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Note

Chromatofocusing as a simple method of purification of two bacterial photosynthetic proteins: cytochrome c_2 and reaction centre of *Rhodopseudomonas viridis*

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There are well estalished purification schemes for cytochrome c_2 (cyt.c_2) of photosynthetic bacteria[1–3], which require several final chromatographic separation steps based on ion exchange and gel permeation. In this paper we report the successful use of chromatofocusing, which separates the proteins according to their isoelectric points, as the final separation step for the purification of cyt. c_2.

Purification schemes for reaction centres of photosynthetic bacteria[4–10] usually require several chromatographic purification steps based on ion-exchange and hydroxyapatite columns. This classical scheme, which takes 2 days to perform, was recently simplified greatly and its duration reduced to 20 min by using a single high-performance liquid chromatographic (HPLC) gel-permeation separation with a TSK (Toyo Soda, Japan) column[11]. Other workers have reported a one- step purification by affinity chromatography of the reaction centre with immobilized cyt.c_2[12,13].

In this paper we report the successful use of chromatofocusing[14] as the single separation step for isolating the reaction centre of *Rhodopseudomonas viridis* from purified thylakoids, requiring about 4 h to perform.

EXPERIMENTAL

Bacteria were grown in 10 l of culture medium as described previously[15] and washed twice with 0.2 *M* Tris–HCl (pH 8) by centrifugation at 3000 *g* for 8 min and resuspension of the pellet with buffer. The suspension was finally adjusted to a volume of 40 ml and the bacteria were broken by French press treatment (twice, 16,000 p.s.i., in the presence of 1–2 mg of DNase) and centrifuged at 2000 *g* for 30 min. The supernatant was centrifuged at 44,000 *g* for 3 h. The supernatant from the latter centrifugation was used subsequently for chromatographic purification of cyt.c_2. The pellet containing the thylakoids was resuspended in 50 m*M* Tris–HCl (pH 8) and layered on discontinuous sucrose gradients containing 39%/31% (w/w) concentration steps, and centrifuged at 55,000 *g* for 1.5 h. After the thylakoids found on top

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of the 39% cushion had been washed with 50 m*M* Tris–HCl (pH 8), they were layered on 31–44% continuous sucrose gradients and centrifuged at 30,000 *g* for 8 h to equilibrium. The thylakoid band at 36% (w/w) sucrose was washed once with 50 m*M* Tris–HCl (pH 8), adjusted to a protein concentration of 2 mg/ml[16], and sodium azide was added to a final concentration of 5m*M*.

This purified thylakoid suspension formed the material for chromatographic purification of the reaction centre.

Chromatographic purification of cyt.c_2

In the supernatant containing the water-soluble proteins, the buffer was changed to 10 m*M* Tris–HCl (pH 8.0)–5 m*M* sodium azide by application to a column with Sephadex G25 fine gel (Pharmacia, Uppsala, Sweden). The supernatant was then applied to a DEAE-cellulose column (Whatman DE-52, 10 cm × 25 mm I.D.). Cyt.c_2 passed directly through the column. In the eluate, containing the cyt.c_2, the buffer was changed to 25 m*M* ethanolamine hydrochloride (pH 10.0) and the material concentrated in a ultrafiltration cell with a YM-10 filter (Amicon, Lexington, MA, U.S.A.).

Chromatofocusing of cyt.c_2

A volume containing *ca.* 8 mg of cyt.c_2 was applied to the chromatofocusing column (30 cm × 9 mm I.D.) containing Polybuffer exchanger PBE94 (Pharmacia) and equilibrated previously with 25 m*M* ethanolamine hydrochloride (pH 10.0). The column was subsequently eluted with 120 ml of 1:10 diluted Polybuffer PB96 (Pharmacia) titrated to pH 7.8 with hydrochloric acid. Chromatofocusing was carried out at 4°C.

Crystallization of cyt.c_2

For crystallization, cytochrome fractions were pooled and equilibrated with 0.1 *M* Tris–HCl (pH 8.5)–2.4 *M* ammonium chloride using a YM-10 filter (Amicon). The material was concentrated to about 20 mg/ml of protein and dialysed with a Spectrapor 6000–8000 membrane (Union Carbide) in a Zeppezauer-type cell[17] against 0.1 *M* Tris–HCl (pH 9)–3.6 *M* ammonium chloride. Crystals formed after 2 weeks.

