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Low-temperature photooxidation of cytochrome *c* in reaction centre complexes from *Rhodopseudomonas viridis*

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Low-temperature photooxidation of the high-potential haem c_{380} (*c*-559) and low-potential haem c_{20} (*c*-552) has been studied in isolated RC complexes from *Rps. viridis*. At appropriately low redox potential, complete irreversible oxidation of c_{20} ensues following flash-excitation at 77 K with $t_{1/2} = 20$ μs. Photooxidation of c_{380}, which can be observed at high E_h, is more rapid ($t_{1/2} < 6$ μs) and fully reversible; however, only about 20% of the haem is oxidized at 77–140 K. Full photooxidizability of c_{380} is attained as the temperature is raised in the range 140–200 K. Studies of the charge recombination kinetics indicate the low temperature-induced decrease of c_{380} photooxidizability to originate from the effect of freezing on the equilibrium constant of electron distribution between the haem and the pigment:

$$c_{380}P^{+}Q_A^{-} \overset{K_{eq}}{\rightleftharpoons} c_{380}^{+}PQ_A^{-}$$

K_{eq} decreasing from about 100 at 293 K to about 0.25 below 140 K. A two-step mechanism of c_{380} photooxidation above 200 K is suggested where initial virtually isopotential temperature-independent electron dislocation from c_{380} to P is followed by nuclear medium reorganization which stabilizes the final $c_{380}^{+}P$ state:

$$c_{380}P^{+}Q_A^{-} \underset{K'_{eq} \approx 0.25}{\overset{e\ \text{transfer}}{\rightleftharpoons}} [c_{380}^{+}PQ_A^{-}]^{\ddagger} \xrightarrow[\text{freezing}]{\text{configurational medium reorganization}} c_{380}^{+}PQ_A^{-}$$

Freezing is likely to prevent reorganization of the medium, thus allowing for observation of the initial electron transfer step at low temperatures. Photooxidation of the low-potential haem(s) may occur via the equilibrium fraction of the $c_{380}^{+}P$ state with unrelaxed nuclear environment. In sum, the results of low-temperature experiments are fully consistent with the linear sequence of haems

$$c_{-60} \rightarrow c_{310} \rightarrow c_{20} \rightarrow c_{380} \rightarrow P$$

and there is no need to postulate parallel electron transfer pathways involved in photooxidation of high- and low-potential haems.

Introduction

Reaction centre (RC) complexes from *Rhodopseudomonas viridis* and a number of other bacteria such as *Chromatium vinosum, Ectothiorhodospira shaposhnikovii, Rhodopseudomonas gelatinosa* and some other species contain a tightly bound four-haem cytochrome *c* which

Abbreviations: RC, reaction center; P, bacteriochlorophyll special pair (the pigment); E_h, redox potential; Q_A, primary quinone acceptor.

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serves as an electron donor to a special bacteriochlorophyll pair (P) [1–11]. Two of the haems are high-potential ($E_m \approx 300$ mV) and may serve as electron acceptors for the soluble c_2-type cytochrome in a cyclic photoredox chain, whereas the two low-potential haems ($E_m \approx 0$ mV) are believe to be involved in a non-cyclic oxidation of low-potential substrates like H_2S [12]. All the four haems are capable of rapid photooxidation at room temperature.

Three-dimensional structure of *R. viridis* RCs shows the four iron-porphyrin groups of the cytochrome to be arranged in a roughly linear sequence along the protein long axis with a Fe-to-Fe separation distance of 14–16 Å, the proximal haem being within 21 Å of P [13,14].

Recently individual spectral and redox characteristics of all the four haems of the *R. viridis* RC-bound cytochrome *c* have been resolved [15–21]. On the basis of structural, physicochemical and rapid kinetics data the following sequence of the redox groups has been suggested:

$$\begin{array}{lccccc} & c\text{-}554 \rightarrow & c\text{-}556 \rightarrow & c\text{-}552 \rightarrow & c\text{-}559 \rightarrow & \mathrm{P} \\ E_m\,(\mathrm{mV}): & -60 & +310 & +20 & +380 & +500 \end{array} \qquad (I)$$

where the iron-porphyrin groups are denoted according to their α-absorption maxima in the room temperature difference spectra [16,17].

Within a framework of model (I) favoured at present by most of the groups working on this subject [15–21], the high-potential haem *c*-559 is the only redox centre of the cytochrome capable of direct electron donation to P, and all other haems can be photooxidized only indirectly via *c*-559. Photooxidation of *c*-556 via *c*-559 has been indeed demonstrated by room-temperature rapid kinetics studies [15–18].

However, the model (I) is questioned by the results of low-temperature cytochrome *c* photooxidation studies. According to the data from several laboratories obtained on a number of bacterial species, low-potential haems remain fully photooxidizable at low temperatures, whereas significant or even major part of the high-potential haems is no longer capable of electron donation to the photooxidized pigment in the frozen samples [22–30].

Conceivably, it is not easy to visualize within a framework of scheme (I) how can low-potential haems, e.g., *c*-552, be oxidized by P^+ via *c*-559 at cryogenic temperatures if the latter does not itself transfer electron to the pigment under these conditions. This fact has long served as an argument for independent pathways of low- and high-potential haem oxidation by P^+, and the concept of parallel pathways received considerable attention in literature [1–5,30–33].

Thinking over this discrepancy led us to a conclusion that it may be more interpretation of the low-temperature experiments than the results themselves which does not fit model (I). Besides, most of the above-mentioned data on the low-temperature cytochrome *c* photochemistry have been obtained on bacteria other than *Rps. viridis* for which the three-dimensional structure and the most detailed characterization of the individual haem redox, spectral and kinetics properties are available.

Therefore, we considered it worthwhile to undertake a study of photoinduced redox transitions of *Rps. viridis* RC-bound cytochrome *c* at low temperatures.

The data obtained in this work confirm some of the basic earlier observations, including low apparent photooxidizability of *c*-559 at cryogenic temperatures. Nevertheless, they appear to be fully consistent with the sequence of haems described by scheme (I). Evidence is presented that freezing of RCs may result in a shift of redox equilibrium between haem *c*-559 and P, making flash-oxidized pigment re-reduction by the haem thermodynamically unfavourable. This allows us to explain poor photooxidizability of the high-potential haem in *Rps. viridis* RCs, and perhaps in other species as well, without recurring to the postulate of electron exchange between the high-potential haem(s) and P being specifically inhibited at low temperatures [32,33].

The effect of low-temperatures may consist in 'freezing out' molecular motions involved in medium reorganization following virtually isopotential initial electron transfer from the proximal heme to P^+. This medium reorganization could provide a major contribution to the overall thermodynamics of the energetically favourable pigment re-reduction by the haem at room temperature.

