Biochimica et Biophysica Acta, 440 (1976) 765—771

BBA Report

BBA 41283

KINETICS OF POPULATING AND DEPOPULATING OF THE COMPONENTS OF THE PHOTOINDUCED TRIPLET STATE OF THE PHOTOSYNTHETIC BACTERIA *RHODOSPIRILLUM RUBRUM*, *RHODOPSEUDOMONAS SPHEROIDES* (WILD TYPE), AND ITS MUTANT R-26 AS MEASURED BY ESR IN ZERO-FIELD

A.J. HOFF

Department of Biophysics and Centre for the Study of the Excited States of Molecules, Huygens Laboratory, State University of Leiden (The Netherlands)

(Received April 16th, 1976)

Summary

Optically detected ESR spectra in zero magnetic field of the triplet state of three photosynthetic bacteria are presented. The zero field splitting parameters $|D|$ and $|E|$ and the widths of the resonances show small but significant differences for the three bacteria. The resonance lines are inhomogeneously broadened as demonstrated by hole-burning experiments. The populating probabilities and depopulating rates for the triplet sublevels have been measured. The populating kinetics are very similar for the three bacteria. The depopulating rates are more than one order faster than those of chlorophyll *a* and chlorophyll *b* and of porphyrin model systems. The populating probability of the lowest level is about 6 times less, and the depopulating rate about 6 times slower, than for the upper levels, identifying this level as the level connected to the molecular z-axis perpendicular to the plane of the molecule. The relative populations of the triplet sublevels are almost equal in zero magnetic field.

Recently it has been shown that it is possible to observe electron spin resonance signals by optical detection in zero magnetic field of the photoinduced triplet in chloroplasts [1] and in the photosynthetic bacterium *Rhodospirillum rubrum* [2]. A study of the kinetic response of the triplet system to perturbations caused by resonant microwaves yields data on the rates of population and depopulation of the triplet sublevels. Such information is important to our understanding of the role of the triplet state in the photosynthetic charge separation process. In this report firstly zero field resonance spectra are presented of three photosynthetic bacteria. Small, but significant differences are found in the zero field splitting parameters $|D|$ and $|E|$ between the two non-related bacteria *Rhodospirillum rubrum* and *Rhodo-*

pseudomonas spheroides (wild type) and between the latter and its carotenoid-less mutant R-26. The resonance lines are very narrow, purely Gaussian and inhomogeneously broadened. Secondly, the populating probabilities and the rates of depopulating for the three triplet sublevels x, y and z are reported. The depopulating rates are unusually fast (see Table II); they do not differ appreciably for the three bacteria investigated. The relative population of the two upper levels is found to be almost the same, that of the lowest level being only slightly smaller.

TABLE I

FREQUENCIES, LINEWIDTHS AND ZERO FIELD SPLITTING PARAMETERS DERIVED FROM ZERO FIELD RESONANCE SPECTRA

x, y, z	MHz (± 0.5)			MHz (± 0.2)			10^{-4} cm^{-1} ± 0.2	
	ν_1	ν_2	ν_3	$\Delta\nu_1$	$\Delta\nu_2$	$\Delta\nu_3$	$\lvert D\rvert$	$\lvert E\rvert$
R. spheroides R.26	468.0	655.5	—	13.4	19.1	—	87.2	31.2
R. spheroides (wild type)	467.0	660.0	193.6*	12.2	17.2	—	187.8	32.2
R. rubrum	466.0	668.5	—	9.7	18.6	—	189.1	33.7

*From an "inverse hole-burning" experiment. The apparent linewidth is not the true inhomogeneous linewidth.

