$ [reviewed in Rutherford and Heathcote (1985) and Golbeck (1987)]. The pure difference spectrum of F_X in PS I can be obtained after chemically prereducing centers F_A and F_B prior to the photoaccumulation protocol. Unfortunately, due to the inability of F_A and F_B in green sulfur bacteria to be chemically reduced, this method cannot be applied in this case.

Swarthoff et al. (1981a) observed a slight shift of the $g = 1.88$ feature in the spectrum of their FeS center depending on whether the samples were frozen under "high"- or "low"-light conditions. The authors invoked an interaction with either a more primary acceptor (considered to be a BChl *a* molecule) or the FeS center which they consider to be the terminal acceptor. However, according to our data, this change is more likely to be due to a superimposition of the broad g_y derivative-shaped signal of center F_X.

In the oriented samples, the g_x trough of center F_X is maximal at 90° (Figure 3b). This is identical with the situation in PS I, where the g_x orientation of F_X is perpendicular to the membrane plane.

In the case of PS I, center F_X is proposed to be either a 2Fe2S center with unusual electronic parameters (Bertrand et al., 1988) or an 4Fe4S cluster with rather distorted geometry (McDermott et al., 1988). Chemical determination of acid-labile sulfur and iron content favors the latter hypothesis (Scheller et al., 1989). In spite of this ambiguity, it seems clear that center F_X found in green sulfur bacteria is structurally homologous to its PS I counterpart.

Spin-Polarized Triplet. The spin-polarized triplet signal in photosynthetic reaction centers is commonly considered to be generated by radical pair recombination of a photoreduced primary acceptor and the photooxidized donor (Thurnauer et al., 1975).

Surprisingly, in green sulfur bacteria, the observation of a photoinduced triplet required a preincubation step at low potentials in the presence of mediators. This suggests that a component was chemically reduced under these conditions and that the presence of the unreduced form prevented triplet formation. To explain its influence on the triplet, this component could be an intermediate electron acceptor functioning between the primary acceptor and the iron–sulfur centers. Such an acceptor would be expected to have a functional one-electron redox couple at very low potential, much too low to be chemically reduced in our conditions. This apparently paradoxical situation can be rationalized if one assumes that this component is a quinone, because the double reduction of quinones to the fully reduced and fully protonated state can occur at much higher potentials than the one-electron reduction to the semiquinone.

A quinone-type acceptor has been proposed to exist in PS I functioning between the primary acceptors and the FeS centers, and it has been recently suggested that it can indeed undergo chemical double reduction at potentials much higher than that of its operative one-electron couple (Sêtif & Bottin, 1989).

It is of note that in PS II the triplet can only be produced after Q_A is doubly reduced (van Mieghem et al., 1989).

An earlier acceptor, A_0, must be involved in radical pair recombination to produce the triplet. The A_0-type acceptor is presumably the same component, a BChl species, identified

as a primary acceptor by picosecond absorption spectroscopy (Nuijs et al., 1985).

The presence of A_0- and A_1-type acceptors in *Chlorobium* has been inferred from the observation of EPR signals originally attributed to these species (Nitschke et al., 1987). Almost identical phenomena were previously observed in PS I (Gast et al., 1983; Bonnerjea & Evans, 1982), but their attribution to the actual acceptors A_0 and A_1 is currently in doubt (Hauska, 1988).

The splitting parameters of the triplet signal seen in *Chlorobium* are clearly different from those determined for photosystems I and II in higher plants and cyanobacteria (Frank et al., 1979; Rutherford & Mullet, 1981). Instead, they are remarkably close to the values found in purple bacteria (Dutton et al., 1971). Comparative studies of the photoinduced spin-polarized triplet in single crystals of the two purple bacteria, of which the structure has been determined, have been published recently (Norris et al., 1989). From these data, the conclusion was drawn that the observed decrease in zero-field splitting parameters (as compared to monomeric chlorophyll) is (at least in the studied cases) more influenced by a charge-transfer character within the two monomers than by delocalization of the triplet. Thus, a decrease in the ZFS can be taken as evidence for a dimeric structure whereas the absence of such a decrease does not necessarily imply a monomeric donor. This infers a dimeric structural organization of P_{840} since the triplet ZFS is reduced from the values of the monomers (Levanon & Norris, 1978) to almost the same degree as in purple bacteria.

