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HPLC PURIFICATION OF A BIOLOGICALLY ACTIVE MEMBRANE PROTEIN : THE REACTION CENTER FROM PHOTOSYNTHETIC BACTERIA

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Reaction centers from Rhodopseudomonas sphaeroides R 26 have been isolated from a crude extract obtained by lauryldimethylamine oxide extraction of chromatophore membranes, by HPLC using a combination of surface-mediated and size exclusion chromatography.

The eluted RCs exhibit a normal activity ($t_{1/2}$ of the back-reaction is 70 ms) and are recovered in good yield (over 50 % based on the activity) and purity (based on the A 280 nm/A 800 nm = 1.30±0.05 ratio and the characteristic 3 polypeptides SDS-PAGE pattern). The elution time (5-10 mn) is about two orders of magnitude faster than for the classical purification techniques.

Beside the most common water-soluble proteins there exists a large variety of water insoluble proteins like the keratin, the histones and the intrinsic membrane proteins. This last class of proteins, which are involved in a number of important membrane bound biological processes (receptors, transport, energy transduction ...) are strongly amphiphilic molecules. Hydrophilic surfaces are found where the protein protrudes out of the lipid bilayer, while an hydrophobic core is buried within the hydrocarbon region of the lipids. The amphiphilic nature of membrane proteins explains why detergents, which are also amphiphilic molecules, have been extensively used to solubilize the biological membranes. Although this solubilization under conditions which preserve the biological activity has been successfully achieved for most of the important membrane proteins, the purification techniques which are subsequently utilized are frequently slow and quite inefficient. Many of these proteins are still purified by relatively unresolving techniques

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such as ammonium sulphate precipitation, sucrose density gradient centrifugation and classical liquid chromatography on a variety of supports. Although the high performance liquid chromatography of peptides and soluble proteins is growing exponentially [1], only very few applications of this technique to membrane proteins have been described. Cytochrome P 450 has been purified by ion exchange chromatography [2, 3] and by reverse phase chromatography [4], and membrane proteins from Halobacterium halobium have been separated by size exclusion chromatography [5].

The reaction center from photosynthetic bacteria is a typical intrinsic membrane protein. Both its role in the initial steps of charge separation from which whole photosynthesis proceeds and its composition in terms of pigments and proteins have been well characterized [6-8] since its first isolation [9]. Although this protein (M.W. 80.000) represents only a few percent of the total protein content of the photosynthetic membrane, reaction center preparations with a high level of purity have been obtained [8]. The purification procedures usually take one to three days, but a recent technique utilizing cytochrome C affinity chromatography indicates the possibility of reducing this time to several hours [10]. However this last technique is not of universal applicability.

Due to its biological importance and to the availability of simple criteria of purity we have selected this intrinsic protein to explore the possibilities of purifying such membrane constituents by HPLC. Furthermore, a preliminary report by Michel [11] indicated that the reaction center of Rhodopseudomonas viridis could be purified by HPLC using size exclusion chromatography. Starting from a crude detergent extract and using the newly developped phases of intermediate polarity, we report here on the rapid isolation (< 10 min) of highly purified and photochemically active reaction centers by HPLC.

MATERIALS AND METHODS

The photosynthetic bacteria (Rhodopseudomonas sphaeroides R-26) was grown and the chromatophore membranes prepared as described earlier [12]. The membranes were suspended in 10 mM Tris HCl, 100 mM NaCl, pH 8.0, and their concentration adjusted to give an absorbance $A_{865\ nm}$ = 50 cm^{-1}. Loosely bound, peripheral membrane proteins were removed by adding the detergent lauryl dimethylamide oxide (LDAO) (Onyx Chemical, Jersey City) to give a final concentration of 0.05 % (v/v) [10]. The washed chromatophore membranes were then pelleted by centrifugation at 200,000 x g for 60 min, resuspended in a buffer containing 10 mM Tris HCl, pH 8.0 and adjusted to give $A_{865\ nm}$ = 50 cm^{-1}. The chromatophore membranes were dissolved by the addition of 30 % LDAO to this buffer to yield a final concentration of 0.75 %. After stirring for 30 min, the suspension was centrifuged at 200,000 x g for 60 min. The supernatant containing the detergent-solubilized reaction center as well as other contaminating proteins was drawn off, divided into 2 ml quantities and stored at - 70°C. This crude extract was used as the starting material for the HPLC protein separation. Comparison of the chromatograms of the stored material with that of freshly prepared extracts showed no difference, and material was routinely stored in this way until just prior to use.