The bacteria *R. rubrum* and *R. spheroides* (wild type) were grown as described [3]. The *R. spheroides* mutant R-26, kindly supplied by Dr. G. Feher, was anaerobically grown in modified Hutner medium. After centrifugation, the cells were taken up in growth medium and 10 mM morpholinopropane sulfonic acid (pH 8) to an absorbance of 2 in 2 mm. Reduction was carried out under N_2 atmosphere by adding excess solid dithionite. After two-fold dilution with glycerol, the samples were quickly frozen at 77°K. Zero field resonance was detected via the Microwave Induced Fluorescence technique developed by van Dorp et al. [4,5]. The samples were excited by broadbanded light centered at 400 nm, detection took place via narrow band interference filters or an Ebert monochromator. Spectra were recorded in a single scan using amplitude modulation of the microwaves and lock-in detection. Kinetic experiments were carried out following the procedure described in detail by van Dorp et al. [5] with slight modifications necessitated by the fast depopulating rates encountered.

In Fig. 1a, b and c, zero field resonance spectra for the three bacteria are displayed. The lines are exceedingly narrow compared to zero field resonance lines normally encountered in glassy solution. Small differences in linewidth are discernable (Table I). The zero field splitting parameters D and E were derived from the frequencies of the lines by identifying the low and high frequency line with the $D - E$ and $D + E$ transitions respectively. Although the values of D and E are significantly different for the three bacteria, they are sufficiently close to indicate that the molecular complex on which the triplet resides has the same structure for the three bacteria. This, together with the fact that the values of D and E are about 20% smaller than those derived from ESR spectra of the triplet state of bacteriochlorophyll in glassy

