PROTEINS OF *R. SPHEROIDES* Y* REACTION CENTER : GEL ELECTROPHORESIS AND ELECTROFOCUSING STUDIES.

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SUMMARY : The reaction center preparation isolated from *R. spheroides* Y was analyzed by polyacrylamide gel electrophoresis and gel electrofocusing procedures. Prior to electrofocusing it migrated in at most two bands, either in presence of detergent or of urea-phenol-acetic acid mixtures. In the presence of SDS, the protein bands were split into 4 subunits (Mw respectively 12 000, 30 000, 61 000 and 74 000). The band at Mw 30 000 was the major component. After gel electrofocusing, a reaction center particle (pI = 6.5) retaining activity was isolated; it contained only the 30 000 Mw subunit. It is proposed that the protein binding the pigment in the native reaction center is either the monomer, or the dimer of this subunit.

INTRODUCTION : We have recently isolated and purified a reaction center from *R. spheroides* Y. cells, normal or deficient in iron content (1). This lipoprotein complex contains a bacteriochlorophyll (P 865) reversibly bleached by light; its particle weight, determined by gel filtration and equilibrium sedimentation, is 150 000 daltons (1). Bound detergent (CTAB) and lipids contribute largely to this value (2). In this report, we present additional data on the purity and isoelectric point of the reaction center isolated from cells normal in Fe content, and on the molecular weight of its protein components.

METHODS : Reaction centers were isolated from *R. spheroides* Y as described previously (1) or according to a slightly modified procedure (2). Essays of P 865 activity, spectroscopic techniques and protein determination were as described in (1).

Several methods for disc gel electrophoresis were used. 1/ The first gel system used was essentially as described by Davis (3). It contained a 1 cm long, 3.75 % acrylamide stacking gel (0.056 M Tris HCl pH 6.7), a 7 cm 5 % acrylamide running gel (0.38 M Tris HCl pH 8.9) and electrode buffer (0.005 M Tris, 0.039 M glycine pH 8.3). A non-ionic detergent, either Triton X-100 or Brij 35, was added to give a final concentration of 0.25 % during the polymerization and of 2 % in the buffer. Reaction center samples (50-150 ug) were applied in 0.05 M Tris HCl pH 8, 10 % sucrose, and electro-

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* Abbreviations : *R* =*Rhodopseudomonas*; SDS = Sodium dodecylsulfate
CTAB = Cetyltrimethyl ammonium bromide.

phoresed at 1,5 mA per tube at 4° for 5 h. Gels were stained with an Amido Black solution (3) for proteins and with an oil red O solution (4) for lipids. 2/ The method of Takayama (5) slightly modified was used on the reaction center preparation after previous extraction with chloroform-methanol 2: 1 (v/v). The extract was dissolved in phenol-acetic acid-water (2: 1: 1 w/v/v) and urea was added to give a final concentration of 4 M. The inclusion of 2,5 % Triton X-100 in the sample solvent was necessary for solubilization of the sample. A preelectrophoresis step of 20 mn was used at 1 mA per gel polymerized at 25° for 3 hr in presence of 0,25 % detergent. Electrophoresis was carried out with 10 % acetic acid as buffer; the current was set at 1 mA per tube for 30 mn and then at 4 mA/tube for 4 to 5 hr. Gels were stained overnight with 0.25 % Coomassie brilliant blue or 1 % Amido Black in 7 % acetic acid, and destained by electrophoresis in 7 % acetic acid. 3/ Solubilization of reaction center proteins was tried under alkaline conditions according to a variation of the procedure described by Neville (6), by suspending the reaction center extract in 50 mM K_2CO_3, 8 M urea, 10 % β-mercaptoethanol and 5 % Triton X-100. Separation was effected in polyacrylamide gels containing 5 M urea, 0.25 % Triton X-100, under a discontinuous acidic pH buffer (lower buffer : pH 2.7 upper buffer : pH 4). 4/ Polyacrylamide gel electrophoresis in SDS was performed as described by Weber and Osborn(7). Two different procedures were used for the preparation of the samples. The sample, dissolved in 1 % SDS, 1 % β-mercaptoethanol 0.1 M sodium phosphate buffer, pH 7.0, was incubated at 37° for 3 hr, or heated for 3 mn in a boiling water bath. Then it was dialyzed overnight at 25° against 0.1 % SDS, 0.1 % β-mercaptoethanol in 0.01 M sodium phosphate buffer pH 7.5. In both cases the incubation was performed with or without preliminary extraction of lipid by chloroform-acetone. Samples were loaded directly onto the gels (10 % acrylamide, 0.25 % bis acrylamide) in 10 % glycerol, 5 % β-mercaptoethanol and run for 3 hr at 8 mA/gel. The gels were stained with Coomassie brilliant blue and destained with a mixture of 25 % ethanol, 8 % acetic acid and water with gentle stirring. Densitometric analysis was performed with a Chromoscan densitometer (Joyce-Loebl) with the gel attachment operated in reflectance. Calibration curves were obtained by using proteins of known molecular weights.