Materials and Methods

Lauryldimethylamine oxide, 1,4-benzoquinone and duroquinone were from Fluka; Triton X-100, ubiquinone-6, diaminodurene, phenazine ethosulphate from Sigma; Tris and Hepes from Serva; ascorbic acid from Koch-Light; DEAE-cellulose from Whatman; sodium dithionite from Merck; *o*-phenanthroline from Chemapol; the tris-phenanthroline complex of Co(III) was synthesized as described [34].

Reaction centre complexes were isolated from *Rps. viridis* cells as described in Ref. 15. RC concentration was determined spectrophotometrically using a $\Delta\varepsilon_{830}$ value of 300 $mM^{-1} \cdot cm^{-1}$ [35].

Redox potential was measured at 25 °C with a platinum electrode vs. the Ag|AgCl reference.

Low-temperature experiments were carried out in a custom-built cryostat described in Ref. 36. Temperature of the sample was controlled with the aid of a copper/constantan thermocouple mounted at the bottom of the cryostat.

Static absorption spectra of RCs at room and low temperature were recorded in a Specord UV-VIS M-40

spectrophotometer (Carl Zeiss Jena, D.D.R.) with a resolution of 0.12 nm.

Measurements of photoinduced absorption changes induced by continuous illumination (as referred in the text) were made in a phosphoroscope device described in Ref. 37 (a stroboscopic system with a spinning cell jacket with four windows allowing for alternating 2 ms white light illumination/2 ms light absorption measurement periods).

Rapid kinetics of flash-induced absorption changes was measured in a home-built single-beam spectrophotometer constructed in the laboratory. Optical slit width was 0.8 nm. The photomultiplier output was fed into a transient recorder NEO-200 (Hungary), and the transients were averaged with the aid of Nicolet-527. Flash excitation was performed with a xenon flash-lamp ISSh-100-3M (pulse half-width, 6 μs).

The basic incubation medium for low-temperature experiments contained 40 mM Hepes-buffer (pH 7.2), 0.1% Triton X-100 and 60% (v/v) glycerol.

For averaging of the kinetics of irreversible photooxidation of the low-potential cytochrome *c* haem at 77 K, the sample was warmed to room temperature and then frozen back to 77 K after each flash-induced redox transition. In these experiments, reaction mixture was supplemented with ubiquinone-6 and excess duroquinone to facilitate reoxidation of photoreduced Q_A^-.

Results

Resolution of absorption characteristics of the four haems at 77 K

Fig. 1 shows low-temperature absolute absorption spectra of *Rps. viridis* RCs in the cytochrome α-band region at several characteristic redox potentials.

At +431 mV all the haems are oxidized (spectrum a); the broad maximum at about 544 nm belongs to bacteriopheophytin. As the redox potential decreases, absorption bands of the four ferrous haems appear in sequence (b–e). Notably, resolution of the α-band into Q_x and Q_y transitions is observed at 77 K for each of the four iron-porpyrin groups of the cytochrome.

The splitting is particularly large for the haem with the highest E_m (380–400 mV [15,21]) which shows a major peak at about 557 nm and a rather weak second band at about 548 nm (spectrum b). The latter is largely obscured by the bacteriopheophytin absorption and, in fact, is barely discernible in the absolute spectra; nevertheless, it can be seen quite clearly in the difference spectra (reduced minus oxidized), either static (e.g., b minus a in Fig. 1, not shown; cf. Ref. 20) or photoinduced (Fig. 2B). Accordingly, partial resolution of the Q_x and Q_y transitions of this haem can be observed even at room temperature in the difference absorption

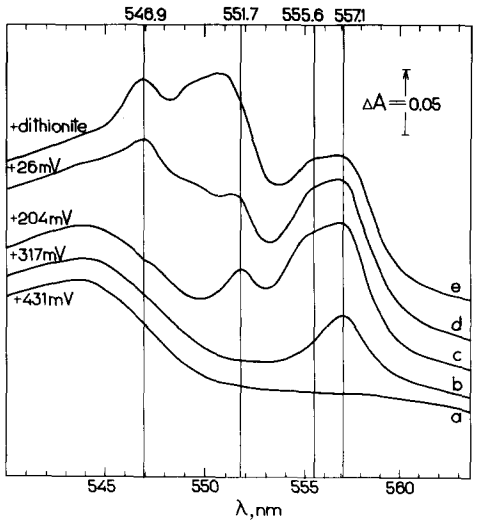

Fig. 1. Low-temperature absolute absorption spectra of *Rps. viridis* reaction centre cytochrome *c*. The spectrophotometric cell (1.5 mm optical pathway) contains 9 μM RC complex in the basic medium. The following additions have been made. (a) 2.5 mM ferricyanide + ascorbate to $E_h = 431$ mV; (b) 2.5 mM ferricyanide, 100 μM 1,4-benzoquinone + ascorbate to $E_h = 317$ mV; (c) 100 μM diaminodurene, 100 μM 1,4-benzoquinone + ascorbate to $E_h = 204$ mV; (d) 14 mM ascorbate and 100 μM phenazine ethosulphate, $E_h = 26$ mV; (e) excess dithionite.

spectra (a peak at 559 nm with a weak shoulder at 550–552 nm [15,17]).

Lowering E_h to approx. +200 mV (complete reduction of the both high-potential haems [15–17]) results in augmenting of haem c_{380} peaks * and in appearance of two new bands at 555.6 and 551.7 nm belonging to haem c_{310} (spectrum c).

The two low-potential haem spectra are characterized by the doublets at 547/549 nm (haem c_{20}) and 551/549 nm (haem c_{-60}) (Fig. 1, spectra d, e). The splitting is rather small in this case in agreement with narrow symmetrical bands observed at room temperature [17].

These results are in good agreement with a very recent report [20] except that splitting of the low-potential haem α-peaks was not obtained in that work, and corroborate the conclusion of Dracheva et al. [15–17] that all the four haems of the RC cytochrome have different redox and spectral characteristics.

In general, redox behaviour of the RC iron-porphyrin groups in the low-temperature studies agrees reasonably well with E_m values determined at room temperature, although a shift to somewhat lower reducibility of the high-potential haems was observed. This shift is perhaps due to the presence of high concentration of glycerol in

* In order to avoid confusion arising from different position of the cytochrome haem absorption peaks at room and low temperature, the four iron-porphyrin groups are referred below according to their E_m values reported in Ref. 17, i.e., c_{380} (*c*-559), c_{310} (*c*-556), c_{20} (*c*-552) and c_{-60} (*c*-554).

