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PROTEOLYTIC ACTIVITIES IN PREPARATIONS OF *RHODOSPIRILLUM RUBRUM* REACTION CENTERS

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Proteolytic activities have been detected in preparations of isolated reaction centers of *Rhodospirillum rubrum* by the use of ^{125}I-labeled casein and insulin as substrates. The activities became undetectable after gel filtration and were partially impaired by several commercially available inhibitors that did not modify the primary photochemical properties of the reaction centers. The electrophoretic pattern of the reaction center proteins became modified during storage, even at 4°C in the dark. These alterations appeared considerably attenuated in gel-filtered reaction centers and were totally suppressed by some proteinase inhibitors. Thus, it seems that the proteolytic activities that accompany the isolated reaction centers modify their protein complement, giving rise to a certain variability from one batch to another. As far as we know, the present paper is the first report on *R. rubrum* proteolytic activities.

Introduction

Any work on protein characterization should take into account the possible artifacts which may be generated by contaminant proteinases. Since the indiscriminate addition of proteinase inhibitors may not prevent such artifacts [1], it seems that some previous characterization of such contaminant proteolytic activities is required in order to avoid undesired alterations of the protein under study.

The description of contaminant proteinases may have further physiological interest. Thus, some proteins have been shown to copurify with highly specific proteinase activities which appear to constitute intrinsic properties of the proteins themselves [2–5]. In those cases, it seems likely that the proteolytic activities may be physiologically involved in the regulation of the cellular levels of such proteins [2]. In prokaryotic organisms studies on proteinases have still a more immediate interest because the general mechanisms of proteolysis, especially of membrane bound proteins, remain largely unknown.

It has been reported that the reaction center of Rhodospirillaceae includes three different polypeptides [6]. Since its first reported isolation [7], the relative stoichiometry of those polypeptides is generally admitted to be 1 : 1 : 1. However, with the exception of that first report, complicated calculations were necessary to obtain this stoichiometry [8,9]. Certain anomalies in the relative levels of *R. rubrum* reaction center proteins led us to conclude that their stoichiometry changed with the preparation. As we show here, those changes seem to be due to the presence of proteolytic activities in chromatophores and detergent-solubilized fractions. We describe how those activ-

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Abbreviations: LDAO, dodecyldimethylamine *N*-oxide; PCMB, *p*-chloromercuribenzoic acid; P870, primary electron donor of photoreaction centers; PMSF, phenylmethylsulfonyl fluoride; TLCK, tosyllysylchloromethyl ketone.

ities can be extensively prevented without inducing any apparent alteration of the solubilized reaction centers. As far as we know, this is the first report on proteinases of *R. rubrum*.

Materials and Methods

Strains, growth conditions and purification of photoreaction centers

Rhodospirillum rubrum strain S1 (wild type) was cultured and chromatophores were obtained as reported previously [10]. Reaction centers were solubilized from chromatophores and purified by the method of Vadeboncoeur et al. [8]. The process is summarily outlined in Fig. 1. 10 mM Tris-HCl (pH 8)/0.1% Triton X-100 was used through steps 9 to 12. Detergent was omitted during Sephadex G-25 filtration and final dialysis.

Assay of proteolytic activity

The assays were performed as previously described [1]. The reaction mixture contained 32 μg of ^{125}I-casein (8500 cpm $\cdot \mu g^{-1}$) or 8 μg ^{125}I-insulin (12000 cpm $\cdot \mu g^{-1}$). The buffer was 10 mM Tris-HCl (pH 8). Prior to the assay the samples were dialyzed (24 h) against this buffer.

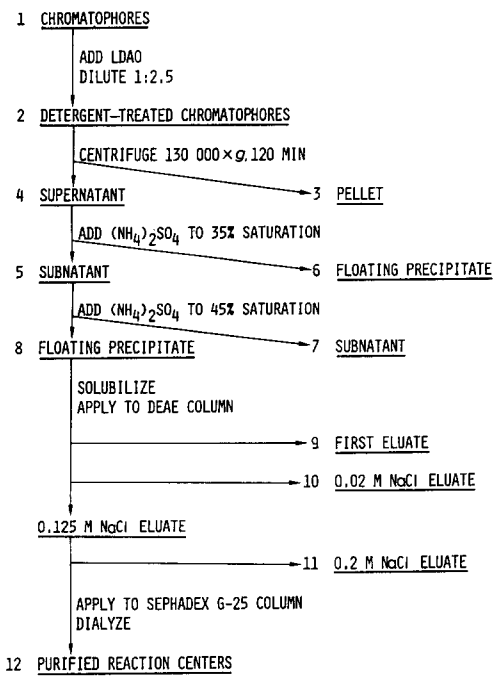

Fig. 1. Flow chart of the reaction center isolation procedure.

