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NITRATE REDUCTASE AND THE MEMBRANE COMPOSITION OF PLEIOTROPIC CHLORATE RESISTANT MUTANTS OF ESCHERICHIA COLI K-12

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SUMMARY

The protein composition of the membrane fraction of pleiotropic chlorate resistant mutants of E. coli K-12 was analyzed as a function of Nitrate Reductase and other components. The results for all pleiotropic chlorate resistant mutants show: the presence of the Nitrate Reductase structural protein, an increase in the relative protein content in an inactive complex of this enzyme, and the disappearance of a low molecular weight protein. The later protein is lost from the wild type membrane fraction by treatment at 60°C, 20 min. It is concluded that Nitrate Reductase is present in the cytoplasmic membrane of all pleiotropic chlorate resistant mutants in a form structurally and functionally different from the wild respiratory enzyme coupled to the electron transport chain.

INTRODUCTION

Nitrate Reductase (NR, E.C. 1.9.6.1.)** is a membrane bound respiratory enzyme of Escherichia coli K-12 (1,2). It reduces nitrate to nitrite, generating energy for anaerobic growth. It also reduces chlorate to chlorite which is further reduced to toxic products (3). NR-less mutants have been obtained under anaerobic conditions in the presence of chlorate (3). These mutants were named chlorate resistant (chl^r), and were shown to belong to two categories: non pleiotropic or specific for mutations which affect NR activity, and pleiotropic mutants, those which have also lost FDH activity (3). chlC (4) is the only locus controling the first category of mutants, and chlA, B, (4), chlD (5), chlE (6), and chlG (7) were shown as loci controling pleiotropic effects. Another mutant, chlF (7) has completely lost FDH, and partially NR activity. Pleiotropy has been explained in several ways, the most important of which have been the "particle integrity hypothesis" of Azoulay et al. (see 8 and 9) and the "Molybdenum-cofactor procesing hypothesis" (10,11,12).

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** Abreviations: NR, Nitrate Reductase; FDH, Formate Dehydrogenase; WT, wild type strain; PAGE-SDS, polyacrylamide gel electrophoresis containing Sodium Dodecyl Sulfate; MVH, reduced Methyl Viologen.

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The membrane composition of *chl*^{r} mutants is important in order to determine: i) presence or absence of NR structural protein, ii) role of different *chl*^{r} loci in the construction of NR structural protein, and in its activity, and iii) role of NR in the construction of the cytoplasmic membrane of *E. coli*. In previous studies there have been contradictory results:absence of NR structural protein in the membrane of *chlA* (13), *chlD* and *chlE* (14,15) and absence of five polypeptides in membranes of *chlA* and *chlD* (16), while an inactive structural component of NR has been reported in our laboratory in all mutants studied (12). In this paper we demonstrate that all structural components of NR protein are present in the membrane of all pleiotropic mutants studied (*chlA*, B, D and E) in an inactive form.

MATERIALS AND METHODS

The following strains were used: *E. coli* K-12 PA601 (356 in our collection) and its pleiotropic chlorate resistant mutants: *chlA*, *chlB* (4); *chlD* (unpublished) and *chlE* (17). Cells of each strain were grown anaerobically at 32°C in 2l of nutrient broth, buffered with Na-K phosphate 25mM, pH 7,2 containing 0.2 % glucose, 0.2% KNO_3 and 0.1mM Na-azide. After 15h, the cells were harvested by centrifugation and washed 3 times with K-phosphate buffer 66mM pH 7.2 containing 5mM $MgSO_4$, and disrupted by passage through a Yeda pressure cell with 1500psi. The crude extract was ultracentrifuged at 95,000xg for 75 min and the membrane fraction was pelleted. This fraction was suspended in Tris-HCl buffer 0.1M pH 8,3 at 5-7.6 mg protein/ml. A part of this fraction was heated to 60°C for 20 min (1), cooled rapidly, and left overnight at 4°C. The heated membrane fraction was recovered by another ultracentrifugation, as above. Heated and unheated membrane fractions were dissociated with 1% SDS, 50mM β-mercaptoethanol and 50mM EDTA at 20°C during 1h, as in (12). Samples of 0.1ml of each strain and each treatment (except WT-unheated for which 0.05ml were used), were applied on top of an SDS containig gel that had been prepared as in (12). The electrophoretic run was at 5mA/gel and lasted until the tracking dye (Bromophenol blue) reached the bottom of the gel. NR activity was assayed in all gels by a procedure previously described (12), and then stained with 1% Amido black in 7% acetic acid. The colored gels were scanned at 600nm in a gel scanner. Purified NR was obtained by the method of Lund & DeMoss (18) slightly modified. This protein was treated with dissociating agents in the same manner as the membrane fractions. 51 µg of treated and untreated samples were applied on SDS gels, as above.