Moreover, the orientations which we determined for the three canonical triplet axes are very similar to the orientations found in purple bacteria (Hales & Gupta, 1979; Tiede & Dutton, 1981). Therefore, we assume a geometry very similar to that elucidated for the purple bacterial reaction center by X-ray crystallography (Deisenhofer et al., 1985; Allen et al., 1986; Chang et al., 1986), i.e., a dimer of BChl molecules, both monomers being oriented approximately perpendicular to the membrane and with their optical Q_y transition [which is parallel to the x axis of the triplet as has been shown by Thurnauer and Norris (1977)] nearly in the plane of the membrane. It is of note that the optical Q_y transition for P_{840} has been reported to be parallel to the membrane (Swarthoff et al., 1981c).

In PS I, the z-axis (i.e., the axis perpendicular to the ring plane) orientation to the membrane is the same as in purple bacteria and green sulfur bacteria; the orientations of the x and y axes, however, appear to be different (Rutherford and Sêtif, unpublished results).

Model for the Green Sulfur Bacterial Reaction Center. Three acceptors in the reaction center from green sulfur bacteria have been characterized in this study. Two of them (centers F_A and F_B) are 4Fe4S centers; the third one corresponds to the FeS center F_X in photosystem I, the detailed chemical nature of which is still a matter of debate. According to our results, centers F_A and F_B are significantly more reducing than their counterparts in PS I. We are unable to determine if center F_X is shifted to more negative midpoint potentials as well. However, if it functions as an electron acceptor prior to F_A/F_B, such a shift probably occurs because otherwise F_X would lie about equipotential to F_A/F_B. Recent work in PS I indicates that F_X is not an obligate electron acceptor (Brettel, 1989; Brettel and Sêtif, unpublished results). It is possible then that the E_m of F_X is not shifted to match the other components in green bacteria.

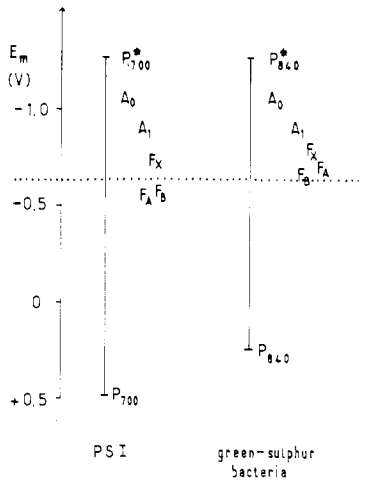

FIGURE 6: Schematic comparison of the approximate midpoint potentials of the electron donor and acceptors in PS I and green sulfur bacteria. The potentials of the excited donors were calculated from the midpoint potential of the P/P^+ couples and their excitation wavelengths. The potentials of acceptors A_0 and A_1 are only rough estimates in the case of both PS I and *Chlorobium*. The dotted line represents the theoretical limit for chemical reduction by sodium dithionite at high pH.

Our results suggest the presence of at least two acceptors in addition to the three FeS centers, analogous to the A_0 (chlorophyll-like) and A_1 (quinone-like) acceptors in PS I. However, since the reducing power of $*P_{840}$ can be calculated from the E_m of the P_{840}/P_{840}^+ couple and the energy of the 840-nm photon to be −1.2 V (which is the same as $*P_{700}$), the relative energy difference between the early electron acceptors must be different from those in PS I. Therefore, it might be predicted that early forward electron-transfer rates and yields in green sulfur bacteria will be different from those in PS I. A schematic comparison between the reaction center in green sulfur bacteria and PS I is depicted in Figure 6.

The presence of a reaction center associated tetraheme cytochrome subunit (Feiler et al., 1989) further stresses the relationship to the purple bacterial RC.

The reaction center of green sulfur bacteria seems to have somewhat chimeric features: its acceptor side very much resembles PS I whereas the primary donor looks almost identical with that of purple bacteria.

These data lead us to speculate that purple bacteria, PS II, green bacteria, and PS I all evolved from a common ancestor. A similar speculation based on proposed homologies of amino acid sequences in PS I and purple bacteria at the level of the primary donors and acceptors has been recently published (Robert & Moënne-Loccoz, 1989).

Extending this idea further, we visualize the acceptor A_1 as an analogue of Q_A [note that a second quinone, which could be the equivalent of Q_B, is also present in PS I as has been shown by Takahashi et al. (1985) and Schoeder and Lockau (1986)] and the FeS center F_X as a very modified counterpart of the Fe^{2+} atom. The dimeric core of the reaction center as shown from the crystal structure of purple bacteria also seems to be present in PS I. The greater molecular mass of the protein subunits could indicate fusion of the reaction center gene with genes coding for antenna chlorophyll proteins which may have occurred in an ancient homodimeric ancestor.

