BBA Report

BBA 41211

Isolation and characteristics of small, soluble photoreactive fragments of *Rhodospirillum rubrum*

WILLIAM R. SMITH, JR., CHRISTIAAN SYBESMA* and KARL DUS[a]

Department of Physiology and Biophysics, and [a]Department of Biochemistry, University of Illinois, Urbana, Ill. 61801 (U.S.A.)

(Received May 3rd, 1972)

SUMMARY

A simple isolation procedure for a photochemically active complex from wild-type cells of the photosynthetic non-sulfur bacterium, *Rhodospirillum rubrum*, is described. The method involves sucrose density centrifugation of chromatophores equilibrated with a large excess of ascorbate and, subsequently, treated with 1% sodium dodecylsulfate in a concentration of 5.4 g/mmole of bacteriochlorophyll. The resulting brown complex has a mol. wt of about 100 000 and a solubility in aqueous buffer of at least 70 mg/ml. 2.7% of the bacteriochlorophyll of the chromatophores was recovered in this preparation. Stoichiometry appears to hold for P870 to cytochrome c_2 (1:1), spirilloxanthin (1:3) and ubiquinone (1:1.7) while cytochrome cc' was observed in variable amounts. Polyacrylamide gel electrophoresis of this complex using 0.05% sodium dodecylsulfate yielded an even smaller photochemically active fraction (mol. wt approx. 35 000) which contained no cytochrome c_2. The amino acid compositions of both fragments are compared.

Using an alkaline–urea–Triton (AUT) treatment, Loach *et al.*[1,2] succeeded in isolating from wild-type *Rhodospirillum rubrum* photoreceptor subunits containing P870, the bacteriochlorophyll–protein complex capable of undergoing reversible light-induced oxidation and, in addition, a large amount of light-harvesting bacteriochlorophyll. However, particles retaining P870 while devoid of light-harvesting bacteriochlorophyll have been obtained from several photosynthetic bacteria[3–8]. These complexes have become known as reaction center preparations.

*Present address: Biophysical Laboratory, Faculty of Sciences, Free University of Brussels, A. Buyllaan 105, 1050 Brussels, Belgium.

Most notable among these reaction center preparations, perhaps, are the complexes isolated from the carotenoidless mutant R-26 of *Rhodopseudomonas spheroides*. Reed and Clayton[3] isolated it using Triton X-100 while Feher[9] employed lauryl dimethyl amine oxide and obtained a preparation of slightly different composition.

Instead of the above-mentioned detergents, Segen and Gibson[10] used sodium dodecylsulfate to separate reaction center preparations from both mutant and wild-type *Rps. spheroides*. In this paper, we describe a similar approach using the detergent sodium dodecylsulfate which facilitates isolation of small reaction center preparations from wild-type *R. rubrum*, an organism with which previous attempts to isolate such a complex were unsuccessful.

R. rubrum, strain IV (Giesbrecht), was grown anaerobically in the light as described previously[11]. Cells from 8-day-old cultures in 50 mM Tris buffer (pH 8.0) were disrupted by sonication (Branson Sonifier, Model S110, 6 d.c.A; 3 × 3 min) and the cell debris removed by centrifugation (15 000 × *g* for 20 min). Further centrifugation (144 000 × *g* for 4 h) sedimented the chromatophores which were subsequently resuspended again in 50 mM Tris buffer (pH 8.0) to an absorbance of 14 at 590 nm. By using a millimolar extinction coefficient (ϵ) of 20 $mM^{-1} \cdot cm^{-1}$ (ref. 7), this corresponded to a bacteriochlorophyll concentration of 0.7 mM.

The preparation of the reaction center preparation (Fig. 1A) was initiated by equilibrating 44-ml portions of the chromatophore suspension with a large excess (60 mg/ml) of ascorbate for approximately 30 min. Then 1% sodium dodecylsulfate was added with stirring to a concentration of 5.4 g/mmole of bacteriochlorophyll. Portions of the homogenate were layered onto each of six discontinuous sucrose gradients prepared from

