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CYTOCHROME PHOTOOXIDATION AT LIQUD NITROGEN TEMPERATURES IN PHOTOSYNTHETIC BACTERIA

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SUMMARY

The whole cells of a number of purple photosynthetic bacteria representing both sulfur and nonsulfur species of these unique microorganisms were examined with respect to their ability to undergo the photooxidation of a cytochrome and other light-induced absorbance changes at 77°K. Out of a total of fifteen species thus far investigated, twelve have been found to possess a *c*-type cytochrome which is capable of undergoing photooxidation at temperatures of liquid nitrogen. Evidence is presented for the reversible oxidation-reduction reaction of cytochrome *c* in *Rhodopseudomonas palustris* at 77°K.

In addition to the cytochrome reaction, absorbance changes caused by other spectral components were also observed in many species at 77°K. These changes were identified with a spectral shift of carotenoid peaks, P435, and tentatively with a minor band of bacteriochlorophyll in the light-*minus*-dark difference spectra.

It is postulated that the difference in the electron transfer times of cytochrome *c* and the diversity of absorbance changes at 77°K noted in a variety of bacteria are due to species variation in the molecular orientation of spectral components within the photosynthetic apparatus.

INTRODUCTION

The photooxidation at temperatures of liquid nitrogen of a *c*-type cytochrome in the photosynthetic sulfur bacterium, *Chromatium* D, was first described by CHANCE AND NISHIMURA[1] in 1960. The absence of such a light-induced cytochrome oxidation at 77°K in the two species of nonsulfur purple bacteria, *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides*[1-5], has raised a question regarding the generality of the collision-independent electron transfer reaction and the role of *c*-type cytochrome during the early events in bacterial photosynthesis.

Recently, DEVAULT *et al.*[6-8] extended the work in *Chromatium* D using a pulsed ruby laser photochemical activation apparatus equipped with a cryostat for work at temperatures of liquid helium. Kinetic analysis of laser-induced oxidation of *c*-type cytochrome over a temperature range of 4.4°K to 300°K has resulted in a theory of electron tunneling in *Chromatium* D (ref. 7).

This paper describes studies on the light-induced oxidation of a *c*-type cytochrome and on other reactions at 77°K in the whole cells of a number of species of nonsulfur and sulfur purple photosynthetic bacteria. The phenomenon of low temperature cytochrome photooxidation has been shown to be a general characteristic of these photosynthetic systems[9].

MATERIALS AND METHODS

The following species of photosynthetic bacteria were examined: *Chromatium vinosum*; *Chromatium violascens*; *Thiocapsa floridana*; *Rhodopseudomonas gelatinosa*, strain I; *Rhodopseudomonas viridis*; *Rhodopseudomonas capsulata*, ATH. 2.3.3; Rhodopseudomonas species NW (isolated by T. Kihara and E. Haskins); *Rhodopseudomonas palustris*, ATH. 2.1.2; *Rhodomicrobium vannielii*, strain 1210D; *Rhodomicrobium* species CK (isolated from a garden soil); *Rhodospirillum fulvum*, strains 1350 and 1360; *Rhodospirillum molischianum*, strain 6960. In addition to the twelve species listed above, *Rhodospirillum rubrum*, strain S-1 and seventeen other strains of this bacterium, ATH. 1.1.2 through 1.1.18, and two strains of *Rhodopseudomonas spheroides*, ATH. 2.4.15 and 2.4.16, all of them obtained from Professor Van Niel's collection, were re-examined with respect to light-induced cytochrome reactions at 77°K. Cultures of all nonsulfur species, except for *R. molischianum* and *R. fulvum*, were grown at 28° in an illuminated water bath in Medium 1 described by HASKINS AND KIHARA[10]. Medium 2 (ref. 9) was used for cultivation of *R. molischianum*. *R. fulvum* was grown in the growth medium described by PFENNIG *et al.*[11]. The sulfur bacteria were cultured in modified Pfennig's medium (see ref. 12). Cultures of the six obligatory anaerobes, *Rm. vannielii*, *R. molischianum*, *R. fulvum*, *C. vinosum*, *C. violascens*, and *T. floridana* were continuously gassed with a mixture of CO_2–N_2 (5:95, v/v). Whole cells for experiments were obtained after 2–3 days of growth. They were harvested by centrifugation and were resuspended in a small amount of native supernatant culture medium to give concentrated samples. The cuvette[7] used in all experiments had Plexiglass windows, 1.6 mm thick and spaced 1.0 mm apart.