Analytical polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out according to the method of Fairbanks et al. [11] for 0.1% SDS, but gels contained 10% acrylamide and 0.26% *N,N'*-methylenebisacrylamide. Prior to electrophoresis, the samples (3 μM P870) were mixed in a 1 : 1 ratio with a solution of 70 mg SDS/ml and 2.25 M glycerol in 0.21 M Tris-HCl (pH 6.8). 10 μl of this mixture were layered on each gel (5.5 cm long). Electrophoretograms were obtained as already described [12], but the Bascom-Turner 8120 recorder was interfaced to a Hewlett-Packard 9826 computer, where the data were stored and processed.

Other assays

Absorption spectroscopy was carried out as reported by Giménez-Gallego et al. [10]. A flash of 10 joules per pulse was used for excitation in kinetic experiments. P870 concentration was estimated at 865 nm using an extinction coefficient of 143 $mM^{-1} \cdot cm^{-1}$ [13]. Protein was assayed according to the method of Lowry et al. [14] as modified by Dulley and Grieve [15]. Gel chromatography was performed as in Ref. 16.

Materials

Reagents were purchased from the following sources: bovine serum albumin, TLCK, bathophenanthroline, ε-aminocaproic acid, leupeptin, aprotinin, chymostatin and pepstatin from Sigma Chem. Co., St. Louis, MO (U.S.A.); PMSF, soybean trypsin inhibitor and iodoacetamide from Calbiochem, La Jolla, CA (U.S.A.); EDTA from Fisher Company, Fair Lawn, NJ (U.S.A.); sodium azide from BDH Chemicals Ltd., Poole (U.K.); K^{125}I (NaOH) from The Radiochemical Centre, Amersham (U.K.); casein, Triton X-100 and sodium deoxycholate from Merck, Darmstadt (F.R.G.); LDAO was a generous gift from the Onyx Chemical Company, NJ (U.S.A.).

Results

In accordance with other authors [9], we observed that the area under the protein bands in

the reaction center electrophoretograms were, within a wide range, linearly related to the amount of P870 introduced in the gel. However, that was not true if different batches of reaction centers were compared, as is shown in Fig. 2. One of the electrophoretograms (Fig. 2a) belongs to a batch whose isolation lasted about 52 h, as is usual with the normal procedure [8]. The other one (Fig. 2b) corresponds to another batch prepared in 8–10 h (dialysis replaced by gel filtration through Sephadex G-25). As is apparent in Fig. 2b, the relative areas of the individual bands keep the same ratio as the M_r of the polypeptides, as should be expected from a 1 : 1 : 1 stoichiometry. Such is not the case for the preparation obtained by the lengthier procedure (Fig. 2a). We suspected that a proteinase might be at the origin of those differences. In order to check that point, we assayed the possible presence of caseinolytic and insulinolytic activities in several fractions along the preparation procedure [1]. The results of those assays (Table I) show that the procedure resulted in the purification of

TABLE I

PROTEOLYTIC SPECIFIC ACTIVITIES AND P870 SPECIFIC CONTENT IN DIFFERENT FRACTIONS ALONG THE REACTION CENTER PURIFICATION PROCEDURE

Units are pmol hydrolized per min per mg of protein or nmol of P870 per mg of protein.

Fraction [a]	Caseinolytic activity (units)	Insulinolytic activity (units)	P870 content (units)
1	0.41	0.44	1.0
2	0.74	0.94	
3	0.98	0.73	
4	1.81	1.81	1.77
5	2.18	2.28	
6	0.77	0.24	
7	1.50	1.67	
8	2.2	4.97	3.27
9	4.4	2.00	
10	2.62	14.8	
11	4.54	6.44	
12	3.78	1.32	4.72

[a] See Fig. 1.