RESULTS

a) In order to identify the components in which the purified NR protein dissociates, native and SDS-mercaptoethanol-EDTA pretreated NR were run in our PAGE-SDS system. The resulting pattern is shown in Fig. 1.

In this system, both, pretreated NR and NR without previous treatment dissociate in several protein bands and partially maintain their activity. Moreover, in the later case, there are three NR activities which migrate at R_f 0.08 (nr_3), 0.13 (nr_2) and 0.26 (nr_1). The later is the most important in protein content and NR activity, and it is the only band of activity present in the gel of pretreated NR, under the conditions used. There is a protein band which corresponds to the nr_1 band of activity, and is be denominated BmA.

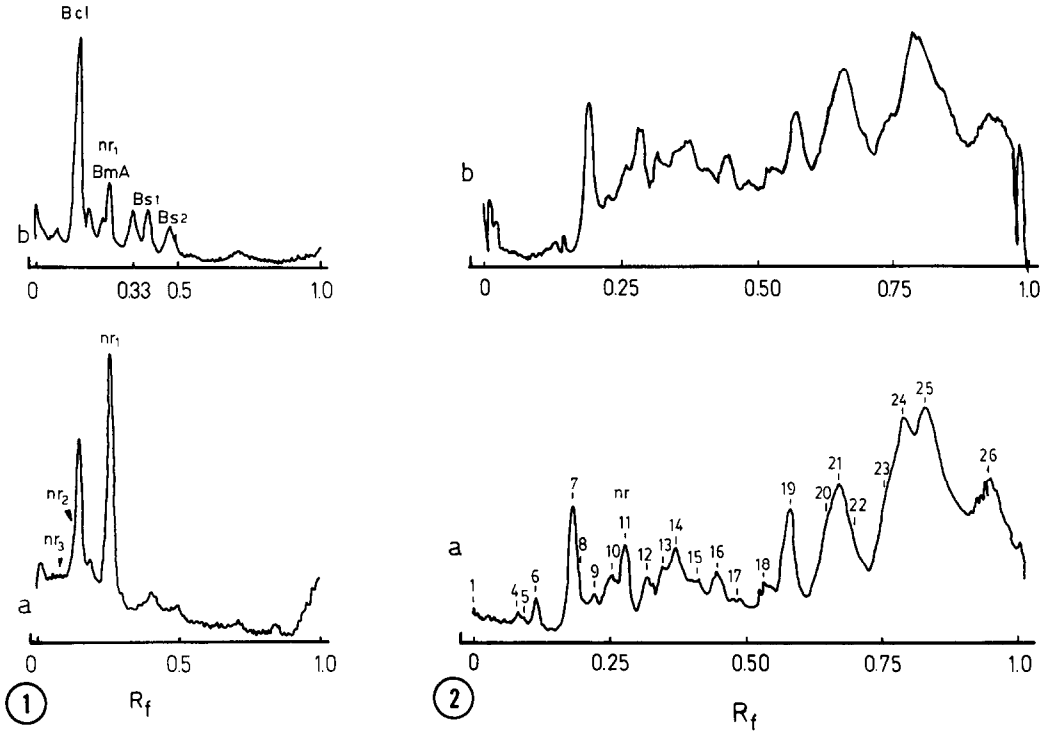

Fig. 1: Gel profiles for PAGE-SDS of purified NR. a, untreated NR and b, SDS-β-mercaptoethanol-EDTA pretreated NR (see Materials and Methods). nr_1, nr_2 and nr_3 indicate NR activities.

