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# Laser-induced optoacoustic calorimetry of primary processes in cells of *Rhodospirillum rubrum*

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**Laser-induced optoacoustic spectroscopy (LIOAS) was used to measure the heat production in intact cells of *Rhodospirillum rubrum* S1 within approx. 1 μs after 15 ns excitation at 590 or 532 nm. In cells with the reaction centers in the open state, P-880IQ$_A$ (prior to the excitation) the photoproducts formed within 1.4 ± 0.1 μs, i.e., P$^+$-880IQ$_A^-$, were found to have a molar energy content $\Delta E_r = 0.62 \pm 0.1$ eV. From this value the midpoint redox potential $E_m(Q_A^-/Q_A) = -(0.17 \pm 0.1)$ eV results. The relative energy storage $\Delta E_r/N_A h\nu_e$ within the observation time, calculated for the excitation at the absorption maximum at 880 nm, is 0.44, i.e., considerably smaller than $\Delta E_r/N_A h\nu_e = 0.65$ obtained for PS II (see previous paper: Nitsch, C., Braslavsky, S.E. and Schatz, G.H. (1988) Biochim. Biophys. Acta 934, 201–212). In cells with the reaction centers in one of the closed states P$^+$-880IQ$_A$ and P-880IQ$_A^-$, prior to the excitation, the quantum yield for the formation of triplets (lifetime > 1.4 μs) was estimated to be $\Phi_T^{cl} = 0.50 \pm 0.15$. The comparison of the heat production after excitation of either the carotenoids at 532 nm or the bacteriochlorophylls at 590 nm, reveals that 34 ± 20% of the quanta absorbed by the carotenoids are transferred to the bacteriochlorophyll in the antenna by single-singlet energy transfer.**

## Introduction

In the reaction centers (RC) of photosynthetic purple bacterium the excitation of the primary donor P is followed within a few ps by the formation of the primary radical pair P$^+$I$^-$, which is stabilized within some hundreds of ps by a further electron transfer from I$^-$ to an ubiquinone, Q$_A$ (see Ref. 1 for a review). The lifetime of the resulting state, P$^+$-880IQ$_A^-$, is in the order of tens of microseconds for *Rhodospirillum rubrum* [2]. The light-harvesting complex in purple bacteria contains BChl and various carotenoids. When the carotenoids (Car) are excited, the energy is first transferred to BChl via singlet–singlet energy transfer and, after further transfer among the BChls, finally trapped in the RCs. The kinetics [1] of the primary photochemical events in bacterial RCs as well as their structural basis [3] have been studied in detail. Information about the energetics of charge separation, i.e., the conversion of light energy to electrochemical energy has been derived from thermodynamic calculations [4,5], from data based on redox titrations of the respective components (see Ref. 6 for a review), and from measurements of delayed fluorescence [7], phosphorescence [8], and triplet states [9]. However, since in most cases these measurements do not yield calorimetric data, the calculation of thermodynamic parameters involves a cumbersome procedure.

In a previous paper we reported the direct measurement of the heat evolved upon excitation of intact cells, of Photosystem I and of Photosystem II particles of the cyanobacterium *Synechococcus* sp. by laser-induced optoacoustic spectroscopy (LIOAS) [10]. This method can distinguish between the heat released occuring on the picosecond-to-nanosecond timescale and the heat released on the microsecond-to-millisecond range [11,12]. Thus, one can separately measure energetics of primary and of secondary electron-transfer steps. By comparing the heat from a photosynthetically active sample to that from an inhibited sample (internal heat calibration) or to that from a calorimetric reference (external heat calibration) the molar energy storage of

Abbreviations: RC, reaction center; BChl, bacteriochlorophyll; Car, carotenoid; SX, spirilloxanthin; LIOAS, laser-induced optoacoustic spectroscopy; ACar, antenna carotenoid; RCCar, reaction center carotenoid; P-880, primary electron donor; I, primary electron acceptor; Q$_A$, ubiquinone; ABChl, antenna bacteriochlorophyll; PAS, photoacoustic spectroscopy.

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the active sample was calculated and midpoint redox potentials of the respective components in both photosystems were estimated.

We have now applied this method to measure in a direct way the energy storage occuring within about 1 μs in intact cells of the photosynthetic bacteria *Rhodospirillum rubrum*. Measurements were performed under various redox conditions, i.e., at different states of the RCs. Approximate values for the midpoint redox potential of $Q_A$ can be obtained by simple thermodynamic considerations. Values for the yields of triplet formation in the RCs and in the antenna are obtained in a straightforward manner. By comparing the heat evolved after excitation of either Car or BChl the efficiency of singlet–singlet energy transfer from Car to BChl could be also estimated.

## Materials and Methods

*Samples*

Whole cells of *Rhodospirillum rubrum* strain S1 (*R. rubrum*, Deutsche Sammlung von Mikroorganismen, Braunschweig) were grown anaerobically (medium 27, German Collection of Microorganisms, Catalogue of strains 1983) at 30 °C under incandescent light. For the measurements the cells were centrifuged, washed twice with a buffer containing 10 mM Tris-HCl, 50 mM NaCl and 5 mM $MgCl_2$ (pH 8), and resuspended in the same buffer. Ethylene glycol (33%) was added in order to obtain larger optoacoustic signals [10]. The absorbance of the samples was adjusted to approx. 0.4 at the excitation wavelength (532 or 590 nm).

To keep the RCs in the open state P-880$IQ_A$, the samples were dark adapted and pumped through the cuvette [10]; no inhibitors and/or redox reagents were added. For internal heat calibration the RCs were either kept in the closed, oxidized state $P^+$-880$IQ_A$ by adding 1 mM $K_3Fe(CN)_6$ and background light (400–700 nm, 1500 $W \cdot m^{-2}$), or in the closed, reduced state P-880$IQ_A^-$ by adding 2 mM dithionite.