Light-induced absorption changes in the suspension of whole cells were measured in a dual wavelength spectrophotometer equipped with two Bausch and Lomb monochromators[13] and fitted with an attachment for cross illumination of the sample and a clear Dewar flask for low temperature work. Sample illumination was provided from a tungsten source filtered through a Wratten 88 A filter. The light intensity measured with a thermopile at the cuvette surface was 44.3 mW/cm^2. A Corning blue-green filter 9788 was placed on an EMI photomultiplier tube (type 9524B) as a guard filter against near-infrared cross illumination.

A fast and sensitive single-beam spectrophotometer developed by DEVAULT AND CHANCE[6,7] was employed for the study of kinetics of cytochrome oxidation at liquid nitrogen and room temperatures induced by a pulsed ruby laser of wavelength 694.3 nm. The laser used in the Q-switched mode gave a pulse approx. 30 nsec in duration and 0.4–1.0 m J/cm^2 at the sample. A capacitance-coupled a.c. transient suppression circuit developed by D. DEVAULT AND J. CAIN (unpublished) was used between the photomultiplier and oscilloscope to obtain oscilloscope traces during experiments with frozen samples.

RESULTS

Eleven different species of sulfur and nonsulfur photosynthetic bacteria exhibited light-induced oxidation of a *c*-type cytochrome at liquid nitrogen temperatures. However, under the same conditions, a spectral shift of the Soret band at 77°K was not observed in the strain of *Rps. capsulata*, in either of the two strains of *Rps. spheroides* or in any of the eighteen strains of *R. rubrum*. Failure to observe cytochrome photooxidation at 77°K in *Rps. speroides* and *R. rubrum* has been previously reported$^{1-5}$.

Light-*minus*-dark difference spectra of frozen samples of representative species under continuous near-infrared illumination are shown in Fig. 1. The oxidation of a cytochrome of the *c*-type is evident in Figs. 1A–1C by the absorbance increase at 408 nm and decrease at 418–420 nm which have shifted slightly to shorter wavelengths at low temperatures^{3,5}. Conspicuous absorption changes in the region between 450 and 540 nm in many of the species typically shown in *Rps. gelatinosa* (Fig. 1C) are assumed to be due to a shift towards longer wavelengths of the absorption peaks of carotenoid components. In these cases the spectral interference by carotenoid obscures the β band of cytochrome *c*, and the shape of the α band is altered. In *T. floridana, C. violascens, R. fulvum, R. molischianum, Rps. palustris, Rps. capsulata*, and *R. rubrum* the bleach-