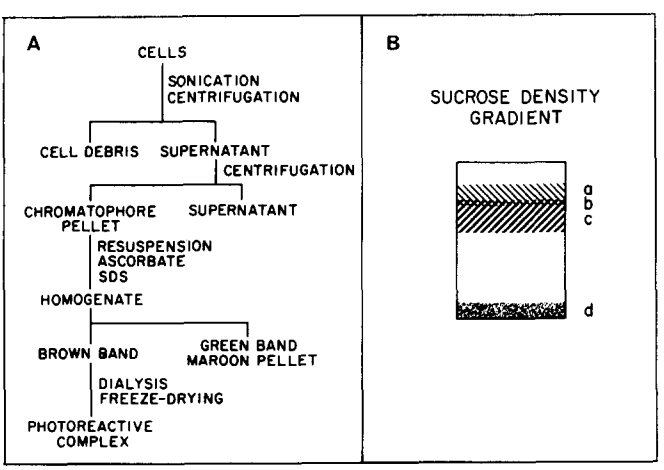

Fig 1. (A) Isolation procedure for the reaction center preparation of *R. rubrum*. All steps were carried out at 0 to 10°C in 50 mM Tris buffer (pH 8.0). SDS = sodium dodecylsulfate. (B) Sucrose density gradient patterns of sodium dodecylsulfate-treated chromatophores of *R. rubrum;* a, green brand; b, 10% overlap of the green and brown bands; c, brown band; d, maroon pellet. The gradients were discontinuous with 13 ml each of 0.6 M and 0.8 M sucrose in 0.1 M phosphate buffer (pH 7.5) using 40 ml cellulose nitrate centrifuge tubes.

13 ml of 0.6 M and 0.8 M sucrose in 0.1 M phosphate buffer (pH 7.5), and centrifuged for 15 h at 144 000 × *g* in an SW-27 rotor.

Measurements of light-induced absorbance changes were made with a split-beam difference spectrophotometer described elsewhere[11]. Absorption spectra were obtained using a Cary 14R recording spectrophotometer. Carotenoids were characterized according to Thornber *et al.*[5], while ubiquinone was determined by the method of Pumphrey and Redfearn[12] as modified by Takamiya and Takamiya[13]. Bound sodium dodecylsulfate was measured according to the method of Reynolds and Tanford[14]. Preparative acrylamide gels were prepared by the method of Fogel and Sypherd[15]. Electrofocusing experiments were performed using apparatus and techniques outlined by Vesterberg and Svensson[16].

Centrifugation of the sodium dodecylsulfate-treated homogenate in the sucrose density gradient outlined above resulted in three bands (Fig. 1B). Spectral data indicated that the upper green band contained mainly bacteriopheophytin and oxidized bacteriochlorophyll while the brown band immediately below it was identified as the reaction center preparation. The third fraction, a maroon pellet at the bottom of the centrifuge tube contained predominantly light-harvesting bacteriochlorophyll. There was approximately a 10% overlap between the green and brown bands while the maroon pellet was well separated from the former two. The reaction center preparation was dialyzed overnight against 3 l of distilled water and then lyophilized. In a representative run, 300 mg of this reaction center preparation were obtained from 26 g wet-weight of cells which represents between 2.6 and 2.7% of the bacteriochlorophyll of the chromatophores. It was further purified by passage through a column of Sephadex G-100 equilibrated with dilute ammonia.

Freeze–dried reaction center preparation containing only traces of phospholipid but as much as 0.55 g of sodium dodecylsulfate per g of reaction center preparation had a solubility of nearly 70 mg/ml in 50 mM Tris buffer (pH 8.0). Amino acid analysis of this material (Table I) revealed a high content of nonpolar amino acid residues which is consistent with reaction center preparations from other bacteria[9].

Electrofocusing experiments of the reaction center preparation demonstrated the presence of at least four components: bacteriochlorophyll–protein complex, cytochrome c_2[17], often referred to as C422[10], cytochrome *cc'*, formerly called RHP[18], and an as yet insufficiently characterized blue-green colored component. While most of the bacteriochlorophyll–protein was present in the column as a precipitate, it seems likely that this blue-green component is a small amount of dissolved bacteriochlorophyll. Electrofocusing experiments with more purified preparations substantiated this assumption. Figs 2A and 2B represent absorption spectra of purified samples of C422 and cytochrome *cc'*, respectively. C422 has a high redox potential of +320 mV and an isoelectric point of 5.9 (ref. 17). It was isolated in the reduced state. Cytochrome *cc'*, with a midpoint potential of approximately 0 mV and an isoelectric point of 5.4, was recovered in the oxidized state. Fig. 2B is a spectrum of this material in the presence of dithionite. The blue-green component which was isolated in very minute quantities showed an isoelectric point between pH 4.5 and 5, which is close to the isoelectric point

TABLE I

COMPARATIVE AMINO ACID ANALYSES

The compositions refer to 24 h hydrolysis with 5.7 M HCl at 105–110°C. $CySO_3H$ and $MetSO_2$ were determined after performic acid oxidation while Trp values were obtained from hydrolyzates made in the presence of thioglycolic acid. Values are expressed in Mole %. N.D. means not determined.