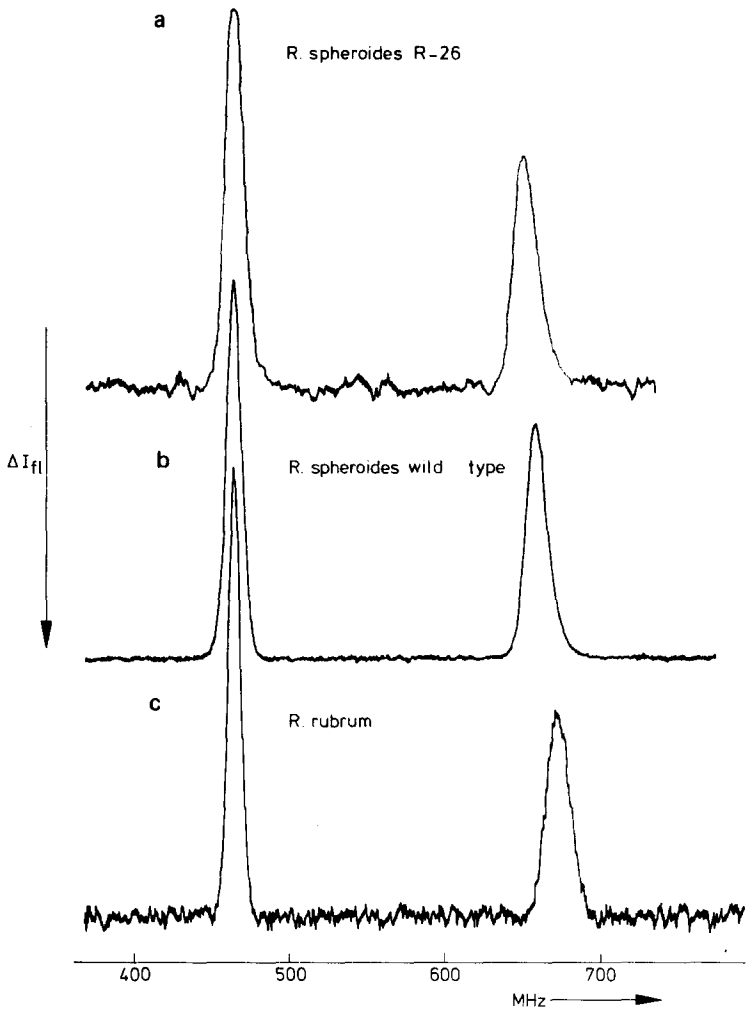

Fig. 1. Zero field resonance spectra at 2°K of (a) *Rhodopseudomonas spheroides* R-26, (b) *R. spheroides* wild type, (c) *Rhodospirillum rubrum*. Excitation: Oriel HBO 1000 Watt mercury arc filtered by 10 cm of a solution of $CuSO_4$ (100 g/l) in H_2O, and Corning 4-76 and Schott BG 25 filters. Detection: Schott RG 715 cut-off and AL 911 nm or AL 905 nm interference filter followed by an EMI type 9685 A photomultiplier. Microwaves were generated by a Hewlett Packard HP 8690 B sweep oscillator, power level 20 mM, amplitude modulation 237 Hz, scanning rate 3 MHz/s, time constant (a) 1 s, (b) and (c) 0.3 s. All spectra are single scans. The decrease in fluorescence for the strongest line amounts to (a) 0.013, (b) 0.04, (c) 0.036%.

solution [6] and the extreme narrowness of the zero field resonance lines, suggests that the triplet is located on the bacteriochlorophyll complex that makes up the primary donor. The third resonance line with a frequency of 2E/h could only be observed by a double resonance technique, in which one of the transitions was saturated with resonant microwaves (Fig. 2a). The intensity of this resonance in an ordinary zero field resonance experiment is less than a few percent of that of the two other resonances.

The lines are almost pure Gaussians (Fig. 2b), which suggests that they are inhomogeneously broadened. This was demonstrated by a "hole-burning" experiment, in which a transition was saturated at one particular frequency, while at the same time it was scanned with a second microwave source (Fig. 2c). The width of the hole, about 1 MHz, is much larger than the spread in frequency of the microwave source (about 50 kHz). The contribution to the spin packet linewidth due to lifetime broadening is only about 7 kHz; apparently most of the width is due to T_2 processes, with T_2 of the order of 1 μs. The depopulating rate of the three triplet sublevels was determined by monitoring the response of the system to a perturbation caused by connecting two or three of the sublevels briefly with resonant microwaves. The rates thus obtained are shown in Fig. 3 for *R. spheroides* wild type. It is seen that they depend on the light intensity I. Extrapolation to zero light intensity gives the true depopulating rates $k_u(0)$ (u = x, y or z). Van Dorp et al. [5] have shown that straight extrapolation may be a hazardous procedure, since the dependence on light intensity can be strongly non-linear at low light intensities. These authors represent the populating and depopulating kinetics by a system of coupled differential equations with $k_u(0)$ and the populating probabilities p_u (u = x, y, z) as parameters. The p_u values were calculated from the changes in steady-state level of fluorescence on connecting the sublevels in different ways with resonant microwaves (Table II). In these experiments, the microwaves were frequency modulated over about double the linewidth in order to avoid complications arising from the inhomogenous nature of the zero field resonance lines. From the solution of the set of differential equations theoretical curves of $k_u(I)$ vs. I were calculated for various values of $k_u(0)$ and compared to the experimental data (Fig. 2). Best values of $k_u(0)$ are collected in Table II, the error representing the limits in $k_u(0)$ for which a reasonable fit was obtained. Again, the various rates of populating and depopulating are very similar for the three bacteria. However, the absolute value of the k_u values are much higher than for free base porphyrin [5], Mg-porphyrin (Jansen,

