ALTERATION OF RESPIRATORY PARTICLES BY MUTATION IN *ESCHERICHIA COLI* K 12

Edgard AZOULAY, Juan PUIG and Francis PICHINOTY
Laboratoire de Chimie Bactérienne, CNRS, Marseille, France

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Piéchaud *et al.* (1967) have recently described a method for isolating a class of mutants based on their ability to grow on nutrient medium containing $KClO_3$. The mutation (*chl*-r) has been mapped (Puig *et al.* 1967) and characterized as pleiotropic because of the simultaneous loss of several enzymatic activities : nitrate and chlorate-reductases (both associated with nitrate-reductase A) and hydrogen-lyase.

This type of mutant (*chl*-r) in some other species of *Enterobacteriaceae* loses, in addition to nitrate-reductase A and hydrogen-lyase activities, other oxidation-reduction activities not found in *E. coli* such as nitrate-reductase B, chlorate-reductase C and tetrathionate- and thiosulfate-reductase activities. All these mutants have retained their fumarate-reductase activity. These reductases which are wide-spread in the *Enterobacteriaceae* have several properties in common : 1) They have an important role in the anaerobic transfer of electrons ; 2) They are all co-repressed by oxygen although each is induced by a different substrate ; 3) They are attached to the cells' membranous structure and therefore are found in the particulate fraction of cell-free extracts.

In order to explain the pleiotropic effect of the mutation to chlorate-resistance (*chl*-r) and in particular the simultaneous loss of NO_3^- reductase and hydrogen-lyase activities in *E. coli* K 12 we have proposed an hypothesis that any mutation which causes a profound alteration either of the structure of the elements constituting the particles, or of the assembling mechanism of these elements, will produce an effect identical to the one we have described.

This report describes a method by which we show that these activities are associated with membranous structures of a certain size. This method also allows us to gauge the extent of the alteration provoked by the mutation at the structural level.

METHODS AND EXPERIMENTAL RESULTS

In this study the strains we have used are the wild Hfr P4x *Escherichia coli* K 12 (*chl*-s), and its mutant 376 (*chl*-r) which is nit$^-$ gas$^-$; *Proteus vulgaris* A. 232 and its mutant (*chl*-r) which is nit$^-$, hydrogen-lyase$^-$, tetrathionate red$^-$, thiosulfate red$^-$.

The bacteria were grown anaerobically in complex medium containing glucose and the substrate of the reductase to be studied (nitrate in the case of nitrate-reductase, etc.).

Crude extracts were made by breaking cells suspended in tris buffer (0.01 M pH 7,8) - magnesium acetate (0.014 M) - KCl (0.06 M) in a French press. Bacterial debris was removed by centrifugation at 18 000 g for 20 minutes.

The activities of nitrate, tetrathionate and thiosulfate-reductases as well as of fumarate-reductase, which is present in both wild and mutant strains, were measured by sensitive and specific manometric techniques (Pichinoty 1966). Unfortunately we have not been able to assay hydrogen-lyase activity because the method usually used is not sensitive enough and in addition gives unreliable values when applied to extracts.

1 - In a series of preliminary experiments we have shown that the enzymatic activities affected by the mutation are attached to the bacterial membrane. This was done by separating the cytoplasmic proteins from "osmotically sensitive sphere" preparations of *E. coli* by the technique of Sistrom (1958) as modified by Kolber and Stein (1966) ; no nitrate-reductase activity was found. However this activity was recovered intact in the "membranes" prepared by the method of Kaback and Stadtman (1966).

2 - In another series of experiments the crude extract was centrifuged in a Spinco model L for 30 minutes at 110 000 g. The sedimented particles were washed twice and analyzed on a linear sucrose gradient according to the technique of Martin and Ames (1961), slightly modified : the particle preparation (3 to 5 mg of protein) was layered on a 4 ml gradient 20 to 80 % (w/v) of sucrose (purest grade), and centrifuged at 39 000 rpm in the SW39L rotor for 5 hours. Two drop fractions (0.13 ml) were drawn to which 0.5 ml of buffer were added before measuring nitrate-reductase activities as a function of protein content. Figure 1 shows that the particulate proteins are distributed into several groups (curve 1) and the nitrate-reductase activity of this

organism is concentrated in one symmetrical peak (curve 3).