Electrofocusing was performed using the electrophoresis apparatus, with slight modifications of the method of Wrigley (8). The gels contained 6,5 % acrylamide, 0.2 % bis acrylamide, 1 % ampholytes (LKB) (pH range 3-10, 3-6, 5-8) and 0.5 % Brij 35, the latter detergent being necessary to prevent precipitation of proteins and increase resolution. They were photopolymerized with 0.18 mg riboflavine and 0.01 ml Teemed (N'N'-tetramethyl-ethylenediamine)

per 20 ml gel solution in 7x100 glass tubes containing 1,5 ml gel solution. The samples (100-250 µg of protein) were dissolved in diluted buffer (< 0.05M) and mixed with an equal volume of 50 % w/v sucrose. They were layered on the top of the gels under 20 % sucrose and 2 % ampholine solution. The anodic and cathodic vessels were filled with 0.2 % sulfuric acid and 0.4 % triethanolamine respectively. Electrofocusing was done at 4° in the dark, with a maximum intensity of 1 mA/tube at 300 volts; it was achieved in 4-5 hrs.The protein zones were detected as white bands after immersion in 5 % trichloroacetic acid. After several such washes to remove the carrier ampholytes, the gels were stained with Coomassie brilliant blue at 60° for 20 mn and destained as described by Vesterberg (9). Isoelectric points were determined by cutting out the protein bands from several gels run in parallel and eluting the pooled corresponding slices with distilled water for 12 hrs. Entire gels were also sliced into 5 mm sections and eluted in order to define the shape and range of the pH gradient. The protein-bearing slices were soaked in the minimal volume of 0.01 M sodium phosphate buffer pH 7.5, 0.2 % Brij 35, and dialyzed after trituration in same buffer for 48 hrs, with several buffer changes. Eluted material was isolated after centrifugation of the extract (3000 RPM, 20 mn) and subjected to SDS electrophoresis as described above.

RESULTS : In order to determine the purity of reaction centers prepared from crude chromatophores, we first tried analysis without modification of their spectral characteristics, *i.e.*, they were subjected to electrophoresis in polyacrylamide gels containing a non-ionic detergent. The presence of the detergent resulted in solubilization of precipitated material which was formed as soon as the current was applied.

In such a gel, the reaction center migrated in two relatively slow-moving coloured bands. Fig. 1 shows the pattern obtained in Amido Black and oil red stained gels; lipids were present only in the faster migrating band. After elution from the gel, the two compounds showed the same absolute absorption spectrum as before electrophoresis; however, the reversible photobleaching of the 865 nm band was completely absent.

In order to fractionate the reaction center particles further, a variety of solubilizing agents (urea, acetic acid, phenol) were employed. It may be noted that urea plus acetic acid and phenol yielded slightly turbid dispersions; addition of Triton X-100 increased the solubilizing power of the solvent. In this case, only one protein band was apparent (Fig. 1_3) but after its elution, no photobleaching activity was found. Better resolution was obtained when reaction centers were solubilized under alkaline conditions with detergent and mercaptoethanol, and fractionated by electrophoresis in a disconti-