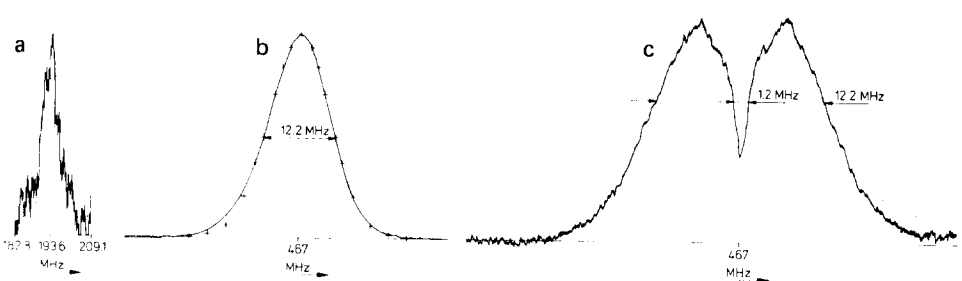

Fig. 2. (a) Double resonance line of *R. spheroides* wild type at a frequency corresponding to 2 E, the energy difference between the triplet x and y level. Excitation and detection as in Fig. 1. Microwaves from one sweep oscillator were set at 467 MHz, while the frequency of a second oscillator was swept at a rate of 7.5 MHz/min. Time constant, 30 s. (b) Comparison of the 467 MHz resonance of *Rps. spheroides* wild type with a Gauss curve. Conditions as in Fig. 1 except scanning rate 0.3 MHz/s. Crosses: computed Gaussian normalized to height and half-width of the experimental curve. The good fit is typical also for the other resonances displayed in Fig. 1. (c) "Hole-burning" experiment on the 467 MHz line of *R. spheroides* wild type. Conditions as in Fig. 1, except scanning rate 0.3 MHz/s. One microwave oscillator was set at 467 MHz, while a second oscillator was slowly swept through the 467 MHz resonance. Similar results were obtained for the other resonances displayed in Fig. 1.

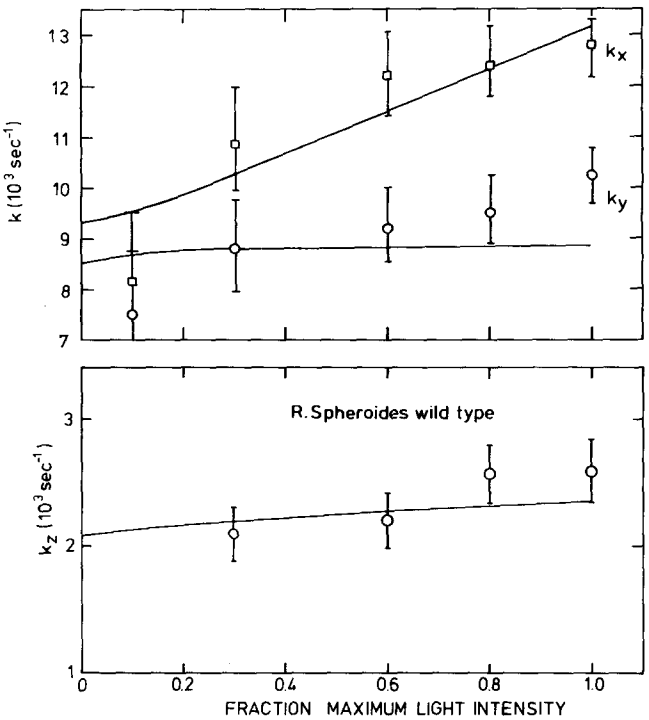

Fig. 3. Dependence of the apparent depopulating rates on light intensity for *R. spheroides* wild type. The rates were determined from the time course of the change in fluorescence following a brief pulse of resonant microwaves, which were frequency modulated over twice the zero field resonance linewidth at 150 kHz. The maximal light intensity corresponds to an excitation rate of 4500 sec^{-1}. Excitation and detection as in Fig. 1. Error bars represent mean deviation for three independent experiments. Drawn curve is theoretically calculated, using $k_x = 9300\ s^{-1}$, $k_y = 8500\ s^{-1}$ and $k_z = 2100\ s^{-1}$. Similar graphs were obtained for the other two bacteria studied.

TABLE II

	Level	Populating* probability p_u	Depopulating rate** k_u sec^{-1}	Relative population***
R. spheroides (wild type)	x	0.457	9300 ± 500	0.336
	y	0.449	8500 ± 500	0.360
	z	0.094	2100 ± 100	0.304
R. spheroides R.26	x	0.484	9000 ± 1000	0.336
	y	0.445	8000 ± 1000	0.347
	z	0.071	1400 ± 200	0.317
R. rubrum	x	0.488	9000 ± 1000	0.339(9)
	y	0.437	8000 ± 1000	0.335(8)
	z	0.075	1400 ± 200	0.330(3)

*Error about 1% based on variation of parameters in computer fit.
**Error based on three independent measurements.
***Because of the large differences in $k_{x,y}$ and k_z, the difference in population of the x or y and z levels is a first approximation of expected zero field resonance line intensity.