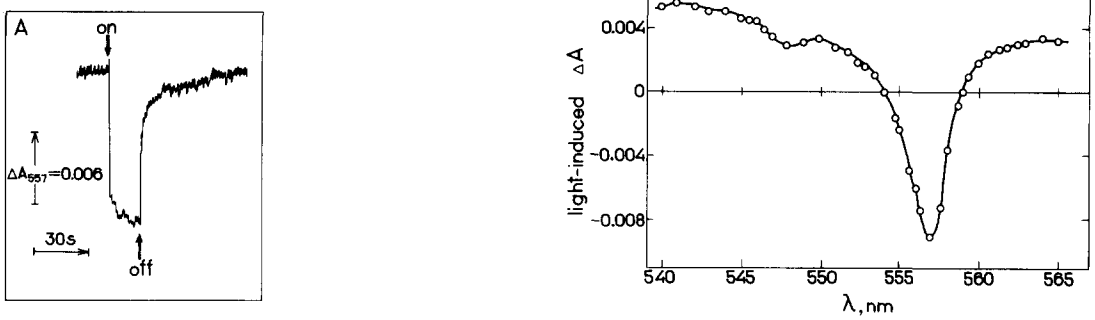

Fig. 2. Reversible photooxidation of haem c_{380} at 77 K. The cell with an optical pathway of 2 mm contains 18 μM RCs in a basic medium supplemented with 1 mM ferricyanide, 100 μM 1,4-benzoquinone and ascorbate to $E_h = 320$ mV. (A) Typical kinetics of the photoinduced absorption changes at the λ_{max} of heme c_{380}; switching on and off the light is indicated by arrows. (B) Difference spectrum of the photoinduced absorption changes.

the medium, rather than to the effect of temperature. Indeed, redox titrations of the haems monitored by EPR spectroscopy at liquid helium temperatures in glycerol-free media [21,38] give E_m values very close to those obtained with the aid of absorption spectroscopy at 25 °C [17].

A specific feature of the experiments at 77 K consists in a better separation of the four-haem individual redox transitions on the E_h scale, as compared to room temperature. In particular, it is possible to quench RC in a state such that haem c_{380} is 70–75% reduced whereas the second high-potential haem c_{310} remains oxidized by at least 90% (e.g., Fig. 1b).

Photooxidation of high-potential haem c_{380} at low temperatures

Until recently, the 'high-potential cytochromes *c*' in *Rps. viridis* RC were considered to be not photooxidizable at low temperatures [21,29,39]. In contrast, we found illumination of RCs frozen in a state with only one haem (c_{380}) reduced (preillumination conditions analogous to those in Fig. 1b) to result in a rapid bleaching at 557 nm (Fig. 2A). Difference spectrum of the photobleaching shows a major trough at 557 nm and a second minor one at 548 nm typical of the split α-band of heme c_{380} (Fig. 2B). The photobleaching is readily reversible in the dark (Fig. 2A). Rereduction of c_{380} (i.e., $c^+_{380}Q^-_A \rightarrow c_{380}Q_A$ charge pair recombination) is dominated by a rapid phase, not resolved under the conditions of Fig. 2A (but see below), followed by some slower optical changes with $t_{1/2} \approx 10$ s (about 20% of the overall response). Facile dark re-reduction of c_{380} may explain why photooxidation of this haem was not observed in a recent low-temperature EPR spectroscopy study [21] (but cf. Ref. 38).

At E_h of +200 mV, when both high-potential haems were reduced, approx. 10% bleaching at 555 and 551.5 nm could be observed at 77 K upon a prolonged illumination which, however, was not reversible in the dark (data not shown). This observation agrees with the earlier data on RCs from *Rps. gelatinosa* and *Rps.* sp. [27,40], where optical changes in the cytochrome γ-band were monitored, and may point to a possibility of the second high-potential haem (c_{310}) partial photooxidation leading to a relatively stable 'tertiary dipole' formation, $c^+_{310}c_{380}PQ^-_A$ *.

Notably, the difference spectrum in Fig. 2B is characterized by an even general increase of absorbance throughout the range 540–565 nm typical of bacteriochlorophyll special pair oxidation. This effect indicates that in a significant fraction of RCs rereduction of the photooxidized pigment by c_{380} does not take place or, in other words, that only part of c_{380} is photooxidizable at 77 K, in contrast to room-temperature experiments.

In order to better characterize c_{380} photooxidation at low temperatures, experiments with flash-excitation were carried out subsequently using an approx. 90%-saturating 6 μs xenon flash.

Fig. 3A shows typical kinetic traces of flash-induced absorption changes in the cytochrome α-band region at 77 K. Both at 557 nm (λ_{max} of c_{380}) and 551 nm (the reference wavelength) a photo-induced increase in absorbance is observed due to the dominating contribution of the pigment oxidation under these conditions. However, the amplitude of the response at 557 nm is about 3-fold lower than at 551 nm pointing to a negative contribution from c_{380} oxidation at the former wavelength. This is demonstrated clearly by the spectrum of the flash-induced absorption changes (Fig. 3B).

On the other hand, there is no significant difference between the kinetics of optical changes at 551 and 557 nm (e.g., Fig. 3A) when the traces are normalized to the

* After the manuscript had been prepared for publication, we were able to resolve an additional more rapid phase of c_{310} photooxidation which was reversible in the dark with $t_{1/2} \approx 1$ min (see also very recent papers [20,38]).

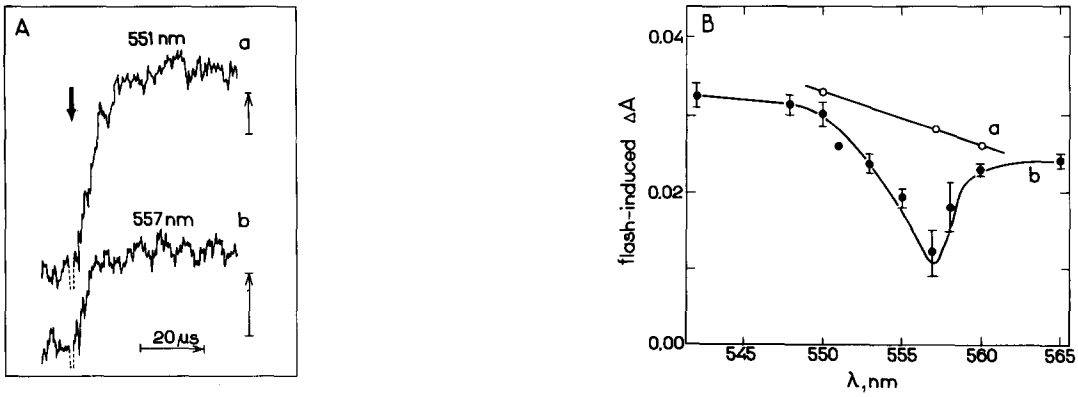

Fig. 3. Flash-induced oxidation of haem c_{380} at 77 K. 20 μM RCs in the basic medium. Optical pathway, 2 mm. (A) Kinetics of the flash-induced optical changes. Redox potential has been adjusted to 320 mV by ascorbate in the presence of 1 mM ferricyanide and 100 μM 1,4-benzoquinone. The vertical bars correspond to $\Delta A = 0.003$ increase at the indicated wavelengths. Twenty or forty-one 30 s spaced transients have been averaged in a and b, respectively. Here and below, start of the 6 μs half-width flash is indicated by an arrow. (B) Difference spectrum of the flash-induced absorbance changes. The amplitude of the photoinduced ΔA has been measured at the indicated λ values 20 μs after the flash start. Each point is the average of 4–20 measurements. (a) in the presence of 2 mM ferricyanide; (b) $E_h = 320$ mV (conditions, as in (A)).

amplitude of the response. Also recordings on an extended time-scale (hundreds of microseconds) reveal no evidence for absorption changes other than the initial approx. 10 μs jump and a backward slow drift due to charge recombination (data not shown, cf. Fig. 4).