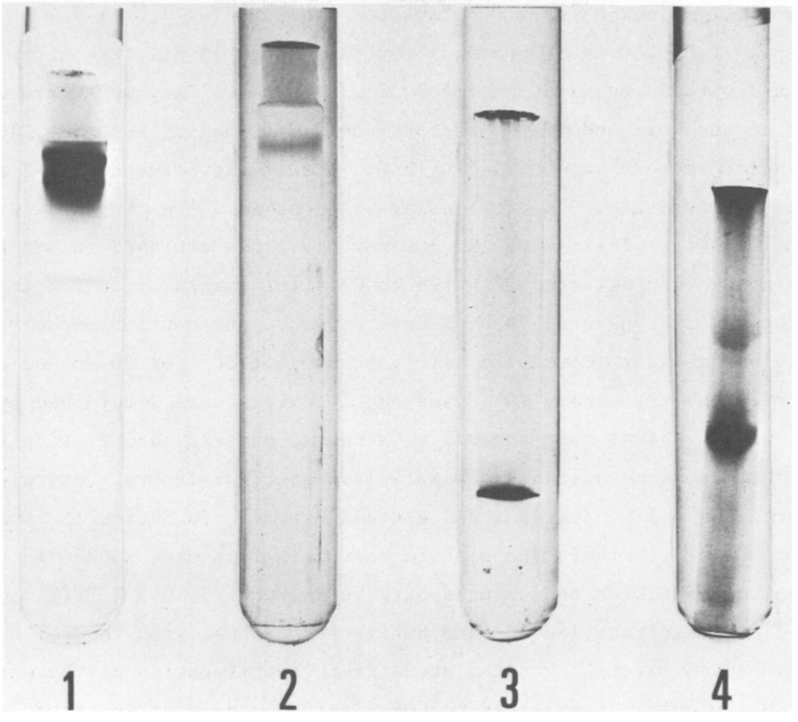

Fig. 1. Electropherograms of *R. spheroides* Y reaction centers. 1: method of Davis (see methods,1) in presence of Triton X-100; staining with Amido Black. 2: Same method but staining with oil red O. 3: Method of Takayama (see methods, 2) staining with Coomassie brilliant blue. 4: Method of Neville (see methods,3) staining with Amido Black.

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nuous acidic pH system. A major band was observed (Fig.1_4), with a second ill-defined band.

In order to dissociate completely the protein moieities of the reaction center, we resorted to SDS gel electrophoresis. Fig. 2 illustrates a series of SDS gels run in parallel, for purified chromatophores (previously lipid-extracted), and reaction centers isolated therefrom, with or without extraction of lipids. One major ("c") and three minor ("a","b","d") bands were obtained in the lipid-extracted reaction center. Identical results were obtained with preparations still containing lipid material, but with changes as seen in the position and the staining intensity of the broad electrophoretic band given by lipids at this SDS concentration (10). The electrophoretic pattern of chromatophores shows many bands and is obviously very different from that of the reaction center. A typical densitometer trace of the reaction center electropherogram, for a Coomassie blue stained gel is given in Fig.3.