G., private communication) and chlorophyll *a* and chlorophyll *b* [7,8]. Since the decay of the triplet is strictly non-radiative, this means that the electron-photon coupling is much stronger in the primary donor than in the chlorophyll molecule in vitro. The average decay rate k (160 μs) which is obtained at higher temperatures when the three sublevels are no longer spin isolated, is much longer than the time allotted for formation of the donor-acceptor pair in the primary process (about 150 ps [9,10]). This means that there is ample time for chemical reactions from the triplet state. On the other hand, from picosecond laser work [9,10], it appears that the triplet state plays a role in the back reaction, and not in the forward process. Recently, variable fluorescence in reduced bacteria at low temperatures has been found (Holmes, Grondelle and Hoff, unpublished) with a decay time of 100—150 μs, corroborating this assignment. As in the porphyrin model systems, the populating probability is lower for the z level than for the other two levels. This is in accord with the recent finding [11] that D is positive for the bacterial system, which identifies the z level as the lowest one. In planar π-electron systems made up of light elements, the z level has little singlet character, and is consequently populated and depopulated at a low rate.

Contrary to the results for chloroplast [1], the relative populations of the sublevels in zero field as shown in Table II cannot account for the spin polarization of the triplet ESR spectrum of *R. rubrum* observed by Leigh and Dutton [12]. In fact, Schaafsma (private communication) first noted that this spin polarization cannot be obtained by the usual spin orbit coupling scheme for populating the triplet sublevels within a single molecule. The zero field populating parameters presented in this report can serve as a starting point for the various mechanisms which have since been proposed to explain the anomalous polarization in high magnetic field.

A final conclusion from the above zero field resonance experiments is that it allows to estimate the fraction of the intensity of the 905 nm fluorescence band due to the reduced reaction center. The kinetic data could only be fitted for a fraction between 2.5 and 0.25%, with best fit for about 1%. The order of magnitude agrees with the observations made by Slooten [13], who compared the fluorescence yields of intact cells and reduced reaction center particles.

The author is greatly indebted to Drs. W.G. van Dorp and J.H. van der Waals for introducing him to the zero field resonance technique and to Dr. J. Amesz for helpful discussions. Mr. A.H.M. de Wit provided expert microbiological assistance. The investigations were supported by the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

References

1 Hoff, A.J. and van der Waals, J.H. (1976) Biochim. Biophys. Acta 423, 615—620.
2 Clarke, R.H., Connors, R.E., Norris, J.R. and Thurnauer, M.C. (1975) J. Am. Chem. Soc. 97, 7178—7179.
3 Slooten, L. (1972) Biochim. Biophys. Acta 256, 452—466.
4 Van Dorp, W.G., Schaafsma, T.J., Soma, M. and van der Waals, J.H. (1973) Chem. Phys. Lett. 21, 221—225.

5 Van Dorp, W.G., Schoemaker, W.H., Soma, M. and van der Waals, J.H. (1975) Mol. Phys. 30, 1701—1721.
6 Uphaus, R.A., Norris, J.R. and Katz, J.J. (1974) Biochem. Biophys. Res. Commun. 61, 1057—1063.
7 Clarke, R.H. and Hofeldt, R.H. (1974) J. Chem. Phys. 61, 4582—4587.
8 Kleibeuker, J.F. and Schaafsma, T.J. (1974) Chem. Phys. Lett. 29, 116—122.
9 Rockley, M.G., Windsor, M.W., Cogdell, R.J. and Parson, W.W. (1975) Proc. Natl. Acad. Sci. U.S. 72, 2251—2255.
10 Dutton, P.L., Kaufmann, K.J., Chance, B. and Rentzepis, P.M. (1975) FEBS Lett. 60, 275—280.
11 Norris, J.R. (1976) Biophys. J. 16, 224a, abstract F-PM-D11.
12 Dutton, P.L., Leigh, J.S. and Reed, D.W. (1973) Biochim. Biophys. Acta 292, 654—664.
13 Slooten, L. (1973) Dissertation, University of Leiden.