The detection of a new class of photosynthetic organisms, the *heliobacteria* (Gest & Favinger, 1983; Beer-Romero & Gest, 1987), and the proposal that they possess a unique photosynthetic reaction center (van Kan et al., 1989) could provide crucial evidence for or against the above hypothesis.

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REFERENCES

Aasa, R., Bergström, J., & Vänngård, T. (1981) *Biochim. Biophys. Acta 637*, 118–123.

Allen, J. P., Feher, G., Yeates, T. O., Rees, D. C., Deisenhofer, J., Michel, H., & Huber, R. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8589–8593.

Beer-Romero, P., & Gest, H. (1987) *FEMS Microbiol. Lett. 41*, 109–114.

Bertrand, P., Guigliarelli, B., Gayda, J.-P., Sêtif, P., & Mathis, P. (1988) *Biochim. Biophys. Acta 933*, 393–397.

Biebl, H., & Pfennig, N. (1978) *Arch. Microbiol. 117*, 9–16.

Blankenship, R. E. (1985) *Photosynth. Res. 6*, 317–333.

Blankenship, R. E., Trost, J. T., & Mancino, L. J. (1988) in *The Photosynthetic Bacterial Reaction Center* (Breton, J., & Verméglio, A., Eds.) Plenum Press, New York and London.

Blasie, J. K., Erecinska, M., Samuels, S., & Leigh, J. S. (1978) *Biochim. Biophys. Acta 501*, 33–52.

Blum, H., Salerno, J. S., & Leigh, J. S. (1978) *J. Magn. Reson. 30*, 385–391.

Bonnerjea, J., & Evans, M. C. W. (1982) *FEBS Lett. 148*, 313–316.

Brettel, K. (1989) *Biochim. Biophys. Acta 976*, 246–249.

Buchanan, B. B., & Evans, M. C. W. (1966) *Biochim. Biophys. Acta 180*, 123–129.

Cammack, R., Ryan, M. D., & Stewart, A. C. (1979) *FEBS Lett. 107*, 422–426.

Chang, C.-H., Tiede, D. M., Tang, J., Smith U., Norris, J. R., & Schiffer, M. (1986) *FEBS Lett. 205*, 82–86.

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

Dismukes, G. C., & Sauer, K. (1978) *Biochim. Biophys. Acta 504*, 431–445.

Dutton, P. L. (1971) *Biochim. Biophys. Acta 226*, 63–80.

Dutton, P. L., Leigh, J. S., & Seibert, M. (1971) *Biochem. Biophys. Res. Commun. 40*, 406–413.

Evans, M. C. W., & Heathcote, P. (1980) *Biochim. Biophys. Acta 590*, 89–96.

Feiler, U., Nitschke, W., Michel, H., & Rutherford, A. W. (1989) *Symposium on Molecular* Biology *of Membrane-Bound Complexes in Phototrophic Bacteria*, Freiburg, FRG, Abstr. P-6, 19.

Fowler, C. F., Nugent, N. A., & Fuller, R. C. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 2278–2282.

Frank, H. A., McLean, M. B., & Sauer, K. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5124–5128.

Gast, P., Swarthoff, T., Ebskamp, F. C. R., & Hoff, A. J. (1983) *Biochim. Biophys. Acta 722*, 168–175.

Gest, H., & Favinger, J. L. (1983) *Arch. Microbiol. 136*, 11–16.

Golbeck, J. H. (1987) *Biochim. Biophys. Acta 895*, 167–204.

Hales, B. J., & Gupta, A. D. (1979) *Biochim. Biophys. Acta 548*, 276–286.

Hauska, G. (1988) *Trends Biochem. Sci. 13*, 415–416.

Heathcote, P., & Warden, J. T. (1982) *FEBS Lett. 140*, 277–281.

Hootkins, R., & Bearden, A. (1983) *Biochim. Biophys. Acta 723*, 16–29.

Hurt, E. C., & Hauska, G. (1984) *FEBS Lett. 168*, 149–154.

Jennings, J. V., & Evans, M. C. W. (1977) *FEBS Lett. 75*, 33–36.

Knaff, D. B. (1978) in *The Photosynthetic Bacteria* (Clayton, R. K., & Sistrom, W. R., Eds.) pp 629–640, Plenum Press, New York and London.