Fig. 2: Gel profiles for PAGE-SDS of the WT-membrane fraction pretreated with SDS-β-mercaptoethanol-EDTA. a, unheated membrane fraction and b, heated (60°C, 20 min.), membrane fraction (see Materials and Methods). nr indicates NR activity.

Another important protein band is Bc1, which migrates variably between R_f 0.12-0.19, depending on protein concentration. This band does not have activity, however, upon dissociating treatment, its protein content increases while that of BmA decreases (Fig. 1).

The bands that migrate faster than BmA are probably subunits of NR (Bs1 and Bs2) except band 0.33 which appeared only in the first run.

The other NR activities (nr_2 and nr_3) correspond to bands with low protein contents.

b) Fig. 2 shows the distribution of protein bands which results from running a WT membrane fraction in our PAGE-SDS system. In the unheated membrane fraction (Fig. 2a) there are 24 discrete bands. Fig. 2b shows the band profile for the heat-treated WT membrane fraction. Band 25 is evidently lost in the later case, which contains twice as much protein as the former. This band will be denominated BpP.

Table 1: Band distribution for heated (h) and unheated (uh) membrane fractions of WT, chlA, chlB, chlD and chlE. Numbers indicate R_f. + and - indicate presence and absence of any band, respectively.

Band N°	WT		chlA		chlB		chlD		chlE	
	uh	h	uh	h	uh	h	uh	h	uh	h
1	0.01	+	-	-	-	+	-	-	+	+
2	--	-	0.07	+	+	-	-	-	-	-
3	--	-	0.08	+	+	-	+	-	+	+
4	0.09	+	+	+	+	+	+	+	+	+
5	0.10	+	+	+	+	+	+	+	+	+
6	0.12	+	+	+	+	+	-	-	+	+
7	0.19	+	+	+	+	+	+	+	+	+
8	0.20	+	+	-	+	+	-	-	+	+
9	0.22	+	+	+	+	+	+	+	+	+
10	0.25	+	+	+	+	+	+	+	+	+
11	0.28	+	+	+	+	+	+	+	+	+
12	0.31	+	+	+	+	+	+	+	+	+
13	0.34	+	+	+	+	+	+	+	+	+
14	0.36	+	+	+	+	+	+	+	+	+
15	0.40	+	+	+	+	+	+	+	+	+
16	0.44	+	+	+	+	+	+	+	+	+
17	0.47	+	+	+	-	+	+	+	+	+
18	0.52	+	+	+	+	+	+	+	+	+
19	0.56	+	+	+	+	+	+	+	+	+
20	0.62	+	+	+	+	+	+	+	+	+
21	0.65	+	+	+	+	+	+	+	+	+
22	0.68	+	-	+	-	+	-	+	-	-
23	0.73	+	+	+	+	+	+	+	+	+
24	0.76	+	+	+	+	+	+	+	+	+
25	0.81	-	-	-	-	-	-	-	-	-
26	0.91	+	+	+	+	+	+	+	+	+

NR activity migrates in band 11 in the unheated fraction and there is no activity in the heated sample. This activity corresponds to nr_1 and therefore, band 11 corresponds to BmA.