The HPLC was performed on a Waters apparatus consisting of an injector U_6K, pump 6000 A and detectors M 440 and M 481. The elution profile was monitored at 254 or 280 nm. The reaction center spectra and index of purity derived from the 280 nm and 800 nm absorbance ratio were obtained on a Cary 17 spectrophotometer. The reaction center activity was measured by monitoring the flash induced 860 nm absorbance transient with a kinetic spectrophotometer as described earlier [13].

RESULTS

Several types of HPLC columns were tried for the purification of the reaction center protein from the crude membrane extract. In every case the detergent LDAO was used in the elution buffers and the quality of the reaction centers was judged from the optical absorption spectrum and the $\frac{280nm}{800nm}$ absorption ratio as a criterion of purity, where a value of 1.20 - 1.30 is found for purified reaction centers [8].

Reverse phase columns like Protesil 300 diphenyl (Whatman) were found not to be useful for the purification of an active reaction center fraction. However, purification was achieved by combination of size exclusion chromatography (TSK G 4000 SW from Toyo Soda) and surface mediated chromatography (Lichrospher diol 1000 from Merck). The influences of the different parameters (column length, pH, ionic strength, LDAO concentration) have been determined only on the diol columns. They possess both hydrophilic (diol) and apolar (propyl

chains) groups grafted on a silica matrix and the pore size allows the proteins to enter the gel. Thus, according to the elution conditions, these columns function in size exclusion and surface-mediated modes (hydrophobic and - or - ion exchange interaction).

a) Column length : the separation of protein peaks and the purity of the recovered reaction centers is improved when several columns are placed in series. A compromise between the protein purity and the chromatographic rapidity and inlet pressure led us to use a length of 50 cm (2X25 cm).

b) pH : as the pH was increased, a better separation of protein components was achieved. These experiments were performed with 0.01 M phosphate buffers (+ 0.05 % LDAO) between pH 5.5 and 8. However since the silica support of the diol phase is solubilized in alkaline medium, the limit of pH 8 was choosen using 0.01 M dipotassium phosphate. The chromatograms were not appreciably changed by substituting Tris buffer of the same pH. It should be pointed out that under these conditions of pH, ion exchange properties of the column are not negligible since the silica is negatively charged.

c) ionic strength : concentrations of phosphate buffers (K_2HPO_4 from 0.002 M to 0.04 M) or of sodium chloride (from 0.01 M to 1 M) were tried. The chromatogram showed a better retention of certain protein components at high ionic strength suggesting a suppression of repulsive ionic interactions, but no significant variations in the 280 nm/800 nm ratio of the recovered reaction centers were found.

d) concentration of the detergent LDAO : the retention time of the proteins through the column was shortened by raising the concentration of detergent (from 0.05 to 4.5 %). Paradoxically the purity of the reaction center was higher for the high detergent concentrations ($\frac{A\ 280nm}{A\ 800nm}$ from 1.7 to 1.4). However, in order to avoid the destabilizing effects of high detergent concentration, a concentration equal to that in the membrane solubilization buffer (0.75 %) was

TABLE 1

% LDAO 1st chromatography on diol column	% LDAO 2nd chromatography on TSK column	280nm/800nm ratio
0.75	0.75	1.25 - 1.28
0.75	0.37	1.31
0.75	0.18	1.36

Reaction center purities for different combined diol plus gel filtration chromatographies and various concentrations of LDAO.

choosen for the final purification conditions. In these conditions reaction centers were exclusively found to elute in a single major peak at about 4 min after the injection. An optical absorption spectrum of this material in the visible and near infrared regions was essentially identical to that of purified reaction center preparations [8, 10, 14], suggesting that there is no significant contamination by other pigmented proteins. However the elevated 280 nm/800 nm ratio (1.6) indicated that there is an appreciable non-pigmented protein contamination.

A complete purification of reaction centers could be achieved by a combination of diol plus gel filtration columns. The gel filtration can be performed as a second chromatography by injection of the partially purified reaction center fraction eluted from the diol columns. LDAO concentration can be lowered to 0.2 % (Table 1, purity ratio ranging from 1.36 to 1.25). The diol and gel filtration columns can be also mounted in series. This latter method has the advantage of decreasing the purification time and avoiding additional sample dilution.