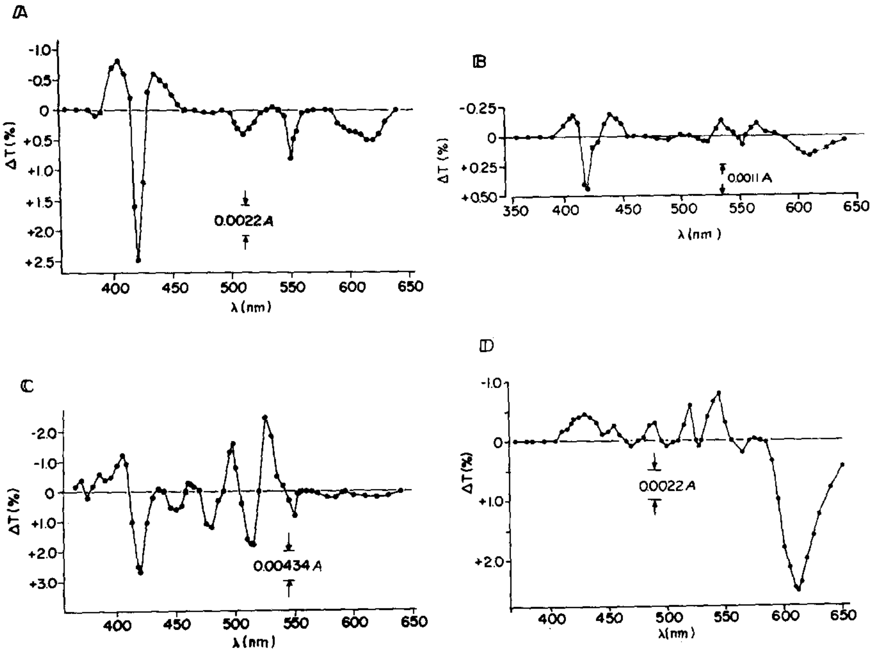

Fig. 1. A. Low-temperature (77°K) light-*minus*-dark difference spectrum for the 'anaerobic' whole cells of *T. floridana*. Light-induced absorbance changes of a *c*-type cytochrome observed in this species are not reversible in the dark. 0.15 mM bacteriochlorophyll. B. Same as A for *Chromatium violascens*. The photooxidized cytochrome recovers in the dark 20–50 % of the original change at the Soret band. 0.25 mM bacteriochlorophyll. C. Same as A for *Rps. gelatinosa*. All of the absorbance changes in this species undergo partial dark recovery to a varying degree. 0.14 mM bacteriochlorophyll. D. Same as A for *Rps. capsulata*, showing absence of apparent cytochrome oxidation in this bacterium. All of the changes are reversed rapidly and completely when actinic light is turned off. 0.2 mM bacteriochlorophyll.

ing of the minor bacteriochlorophyll band at about 590 nm is thought to be responsible for the broad absorbance decrease peaking between 600 and 612.5 nm. Often conspicuous absorbance increases in the region around 435 nm can be observed in most of the species examined, which are believed to be due mainly to the pigment P435. Light-on and -off responses of P435 and bacteriochlorophyll are very rapid and completely reversible at 77°K.

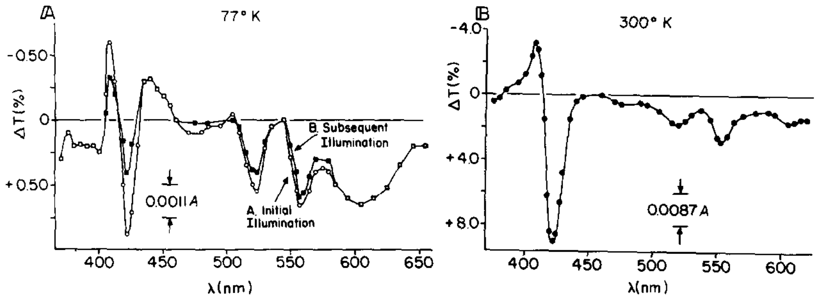

Fig. 2. A. Low temperature (77°K) light-*minus*-dark difference spectra for *Rps. palustris*, ATH. 2.1.2. Curve A was obtained by plotting light-induced absorbance changes attained after initial illumination. Curve B represents a difference spectrum of reversible absorbance changes in the same sample obtained after subsequent illumination. 0.13 mM bacteriochlorophyll. B. Light-*minus*-dark difference spectrum (300°K) for *Rps. palustris*, ATH. 2.1.2. 0.1 mM bacteriochlorophyll.

In *C. vinosum*, *T. floridana*, *R. fulvum*, Rhodopseudomonas sp. NW, and *Rm. vannielii*, the photooxidation of the cytochrome as monitored at 418–420 nm at temperatures of liquid nitrogen is not reversed in the dark. Fig. 3A typically illustrates this one-way electron donation and absence of a subsequent dark reduction of cytochrome *c* at 77°K. In this respect species are much like *Chromatium* D. Absorption changes due to carotenoids are absent in the three species of sulfur bacteria as typically shown in *T. floridana* (Fig. 1A). The aeration of the suspensions of the five species mentioned above as having no reversible electron flow results in the elimination of the phenomenon of low-temperature cytochrome oxidation.

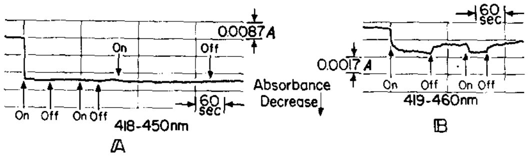

Fig. 3. A. Time-course of the decrease in absorbance at 418 nm relative to 450 nm of a whole cell suspension of Rhodopseudomonas species NW induced by near-infrared illumination at 77°K, showing one-way electron donation by a *c*-type cytochrome. B. Time-course of the light-on and -off response at 419 nm relative to 460 nm of a suspension of *Rps. palustris* at 77°K, showing possible dark reduction of part of the photooxidized cytochrome.