Apparently, haem c_{380} low-temperature photooxidation takes place within the apparatus rise-time limited by the 6 μs half-width of the light pulse.

The rapid photooxidation of c_{380} at 77 K is in line with the results obtained by Chamorovsky et al. on *Ect. shaposhnikovii* [32,33]. On the other hand, our data are in disagreement with the at least 1000-fold slower photooxidation of the high-potential haem *c*-558 in *Rps. viridis* RCs as reported by Shopes et al. [18] at 125 K. However, in the latter work both high-potential haems were reduced before the flash and no difference spectra of the photoresponse were given which strongly complicates interpretation of the multiphasic absorbance changes at 558 nm given in Fig. 3 of Ref. 18. In particular, the millisecond part of the ΔA_{558} kinetic trace in this figure with $t_{1/2} \approx 2$ ms according to our experience is very likely to originate largely from P^+ dark re-reduction-associated absorption changes at this wavelength and temperature.

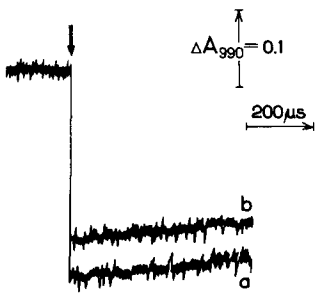

Fig. 4. Flash-induced oxidation of the bacteriochlorophyll special pair at 77 K. (a, b) Conditions as in Fig. 3B (a) and (b), respectively. Four 30 s spaced traces have been averaged in each case.

The amplitude of the difference spectrum b in Fig. 3B corresponds to photooxidation of about 15% of c_{380} present in the reduced form before the flash. Taking into account incomplete light saturation (≈ 90%) we conclude that about 17% of c_{380} is rapidly photooxidizable at 77 K.

This conclusion is corroborated by complementary measurements in the bacteriochlorophyll special pair absorption band (Fig. 4). One can see photooxidation of the pigment at 77 K (monitored as bleaching at 990 nm) to be slightly but distinctly less in the sample with c_{380} pre-reduced (trace b) as compared to RCs with the oxidized cytochrome (trace a). Evidently the difference is due to rapid (non-resolved) re-reduction of P^+ by c_{380} in a part of RCs. This part was on the average 18%; after correction for about 75% reduction of the heme this gives an estimate of about 24% for the c_{380} photooxidizability at 77 K, which is not too much different

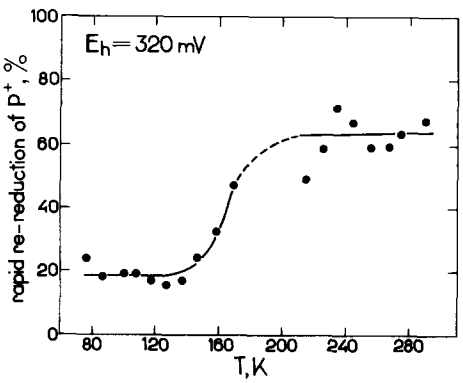

Fig. 5. Temperature dependence of the bacteriochlorophyll special pair fraction rapidly re-reduced by haem c_{380}. The experiments analogous to that in Fig. 4 was carried out at different temperatures. Photoinduced ΔA_{990} was measured 20 μs after the flash. In the temperature interval corresponding to the dashed part of the curve the measurements could not be carried out because of a strong ice-nucleation.

from the evaluation based on haem photobleaching measurements.

Subsequently we investigated temperature dependence of the amplitude of P^+ rapid re-reduction by haem c_{380}. To this end, experiment similar to that shown in Fig. 4 was repeated at different temperatures. As one can see from the data in Fig. 5, the fraction of the pigment re-reduced by the cytochrome remains constant from 77 to approx. 140 K, increases abruptly in a narrow temperature range and reaches a plateau above 200 K; the plateau level agrees with the approx. 60% reduction of c_{380} in the sample at those temperatures.

This temperature profile is rather similar to that reported originally for 'high-potential cytochrome *c*' photooxidation in *Ect. shaposhnikovii* [32]. Somewhat different results published by the same group more recently [33] may be a consequence of glycerol omitted from the reaction medium. However, it has to be emphasized that in Refs. 32, 33 differentiation was not yet made between the two high-potential haems and reconsideration of the data may be required for tenable analysis.

Kinetics of charge recombination

In the earlier studies, dark re-reduction of the 'high-potential cytochrome *c*' in RCs from *Rps. gelatinosa* [25,26], *Ect. shaposhnikovii* [40] and, very recently, in *R. viridis* [38] was reported to occur at 77 K on a time-scale of seconds or even minutes. In contrast, our preliminary experiments with continuous illumination revealed a much faster $c^+_{380}Q^-_A$ secondary dipole recombination in *Rps. viridis* RCs (e.g., Fig. 2A). Therefore we considered it worthwhile to extend the studies on this point with the use of flash-excitation.

Room-temperature studies. Co(phenanthroline)$_3^{2+/3+}$ was employed in these experiments as a redox buffer instead of the commonly used ferricyanide because the latter can serve as an efficient electron acceptor for Q^-_A [41], diminishing the amplitude of the rapid ($t_{1/2} \approx 1$ ms) $P^+Q^-_A$ charge recombination. In our hands, 300 μM Co(phen)$_3^{3+}$ ensured complete oxidation of the RC cytochrome *c* but, in contrast to ferricyanide, had no effect on the yield of the $Q^-_A \rightarrow P^+$ rapid recombination (data not shown).

Under these conditions, P^+ dark re-reduction at room temperature following flash-excitation proved to be essentially monophasic with $t_{1/2} = 0.9$ ms (not shown), which is in good agreement with the data in Ref. 42 and indicates virtually complete absence of the secondary quinone acceptor in the preparation.