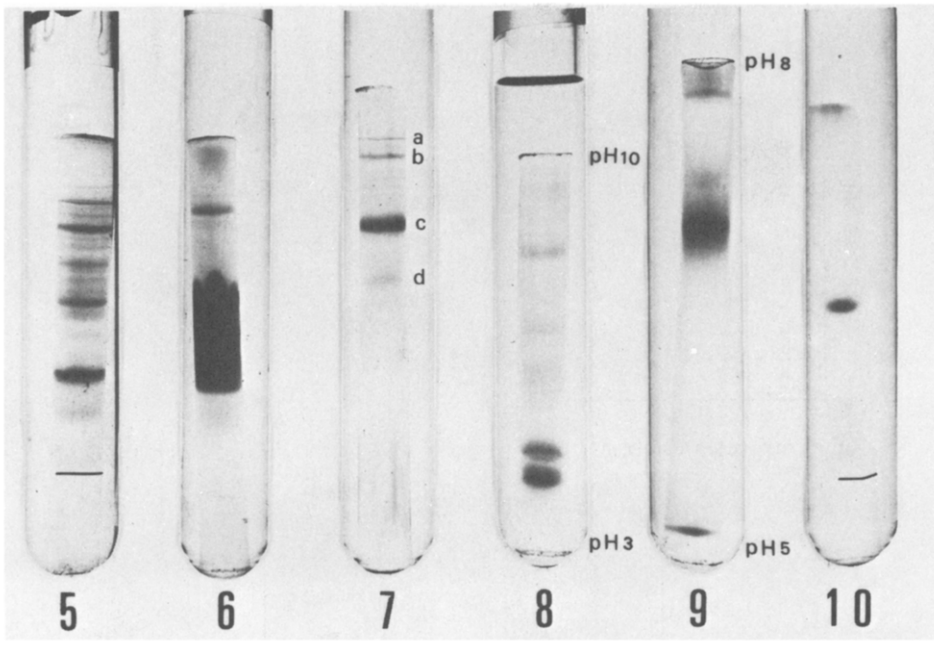

Fig. 2. SDS gel electrophoresis and gel isoelectrofocusing. 5 : SDS gel electrophoresis of purified chromatophores. 6,7 : SDS gel electrophoresis of whole reaction center; in 7 lipids have been extracted before electrophoresis. 8 : Isoelectrofocusing of reaction center in the pH range 3-10. 9 : Isoelectrofocusing of reaction center in the pH range 5-8. 10 : SDS gel electrophoresis of the main protein component (pI : 6.5) recovered from gel isoelectrofocusing in the pH range 5-8.

The major band "c" and the minor bands ("a" +"b" +"d") amounted for 90 % and 10 % respectively of the total intensity in the staining profile.

Apparent molecular weights of components "a","b","c","d", were obtained by careful calibration of the gels with molecular weight markers (Fig. 4). Apparent subunit molecular weights were as follow : band "a" : 74,000 band "b" : 61,000 band "c" : 30,000 band "d" : 12,000 (maximum deviation : 5 %). This pattern was quite reproducible, but the sharpness and intensity of the minor bands were affected by changes in conditions of electrophoresis. Both band "a" and "b", eluted together from the gel and rerun on a SDS gel produced one band corresponding to a subunit of molecular weight 61,000. After elution and electrophoresis the band "d" always showed an unchanged subunit of 10,000 to 12,000 in molecular weight. Moreover, when subjected to a second electrophoresis without sufficient mercaptoethanol, the major band "c" yielded a broad band of similar mobility in addition to two

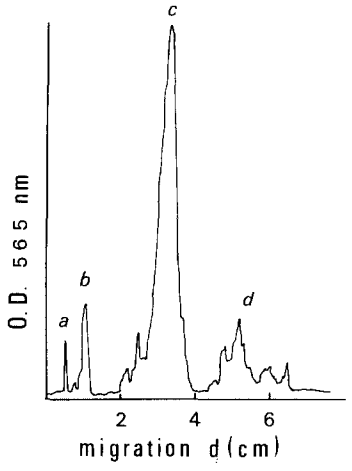

Fig. 3.

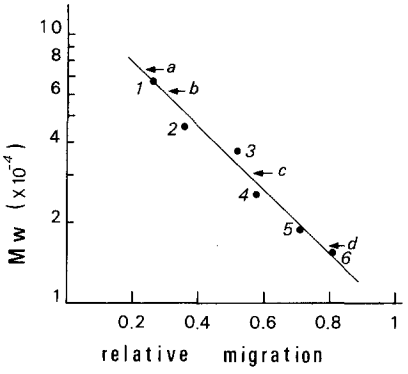

Fig. 4.

Fig. 5.

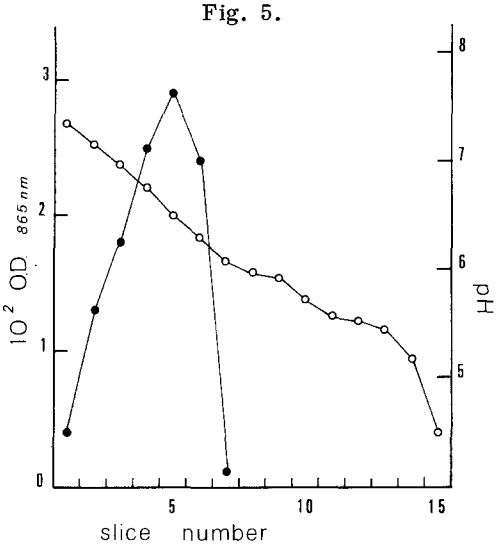

Fig. 3. Densitogram of proteins from *R. spheroides* Y reaction center. Gels stained with Coomassie brilliant blue were scanned at 565 nm through a 0,3 mm slit in a solution containing 9 % sucrose, 5 % methanol, 7,5 % acetic acid.