In contrast to the above species, aeration of the suspension of *R. molischianum*, *Rps. viridis*, *Rps. gelatinosa*, Rhodomicrobium sp. C, *Rps. palustris*, and *C. violascens* does not have appreciable effect on the cytochrome photooxidation. The actinic light-on and -off kinetics of the low-temperature absorption changes at 418–420 nm of the unaerated whole cells of these bacteria show that the changes are partially reversible to a varying degree in the dark (Fig. 3B). The partial recovery monitored in the Soret region in some of these cases may not be entirely attributed to the dark reduction of the photooxidized cytochrome *c*; there is a possibility of interfering absorption changes of carotenoids as spectral shift of carotenoid peaks are also partially, or in some cases completely, reversible at temperatures of liquid nitrogen. This reverse reaction identifies a new group of photosynthetic bacteria in which dark reduction of part of the photooxidized cytochrome takes place at 77°K.

Cytochrome *c* photooxidation in *Rps. palustris* proceeds in much the same way as in the other species that exhibit some recovery at the Soret band upon cessation of illumination at 77°K. Initial light-induced absorbance changes and subsequent light and dark reactions were studied in the visible region from 360 to 640 nm in *Rps. palustris*. The partial dark recovery after the initial illumination and completely reversible changes after subsequent illuminations in the α, β, and γ regions are apparently caused largely, if not entirely, by the reduction of the photooxidized cytochrome. The kinetics of light-on and -off response (Fig. 3B) indicate 40–50 % recovery at 419 nm. The two difference spectra shown in Fig. 2A were obtained by plotting the steady level of absorption changes attained with initial illumination (Curve A) and during subsequent illumination after a dark period (Curve B). Curve B, therefore, represents the total spectral changes that are repetitively and completely reversible at 77°K. It is to be noted in these spectra that the α and β bands with peaks at 557 and 523 nm, respectively, are disproportionately large with respect to the γ band. The positions of the α and β bands may have been displaced towards the longer wavelengths due in part to the large bacteriochlorophyll bleaching. The light-*minus*-dark low temperature difference spectrum of cell-free extracts of the same species in which absorbance changes peaking at 435 nm and at 612.5 nm are nearly eliminated show the α band at 548 nm and the β band at 516.5 nm (ref. 14). In addition to the marked deviation from the normal light-*minus*-dark difference spectrum of a *c*-type cytochrome at 300°K, the reversible changes of the α and β bands amount to about 80 % of the initial changes. Of the other spectral components often found to undergo absorbance changes at 77°K, band shifts of carotenoid pigments are apparently absent in this species[14,15]. The difference spectrum of the same bacterium at 300°K also lacks carotenoid absorption changes (Fig. 2B).

Those changes, which are completely reversible at 77°K, in the region between 430 nm and 450 nm in Fig. 2A can be attributed to the as yet unidentified pigment P435 observed originally in *R. rubrum*[3,16–18]. The scanning of the spectrum in the visible region, carried out at 77°K on the single-beam rapid spectrophotometer in an attempt to determine the shape of P435 absorption changes[15], reveals that the changes attributable to P435 are maximal at 440 nm and minimal at 405 nm and 520 nm. No increase in transmission was found associated with P435 following a Q-switched ruby

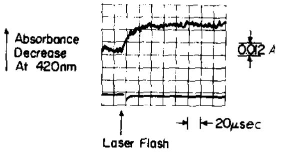

Fig. 4. Q-switched, ruby laser-induced oxidation at 77°K of *c*-type cytochrome in an anaerobic whole cell suspension of Rhodopseudomonas sp. NW at 420 nm. The most extensive laser-induced low-temperature cytochrome oxidation and shortest half-rise times were obtained with this bacterium. 0.28 mM bacteriochlorophyll.

laser flash. From these observations it is concluded that P435 changes do not make a spectroscopic contribution to the dark recovery of photooxidized cytochrome change at 419 nm since the changes due to these two pigments are opposite in direction at this wavelength.

The prominent band with a peak at about 605 nm in Fig. 2A is assumed as in the other cases to result from the decrease in absorption of the minor bacteriochlorophyll band; these changes are also completely reversible at 77°K. Because of its proximity to the α band of cytochrome at 557 nm, it is probable that the extent of recovery of the α band is somewhat modified by the concomitant bacteriochlorophyll changes taking place in the same direction.