Fig. 6A shows typical kinetics of the secondary dipole ($c^+_{380}PQ^-_A$) charge recombination at room temperture. RCs were poised at the redox potential $E_h = +380$ mV, such that haem c_{380} showed about 40% reduction, whereas the second high-potential haem c_{310} was almost fully oxidized. Under these conditions, dark relaxation

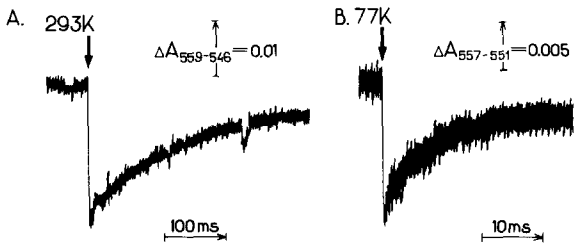

Fig. 6. Kinetics of the $c^+_{380}Q^-_A$ charge pair recombination at room temperature (A) and 77 K (B). (A) 10 μM RCs in a reaction medium containing 100 mM Hepes (pH 7.2), 0.1% Triton X-100, 300 μM Co(phenanthroline)$_3^{3+}$. E_h has been adjusted to 382 mV by ascorbate. Optical pathway 2.8 mm, $t = 20\,^\circ$C. The kinetics given is the average of seven individual traces induced by 1 min spaced flashes. (B) Conditions as in Fig. 3B, curve b. Optical pathway, 2 mm; 77 K. Twenty-four 30 s spaced flash-induced transients have been averaged.

of the flash-induced c_{380} photobleaching is dominated ($\approx 80\%$) by an exponent with $t_{1/2} = 100$ ms.

It has to be emphasized that the kinetics shown in Fig. 6A decelerate greatly upon reconstitution of the secondary quinone acceptor function by added UQ_6 (not shown). Hence, the flash-oxidized heme c_{380} dark re-reduction is indeed due to charge recombination within RC complex rather than to electron donation from exogenous redox compounds present in the medium.

A rather similar value of $t_{1/2} = 60$ ms was observed for re-reduction of the tightly-bound cytochrome *c* 'under oxidizing conditions' in *Ect. shaposhnikovii* [5]. At the same time a much slower recombination of the 'cytochrome c^+_{558}'-Q^-_A charge pair in *Rps. viridis* ($t_{1/2} \approx 2$ s) was reported by Shopes and Wraight [42]. Presumably, at the ambient $E_h = +220$ mV the latter authors dealt with RCs in which both high-potential haems were reduced. Accordingly, it was most likely the tertiary dipole $c^+_{310}c_{380}PQ_A$ recombination monitored in Ref. 42. Remarkably, taking into account the E_m difference of 70 mV between the two high-potential haems [17], our value of $t_{1/2} = 100$ ms for $c^+_{380}Q^-_A$ pair discharge allows us to estimate $t_{1/2}$ for the $c^+_{310}Q^-_A$ recombination to be as high as 1.5 s, in good agreement with the experimental data of Shopes and Wraight (2 ± 0.5 s at pH = 6.1, ≈ 1 s at pH 8) [42].

The presence of some minor slow component(s) ($\approx$ 20%) can be seen in the haem re-reduction kinetics in Fig. 6A as well as at low temperatures (Fig. 6B), which is in contrast to monophasic $P^+Q^-_A$ pair recombination in the same RC preparations. As regards room-temperature experiments, this effect might originate at least partly in the presence of a certain equilibrium fraction of RC complexes with both high-potential haems reduced. At the $E_h = 380$ mV this fraction can be estimated to comprise about 10–15% of the complexes with haem c_{380} reduced.

However, this explanation is hardly applicable to our low-temperature experiments where the amount of c_{310}

pre-reduced was negligible. It has to be mentioned that the cytochrome *c* haem properties in isolated frozen RCs show certain heterogeneity revealed by EPR studies as compared to the native *Rps viridis* membranes [21], which may be a more likely reason for heterogeneous kinetics of haem re-reduction at low-temperatures.

Low-temperature studies. Fig. 6B shows typical kinetics of c_{380} dark re-reduction ($c^{+}_{380}Q^{-}_{A}$ pair recombination) at 77 K. Surprisingly, at low temperature about 80% of the reaction proceeds with $t_{1/2} = 5$–6 ms, i.e., more than an order of magnitude faster than at room temperature (cf. Fig. 6A).

In the same sample a very close value of $t_{1/2} = 6$ ms has been measured for $P^{+}Q^{-}_{A}$ pair recombination followed at 990 nm (data not shown), which agrees fairly well with the results of Shopes and Wraight [43], who reported a temperature-independent $t_{1/2} = 6.7$ ms for the 170–100 K interval in 60% glycerol. Thus our data corroborate the finding [43] that freezing decreases about 6-fold the rate of the primary dipole recombination in *R. viridis* RC.

The P^{+} decay measurements at 990 nm give a much better signal-to-noise ratio than recordings in the haem α-band and, accordingly, allow for much more accurate evaluation of charge recombination kinetics in RCs. Careful studies of the pigment dark re-reduction monitored at 990 nm show that the rate of electron return from Q^{-}_{A} to P^{+} at 77 K is slightly but distinctly different for the RC states with haem c_{380} reduced or oxidized before the flash. Thus $t_{1/2}$ of the reaction decreased reproducibly from 5.2 ± 0.9 ms for the samples with c_{380} reduced by 70–80% to 4.6 ± 0.5 ms for RCs in which the cytochrome was fully oxidized. The significance of this effect will be discussed later on (see Discussion).

Low-potential haem c_{20} photooxidation at 77 K

These experiments were carried out at redox potentials such that the low-potential cytochrome *c* haem with higher E_m (*c*-552, c_{20}) was reduced by approx. one-half, the second low-potential iron-porphyrin group (*c*-554, c_{-60}) being almost fully oxidized.

Low-temperature absolute absorption spectrum of such a sample is shown in Fig. 7a. In addition to the α-peaks of the two high-potential haems, the maxima at 547 and 549 nm of the ferrous haem c_{20} can be seen clearly in the spectrum. These maxima disappear upon a single flash illumination of the sample (Fig. 7b), indicating almost complete irreversible photooxidation of c_{20} in agreement with [21,29,39] (and see Refs. 22, 23, 25–28, 30 for related experiments on *C. vinosum* and *Rps. gelatinosa*).

Fig. 8 shows the kinetics of flash-induced optical changes in the absorption bands of heme c_{20} (a) and bacteriochlorophyll special pair (b) in a sample with half-reduced haem c_{20} analogous to that in Fig. 7a.