$CoCl_2$ and $CuCl_2 \cdot 2H_2O$ (both Merck z.A.), dissolved in a 1 : 2 (v/v) EG/water mixture, were used as external calorimetric references at 532 and 590 nm, respectively.

*Optoacoustic measurements*

The LIOAS set-up resembled that described previously [10,12] with the following changes:

(a) The second harmonic of a Nd-YAG laser was used either for direct excitation at 532 nm or to pump a dye laser (JK Lasers, System 2000). Rhodamine B (Lambda Physics) in methanol was used as laser dye for excitation at 590 nm. The pulse duration was about 15 ns.

(b) The external reservoir was equipped with a calomel and a platinum electrode (K401 and P101, Radiometer Copenhagen) in order to control the redox potential in the medium during measurements under reducing conditions. With 2 mM dithionite and under $N_2$ atmosphere a potential of −0.5 V, which is sufficiently low to convert $Q_A$ to its reduced state $Q_A^-$ (vide infra), was maintained for more than 1 h.

(c) A fixed laser beam diameter $2R = 2$ mm (effective transit time of the heat-produced acoustic wave through the laser beam radius $\tau_a' = 1.4 \pm 0.1$ μs [10]) was used for excitation at both wavelengths. The laser output was varied between 0.3 and 50 μJ per pulse, corresponding to fluences ranging from $3 \cdot 10^{13}$ to $5 \cdot 10^{15}$ photons per $cm^2$ per pulse for $\lambda_{exc} = 590$ nm. From the fluence the number of photons absorbed per RC per pulse can be calculated. Neglecting carotenoid absorption at 590 nm (vide infra), and assuming a BChl absorption cross section $\sigma_{590}$ of $9 \cdot 10^{-17}$ $cm^{-2}$, and given a unit size of 50 BChl per RC [13] and a sample absorbance of 0.4, 0.08 and 14 photons are calculated to be absorbed per RC per pulse for the two extremes of the fluence range given above. The value for $\sigma_{590}$ is estimated from the antenna BChl absorption coefficient of 140 $mM^{-1} \cdot cm^{-1}$ at 880 nm [14] and an absorbance ratio $A_{590}/A_{880} = 0.17$ for *R. rubrum* chromatophores.

(d) The signals from intact *R. rubrum* cells (size about 10 μm [15]) needed correction for the contributions due to scattered light as described in the previous paper [10]. Signals only due to scattered light were generated using emulsions of phospholipids as $\lambda^{-1}$-scatterers in EG/water mixtures. The turbidity was matched at 700 nm, where the *R. rubrum* cells have negligible absorption. This approximation cannot introduce more than approx. 5% error, since scattering in this region amounts to 50% and more of the attenuation. Thus, the scattering-induced signals were considerably larger than those from cyanobacteria [10]. For external heat calibration, the absorbances of the non-scattering reference solutions were adjusted to the difference in absorbance between *R. rubrum* cells and the phospholipids at the respective measuring wavelength. The alternative method of preparing scattering reference solutions with the same total attenuation and the same contributions due to scattering and absorption and comparing the respective scattering-corrected signals induces a larger error than the method described above. Absorbances were measured with a Perkin-Elmer 356 spectrophotometer.

Optoacoustic signal amplitudes for *R. rubrum* cells with open and closed RCs were measured over a wide energy range (see above), excited with either 532 or 590 nm. 532 nm is well within the triple-banded absorption of spirilloxanthin (SX), which is the major (91%) carotenoid in *R. rubrum* S1 [16], whereas bacteriochlorophyll (BChl) is not excited at this wavelength. At 590 nm preferentially BChl is excited, which can be deduced from the absorption spectrum of *R. rubrum*

chromatophores after removal of spirilloxanthin by extraction [17]. The absorbance due to SX in vivo at 590 nm is estimated to amount to 5–10% of the total. Optoacoustic measurements with excitation at 600 nm, where only BChl absorbs, were not possible, since scattering causes more than 80% of the attenuation at this wavelength and the signals due to absorption are too small.

*Signal handling*

For dilute solutions the normalized optoacoustic signal $H_N = (H/E_0)$ is given by Eqn. 1 [11];

$$H_N = K \cdot \alpha \cdot (1 - 10^{-A}) \tag{1}$$

where $K$ is determined from instrumental and thermoelastic properties, $E_0$ is the excitation energy, $A$ is the absorbance at the excitation wavelength, and $\alpha$ is the fraction of the absorbed energy that is dissipated as heat within the acoustic transit time $\tau_a'$ [18]. For the calorimetric references $CoCl_2$ and $CuCl_2$ $\alpha = 1$ [19]. For *R. rubrum* with open RCs in which some energy may be stored by products for a time longer than $\tau_a'$ or may be lost by fluorescence, $\alpha$ becomes less than 1 and $H_N$ is decreased. Thus, with external heat calibration $\alpha$ is equal to the ratio of the normalized signals from a sample with open RCs, $H_N^{op}$, and from the reference, $H_N^{ref}$:

$$\alpha = \frac{H_N^{op}}{H_N^{ref}} \tag{2}$$

However, for the case of internal heat calibration also the processes occurring in samples with closed RCs, i.e., increased fluorescence and energy storage by long-lived (greater than $\tau_a'$) triplet states, should be taken into account. Then $\alpha$ is given by Eqn. 3:

$$\alpha = \left(1 - \Phi_f^{cl}\frac{\nu_f}{\nu_e} - \frac{\Phi_T^{cl}\Delta E_T}{N_A h \nu_e}\right) \cdot \frac{H_N^{op}}{H_N^{cl}} \tag{3}$$

where $\Phi_f^{cl}$ and $\Phi_T^{cl}$ denote the fluoresence quantum yield and the triplet yield for closed RCs, respectively, $\nu_e$ and $\nu_f$ are the radiation frequencies of the excitation and of the weighted average of the fluorescence, respectively, $N_A$ is Avogadro's number and $h$ is the Planck constant, $H_N^{cl}$ is the normalized signal for closed RCs, and $\Delta E_T$ the molar energy content of the triplet state.