Fig. 4. Determination of the molecular weights of *R. spheroides* Y proteins by SDS polyacrylamide gel electrophoresis. The marker proteins are : 1: Bovine serum albumin (M_w : 67,000) 2: Ovalbumin (M_w : 45,000) 3: glyceraldehyde-3-phosphate dehydrogenase (M_w : 36,000) 4: Chymotrypsinogen (M_w : 25,000) 5: Myoglobin (M_w : 17,800) 6: Cytochrome c (M_w : 12,400) Relative migration is the migration of the protein divided by bromophenol migration.

Fig. 5. Isoelectric focusing of reaction center from *R. spheroides* Y at 300 V in 1 % ampholine pH 5-8. • : Optical density at 865 nm. o : pH. Average values of 0,2 pH unit/5 mm were found when the pH was measured at constant intervals.

new bands, the major one corresponding to a molecular weight ∿ 60,000, the other one to a molecular weight ∿ 74,000. This result suggests that the "b" component is a dimer of a "c" subunit. It may be noted that "b" and "c"bands contained iron as shown by staining with bathophenanthroline.

Fig. $2_{8,9}$ exhibits results of isoelectric focusing of reaction centers on polyacrylamide gels. In a 3 to 10 pH ampholyte system two major components with low isoelectric points (between pH 5.4 and 6.5) could be detected along with a few additionnal minor components with higher isoelectric points (between 7.25-8). The pattern given by electrofocusing in this wide pH range, was similar to the electrophoretic pattern obtained at pH 8.9 in presence of detergent (Fig. 1_1). In a 3 to 6 pH system the components did not migrate in the gel, and the protein remained at the top at the highest pH. Optimum separation was obtained in the narrower range 5-8 (Fig. 2_9). A major band was detected, together with bands at each end of the gradient.Occasionally, a very faint band(possibly a dimer)was also seen near the major band. This major component of the reaction center possessed a pI of 6.5 at 20° (Fig. 5). After elution, its absorption spectrum was identical to the spectrum of the reaction center before electrofocusing; the photobleaching of the 865 nm band was complete and reversible, but only after elimination of the ampholytes. No such recovery of spectral properties was found in the components focused at other pI values. Furthermore, we noted that the optical spectrum was only preserved in a narrow pH range around 6.5; at lower pH values, the absorbance at 865 nm together with the characteristic photobleaching disappeared completely.

We analyzed the protein components of this pI 6.5 particle, by SDS gel electrophoresis of the eluted band, after heating in presence of 1 % SDS and 1 % mercaptoethanol. Only one protein species was found, with a $M_w \sim$ 30,000 daltons, identical to the "c" component detected in reaction center before electrofocusing (Fig. 2_{10}).

DISCUSSION : Two main points emerge from these results : 1/ isoelectric focusing,at least for analytical purpose, seems the best tool for purifying a reaction center from aggregates or contaminants. As the P 865 activity is preserved during electrofocusing in the 5 to 8 pH range, one may conclude that the pigment is still in a native state in the particle of pI = 6.5 2/ In such a particle, only one protein subunit, with a apparent $M_w \sim$ 30,000 is present. Thus it represents the smallest unit of the protein which binds the pigments (bacteriochlorophyll and possible bacteriopheophytin); this protein could be either a monomer, or, more probably, a dimer. This conclusion is at variance with the structure proposed for the reaction center protein

of the carotenoidless mutant of *R. spheroides* (11, 12); in that case, the protein is considered to be a trimer built from three different subunits, of apparent M_w 19,000, 22,000 and 27,000. Further studies of this protein species, and of its linkages with the pigments, are now in progress.

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