Amino acid	*R. rubrum reaction center preparation fragments*		*Rps. spheroides bacteriochlorophyll–protein complexes*	
$CySO_3H$	0.8	0.9	0.7	–
Asp	10.4	9.5	8.9	5.8
$MetSO_2$	2.5	2.1	1.7	2.3
Thr	5.9	6.2	5.8	5.8
Ser	5.3	5.8	6.3	7.2
Glu	10.0	9.9	8.9	7.3
Pro	4.7	5.4	4.9	4.7
Gly	9.7	10.8	14.1	7.3
Ala	12.6	12.7	15.8	13.3
Val	6.9	6.4	5.9	11.2
Ile	4.4	4.3	3.2	5.7
Leu	9.1	9.6	9.1	11.4
Tyr	1.9	2.2	2.5	2.8
Phe	2.9	3.6	4.3	5.1
His	1.0	1.1	1.1	–
Lys	5.4	4.2	3.5	4.3
Trp	0.8	1.1	N.D.	1.5
Arg	5.7	4.2	3.5	4.0
Mol. wt	≈100 000	≈35 000	≈11 000	*

*References 26 and 27.

of bacteriochlorophyll–protein. In a representative experiment, from 312.8 mg of the reaction center preparation placed on the electrofocusing column, 19.72 nmoles of C422 and 7.07 nmoles of cytochrome *cc'* were recovered. However, due to the large amount of precipitate in the electrofocusing column, it was recognized that the above quantities represent only a small percentage of the total amounts of these components present in the reaction center preparation.

Purification on Sephadex removes some ultraviolet-absorbing contaminants present in the "crude" reaction center preparation (Fig. 3A, solid line). The best A_{280}/A_{800} ratio after Sephadex purification was approximately 3.1. The spectral maxima at 870 nm, 802 nm, 595 nm, and 370 nm are indicative of reaction center bacteriochlorophyll[19]. The small peaks at 755 nm and 670 nm are due to bacteriopheophytin and oxidized bacteriochlorophyll, respectively. The small maxima at 540 nm and 505 nm are most likely due to carotenoids. The Soret absorptions of the hemeproteins present in the preparation contribute to the large peak at 400 nm and the shoulder at

410 nm. Protein absorption accounts for most of the absorbance at 280 nm of the Sephadex-purified reaction center preparation. A possible contribution of quinones at 290 nm cannot be ruled out.

The dashed line in Fig. 3A shows the absorption spectrum of the reaction center preparation under illumination with far-red light (using a Wratten 89B filter) with an intensity of $3.5 \cdot 10^6$ ergs/cm^2. The difference spectrum plotted in Fig. 3B is the same as obtained with a split-beam difference spectrophotometer. The characteristic absorption at 870 nm is reversibly lost with a concomitant shift in the absorbance maximum from 802 to 795 nm. In addition to this near-infrared pattern characteristic of P870, absorption decreases at 600 nm and 380 nm may also be due to reaction center bacteriochlorophyll. The troughs at 422 nm, 523 nm, and 551 nm are due to C422. Based on the $\Delta\epsilon$ values of 23 $mM^{-1} \cdot cm^{-1}$ at 551 nm for C422[20] and 93 $mM^{-1} \cdot cm^{-1}$ for P870[19], the C422 and P870 appeared to be reacting in a one-to-one ratio. No evidence of any light-induced reaction of cytochrome *cc'* was observed even when the reaction center preparation was dissolved in the low potential buffer system of Cusanovich *et al.*[21].