From Eqns. 2 and 3 Eqn. 4 is obtained:

$$\frac{H_N^{cl}}{H_N^{ref}} = 1 - \Phi_f^{cl}\frac{\nu_f}{\nu_e} - \frac{\Phi_T^{cl}\Delta E_T}{N_A h \nu_e} \tag{4}$$

Thus, provided two of the three values $\Phi_f^{cl}$, $\Delta E_T$ and $\Phi_T^{cl}$ are known, the third can be calculated with Eqn. 4.

With $\alpha$ the relative energy storage $\Delta E_r / N_A h \nu_e$ by photoproducts formed within $\tau_a'$ in open RCs [20] can be calculated, using the energy balance equation [5]:

$$N_A h \nu_e = \alpha N_A h \nu_e + \Phi_f^{op} N_A h \nu_f + \Phi_T^{op} \Delta E_T + \Phi_r \Delta E_r \tag{5}$$

which includes all processes taking place in samples with open RCs. $\Phi_f^{op}$ and $\Phi_T^{op}$ are the quantum yields of fluorescence and triplet formation for open RCs, respectively, $\Phi_r$ is the quantum yield of the photoreaction, and $\Delta E_r$ the molar energy content of the photoproduct living longer than $\tau_a'$. Rearrangement of Eqn. 5 yields expression [6] for the relative energy storage:

$$\frac{\Delta E_r}{N_A h \nu_e} = \frac{1 - \alpha - \Phi_f^{op}\frac{\nu_f}{\nu_e} - \frac{\Phi_T^{op}\Delta E_T}{N_A h \nu_e}}{\Phi_r} \tag{6}$$

*Curve fitting*

A nonlinear least-square optimization program (Levenberg–Marquardt algorithm) was used to fit the energy-dependent normalized optoacoustic signals. A cumulative one-hit Poisson distribution according to Eqn. 7 [21]

$$H_N = A + B \cdot \left(1 - \frac{1 - e^{-CE_0}}{CE_0}\right) \tag{7}$$

was assumed. This method of curve fitting has already been successfully used in our previous studies [10]. The parameter $A$ is the signal offset at low fluence, and $B$ is the signal difference $\Delta H_N$ between low and high fluence (vide infra). A and B have units of $10^2$ $V \cdot J^{-1} \cdot C$ has units of $\mu J^{-1}$ and is related to a'critical' energy, above which double and multiple hits per RC occur. For a pulse energy $E_0 = C^{-1}$ the signal has the value $A + B/e$.

## Results and Discussion

*Bacteriochlorophyll excitation – open reaction centers*

Fig. 1 shows a semilogarithmic plot of the scattering-corrected and energy-normalized signals $H_N$ vs. $E_0$ obtained from intact *R. rubrum* cells with different redox states of the RCs prior to the excitation at 590 nm. For the signals from samples with initially open RCs (○), $H_N^{op}$, a sigmoidal curve is obtained which increases with fluence. This was already observed for various photosynthetic systems [10,22] and was ascribed to a gradual closure of the RCs by multiple hits increasing with the excitation energy of the laser. Further charge separation and energy storage is thus inhibited at high fluences. The value for $H_N^{op}$ that enters in Eqn. 2 or 3 therefore is the low $H_N^{op}$-level. The Poisson fit according to Eqn. 7 yields, for the low level, $A = 2.79 \cdot 10^2$

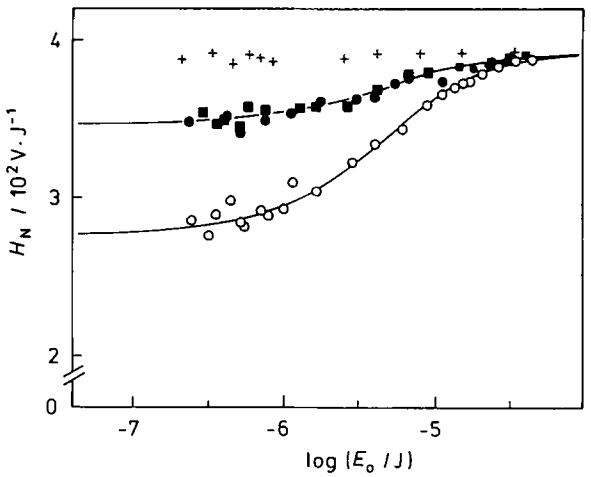


Fig. 1. Energy-dependence of the scattering-corrected and energy-normalized LIOAS signals $H_N$ from intact *R. rubrum* cells excited at 590 nm. $A_{590} = 0.4$ and $2R = 2.0$ mm ($\tau_a' = 1.4$ μs). Redox states of the RC: P-880IQ$_A$, dark adapted (○); P$^+$-880IQ$_A$, 1 mM $K_3Fe(CN)_6$ and background light (■); P-880IQ$_A^-$, 2 mM dithionite (●). (+): Energy-normalized LIOAS signals $H_N^{ref}$ from a $CuCl_2$ reference solution (see Materials and Methods).