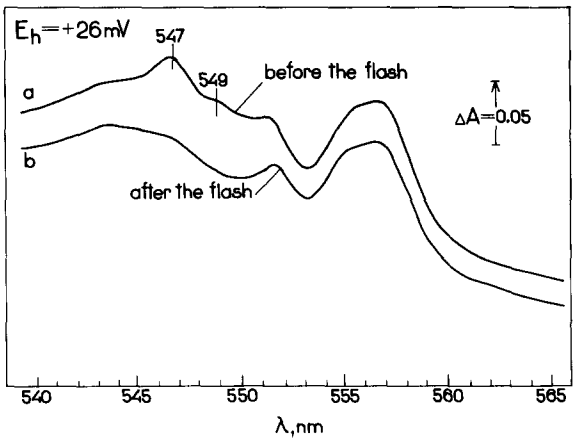

Fig. 7. Irreversible photooxidation of the low-potential haem c_{20} at 77 K. (a) The sample containing 9 μM RCs in the basic medium supplemented with 14 mM ascorbate and 100 μM phenazine ethosulphate ($E_h = +26$ mV) has been frozen and cooled down to 77 K in the dark. (b) The same after single-flash illumination at 77 K. Absolute absorption spectra have been recorded in the cell with 1.5 mm optical pathway.

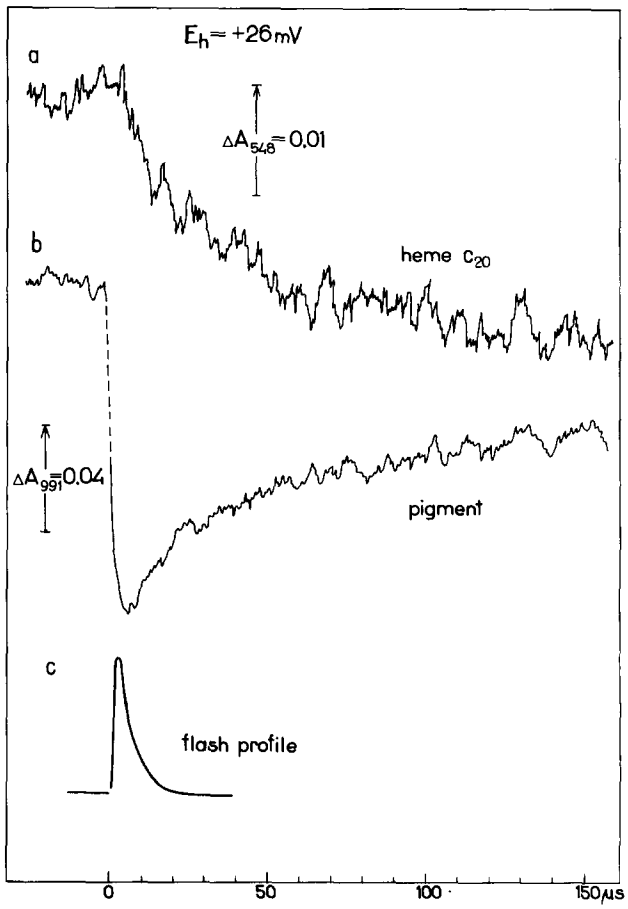

Fig. 8. Kinetics of low-potential haem c_{20} photooxidation (a) and bacteriochlorophyll special pair re-reduction (b) at 77 K. (a, b) 18 μM RCs in the basic medium supplemented with 50 mM ascorbate, 200 μM duroquinone, 30 μM ubiquinone-6 and 100 μM phenazine ethosulphate; $E_h = 26$ mV. Three individual traces have been averaged for each curve (see Materials and Methods). Optical pathway, 2 mm. (c) Time-profile of the flash is shown.

One can see that as soon as the light pulse is over, the kinetics of c_{20} oxidation matches that of P^+ partial re-reduction fitting to an exponent with $t_{1/2} = 20$ μs. A rather similar value of $t_{1/2} = 10$ μs was reported for photooxidation of the tightly-bound cytochrome *c* in the whole *Rps. viridis* cells as measured at 77 K in the Soret band [39].

The amplitude of the photobleaching at 548 nm in Fig. 8a corresponds to oxidation of at least 80% of c_{20} initially reduced. Accordingly, the 20 μs phase of P^+ re-reduction is observed for approx. one-half of the photobleached pigment molecules (Fig. 8b) in agreement with c_{20} half-reduction before the flash.

Discussion

There have been numerous studies on low-temperature photooxidation of the four-haem cytochrome *c* tightly bound to RC complexes of a number of photosynthesizing bacteria. However, in the earlier works the inequivalence of the two high-potential as well as of the low-potential haems, discovered only recently [15–21], was not taken into consideration, which entails significant complications in interpretation and comparison of those results. A distinctive feature of the present work is that it focuses on low-temperature photochemistry of individual cytochrome *c* haems, i.e., the high-potential c_{380} (*c*-559) and low-potential c_{20} (*c*-552).

As found in this work (and see also Ref. 44), cryogenic temperatures affect photooxidation of both high- and low-potential haems in *Rps viridis* RC but in a different way. In case of the high potential c_{380}, it is the amount of flash-oxidized haem which decreases about 4–5-fold upon freezing, the oxidation remaining rapid enough to be complete within the dead time of our apparatus. In contrast, full photooxidizability is retained by low-potential heme c_{20} at 77 K, but the reaction decelerates about 200-fold as compared to room temperature. These data are more or less consistent with the basic earlier observations on various bacteria [22,23,28,45], and in particular corroborate results of Charmorovsky et al. on *Ect. shaposhnikovii* [32,33] and *Chromatium minutissimum* [46].

Such diverse effect of freezing on photooxidation of the high- and low-potential haems has been interpreted earlier as a strong evidence for parallel pathways of electron transfer to the pigment from the low- and high-potential 'cytochromes' in *C. vinosum* or *Ect. shaposhnikovii* [5,30,32,33].

However the three-dimensional structure of the *Rps. viridis* RC complex [13,14] indicates that direct electron transfer to the pigment from any of the cytochrome haems except for the nearest one, identified by Dracheva et al. as *c*-559 [15] (here, c_{380}), is unlikely.

According to the model currently favoured [15–21] electron transfer on the donor side of *R. viridis* RC is described by an alternating sequence of high- and low-potential haems.

$$c_{-60} \rightarrow c_{310} \rightarrow c_{20} \rightarrow c_{380} \rightarrow P \qquad \text{(II)}$$

Photooxidation of c_{380} at 77 K being considerably faster than that of c_{20} as observed in this work (and see also Ref. 44) gives further support to this model. On the other hand, it remains to be explained how can complete photooxidation of c_{20} via c_{380} occur at 77 K if the latter is only about 20% photooxidizable.

Here we come to a central point of the discussion – why is it only a fraction of c_{380} in *Rps. viridis* (or of its counterpart in other bacteria) which is photooxidizable at low temperatures? The following alternatives may be considered.