Knaff, D. B., & Malkin, R. (1976) *Biochim. Biophys. Acta 430*, 244–252.

Knaff, D. B., Olson, J. M., & Prince, R. C. (1979) *FEBS Lett. 98*, 285–289.

Komiya, H., Yeates, T. O., Rees, D. C., Allen, J. P., & Feher, G. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 9012–9016.

Lagoutte, B., Sêtif, P., & Duranton, J. (1984) *FEBS Lett. 174*, 24–29.

Levanon, H., & Norris, J. R. (1978) *Chem. Rev. 78*, 185–189.

Malkin, R., & Bearden, A. J. (1978) *Biochim. Biophys. Acta 505*, 147–189.

McDermott, A. E., Yachandra, V. K., Guiles, R. D., Britt, R. D., Dexheimer, S. L., Sauer, K., & Klein, M. P. (1988) *Biochemistry 27*, 4013–4020.

Meyer, T. E., Bartsch, R. G., Cusanovich, M. A., & Mathewson, J. H. (1968) *Biochim. Biophys. Acta 153*, 854–861.

Nitschke, W., Feiler, U., Lockau, W., & Hauska, G. (1987) *FEBS Lett. 218*, 283–286.

Norris, J. R., Budil, D. E., Gast, P., Chang, C.-H., El-Kabbari, O., & Schiffer, M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4335–4339.

Nuijs, A. M., Vasmel, H., Joppe, H. L. P., Duysens, L. N. M., & Amesz, J. (1985) *Biochim. Biophys. Acta 807*, 24–34.

Ohyama, K., Fukuzawa, H., Kohchi, T., Shirai, H., Sano, T., Sano, S., Umesono, K., Shiki, Y., Takeuchi, M., Chang, Z., Aota, S., Inokuchi, H., & Ozeki, H. (1986) *Nature 322*, 572–574.

Ovchinnikov, Yu. A., Abdulaev, N. G., Zolotarev, A. S., Shmukler, B. E., Zargarov, A. A., Kutuzov, M. A., Telezhinskaya, I. N., & Levina, N. B. (1988a) *FEBS Lett. 231*, 237–242.

Ovchinnikov, Yu. A., Abdulaev, N. G., Shmukler, B. E., Zargarov, A. A., Kutuzov, M. A., Telezhinskaya, I. N., Levina, N. B., & Zolotarev, A. S. (1988b) *FEBS Lett. 232*, 364–368.

Prince, R. C., & Olson, J. M. (1976) *Biochim. Biophys. Acta 423*, 357–362.

Prince, R. C., Crowder, M. S., & Bearden, A. J. (1980) *Biochim. Biophys. Acta 392*, 323–337.

Robert, B., & Moënne-Loccoz, P. (1989) *VIIIth International Congress on Photosynthesis*, Stockholm, Sweden, Abstr. 108.

Rutherford, A. W., & Mullet, J. E. (1981) *Biochim. Biophys. Acta 635*, 225–235.

Rutherford, A. W., & Heathcote, P. (1985) *Photosynth. Res. 6*, 295–316.

Scheller, H. V., Svendsen, I., & Møller, B. L. (1989) *J. Biol. Chem. 264*, 6929–6934.

Schmidt, K. (1980) *Arch. Microbiol. 124*, 21–31.