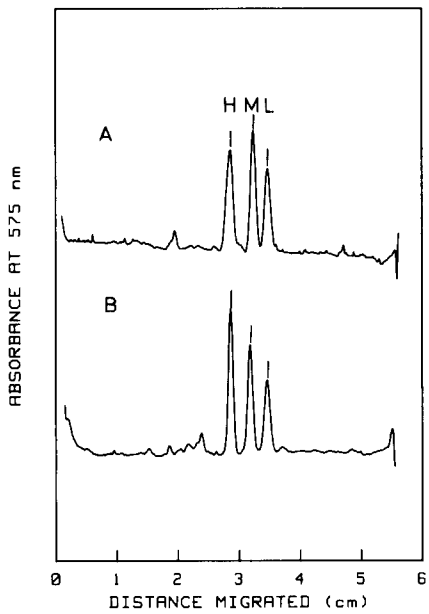

Fig. 2. Electrophoretograms of two different batches of reaction centers. (A) Preparation performed within 52 h. (B) Preparation carried out within 8–10 hours (see the text). The relative areas (L taken as 25) are: (A) 33.0 for H, 33.2 for M and 25 for L; (B) 36.6 for H, 31.9 for M and 25 for L. SDS-polyacrylamide gel electrophoresis was carried out as described under Methods.

both activities, the specific levels of which increased at least as much as that of the reaction center itself.

In order to learn whether the contaminant proteinases modified the peptidic composition of the reaction center such activities should be removed from the preparation. We tried to achieve that by several approaches.

First, we subjected a reaction center suspension to several consecutive freeze-thawing cycles. We were not able to detect any decrease in the proteolytic activities, either caseinolytic or insulinolytic, in those preparations.

We also determined whether the proteinases were altered by several detergents which are usually employed to keep reaction centers in solution. It was found that sodium deoxycholate did not affect either the caseinolytic or the insulinolytic activities, Triton X-100 did not modify the first one but highly stimulated the second, and LDAO strongly inhibited both activities (Fig. 3).

The reaction center suspension was also treated with Amberlite XAD-2, a treatment which removes Triton X-100 [17]. We found that the treat-

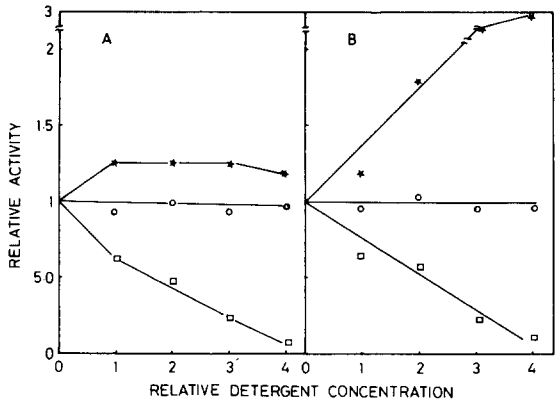

Fig. 3. Effect of different detergents on the caseinolytic and insulinolytic activities of a reaction center preparation. One concentration unit of detergent is 0.1% for deoxycholate (○); 0.025% for Triton X-100 (★); and 0.0125% for LDAO (□). One relative activity unit is 3.78 pmol of casein hydrolyzed · min^{-1} per mg protein in A, and 1.32 pmol of insulin hydrolyzed · min^{-1} per mg protein in B.

ment induced, at the same time, the disappearance of the insulinolytic activity (Table II).

Finally, we tried also to eliminate the proteinase activities from the reaction center preparation by gel chromatography. We used Sephacryl S-400, since with gels of smaller exclusion limit the reaction centers appeared in the void volume. As Table II shows, we failed to detect either caseinolytic or insulinolytic activity after gel filtration.

TABLE II

EFFECT OF AMBERLITE XAD-2 TREATMENT AND SEPHACRYL S-400 GEL FILTRATION ON PROTEOLYTIC ACTIVITIES OF REACTION CENTER PREPARATIONS

3.3 g of Amberlite XAD-2 per g Triton X-100 were added and later eliminated by decantation. Units are pmol hydrolyzed per min per nmol of P870.