$V \cdot J^{-1}$, at an amplitude $B = 1.17 \cdot 10^2$ $V \cdot J^{-1}$ and $C = 0.31$ $\mu J^{-1}$. From $C^{-1} = 3.2$ μJ, corresponding to a fluence of $3.2 \cdot 10^{14}$ photons per cm$^2$ per pulse, a value of 0.85 photons absorbed per RC per pulse is calculated (see Materials and Methods). This number is close to unity demonstrating the validity of a one-hit Poisson distribution model.

For the $CuCl_2$ reference signals (+), $H_N^{ref}$, shown in the same figure, no signal change is observed with varying fluence. A value $\alpha = (H_N^{op}/H_N^{ref}) = 0.70 \pm 0.05$ is obtained for low fluences. The same value is also calculated for the ratio $A/(A+B)$, i.e., $\alpha$ can also be obtained from internal heat calibration without adding chemicals just by varying the fluence over a sufficiently wide range.

The contributions due to fluorescence and triplet state formation, needed in order to evaluate the relative energy storage $\Delta E_r/N_A h\nu_e$ using Eqns. 5 and 6, are evaluated as follows.

From the average fluorescence lifetime $\tau_f = 60$ ps, measured for *R. rubrum* cells with open RCs [23], and the radiative lifetime $\tau_0 = 18$ ns, calculated for BChl a in vivo from the integrated absorption coefficient of *Rhodobacter sphaeroides* chromatophores [24], $\tau_f/\tau_0 = \Phi_f^{op} = 0.0033$ is calculated. For $\lambda_f^{max} = 900$ nm [23,24] $\nu_f/\nu_e = 0.66$ results. Thus, the fluorescence term in Eqn. 6, $\Phi_f^{op} \cdot \nu_f/\nu_e$, is 0.0022 and practically negligible.

The second term in Eqn. 6 accounts for the formation of triplet states. In carotenoid-containing purple bacteria BChl triplets in the antenna ($^3$ABChl) are efficiently and rapidly quenched by antenna-carotenoids (in 20 ns [25], i.e., very much smaller than $\tau_a'$) leading to the formation of antenna-carotenoid triplet states ($^3$ACar). This has been shown by difference absorption spectroscopy using nanosecond excitation flashes [26,27]. For chromatophores of *R. rubrum* with the RC-state P-880IQ$_A$, $^3$ACar with a lifetime of 3.6 μs (greater than $\tau_a'$) are formed with a yield of $\Phi_T^{op} = 0.06$ [27]. Therefore, part of the absorbed energy is stored by the triplets. With the estimated triplet energy $\Delta E_T$ for SX of 63 $kJ \cdot mol^{-1}$ (0.65 eV) [28] and $N_A h\nu_e = 2.1$ eV at 590 nm the relative energy storage due to triplet formation in Eqn. 1, $\Phi_T^{op}\Delta E_T/N_A h\nu_e$, is about 0.019.

Assuming $\Phi_r = 0.92$ [26], a value of $0.30 \pm 0.05$ is calculated for the relative energy storage $\Delta E_r/N_A h\nu_e$ corresponding to a molar energy content $\Delta E_r = 0.62 \pm 0.1$ eV of the photoproducts formed within $\tau_a'$ (1.4 μs) and living longer than this time, i.e., of P$^+$-880IQ$_A^-$ (vide infra). Extrapolation of the measured values permits the calculation of a value for the energy stored $\Delta E_r/N_A h\nu_e = 0.44$, should direct excitation at the BChl *a* absorption maximum at 880 nm (corresponding to $N_A h\nu_e = 1.4$ eV) would have been made. For PS II particles from *Synechococcus* sp. excited at 677 nm, i.e., at the Chl *a* absorption maximum a larger value of 0.65 was obtained [10]. This implies that in the bacterial photosystem, which is often considered to be related to PS II, the relative energy storage within approx. 1 μs turns out to be considerable smaller than in PS II.

*Bacteriochlorophyll excitation – closed reaction centers*

The energy dependence of the signals from samples with closed RCs, $H_N^{cl}$, obtained under the same optical conditions as for open RCs is also shown in Fig. 1. A curve similar to that from open RCs but starting at a higher low-fluence level is obtained. The fit yields $A = 3.47 \cdot 10^2$ $V \cdot J^{-1}$, $B = 0.46 \cdot 10^2$ $V \cdot J^{-1}$ and $C = 0.32$ $\mu J^{-1}$. No difference is observed between samples with the RCs in the redox states P$^+$-880IQ$_A$ (■) or P-880IQ$_A^-$ (●), i.e., with either the electron input or output inhibited. At low fluences $H_N^{cl}/H_N^{ref} = 0.88 \pm 0.05$ and $H_N^{op}/H_N^{cl} = 0.80 \pm 0.05$. Although the inflexion points of the curves for open and closed RCs seem to be at the same fluence, there is no reason to expect this behaviour. This might be a result of either coincidence or of the errors involved in the determination and subsequent mathematical fitting.

Inserting $H_N^{cl}/H_N^{ref}$ into Eqn. 4 the fraction of the absorbed energy not released as prompt heat may be calculated. From $\tau_f = 200$ ps measured for closed RCs [23] and $\tau_0$ (see above value) $\Phi_f^{cl} = 0.011$ results. With $\nu_f/\nu_e = 0.66$, the fluorescence term $\Phi_f^{cl} \cdot \nu_f/\nu_e$ in Eqn. 4 amounts to 0.0073.