Schoeder, H.-U., & Lockau, W. (1986) *FEBS Lett. 199*, 23-27.
Sêtif, P., & Bottin, H. (1989) *Biochemistry 28*, 2689-2697.
Shiozawa, J., Lottspeich, F., & Feick, R. (1987) *Eur. J. Biochem. 167*, 595-600.
Shuvalov, V. A., Shkuropatov, A. Ya., Kulakova, S. M., Ismailov, M. A., & Shkuropatova, V. A. (1986) *Biochim. Biophys. Acta. 844*, 337-346.
Swarthoff, T., & Amesz, J. (1979) *Biochim. Biophys. Acta 548*, 427-432.
Swarthoff, T., Gast, P., Hoff, A. J., & Amesz, J. (1981a) *FEBS Lett. 130*, 93-98.
Swarthoff, T., Gast, P., & Hoff, A. J. (1981b) *FEBS Lett. 127*, 83-86.
Swarthoff, T., Amesz, J., Kramer, H. J. M., & Rijgersberg, C. P. (1981c) *Isr. J. Chem. 21*, 332-337.
Takahashi, Y., Hirota, K., & Katoh, S. (1985) *Photosynth. Res. 6*, 183-192.
Thurnauer, M. C., & Norris, J. R. (1977) *Chem. Phys. Lett. 47*, 100-105.
Thurnauer, M. C., Katz, J. J., & Norris, J. R. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3270-3274.
Tiede, D. M., & Dutton, P. L. (1981) *Biochim. Biophys. Acta 637*, 278-290.
Trumpower, B. L. (1981) *Biochim. Biophys. Acta 639*, 129-155.
van Kan, P. J. M., van de Meent, E. J., Kleinherenbrinck, F. A. M., Aartsma, T. J., & Amesz, J. (1989) *Symposium on Molecular Biology of Membrane-Bound Complexes in Phototrophic Bacteria*, Freiburg, FRG, Abstr. P-6, 59.
van Mieghem, F. J. E., Nitschke, W., Mathis, P., & Rutherford, A. W. (1989) *Biochim. Biophys. Acta 977*, 207-214.
Widdel, F., Kohring, G. W., & Mayer, F. (1983) *Arch. Microbiol. 134*, 286-294.
Woese, C. R. (1987) *Microbiol. Rev. 51*, 221-271.
Wynn, R. M., & Malkin, R. (1988) *FEBS Lett. 229*, 293-297.

Pancreatic Cholesterol Esterases. 1. Pancreatic Cholesterol Esterase Induction during Maturation

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ABSTRACT: The activities of pancreatic cholesterol esterase from calf and cow pancreas were examined in detail. A 1300-fold enhancement of enzymatic activity was found after maturation, even though cholesterol esterase activity levels in other organs did not change from the juvenile to the adult species. Radioimmunoassays also showed that the calf pancreas contained at least 100-fold less cholesterol esterase protein. Decreased amounts of protein were not due to enhanced proteolysis, since cytosol from cow pancreas degrades exogenously added cholesterol esterase faster than that from calf pancreas. Rather, enhancement of pancreatic cholesterol esterase activity associated with bovine maturation was the result of specific, increased synthesis of a 72-kDa enzyme. This labile 72-kDa cholesterol esterase species was purified to homogeneity by a two-step process in 75% yield and is the major form of bovine pancreatic cholesterol esterase (99%). A much less abundant 67-kDa species, accounting for less than 1% of total pancreatic cholesterol esterase activity, was also purified to homogeneity in a similar two-step process. These results demonstrate that a specific form of pancreatic cholesterol esterase is induced during maturation, and they bear importantly on understanding juvenile cholesterol metabolism as related to dietary absorption of this sterol.

Coronary artery disease is caused by progressive occlusion of the coronary arteries, and its incidence and severity can be correlated with the plasma concentration of cholesterol (Kannel et al., 1971; Goldbourt et al., 1985; Goodman, 1988). Since the biochemical mechanism for the induction of atherosclerosis is not known, dietary manipulation has been the only available recourse for early treatment or prevention. There is increasing evidence that the disease begins early in life, and many of the dietary recommendations proposed for adults have been suggested for children as well, on the assumption that the mechanism of cholesterol uptake is the same in adults as in children (Kwiterorich, 1986; McNamara et al., 1974).

One of the critical steps in cholesterol absorption is the pancreatic cholesterol esterase catalyzed cleavage of cholesteryl esters, one of the dietary forms of ingested cholesterol (Vahouny & Treadwell, 1964). This lipolytic enzyme has been extensively investigated from a variety of adult species, including man, pig, rat, and cow (Lombardo et al., 1978; Rudd et al., 1987; Calame et al., 1975; Van Den Bosch et al., 1973), but there have been no studies from the corresponding juvenile animals or children. Thus, biochemical details of cholesterol absorption have remained largely speculative in children, without comparative knowledge of enzyme structure, function, and regulation.

In this work, we examine the pancretic cholesterol esterase activities in cow and calf and find a nearly 1300-fold enhancement of enzymatic activity during maturation. Moreover, this effect seems to be a characteristic of pancreas, since the cholesterol esterase levels in other organs do not change from the juvenile to the adult species. Using purification methods developed in this paper, we show that induction is selective for the 72-kDa form of the enzyme. Study of the regulation of this enhancement by growth factors, hormones, or other moelcules is feasible now that the enzyme has been cloned (Kyger et al., 1989), and it bears further scrutiny since such induction will influence the pattern of cholesterol ab-

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