	Insulinolytic activity (units)	Caseinolytic activity (units)
Amberlite XAD-treatment		
untreated	0.8	1.41
treated	0.03	1.05
Sephacryl S-400 gel filtration		
unfiltered	1.25	0.78
filtered	0.02	0.1

Fig. 4 shows the effect of prolonged incubation at 4°C in the dark on the protein pattern of reaction centers. When the preparation had not been gel-filtered through Sephacryl, incubation apparently caused the disappearance of the 36-kDa band and an alteration of the 29-kDa one, which increased its mobility and migrated together with that of 25-kDa. In the gel-filtered preparation, the disappearance of the 36-kDa band was retarded and the two lighter bands remained unaltered. Therefore, it seems that proteolytic activities that copurify with reaction centers originated a deep modification of their protein complement. As in the case of certain digestions with exogenous proteinases [16], the alteration of the proteins of reaction centers that appears in Fig. 4 did not affect either the spectrum of the centers or the light-induced changes in their infrared spectra (not shown).

The disappearance of the 36-kDa band in the gel-filtered reaction centers raised some doubts as to whether this modification could also be attributed to any proteolytic activity. So, in order to check that, we tried to inhibit the proteolytic activities by using a wide set of commercially available inhibitors of different proteinase types (Table III). The typical inhibitors of serine proteinases, metalloproteinases, aspartic proteinases, trypsin and chymotrypsin affected to different extents, but

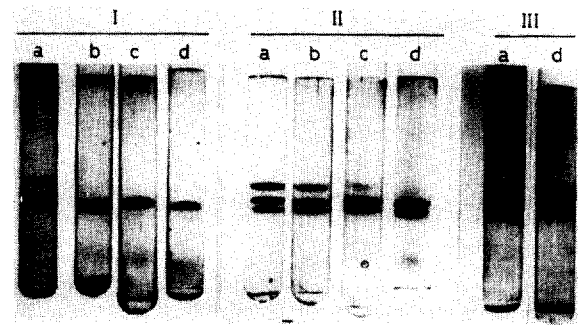

Fig. 4. Modification of the polypeptide pattern of reaction centers during 15 days of incubation at 4°C. Reaction centers (3 μM P870) were kept in the dark for 0 (a), 4 (b), 8 (c) and 15 (d) days. (I) Untreated centers; (II) gel-filtered centers; (III) centers incubated in the presence of 3 μg/ml leupeptin, 3 μg/ml chymostatin and 4 mM PMSF (see Table III). SDS-polyacrylamide gel electrophoresis was carried out as described under Methods.

TABLE III

EFFECT OF SEVERAL PROTEINASE INHIBITORS ON PROTEOLYTIC ACTIVITIES OF REACTION CENTER PREPARATIONS

Assays were carried out as described in Ref. 1.

Inhibitor	Concentration	Proteolysis (% of control)	
		Insulin	Casein
PCMB	2 mM	86.07	100.2
Leupeptin	0.3 $\mu g \cdot ml^{-1}$	40.7	93.4
Iodoacetamide	2 mM	70.7	94.4
EDTA	2 mM	22.4	76.9
Bathophenanthroline	2 mM	60.2	59.8
PMSF	1 mM	67.8	47.9
TLCK	1 mm	55.5	75.8
Aprotinin	0.5 $\mu g \cdot ml^{-1}$	67.9	87.2
Soybean trypsin inhibitor	0.1 $mg \cdot ml^{-1}$	74.6	100.2
Chymostatin	0.3 $\mu g \cdot ml^{-1}$	100.2	65.2
Pepstatin	0.3 $\mu g \cdot ml^{-1}$	100.0	79.8
6-Aminocaproic acid	2 mM	46.9	88.9
Sodium azide	1 mM	93.2	98.7
Leupeptin + chymostatin	– [a]	51.2	72.4
Leupeptin + PMSF	– [a]	32.3	36.08
Chymostatin + PMSF	– [a]	70.0	27.8
Leupeptin + chymostatin + PMSF	– [a]	33.2	20.9

[a] Each inhibitor was present at the same concentration as above.

never completely inhibited, the caseinolytic and insulinolytic activities. Therefore, it seems possible that each of these activities is due to more than one proteinase. For that reason, and to produce a more severe inhibition of the activities, we tested different mixtures of inhibitors (Table III). The strongest inhibition was obtained with a mixture of chymostatin, leupeptin and PMSF. As appears in Fig. 4, this mixture prevented any apparent alteration of the protein pattern of the preparation. Thus, it seems reasonable to conclude that residual proteinase activities after gel filtration were responsible for the disappearance of the 36-kDa band. The experiment was also carried out in the presence of 0.1% Triton X-100, 0.4% sodium deoxycholate or 0.05% LDAO. It should be mentioned that in the presence of sodium deoxycholate the inhibitors were less effective and also that the preparation acquired a soft gel consistency. The addition of the inhibitors to the preparation in the presence of LDAO immediately denatured the protein, which readily floculated.