The main contribution to the deviation of $\alpha$ from unity comes from the triplet term $\Phi_T^{cl}\Delta E_T/N_A h\nu_e$ in Eqn. 4. Long-lived (greater than $\tau_a'$) and thus energy-storing Car triplet states are formed in carotenoid-containing purple bacteria with closed RCs by triplet-triplet energy transfer from BChl [25,26]. For *R. rubrum* cells antenna-associated ($^3$ACar) and RC-associated

($^3$RCCar) triplet states have been distinguished spectroscopically [27]. $^3$RCCar formation follows the recombination of triplet radical pairs in the RC, arising in turn from the initially formed single radical pairs (see equations):

$$^1(P^+\text{-}880I^-) \rightarrow {}^3(P^+\text{-}880I^-) \rightarrow ({}^3P\text{-}880)I$$

$$^3P\text{-}880 + RCCar \rightarrow {}^3RCCar + P\text{-}880$$

Using Eqn. 4 estimates of $\Phi_T^{cl}$ can be made from the optoacoustic measurements taking into account the heat released by the triplets with lifetimes comparable to the transit time of the experiment. Thus, in the third term on the right-hand side in Eqn. 4 $\Delta E_T$ becomes $\Delta E_T^0 \exp(-\tau_a'/\tau_T)$ [12]. Now $\Delta E_T^0$ is the molar energy content of the triplet. With $\Delta E_T^0 = 0.65$ eV for SX (see above) we obtain $\Phi_{^3ACar}^{cl} = 0.50 \pm 0.15$ for cells with the RC state $P^+$-880IQ$_A$. Assuming that the triplet energies of ACar and RCCar do not differ much [28,29], a similar value is obtained for the sum $\Phi_{^3ACar}^{cl} + \Phi_{^3RCCar}^{cl} = 0.35 \pm 0.15$ for cells with the RC state P-880IQ$_A^-$. In [27] $\Phi_{^3ACar}^{cl} = 0.2$ and a lifetime $\tau_T = 3.6$ μs were reported for the RC state $P^+$-880IQ$_A$. In comparison, in cells with the RC state P-880IQ$_A^-$ the total triplet yield was shown to be the sum of $\Phi_{^3ACar}^{cl} = 0.12$ ($\tau_T = 3.6$ μs) and $\Phi_{^3RCCar}^{cl} = 0.12$ ($\tau_T = 3.2$ μs), i.e., 0.24 [27]. Our optoacoustically determined yield is somewhat higher than the literature value. Thus, the whole triplet term $\Phi_T^{cl}\Delta E_T/N_A h\nu_e$ in Eqn. 4 amounts to 0.10 at the present transit time. Inserting the values for the fluorescence and triplet terms (0.0073 and 0.10) and $H_n^{op}/H_N^{cl} = 0.80$ into Eqn. 3, the same $\alpha = 0.70$ is obtained both from internal heat calibration and from external heat calibration according to Eqn. 2.

At high fluences the signals $H_N^{cl}$ approach the reference signal level. Although trapping, charge separation and energy storage by $^3$P-880 and $^3$RCCar formation occur even with multiple photon excitation, these processes should not affect the 'energy-normalized' heat production, since eventually all antenna-associated, excited Chls are quenched emitting all the absorbed energy as prompt heat. This may occur either by singlet–singlet annihilation, which strongly decreases triplet formation [30], or triplet–triplet annihilation, or both.

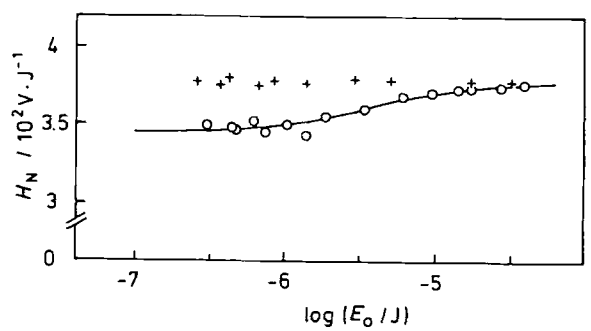


Fig. 2. Energy-dependence of the scattering-corrected and energy-normalized LIOAS signals $H_N$ from (○) intact *R. rubrum* cells excited at 532 nm with the RCs in state P-880IQ$_A$. $A_{532} = 0.45$ and $2R = 2.0$ mm ($\tau_a' = 1.4$ μs) and from (+) from a $CoCl_2$ reference solution.

$^3$Chl–$^3$Chl annihilation was shown to be the major process in the isolated light-harvesting complex of PS II at high fluence [31]. A $\tau_T$ value of 50 ns was found for $^3$Chl. Only at low fluences $^3$Car with a lifetime $\tau_T = 6$ μs could be observed [31]. An analogous situation, i.e., a very short lived triplet state, may explain our observation that no measurable energy storage due to $^3$RCCar formation or $^3$ACar formation is found at high fluences.

*Carotenoid excitation – open reaction centers – Car-to-BChl energy transfer*

Fig. 2 shows a semilogarithmic plot of the scattering-corrected and energy-normalized signals (○) $H_N^{op}$ vs. $E_0$ from intact *R. rubrum* cells excited at 532 nm with the RCs in the open state prior to the measurement. In the same figure the normalized signals (+), $H_N^{ref}$, from a $CoCl_2$-reference solution are shown. As expected, they are constant over the whole fluence range. For $H_N^{op}$, again a sigmoidal curve is observed. The fit yields $A = 3.44 \cdot 10^2$ V·J$^{-1}$, $B = 0.347 \cdot 10^2$ V·J$^{-1}$ and $C = 0.36$ μJ$^{-1}$.