Model A. Upon freezing there occurs disruption of electron transfer pathway between 'high-potential cytochromes' and the pigment in a majority, about 80% in our case, of RC complexes, whereas in the rest 20% the reaction remains essentially unperturbed. This point of view has been favoured by Chamorovsky et al. [32,33]. Clearly, such an explanation is hardly compatible with complete photooxidation of c_{20} via c_{380} in terms of haem sequence (II). Therefore we would like to put forward an alternative interpretation.

Model B. Freezing does not inhibit electron exchange between c_{380} and P in any significant part of RCs, but rather affects equilibrium of the reaction. Following flash-induced oxidation of P, a dynamic equilibrium state is established rapidly in each RC molecule (in less than 6 μs) in which the electron from the reduced c_{380} now spends about 20% of time at P and about 80 at the haem. This is in contrast to room temperature situation, where the equilibrium is shifted towards virtually complete oxidation of c_{380} by P [15–18].

These two models can be distinguished on the basis of the data concerning the kinetics of the photooxidized *c*-380 dark re-reduction by Q_A^- at 293 and 77 K.

At 293 K, dark recombination of the secondary dipole $c^+PQ_A^- \rightarrow cPQ_A$ is about 100 fold slower than the $Q_A^- \rightarrow P^+$ back reaction ($t_{1/2}$ values of about 100 ms and about 1 ms, respectively; this work, Refs. 42, 17). Apparently, relaxation of the secondary dipole proceeds via a thermally excited intermediate state $cP^+Q_A^-$ (e.g., see Refs. 47, 48) according to a scheme:

$$\underset{\text{rapid (μs)}}{c^+PQ_A^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} cP^+Q_A^-} \quad \underset{\text{slow (ms)}}{\overset{k_0}{\rightarrow} cPQ_A} \qquad \text{(III)}$$

In the reaction scheme (III), the effective overall rate constant, k_{eff}, of the secondary dipole recombination (i.e., of the $c^+PQ_A^- \rightarrow cPQ_A$ transition) should be related to the partial rate constants k_1, k_{-1}, k_0 by an equation

$$k_{eff} = k_0 \cdot \frac{k_{-1}}{k_1 + k_{-1}} = \frac{k_0}{1 + K_{eq}} \qquad \text{(IV)}$$

where $K_{eq} = k_1/k_{-1}$ is an equilibrium constant for electron distribution between heme c_{380} and the pigment. This allows for determination of K_{eq} from the kinetics of charge recombination in a singly and doubly reduced RC [47,48].

At room temperature $K_{eq} \gg 1$, so that

$$k_{eff} \approx k_0/K_{eq} \quad (V)$$

and, accordingly,

$$K_{eq} \approx k_0/k_{eff} \quad (VI)$$

Indeed the value of $K_{eq} \approx 100$ estimated from the kinetics of the primary ($P^+Q_A^-$) and secondary ($c_{380}^+Q_A^-$) dipole recombination in *R. viridis* RCs at room temperature according to Eqns. IV–VI is in very good agreement with the approx. 120 mV E_m difference between the pigment (500–515 mV [49,50]) and haem c_{380} (380–400 mV [15,17,21]) as determined by equilibrium redox titrations.

Within a framework of Model A, lowering the temperature may be envisaged to decrease the rate of the secondary dipole recombination via the thermodynamically unfavourable thermally excited intermediate $c_{380}P^+Q_A^-$. It is then remarkable that experimental data show the opposite: there occurs a dramatic increase of the reaction rate (Fig. 6).

As found in this work, at 77 K k_{eff} for c_{380} re-reduction becomes very close to k_0, which means according to Eqn. IV that K_{eq} is now significantly less than 1. This fact is hardly compatible with model A, which assumes the c_{380}^+P state to be energetically well below $c_{380}P^+$ (i.e., $K_{eq} \gg 1$) both at room and cryogenic temperatures. On the other hand, it is a straightforward consequence of Model B.

At this point it is noteworthy that, as mentioned above (see Results), at 77 K charge recombination in RCs with c_{380} reduced before the flash is still slightly slower than in the complexes with the fully oxidized cytochromes (average $t_{1/2}$ values of 5.2 ms and 4.6 ms, respectively). Substitution of these values into Eqn. IV gives $K_{eq} = 0.13$ with a maximal scatter 0.05–0.49, which is in reasonably good agreement with the estimate $K_{eq} \approx 0.25$ obtained from the approx. 20% photooxidation of c_{380} at low temperatures within a framework of Model B.

Therefore we resume that Model A advocated by Chamorovsky et al. [32,33] is not correct, at least in the case of *Rps. viridis* RCs, whereas Model B conforms to experimental data.

What could be the mechanism of the dramatic effect of freezing on the equilibrium between c_{380} and P? A trivial possibility that it consists in different temperature dependences of the E_m values of the two redox centres (cf. p. 431 in Ref. 51) is unlikely. Indeed, were it so, one should expect freezing of a singly reduced RC complex c^+PQ_A to result in electron dislocation from P to c_{380}, which has never been observed.

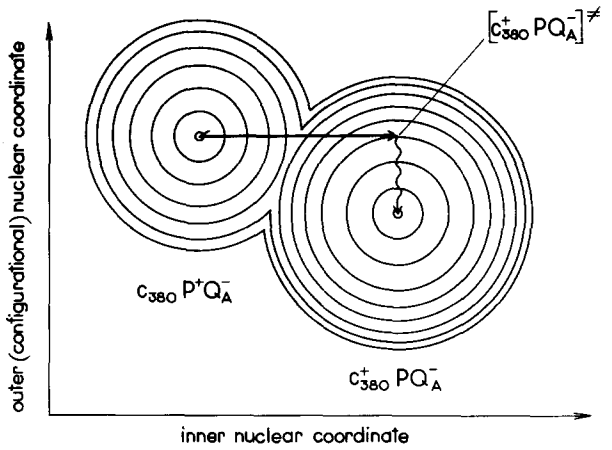

Fig. 9. The diagram of redox reaction between c_{380} and P in *Rps viridis* RC. The diagram is presented according to [69]. The two sets of concentrical circles denote isoenergetic cross-sections of the $c_{380}P^+$ and c_{380}^+P state potential energy surfaces. The straight arrow shows the initial isopotential step of $c_{380}P^+ \rightarrow c_{380}^+P$ redox transition (Scheme VII in the text) which does not involve configurational coordinate changes (medium reorganization) and gives rise to an out-of-equilibrium state $[c_{380}^+P]^{\ddagger}$ stable at low temperatures. The wavy line depicts subsequent transition $[c_{380}^+P]^{\ddagger} \rightarrow c_{380}^+P$ at the expense of nuclear configurational coordinate changes (medium reorganization) occurring at $T > 200$ K.