If we compare the protein patterns of WT membrane fractions (Fig. 2) and NR (Fig. 1), it is evident that band 7 corresponds to Bc1 and bands 15 and 17 correspond to Bs1 and Bs2 respectively.

c) Table 1 summarizes the band distribution pattern in membrane fractions of WT and pleiotropic chl^r mutants (A,B,D,E.) in the same PAGE-SDS system. Results of heated and unheated samples are shown for all membrane fractions. In general, in our PAGE-SDS system, the pattern of slow migrating bands (1 to 6) is quite irregular, however, there are bands such as 6 of the WT which clearly diminish by heating. Band 11 (BmA) is present in heated and unheated membrane fractions of pleiotropic mutants in similar concentrations, but is inactive in all cases. Band 7(Bc1) is also present in all mutant strains examined, however, the ratio of protein quantity in Bc1 to BmA, in unheated membranes, is 2.5 for the WT, 7.6 for chlA, 12.4 for chlB, 10.8 for chlD and 11.0 for chlE. Bands 15 and 17 are present in all cases except in the gel of unheated membrane

fraction of chlB. The most conspicuous result is the absence of band 25 (BpP) in gels of heated and unheated membrane fractions of all pleiotropic mutants. Band 22 has a singular behaviour in most mutants since it appears only after heating the samples.

DISCUSSION

Under the conditions used for these experiments,NR retains its activity in the presence of SDS. This is noteworthy since it permits the detection of the active protein band in an SDS-containing gel. This had been previously observed (12,19). Moreover, purified NR exhibits up to three bands of activity when applied on such gel. These bands may be the product of associations and/or dissociations of a unique form of NR, thus resulting in activities with differences in electrophoretic migration (19).

The four major protein bands, (Bc1, BmA, Bs1, Bs2) which become evident upon the electrophoretic run of NR, are present in the membrane fractions of WT and the pleiotropic chl^r mutants. BmA is thought to be the minimal sized NR activity. Bc1 may possibly be an inactive complex of NR protein. This is supported by three facts: first, a relative increase of Bc1 in the membrane fractions of pleiotropic mutants, second, the increase in protein content of Bc1 when BmA decreases after dissociation of purified NR, and third, that Bc1 accumulates MVH during the activity assay in the gel (unpublished results). We think that Bc1 may not be a physiological component of NR, but it may be representative of a physiological characteristic of each NR. Bs1 and Bs2 are thought to be subunits of NR.

BpP must be a constituent of the NR system since it is absent in all pleiotropic mutants but it is not present in purified NR, therefore not necessary for MVH-NR activity. Thus BpP could be the cytochrome b which is a necessary component of the NR electron transport chain, or another component of the same system, which dissociates from the NR complex upon SDS or heat treatment. This interpretation agrees with previous studies (20). Contrary to previous results (13-16), the membrane fractions of all pleiotropic mutants have the NR structural protein. Although BmA is inactive in all mutant membrane fractions, it is present in all cases, as previously reported (12). The differences between these and previous results may be explained in two ways: first, mutants may not be comparable if they were selected by different methods or if they fall in different complementation groups in each loci, and, second, the results obtained may depend on the method of analysis used (21).

From the presence of the major NR components in all our mutants,and contrary to previous conclusions (8,9,13), we conclude that the cytoplasmic membrane of chlA, B, D, and E is not fundamentally altered and that the pleiotropic chl^r

loci studied may play a role in the processing and incorporation in the membrane of a common Molybdenum cofactor that activates NR and FDH (12) rather than having anything to do with the synthesis of NR structural protein or a respiratory particle. However, the disappearance of BpP and the increase of Bcl in the mutants could indicate that there is another type of structural alteration, such as in quaternary structure. We propose that NR structural protein is present in the cytoplasmic membrane of pleiotropic chl^r mutants (A,B,D,E,) in a form that is structurally and functionally different to the WT enzyme, i.e. an oligomeric structure which is different from that of the WT (22) and without physical and functional association with the anaerobic electron transport chain. This would mean that the pleiotropic chl^r loci are indirectly involved in the assembly of NR respiratory particle.

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