Using Eqn. 2 $\alpha = 0.91 \pm 0.05$ results for 532 nm at low fluences, i.e., a value considerably higher than 0.70 which was obtained for $\lambda_{exc} = 590$ nm. Since at 532 nm the carotenoid is excited, the quantum efficiency of singlet–singlet energy transfer from ACar to ABChl, $\Phi_{ET}$, should be taken into account and Eqn. 5 should thus be expanded into Eqn. 8 in order to consider such transfer.

$$N_A h\nu_e = (1 - \Phi_{ET}) \cdot N_A h\nu_e + \Phi_{ET} \cdot (\alpha' \cdot N_A h\nu_e + \Phi_f^{op} \cdot N_A h\nu_f + \Phi_T^{op}\Delta E_T + \Phi_r \cdot \Delta E_r) \quad (8)$$

As a first approach we assume that the portion $1 - \Phi_{ET}$ of the energy absorbed by the carotenoids, but not transferred to ABChl, is totally released as heat within $\tau_a'$ (1.4 μs). Then $\alpha'$ is the value that would be obtained, should all the energy absorbed at 532 nm be transferred to ABChl. The observed $\alpha = 0.91 \pm 0.05$ corresponds to the sum of all prompt heat released by ACar, ABChl, and during the product stabilization in the RCs, and is therefore equal to $(1 - \Phi_{ET}) + (\alpha' \cdot \Phi_{ET})$. Eqn. 8 is thus simplified to Eqn. (9):

$$N_A h\nu_e = \alpha \cdot N_A h\nu_e + \Phi_{ET} \cdot (\Phi_f^{op} \cdot N_A h\nu_f + \Phi_T^{op}\Delta E_T + \Phi_r \Delta E_r) \quad (9)$$

Rearrangement of Eqn. 9 leads to Eqn. 10:

$$\Phi_{ET} = \frac{1 - \alpha}{\Phi_r \frac{\Delta E_r}{N_A h\nu_e} + \Phi_f^{op} \frac{\nu_f}{\nu_e} + \Phi_T^{op} \frac{\Delta E_T}{N_A h\nu_e}} \quad (10)$$

From Eqn. 10 $\Phi_{ET}$ can be calculated taking for all other parameters the values given above ($N_A h\nu_e = 2.33$ eV at 532 nm) and assuming that the molar energy stored by the product, $\Delta E_r = 0.62 \pm 0.1$ eV is the same for both wavelengths. $\Phi_{ET} = 0.34 \pm 0.2$ results, which is in good accordance with the value of 0.3 obtained from fluorescence measurements for *R. rubrum* [32,33]. Boucher et al. [34] determined $\Phi_{ET} = 0.5 \pm 0.1$ for the same energy transfer process in *R. rubrum* using conventional photoacoustic spectroscopy (PAS), which has a much larger time window [20] than the $\tau_a'$ values in LIOAS. This higher value compared to the literature value (0.3, see above) was explained by a non-negligible percentage of RC kept closed by the excitation light, thus leading to prompt heat dissipation in the BChl antenna. A major reason, however, is related to the $\Delta E_r$ sensed in each case. This may also explain the difference in the $\Phi_{ET}$ determined by both photothermal techniques. With larger heat integration times $\alpha$ tends to unity, irrespective of the exciting wavelength. This can be rationalized by inspection of Eqns. 5 and 9. Neglecting the fluorescence, $\Phi_f^{op} \cdot \nu_f/\nu_e$, and triplet term, $\Phi_T^{op} \cdot \Delta E_T/N_A h\nu_e$, for a given $\alpha$, $\Phi_{ET}$ is inversely proportional to $\Delta E_r$. The 'true' value of $\Phi_{ET}$ obtained by a photothermal measurement should be that extrapolated to zero integration time, i.e., corresponding to the heat stored by the primary radical pair.

The good accordance between our $\Phi_{ET}$ and the literature value supports the assumption made above of a complete and fast deactivation of all excited carotenoids by internal conversion with efficiency $1 - \Phi_{ET}$. Taking into account that the fluorescence quantum yield of carotenoids is vanishingly small [35], this means that either long-lived (greater than $\tau_a'$) triplets ($^3$ACar) are not formed in our experiment upon direct carotenoid excitation or, if they are formed, their lifetimes are not greater than $\tau_a'$ (1.4 μs). Magnetic-field-dependent $^3$ACar formation upon 532 nm excitation has been reported for *R. rubrum* chromatophores with oxidized RC-state $P^+$-880$IQ_A$ and was explained by a *Car fission mechanism yielding the triplet states [27,36,37].

A value of $C^{-1} = 2.78$ μJ obtained from the fit corresponds to a fluence of about $2.34 \cdot 10^{14}$ photons per cm$^2$ per pulse. From the molar absorption coefficient $\varepsilon_{512} = 90$ mM$^{-1} \cdot$ cm$^{-1}$ for SX in vitro [28] and the spectral shift of +20 nm in *R. rubrum* chromatophores (see above) $\sigma_{532} = 3.4 \cdot 10^{-16}$ cm$^2$ was estimated for SX in vivo. A value of 0.08 photons per SX results. With $\sigma_{ET} = 0.34$ and neglecting BChl absorption at 532 nm, this corresponds to 0.027 photons per BChl. For a unit size of 50 BChl/RC [13] and a sample absorbance of 0.45 a value of 0.9 photons absorbed per RC per pulse is calculated. This number is very close to 0.85 obtained with direct excitation of BChl at 590 nm and independently corroborates the concept of the cumulative one-hit Poisson distribution.

*Thermodynamic considerations*

The value $\Delta E_r = 0.62 \pm 0.1$ eV for *R. rubrum* cells with open RCs is the molar energy content of the energy-storing photoproducts formed within the experimental time window $\tau_a'$ of 1.4 μs and living longer than this time. In purple bacteria this time window includes the formation of the secondary radical pair $P^+$-880$IQ_A^-$ (see Ref. 1 for a review):

$$P\text{-}880IQ_A \xrightarrow{\text{few ps}} P^+\text{-}880I^- Q_A \xrightarrow{200\ \text{ps}} P^+\text{-}880IQ_A^-$$

Further electron transfer to the second quinone acceptor $Q_B$ occurs in greater than 100 μs, a time much longer than $\tau_a'$ [1]. Also the $P^+$ reduction by the *c*-type cytochrome in the cyclic pathway is slow compared to $\tau_a'$. The high-potential cytochromes $c_2$ in *R. rubrum* [2] and *Rb. sphaeroides* [2,38] donate electrons to $P^+$ in the order to tens of microseconds (in contrast to, e.g., *Rhodopseudomonas viridis*: 270 ns [39] and *Chromatium vinosum*: less than 2 μs [2]).