Chromatofocusing of the reaction centre

Purified thylakoids were held at the desired temperature in a water-bath and adjusted to the desired detergent concentration by adding 30% (w/v) N,N-dimethyldodecylamine N-oxide (LDAO) (Fluka, Buchs, Switzerland) dropwise at intervals of 4 sec under dim light. Immediately after reaching the end concentration, the sample was chilled to 0°C by dipping it into an ice-cold water-bath for 2 min. The solubilized membranes then were centrifuged at 60,000 *g* for 30 min at 4°C and 10 ml of supernatant were applied to the chromatofocusing column at a flow-rate of 1 ml/min. The latter was previously equilibrated with 25 m*M* imidazole hydrochloride (pH 7.3)–0.1% LDAO. Subsequently, the column was eluted at a flow-rate of 0.8 ml/min with 96 ml of a 1:8 dilution of Polybuffer PB74 (Pharmacia) (pH 7.45) with 0.1% LDAO added. The absorption of the eluate was measured at 280 nm. The whole chromatofocusing was carried out at 4°C under dim green light. Immediately after elution,

the pH of the fractions was measured in order to evaluate the course of the pH gradient.

Characterization methods

The cyt.c_2 fractions and the reaction centre fractions were characterized by SDS–polyacrylamide gel electrophoresis[18] with an 11.5–16.5% (w/v) linear acrylamide gradient and by spectroscopy between 280 and 1100 nm with a Zeiss spectrophotometer at room temperature. In order to have a simple purity criterion, the ratio $A_{273/415}$ for cyt.c_2 and the ratio $A_{280/830}$ for the reaction centre were used.

RESULTS

Cyt.c_2

The red-brown cyt.c_2 band eluted from the chromatofocusing column at pH 8.7. The purity coefficient was 1.9 for the best fractions.

Isoelectric focusing[19] of this material in a pH gradient from pH 7 to 9 gave

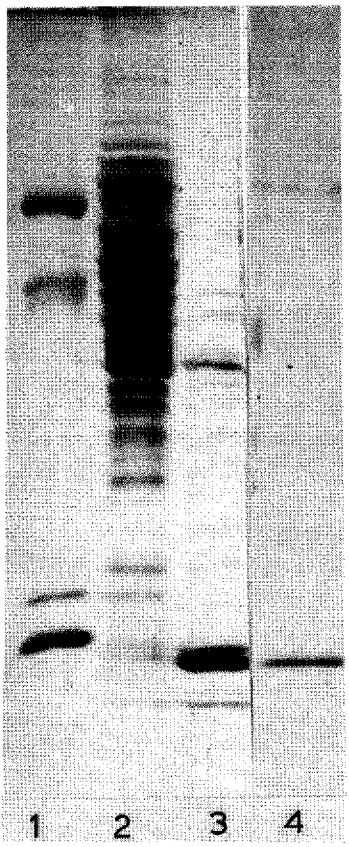

Fig. 1. SDS gels of cyt.c_2 fractions. Track 1, molecular weight standards of 64, 43, 17.2 and 12.5 kdalton; track 2, supernatant obtained after centrifugation of broken cells; track 3, proteins passing unbound through the DEAE-cellulose column; track 4, cyt.c_2 after chromatofocusing and crystallization.

one red-brown band at pH 8.5 In order to remove the Polybuffer and to purify further the cyt.c_2, the protein was crystallized as described under Experimental. The washed cyt.c_2 crystals showed only one band in the SDS gel at an apparent molecular weight of 12 kdalton, as is shown in Fig. 1.

Reaction centre

The result of chromatofocusing the reaction centre depends upon the solubilization conditions of the membranes with LDAO. Three parameters were varied: LDAO concentration, temperature and duration of detergent exposure before centrifugation.

The best purities of $A_{280/830}$ = 2.3 were observed after solubilization with 4.5% (w/v) LDAO at 17°C. The reaction centre eluted as a brown band at pH 6.5 under these conditions. A spectrum of this fraction is shown in Fig. 2.