Rates of laser-induced cytochrome oxidation at temperatures of liquid nitrogen were studied with some of the eleven species which exhibited oxidation of cytochrome *c* at 77°K under continuous illumination. In addition, the half-times of cytochrome oxidation induced by a Q-switched laser flash were also determined at 300°K with four species of sulfur bacteria and eleven species of nonsulfur purple bacteria (Table I). Electron transfer times of a reduced *c*-type cytochrome in some species such as *Rps. gelatinosa* and Rhodopseudomonas sp. NW (Fig. 4) were found to be considerably faster than those of other bacteria. *Rps. gelatinosa* gave half-times of 10 to 20 µsec, but the shortest half-rise time was obtained with Rhodopseudomonas sp. NW, which ranged from less than 2 to 6 µsec at 77°K, as estimated from a number of measurements with different samples, while the values in other species ranged from 400 µsec to 12 msec.

A summary of data obtained with the whole cells of various species of photosynthetic bacteria is presented in Table I.

TABLE I

A SUMMARY OF CURRENTLY AVAILABLE DATA ON LIGHT-INDUCED CYTOCHROME OXIDATION IN FIFTEEN SPECIES OF PHOTOSYNTHETIC BACTERIA STUDIED AT LIQUID NITROGEN TEMPERATURES

Name of species	*Obligatory (O) or facultative (F) anaer(*	*Cytochrome oxidation (77° K)*	*Sensitive to mild aeration*	*Partially reversible*	*Laser-induced cytochrome c oxidation half-time (µsec)*	
					300° K	*77° K*
Sulfur bacteria						
Chromatium D	O	$+(\alpha,\beta,\gamma)$	+	–	2	2000
Chromatium vinosum	O	$+(\alpha,\gamma)$	+	–	< 2	500–900
Chromatium violascens	O	$+(\alpha,\beta,\gamma)$	–	+	< 2	
Thiocapsa floridana	O	$+(\alpha,\gamma,\beta?)$	+	–	< 2	400–800
Non-sulfur bacteria						
Rhodospirillum rubrum	F	—				—
Rhodospirillum fulvum	O	$+(\alpha,\gamma)$	+	–	approx. 8	
Rhodospirillum molischianum	O	$+(\gamma,\alpha?)$	–	+	approx. 4	
Rhodopseudomonas spheroides	F	—			approx. 8	—
Rhodopseudomonas palustris	F	$+(\alpha,\beta,\gamma)$	–	+	< 2	8000–12 000
Rhodopseudomonas capsulata	F	—			20–40	—
Rhodopseudomonas gelatinosa	F	$+(\gamma,\alpha?)$	–	+	0.5–0.8	10–20
Rhodopseudomonas viridis	F	$+(\alpha?,\beta?,\gamma)$	–	+	< 2	
Rhodosporomonas sp. NW	F	$+(\gamma,\alpha?)$	+	–	0.3–0.6	< 2–6
Rhodomicrobium vannielii	O	$+(\alpha,\gamma,\beta?)$	+	–	approx. 4	
Rhodomicrobium sp. CK	F	$+(\alpha,\gamma,\beta?)$	–	+	approx. 4	

DISCUSSION

The present investigation has shown cytochrome photooxidation at 77°K to be a phenomenon common to a number of species of both obligate and facultative photosynthetic anaerobes. However, there are three species known to date, namely, *R. rubrum, Rps. spheroides*, and *Rps. capsulata,* that do not undergo such a low-temperature reaction. Our examination of different strains of *R. rubrum* and *Rps. spheroides* confirmed the absence of a spectral shift at the Soret band at 77°K in these species, suggesting possible species dependence of this phenomenon among photosynthetic bacteria.

The earlier low temperature studies on cytochromes in the chromatophore fractions of *Chromatium* D (ref. 5) showed that cytochrome *c* photooxidation was diminished to approximately half-maximal amplitude at 213°K, but at 79°K no response was observed. The cell-free extracts prepared in the presence of air from a nonsulfur bacterium, *Rps. palustris*, ATH. 2.1.2, by sonicating for 5 min or by rupturing intact cells in a French pressure cell at 10000 lb·inch^{-2} are no longer capable of exhibiting photooxidation of cytochrome *c* at 77°K under the same conditions. It is evident that destruction of whole cells by the drastic methods employed for the preparation of cell-free extracts results in the loss of this reaction at low temperatures. This observation and more recent results with cell-free preparations (P.L. DUTTON, T. KIHARA AND B. CHANCE, unpublished results) indicate that aeration, which occurs during the preparation of the extracts, may cause extensive oxidation of endogenous reducing components including *c*-type cytochromes.

In some species of bacteria a conspicuous response of carotenoid components to strong illumination is temperature insensitive at 77°K (Fig. 1C) while absorption changes of carotenoids are absent or inconspicuous at 77°K in *Chromatium* D, *C. vinosum, C. violascens, T. floridana,* Rhodomicrobium sp. CK, and *Rps. palustris,* ATH. 2.1.2.