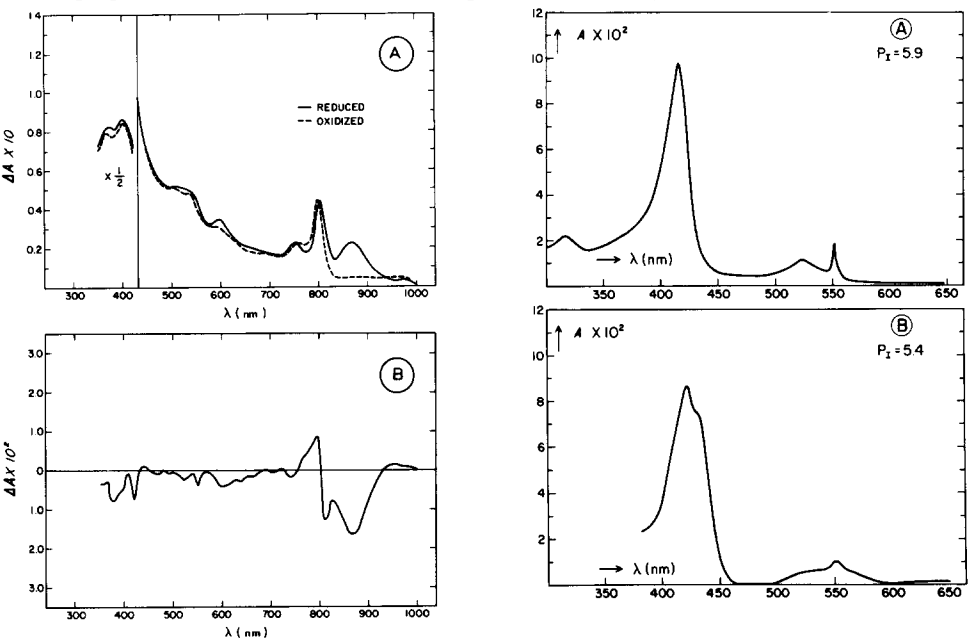

Fig. 2. (A) Absorption spectrum of purified cytochrome c_2 (*i.e.* C422). This hemeprotein was obtained from electrofocusing experiments on the reaction center preparation of *R. rubrum*. (B) Absorption spectrum of purified cytochrome *cc'*. This hemeprotein was obtained from electrofocusing experiments on the reaction center preparation of *R. rubrum*.

Fig. 3. (A) Absorption spectra at room temperature of the reaction center preparation of *R. rubrum* in 50 mM Tris buffer (pH 8.0). ——, spectrum of the reaction center preparation in the "dark"; - - -, spectrum of the sample when illuminated with $3.5 \cdot 10^6$ ergs/cm^2 per s of near-infrared light through a Wratten 89B filter. The spectral ratio of 800 nm/870 nm is 2.00. (B) Light-minus-dark difference spectrum of the reaction center preparation of *R. rubrum* in 50 mM Tris buffer (pH 8.0). The absorbance of the sample in a 1-cm cuvette at 590 nm was 0.035.

Paper chromatography[22] revealed the presence of one major carotenoid, spirilloxanthin. Using an $\epsilon_{1\%} = 2350$, the molar ratio of spirilloxanthin to P870 was calculated to be 3. There were also 1.7 moles of ubiquinone per mole of P870. Results of sedimentation velocity and equilibrium experiments[23], as well as of Sephadex G-100 chromatography[24] indicated molecular weights in the range of 80 000–100 000 for the reaction center preparation. The effect of sodium dodecylsulfate on this mol. wt determination, however, is yet to be evaluated.

The pattern obtained with our reaction center preparation using acrylamide gel electrophoresis in the presence of 0.05% sodium dodecylsulfate and 0.05% β-mercaptoethanol[25] shows a brown band corresponding to a mol. wt approx. 50 000, a band with a mol. wt approx. 35 000 which was also brown, a reddish band which on spectroscopic examination indicated cytochrome c_2 and (in trace quantities) a lower green band with a mol. wt approx. 11 000. Only the fraction with a mol. wt of 35 000 was photoactive (Fig. 4). It contained little or no cytochrome c_2. In order to investigate the

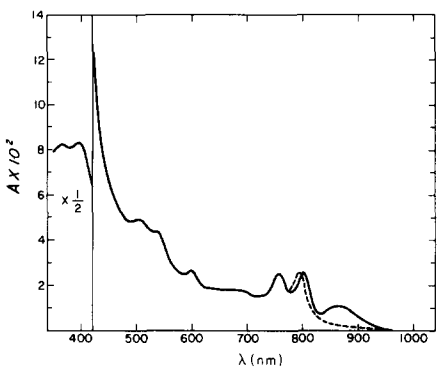

Fig. 4. Absorption spectra of acrylamide gel-purified reaction center preparation of *R. rubrum* in 50 mM Tris buffer (pH 8.0) at room temperature. ——, spectrum of the reaction center preparation in the "dark". - - -, spectrum of the sample when illuminated with $3.5 \cdot 10^6$ ergs/cm² per s of near-infrared light through a Wratten 89B filter. The spectral ratio of 800 nm/870 nm is 2.16 reflecting the presence of less light-harvesting bacteriochlorophyll relative to Fig. 3A.