An example of a chromatogram is shown in figure 1 monitored at 280 nm. Although the elution profile varies from batch to batch (but is perfectly reproducible from one chromatography to the other), the reaction center elutes as a major peak at 8.5 mn after the injection of the crude extract, with a flow-rate of 1.5 ml/mn. Monitoring the

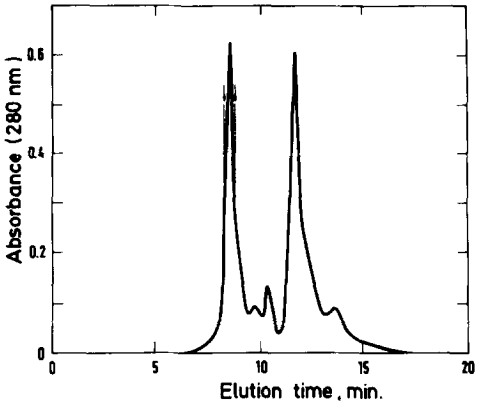
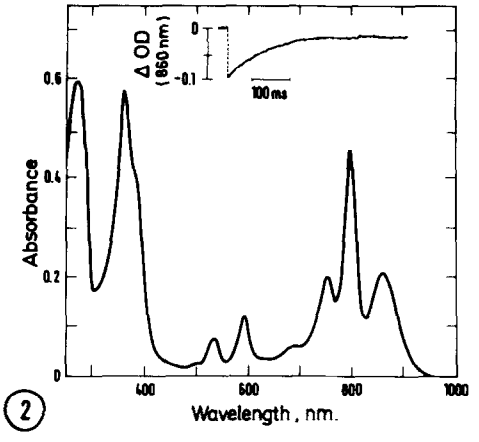


Figure 1 : Combined chromatography on diol plus TSK columns.
Columns : Lichrospher 1000 diol 2x25 cm + TSK G 4000 SW 30 cm
Elution buffer : K_2HPO_4 + LDAO 0.75 %,
flow rate : 1.5 ml/min

optical absorption of the other fractions showed that the reaction centers were only present in this first peak. The vertical lines mark the fraction which was collected. With a series of different batches, the $\frac{A\ 280nm}{A\ 800nm}$ ratio of the recovered reaction centers ranged between 1.23 and 1.33 with 0.75 % LDAO and the yields of the collected fractions averaged about 55 % based on measurement of the 860 nm reaction center absorption.

The reaction centers purified in this manner show normal functional photochemical activity. The optical absorption spectrum for these reaction centers is shown in figure 2. This spectrum is undistinguishable from those of reaction centers purified by other techniques. The flash induced oxidation of the bacteriochlorophyll dimer has been measured at 860 nm. The 70 ± 10 ms half-time for the $[B_2^+Q^-]$ state indicates that these reaction centers contain only the primary ubiquinone molecule (Q_A) [6-8] (Fig. 2). Secondary quinone activity could be reconstituted by the addition of ubiquinone. SDSPAGE electrophoresis under denaturing conditions of these purified reaction centers shows the standard 3 subunits pattern [8] (Fig. 3) with no appreciable contamination by other polypeptides.



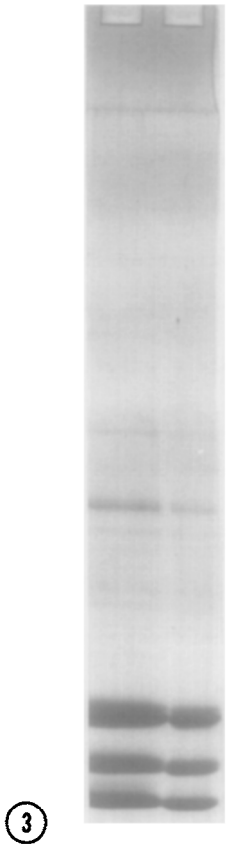


Figure 2 : Absorption spectrum of a reaction center preparation purified according to conditions described in figure 1. Insert : reversible bleaching of reaction center preparation at 860 nm.

Figure 3 : SDS polyacrylamide gel electrophoresis of reaction center preparation showing the characteristic 3 bands pattern.

DISCUSSION

This work shows the utilization of diol columns for the purification of reaction centers of Rhodopseudomonas sphaeroides R-26. Although the relative contributions of size exclusion, hydrophobic interaction and cation-exchange have not been determined in these conditions of pH, ionic strength and LDAO concentration, the method is reproducible and gives sufficiently pure and biologically active reaction centers in a very short time. The same procedure has also been successful with reaction centers of Rhodopseudomonas sphaeroides 2.4.1. At the present time, the quantities involved are relatively small (injection

volume of crude extract : 200 µl, purified reaction center : 600 µl with A_{800nm} = 0.5), but preliminary experiments with reaction centers on the available preparative columns have already yielded reaction centers free of contaminating pigmented proteins (unpublished results). The rapidity of this isolation technique also offers the possibility of investigating the use of short exposures to severe conditions as a means to extract protein complexes or to modify their composition. For example a brief exposure to a high detergent concentration could be used to liberate tightly bound subunits and their separation be achieved before denaturation occurs.

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