The subject of collision-independent reactions in photosynthetic systems has generated considerable interest and speculation. The difficulty in explaining the anomalies concerning the presence or absence of a particular light-induced absorbance change at 77°K in various species evidently stems from the fact that individual factors in different bacteria governing light response of spectral components are poorly understood.

Rps. palustris, ATH. 2.1.2 may prove to be a useful tool for studying light-induced electron transfer reactions at very low temperatures since in this species, not only does the *c*-type cytochrome undergo reversible oxidation–reduction changes, but also P435 and bacteriochlorophyll undergo rapid reversible changes at 77°K upon illumination. Neither the mechanism nor the source of electrons for the cytochrome back reactions is known at present, although reverse electron flow from reaction center bacteriochlorophyll by reverse tunneling may be proposed. However, kinetic analysis on the low temperature absorption changes of these spectral components in the ultraviolet, visible and near-infrared regions would be expected to yield information on the problem.

Estimated values for half-times of cytochrome photooxidation at 300°K range from 300 nsec in Rhodopseudomonas sp. NW to 40 μsec in *Rps. capsulata* (Table I). A possible consequence of this exceptionally fast electron transfer reaction by the *c*-type cytochromes might be rapid stabilization of initial charge separation in light-activated chlorophyll complex by preventing possible back reaction. A pool of the reduced *c*-type cytochromes, assuming their close association with the primary reaction center complex, may also function effectively in preventing photooxidative destruction of bacteriochlorophyll from excessive radiation.

The preliminary data on the kinetics of laser-induced cytochrome oxidation at 77 °K reported here give values for half-times ranging from less than 2 to 12 msec in a variety of bacteria (Table I). The electron tunneling mechanism[7,8] was originally proposed to account for a 2-msec half-time of temperature-independent cytochrome oxidation in *Chromatium* D over a 20-fold range of absolute temperatures from 4.4 to 100 °K corresponding to an activation energy not larger than 4 cal/mole. The wide range of oxidation rates reported here would suggest that the parameters of electron tunneling may be different among a variety of species. Electron transfer times observed with some of the bacteria are considerably shorter than that of *Chromatium* D. These more rapid rates would suggest a barrier narrower than in *Chromatium* D, for which a barrier 1 eV high and 30 Å wide or any other feasible sets of parameters such as 4 eV and 15 Å can be computed.

The width of a barrier through which tunneling occurs may be calculated for a wide range of half-times from the following equation[7] which, for simplicity, assumes the barrier to be a rectangular structure of sufficient height and width:

$$a = \frac{\hbar}{\sqrt{8m(V-E)}} \ln \frac{16\, f\, t_{1/2}\, (V-E)\, E}{0.693\, V^2}$$

where a = barrier width; $\hbar$ = Planck's constant/2π; m = mass of electron; f = frequency of approach of electron to barrier; $t_{1/2}$ = half-time of reaction; V = potential energy of electron inside barrier; E = kinetic energy of electron outside barrier; and $(V-E)$ = barrier height.

If a is in Å, V and E in eV, then:

$$a = \frac{1}{\sqrt{V-E}} \ln \frac{16\, f\, t_{1/2}\, (V-E)\, E}{0.693\, V^2}$$

Assuming that $f = 10^{15}$, $E = 1$ eV and $V - E = 1$ eV, a 2-μsec $t_{1/2}$ would indicate a barrier width of 25 Å, while a $t_{1/2}$ of 12 msec would indicate a width of 33 Å. Thus, a small difference in barrier width (compared to protein dimensions) would readily account for the large variations of $t_{1/2}$ (Table I).

It is possible to speculate that the differences in the low temperature light responses of these bacteria represent steric variations among various components associated with the photochemical reaction site. A matter of importance in the cytochrome reaction at 77 °K would be the distance and height of the barrier between the cytochrome and its electron acceptor, which is currently assumed to be a type of bacteriochlorophyll. The nature of the tunneling barrier may also be an important factor since the organization and chemical composition of the lipoprotein matrix of individual species are likely to be different as variations in gross morphological features of photosynthetic apparatus do exist among different species of bacteria.

The observations presented in this paper indicate that a considerable degree of variation exists between different species of photosynthetic bacteria with respect to the macromolecular organization of the bacterial photosynthetic apparatus.

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