possibility that the *green* band is analogous to a bacteriochlorophyll–protein complex recently isolated by Fraker and Kaplan[26,27], we applied the isolation procedure described above to a sample of *Rps. spheroides*, kindly supplied by Dr S. Kaplan. The green band thus obtained represented a much larger amount of the protein complex than the one obtained from *R. rubrum* and had a far-red absorption maximum at 850 nm which is missing in *R. rubrum*. The amino acid composition of this band (and those of the 100 000-dalton and the 35 000-dalton preparations) was compared to that of the Fraker–Kaplan complex (Table I). The green bands obtained from *Rps. spheroides* as well as from *R. rubrum*, therefore, may be part of the light-harvesting bacteriochlorophyll system in both organisms.

A high concentration of ascorbate seemed to be essential to obtain good yields of the larger reaction center preparation (mol. wt approx. 100 000). Low concentrations of sodium dodecylsulfate favor recovery of the smaller reaction center preparation (mol. wt approx. 35 000) and generally avoid pheophytinization.

This work was supported by National Science Foundation Grant GB 30649X, and by funds made available from the Biomedical Research Council of the University of Illinois.

REFERENCES

1 Loach, P.A., Sekura, D.L., Hadsell, R.M. and Stemer, A. (1970) *Biochemistry* 9, 724
2 Loach, P.A., Hadsell, R.M. Sekura, D.L. and Stemer, A. (1970) *Biochemistry* 9, 3127
3 Reed, D.W. and Clayton, R.K. (1968) *Biochem. Biophys. Res. Commun.* 30, 471
4 Gingras, G. and Jolchine, G. (1969) in *Progress in Photosynthesis Research* (Metzner, H., ed.), Vol. I, p. 209, Tübingen
5 Thornber, J.P., Olson, J.M., Williams, D.M. and Clayton, M.L. (1969) *Biochim. Biophys. Acta* 172, 351
6 Jolchine, G., Reiss-Husson, F. and Kamen, M.D. (1969) *Proc. Natl. Acad. Sci. U.S.* 64, 650
7 Slooten, L. (1972) *Biochim. Biophys. Acta* 256, 452
8 Sybesma, C., Govindjee, R. and Smith, W.R., Jr. (1971) *Proc. 2nd Int. Congr. Photosynth. Res., Stresa, Italy*, in the press
9 Feher, G. (1971) *Photochem. Photobiol.* 14, 373
10 Segen, B.J. and Gibson, K.D. (1971) *J. Bacteriol.* 105, 701
11 Sybesma, C. and Fowler, C.F. (1968) *Proc. Natl. Acad. Sci. U.S.* 61, 1343
12 Pumphrey, A.M. and Redfearn, E.R. (1960) *Biochem. J.* 76, 61
13 Takamiya, K. and Takamiya, A. (1969) *Plant Cell Physiol.* 10, 363
14 Reynolds, J.A. and Tanford, C. (1970) *Proc. Natl. Acad. Sci. U.S.* 66, 1002
15 Fogel, S. and Sypherd, P.S. (1968) *J. Bacteriol.* 96, 358
16 Vesterberg, O. and Svensson, H. (1966) *Acta Chem. Scand.* 20, 820
17 Sletten, K., Dus, K., DeKlerk, H. and Kamen, M.D. (1968) *J. Biol. Chem.* 243, 5492
18 Vernon, L.P. and Kamen, M.D. (1954) *J. Biol. Chem.* 211, 643
19 Clayton, R.K. (1966) *Photochem. Photobiol.* 5, 669
20 Kakuno, T., Bartsch, R.G., Nishikawa, K. and Horio, T. (1971) *J. Biochem. Tokyo* 70, 79
21 Cusanovich, M.A., Bartsch, R.G. and Kamen, M.D. (1968) *Biochim. Biophys. Acta* 153, 397.
22 Davies, B.H. (1965) in *Chemistry and Biochemistry of the Plant Pigments* (Goodwin, T.W., ed.), p. 489, Academic Press, London
23 Schachman, H.K. (1959) *Ultracentrifugation in Biochemistry*, Academic Press, New York
24 Andrews, P. (1967) *Biochem. J.* 91, 222
25 Shapiro, A.L., Vinuela, E. and Maizel, J.V., Jr. (1967) *Biochem. Biophys. Res. Commun.* 28, 815
26 Fraker, P. and Kaplan, S. (1971) *J. Bacteriol.* 108, 465
27 Fraker, P. and Kaplan, S., *J. Biol. Chem.*, in the press