The $\Delta E_r$ value can be converted to a corresponding value of midpoint potential difference, $\Delta E_m$, between the components involved (P-880 and $Q_A$), provided the following assumptions and approximations are made. The 'internal energy' $\Delta E_r$ can be set equal to the enthalpy $\Delta H_r$. We further assume that the entropy changes $\Delta S_r$ due to the electron transfer are small as compared to $\Delta H_r$. $\Delta H_r$ can thus be set equal to the free enthalpy, $\Delta G_r$. So far, only standard entropies $\Delta S^0$ have been determined by redox titrations. However, they might not reflect transient high-enthalpy states of the components [6,40]. $T\Delta S^0 = 0.1$ eV was estimated for the process $PQ_A \rightarrow P^+Q_A^-$ from temperature-dependent measurements of the delayed fluorescence from *Rb. sphaeroides* RCs [7]. This correction is therefore small. $\Delta G_r$ is related to $\Delta G_r^0$, the standard free enthalpy, by Eqn. 11:

$$\Delta G_r = \Delta G_r^0 + kT \cdot \ln \frac{[P^+X^-]}{[PX]} \qquad (11)$$

where $k$ is the Boltzmann constant, X an appropriate acceptor and $[P^+X^-]/[PX]$ the ratio of RCs in the charge-separated and ground states. It is difficult to obtain information about this ratio. For the primary charge separation in bacterial RCs (X = I) the ratio was calculated for various illumination intensities by comparing the light absorption rate to that of charge separation and assuming that the excited state P*I and $P^+I^-$ are in equilibrium during the illumination [41]. Values ranging from 0.01 (weak cw-light) up to $10^4$ (strong laser flash) were obtained. However, the system

never really reaches equilibrium due to further forward electron transfer. Since the optoacoustic measurements are performed at very low fluences, the above ratio for $X = Q_A$ is expected to be less than 1 [42]. Thus, $\Delta G_r^0$ represents an upper bound to the free enthalpy requirement $\Delta G_r$ of the $P^+Q_A^-$ formation. Assuming $[P^+Q_A^-]/[PQ_A] = 1$ as a first approximation, thus cancelling the concentration-dependent term in Eqn. 11, leads to $\Delta G_r = \Delta G_r^0$. Neglecting also Coulombic interactions between $P^+$ and $Q_A^-$ it follows that $\Delta E_r = \Delta G_r^0 = \Delta E_m = 0.62 \pm 0.1$ eV $= E_m(P^+\text{-}880/\text{P-}880) E_m(Q_A^-/Q_A)$. With $E_m(P^+\text{-}880/\text{P-}880) = 0.45$ eV [1] a value of $E_m(Q_A^-/Q_A) = -(0.17 \pm 0.1)$ eV is calculated. The midpoint potential of $Q_A$ in purple bacteria has been shown to be strongly pH-dependent [43]. For pH 8, the pH value of our measurements, redox titrations with *R. rubrum* chromatophores yielded a value of $E_m(Q_A^-/Q_A) = -0.15$ eV [43], in good agreement with our present data.

## Concluding remarks

In terms of energy balance, $H_N/H_N^{ref}$ is equal to the yield of heat production and can thus be used to calculate, by difference, the parameters of complementary processes. Since the fluorescence quantum yields in photosynthetic units are well known in most cases, independent information about the energy stored by transients living longer than the transit time $\tau_a'$ can be obtained. The optoacoustic measurements on *R. rubrum* cells with closed RCs support and complete the data about triplet formation obtained from difference absorption and paramagnetic resonance spectroscopic measurements. For the case of open RCs, provided the fluorescence and triplet parameters are known, the molar energy content of the photoproduct can be calculated. From this, midpoint redox potentials of the components can be estimated, which, in spite of the approximations used, reflect the properties of the system in a better way than results obtained from conventional titrations. For a system containing various pigments the quantum efficiency of energy transfer may be estimated in good accordance to fluorescence data. All parameters calculated in this work are summarized in Table I.

TABLE I

*Summary of data determined in this work*

Unless otherwise indicated, $\lambda_{exc} = 590$ nm

| | |
|---|---|
| Fraction of energy dissipated as prompt heat | $\alpha = 0.70 \pm 0.05$<br>$\alpha_{532} = 0.91 \pm 0.05$ |
| total triplet quantum yield in samples with closed RCs | $\Phi_T^{cl} = 0.50 \pm 0.15$ |
| relative energy storage | $\Delta E_r / N_A h\nu_e = 0.30 \pm 0.05$ |
| difference between midpoint potentials of component | $\Delta E_m = 0.62 \pm 0.10$ eV |
| midpoint potential of $Q_A$ | $E_m = -(0.17 \pm 0.10)$ eV |
| quantum efficiency for energy transfer from Car to BChl ($\lambda_{exc} = 532$ nm) | $\Phi_{ET} = 0.34 \pm 0.20$ |