Rather, the effect is specific for RC complex frozen in a state with at least one haem reduced. This peculiar phenomenon can be rationalized in terms of a two-step mechanism of the overall redox reaction between c_{380} and P (see Fig. 9).

$$cP^+Q_A^- \underset{\bar{e}\ \text{transfer}}{\overset{K'_{eq} \approx 0.25}{\rightleftharpoons}} [c^+PQ_A^-]^{\ddagger} \xrightarrow[\text{reorganization}]{\text{medium}\ K''_{eq} \approx 400} c^+PQ_A^- \quad (VII)$$

The first step is virtually pure electron transfer which occurs at a very small mean energy difference between the cP^+ and c^+P states, giving rise to an intermediate $[c^+PQ_A^-]^{\ddagger}$ *. The rate of this step is likely to be almost temperature-independent as demonstrated for *Ect. shaposhnikovii* [32,33] and may require but minimal nuclear displacements (tunnelling), perhaps, within the zero oscillations in the porphyrin/protein system of RC.

At $T > 200$ K the initial electron transfer is followed by 'medium reorganization' [52], i.e., rearrangement of the redox centre local environment partly compensating

* Rigorous discussion of the medium reorganization mechanism with its traditional deconvolution into λ_i and λ_o terms (e.g., see Refs. 52, 53, 67–69) would be beyond the scope of this paper. For simplicity we consider contribution of the inner-sphere modes of motion, λ_i, to the total reorganization energy, λ, to be small, i.e., that $\lambda \approx \lambda_o$.

for the initial charge displacement. This medium reorganization entails relaxation of the $[c^+PQ_A^-]^‡$ intermediate to a thermodynamically favourable equilibrium $c^+PQ_A^-$ state (Fig. 9). In the frozen samples medium reorganization is likely to be prevented, which allows for observation of the isolated initial 'non-equilibrium' reaction step at low temperatures. Notably, the temperature dependence of c_{380} photooxidation quantum yield in a narrow T range (Fig. 5 and see Refs. 32, 33, 46 for the data on other objects) is typical of a phase transition. Similar dependence is inherent in intramolecular dynamics of many proteins [54–57].

The nuclear reorganization at $T > 200$ K appears to be associated mainly with c_{380}, rather than with P. Indeed, electron transfer from the pigment to bacteriopheophytin retains high quantum yield and rate even at liquid helium temperatures [58]. As to the medium reorganization around c_{380}, it could comprise rearrangement of the solvent around the exposed part of the iron-porphyrin group as well as minor displacements of some amino-acid residues and bound water molecules in the nearest vicinity of the heme (e.g., cf. Ref. 59).

Taking a generally accepted value $K_{eq} \sim 100$ for the net equilibrium $c_{380} \overset{e}{\rightleftharpoons} P$ (Refs. 17, 21, 49, 50, this work) and $K'_{eq} \approx 0.25$ as determined here for the initial electron transfer (see Eqn. VII), one can evaluate free energy of medium reorganization in reaction (VII) to be approx. -0.16 eV or approx. 1300 cm^{-1} (i.e., $K''_{eq} \approx 400$ in Eqn. VII), which is comparable to a value of 0.23 eV calculated for mitochondrial cytochrome c [53].

A similar mechanism of biological redox reactions with a rapid electron transfer to/from the redox centre followed by a conformational relaxation of the protein moiety has been discussed in literature quite extensively in connection with energy-coupling problems (e.g., see Refs. 60–63) and evidence was presented for non-equilibrium 'unrelaxed' redox states of some electron transfer proteins, though under rather artificial conditions (reviewed in Ref 64). This work demonstrates the occurrence of an 'unrelaxed' state in a physiological photoredox reaction.

Low-temperature oxidation of low-potential haem c_{20}

Complete flash-induced oxidation of the low-potential haem c_{20} at low temperatures indicates that in this case there is sufficient drop in energy between the haem and the pigment to provide for virtually irreversible e transfer, even in the absence of medium reorganization.

Assuming the linear sequence of the redox centres $c_{20} \rightarrow c_{380} \rightarrow P$, complete photooxidation of c_{20} could be visualized in two ways.

First, the redox state of c_{20} could influence electronic equilibrium between c_{380} and P in such a way that at low redox potentials (c_{20} reduced) complete oxidation of c_{380} by P^+ ensues, even at 77 K, as discussed, for instance, in Ref. 51. Following rapid complete photooxidation, c_{380} would be re-reduced at a lower rate by c_{20}.

Were this model right, P^+ re-reduction at low redox potentials would be considerably faster ($t_{1/2} < 6$ μs) than photooxidation of c_{20} ($t_{1/2} = 20$ μs). However, this is not the case, and the kinetics of the two processes at 77 match each other fairly well (Fig. 8). Moreover, the amplitude of haem c_{380} rapid photobleaching ($\Delta A_{557-551}$) does not depend significantly on the redox state of c_{20} before the flash (data not shown). These results argue against transient full oxidation of c_{380} by P^+ at low redox potentials and favour gradual oxidation of c_{20} via the equilibrium fraction ($\approx 20\%$) of the RC state with c_{380} oxidized

$$c_{20}c_{380}P \overset{h\nu}{\rightarrow} c_{20}c_{380}P^+ \rightleftharpoons c_{20}c_{380}^+P \rightarrow c_{20}^+c_{380}P \qquad \text{(VIII)}$$

A number of physical mechanisms has been considered in the past in connection with the peculiar temperature dependence of the low-potential haem photooxidation in *Chromatium* and other bacteria with the tightly bound four-haem cytochrome c [22,23,51,65–68]. The present work shows that there is at least one more factor contributing to deceleration of low-potential haem oxidation at cryogenic temperatures. It consists in diminution of the equilibrium concentration (probability) of the RC state with the oxidized high-potential haem proximal to P (c_{380}^+ in case of *Rps. viridis*) following flash-induced P oxidation.

This factor appears to be not very high (2–5-fold) in the case of *Ect. shaposhnikovii* [32,33] or *Rps. viridis* (this work, Ref. 44, 38), but can be quite significant in *Chromatium* where low-temperature photooxidation of high-potential haem is virtually undetectable [27,30,46].

Finally, photooxidation of the low-potential haem via the high-potential one implies that the temperature profile of this reaction should depend on the temperature dependence of the high-potential haem photooxidation rate. Although this dependence is quite small in *Ect. shaposhnikovii* [33], this may not be necessarily so in *C. vinosum* and other species.

Thus we feel that simulations of the temperature dependence of low-potential haem photooxidation rate based on purely quantum-mechanical description [65–68] should be revisited taking into consideration elementary kinetic consequences of the intermediate (high-potential haem) being involved in the process.

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