The mixture of proteinase inhibitors described above (Fig. 4) did not seem to alter reaction centers. Thus, their infrared spectrum, a rather faithful probe of their structure, was not modified by the treatment (not shown). Also the light-induced spectral changes which reflect primary donor oxidation were unaltered. Besides, the recovery of photooxidized P870 after a single flash of actinic light exhibited apparent first-order monophasic kinetics with a half-time (93 ms), similar to that estimated previously by Giménez-Gallego et al. for *R. rubrum* [10] and by other authors for *R. sphaeroides* [18,19]. Since this back-reaction seems to reflect very closely the reciprocal situation of the primary reactants and the state of their environment [20], this kinetic datum supports the conclusion that the described inhibitor mixture does not modify the reaction center. However, in the presence of 0.1% Triton X-100, the inhibitors induced an extensive pheophytinization of the centers during the incubation. Thus, no objection apparently exists to using the inhibitor mixture of Fig. 4 in order to avoid the degradation of reaction centers, once the concentration of detergents in the preparation has been lowered as far as possible.

Discussion

Proteolytic activities are usually detected with the help of small synthetic substrates which, although offering advantages in terms of sensitivity and easy detection, may be cleaved by other types of hydrolase [1]. That was the reason why we decided to use a method that, even if more laborious, combines high sensitivity and selectivity [1,21]. Those assays have made evident the existence of proteolytic activities in the preparation of isolated reaction centers. Such activities probably reflect the presence of more than one proteinase because: (i) in other microorganisms a single enzyme does not usually exhibit both insulinolytic and caseinolytic activities [1]; (ii) inhibitors and Triton X-100 affect independently each activity; (iii) Amberlite XAD-2 selectively removes the insulinolytic activity. Besides, the incomplete effect of each proteinase inhibitor perhaps suggests that more than one proteinase is involved in each of the caseinolytic and the insulinolytic activities.

Since the starting material for the purification of reaction centers was membrane vesicles, it seems reasonable to assume that the proteinases are natively membrane-bound enzymes. Besides, it seems possible that the proteinases which copurify with the reaction centers are not the only ones solubilized by LDAO. That would explain why a considerable part of the proteolytic activity present in the crude extract is removed by some of the purifications steps (fraction 10 is specially remarkable in this respect). It may seem contradictory that although gel chromatography removes both insulinolytic and caseinolytic activities, some degradation of reaction center proteins is still observed (Fig. 4, Table II). Nevertheless, this effect may still be attributed to the presence of some silent proteinases. There are reports in the literature on some similar cases, and several explanations have been proposed: (i) unusual specificity of the proteinase; (ii) special high affinity of the proteinase for its substrate; (iii) endopeptidase activity intrinsically associated with different regions of the purified protein [2–5]. Up to now, as in the present work, these hypotheses have remained untested.

The isolation of active reaction centers with only one polypeptide chain was recently reported [12]. In that report it was suggested that a proteinase possibly present in the preparation might have intervened in the isolation procedure. The data we present here, together with a recent study on the controlled proteolysis of reaction center proteins by exogenous proteinases [16], make it quite likely that this hypothesis is correct.

The results of this report show that prolonged storage of reaction center preparations may lead to alterations of their protein complement, which may in turn have secondary effects on other reaction center properties. In order to avoid such degradation, the preparation procedure should be carried out in the cold, as rapidly as possible, and long steps, such as dialysis, should be avoided or replaced by shorter ones. Although the addition of a mixture of proteinase inhibitors after the final step seems not to interfere with reaction center activity, its use may have undesirable side-effects under different conditions, for instance if either LDAO, Triton X-100 or sodium deoxycholate are present (see Results). A modified preparation procedure, which takes into account some of these points, has been published elsewhere [16].

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