SDS gels of these fractions show the four bands of the *R. viridis* reaction centre[4,15] (Fig. 3). In addition, there is a high-molecular-weight band of apparent molecular weight *ca.* 100 kdalton, which can be removed by high SDS concentrations during solubilization of the sample prior to electrophoresis.

The Polybuffer present in the reaction centre fractions can be removed by repeated filtration and dilution with an XM-100 membrane (Amicon), as could be seen in SDS gel electrophoresis. This is not surprising if one considers that the former has molecular weight less than 25 kdalton[14], while the reaction centre eomplex must have a molecular weight of more than 100 kdalton. The best fractions had $A_{280/830}$ = 2.1.

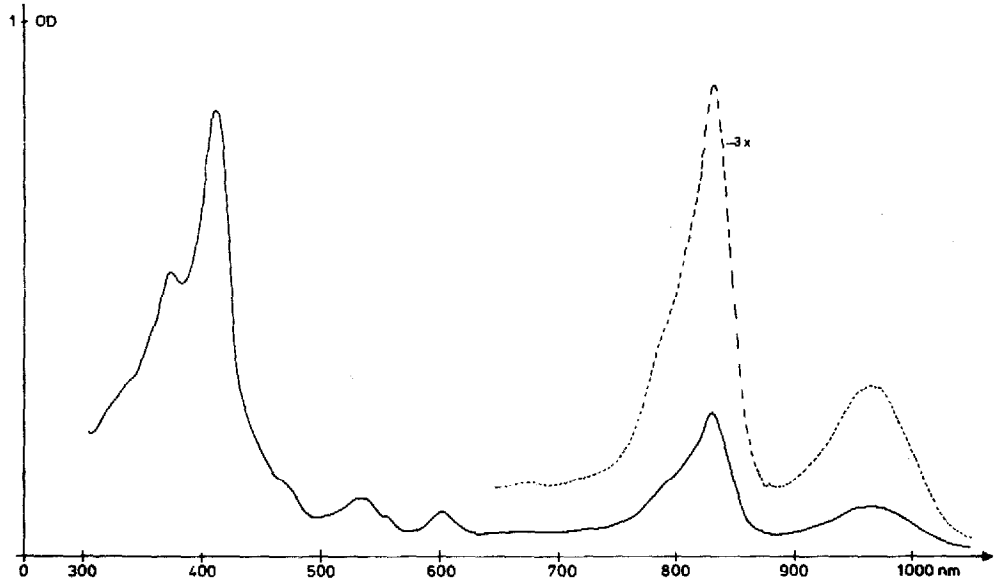

Fig. 2. Spectrum of the reaction centre purified by chromatofocusing.

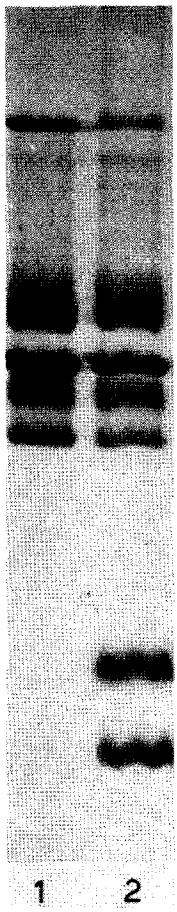

Fig. 3. Gel electrophoresis of purified reaction centres obtained by chromatofocusing. Track 1, reaction centre fraction; track 2, purified thylakoids.

DISCUSSION

The high separation capability of chromatofocusing can be successfully used to purify cyt.c_2 and the reaction centre of *R. viridis*. We consider that this procedure is less labour and time consuming than either of the corresponding classical separation schemes.

HPLC was shown by Michel[11] to be faster than our reaction centre preparation scheme. However, it requires a high capital investment to set up the separation device, whereas chromatofocusing is very economic and can be performed with ordinary liquid chromatographic apparatus. While the affinity approach[12,13] provides high purity, information on the yield of reaction centre is lacking, so that it is difficult to compare this method with the other two schemes.

The results of gel electrophoresis of the reaction centre after solubilization with different SDS concentrations suggest that the 100 kdalton band seen during electrophoresis represents the whole reaction centre complex.

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