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## References

1 Parson, W.W. and Ke, B. (1982) in Photosynthesis, Vol. 1 (Govindjee, ed.), pp. 331–385, Academic Press, New York.
2 Clayton, R.K. (1980) Photosynthesis: physical mechanisms and chemical patterns, Cambridge University Press, Cambridge, pp. 213–227.
3 Deisenhofer, J., Epp, O., Miki, K., Huber, R. and Michel, H. (1984) J. Mol. Biol. 180, 385–398.
4 Duysens, L.N.M. (1959) in The Photochemical Apparatus. Brookhaven Symp. Biol., pp. 10–25, Brookhaven Nat. Lab., Upton, New York.
5 Knox, R.S. (1969) Biophys. J. 9, 1351–1362.
6 Blankenship, R.E. and Parson, W.W. (1979) in Photosynthesis in Relation to Model Systems (Barber, J., ed.), pp. 71–114, Elsevier, Amsterdam.
7 Arata, H. and Parson, W.W. (1981) Biochim. Biophys. Acta 638, 201–209.
8 Woodbury, N.W.T. and Parson, W.W. (1984) Biochim. Biophys. Acta 767, 345–361.
9 Goldstein, R.A., Takiff, L. and Boxer, S.G. (1988) Biochim. Biophys. Acta 934, 253–263.
10 Nitsch, C., Braslavsky, S.E. and Schatz, G.H. (1988) Biochim. Biophys. Acta 934, 201–212.
11 Patel, C.K.N. and Tam, A.C. (1981) Rev. Mod. Phys. 53, 517–550.
12 Braslavsky, S.E. and Heihoff, K. (1989) in Handbook of Organic Photochemistry (Scaiano, J.C., ed.), CRC Press, Boca Raton, FL, in press.
13 Vredenberg, W.J. and Duysens, L.N.M. (1963) Nature 197, 355–357.
14 Clayton, R.K. (1963) in Bacterial Photosynthesis (Gest, H., San Pietro, A. and Vernon, L.P., eds.), pp. 495–500, The Antioch Press, Yellow Springs, OH.
15 Trüper, H.G. and Pfennig, N. (1978) in The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R., eds.), pp. 19–27, Plenum Press, New York.
16 Schmidt, K. (1978) in The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R., eds.), pp. 729–750, Plenum Press, New York.
17 Kito, M., Yamashita, J. and Koyama, Y. (1983) Photobiochem. Photobiophys. 5, 209–217.
18 Lai, H.M. and Young, K. (1982) J. Acoust, Soc. Am. 72, 2000–2007.
19 Braslavsky, S.E., Ellul, R.M., Weiss, R.G., Al-Ekabi, H. and Schaffner, K. (1983) Tetrahedron 39, 1909–1913.
20 Malkin, S. and Cahen, D. (1979) Photochem. Photobiol. 29, 803–813.
21 Mauzerall, D. (1976) J. Phys. Chem. 80, 2306–2309.
22 Jabben, M. and Schaffner, K. (1985) Biochim. Biophys. Acta 809, 445–451.

23 Borisov, A.Y., Freiberg, A.M., Godik, V.I., Rebane, K.K. and Timpmann, K.E. (1985) Biochim. Biophys. Acta 807, 221–229.
24 Zankel, K.L., Reed, D.W. and Clayton, R.K. (1968) Proc. Natl. Acad. Sci. USA 61, 1243–1249.
25 Monger, T.G., Cogdell, R.J. and Parson, W.W. (1976) Biochim. Biophys. Acta 449, 136–153.
26 Kingma, H., Duysens, L.N.M. and Van Grondelle, R. (1983) Biochim. Biophys. Acta 725, 434–443.
27 Kingma, H., Van Grondelle, R. and Duysens, L.N.M. (1985) Biochim. Biophys. Acta 808, 383–399.
28 Bensasson, R., Land, E.J. and Maudinas, B. (1976) Photochem. Photobiol. 23, 189–193.
29 Schenck, C.C., Mathis, P. and Lutz, M. (1984) Photochem. Photobiol. 39, 407–417.
30 Mathis, P. (1969) in Progress in Photosynthesis (Metzner, H., ed.), pp. 818–822, University of Tübingen, Tübingen.
31 Nechushtai,R., Thornber, J.P., Patterson, L.K., Fessenden, R.W. and Levanon, H. (1988) J. Phys. Chem. 92, 1165–1168.
32 Duysens, L.N.M. (1952) Thesis, University of Utrecht.
33 Goedheer, J.C. (1969) Biochim. Biophys. Acta 172, 252–265.
34 Boucher, F., Lavoie, L., Antippa, A.F. and Leblanc, R.M. (1983) Can. J. Biochem. Cell Biol. 61, 1117–1122.
35 Dallinger, R.F., Woodruff, W.H. and Rodgers, M.A.J. (1981) Photochem. Photobiol. 33, 275–277.
36 Rademaker, H., Hoff, A.J., Van Grondelle, R. and Duysens, L.N.M. (1980) Biochim. Biophys. Acta 592, 240–257.
37 Nuijs, A.M., Van Grondelle, R., Joppe, H.L.P., Van Bochove, A.C. and Duysens, L.N.M. (1985) Biochim. Biophys. Acta 810, 94–105.
38 Dutton, P.L., Petty, K.M., Bonner, H.S. and Morse, S.D. (1975) Biochim. Biophys. Acta 387, 536–556.
39 Deisenhofer, J., Michel, H. and Huber, R. (1985) Trends Biochem. Sci. 10, 243–248.
40 Case, G.D. and Parson, W.W. (1971) Biochim. Biophys. Acta 253, 187–202.
41 Parson, W.W. (1978) Photochem. Photobiol. 28, 389–393.
42 Warner, J.W. and Berry, R.S. (1987) Proc. Natl. Acad. Sci. USA 84, 4103–4107.
43 Prince, R.C. and Dutton, P.L. (1987) in The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R., eds.), pp. 439–453, Plenum Press, New York.