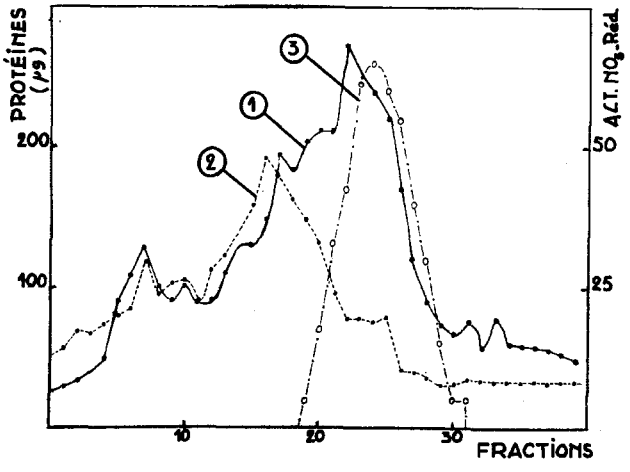

Fig. 1 - Comparison of sedimentation profiles on sucrose gradient of particle preparations of *E. coli* K 12 grown on KNO_3. (1) Wild-type. (2) Mutant (*chl*-r) 376. (3) Nitrate-red activity of the wild-type. Ordinates-Left, protein in µg/fraction measured by the method of Lowry. Right, nitrate-reductase activity expressed as µl H_2 consumed/5 min./ 0.1 ml of a fraction.

This sedimentation profile is perfectly reproducible for different particle preparations from *E. coli*. Therefore we have been able to derive a method of differential centrifugation to achieve a partial purification of nitrate-reductase. Fractions 20-30 of the sucrose gradient contain the major part of the nitrate-reductase activity (60 % of crude extract activity) and 2 % of the total protein ; so the purification is 22-fold. The sedimentation constant of these particles as estimated by using the equations of Martin and Ames (1961) is about 130 S.

3 - We have applied this method to particle preparations made under the same conditions from cells of a wild strain of *Proteus vulgaris* grown anaerobically in the presence of nitrate, tetrathionate, or

thiosulfate. We have found that the three reductases, all of which are lost in the chlorate-resistant mutants (*chl*-r) of this bacterium, sediment at the same rate.

On the other hand, fumarate-reductase, another particulate enzyme found in *E. coli* and *P. vulgaris* but which is not lost in the chlorate-resistant mutants, sediments with a peak different from that which has the nitrate-reductase activity (fractions 6-12).

4 - A particle preparation of mutant 376 (*chl*-r) of *E. coli* grown anaerobically in the presence of KNO_3 was analyzed on a sucrose gradient prepared as before (Figure 1, curve 2). A comparison of the sedimentation profile of this preparation with that of the wild strain (curve 1) shows that the peak associated with "reductases" is absent in the chlorate-resistant mutant. This result is in accord with particulate protein determinations ; that is, for the same dry weight of cells grown in the same conditions we recover 7 % less total particulate protein in the mutant than in the wild.

Identical sedimentation profiles have been obtained for the mutants (*chl*-r) of *P. vulgaris*.

DISCUSSION

Our observations show that the nitrate-reductase-bearing particles come from the cytoplasmic membrane. Centrifugation of particle preparations of the wild-type *E. coli* on a linear concentration gradient of sucrose allows the separation of nitrate-reductase activity with a a 22-fold purification. It effects at the same time a separation of particles which might in our opinion be considered homogeneous. The analysis of sedimentation profiles shows : 1) In extracts of two species of *Enterobacteriaceae* all the enzymatic activities lost in the "pleiotropic" mutation produced by $KClO_3$, are situated in the same symmetrical peak. 2) In the mutants (*chl*-r) the loss of the above activities is always accompanied by the disappearance of that peak.

This work confirms our original hypothesis that the mutation drastically changes one species of membrane particles, but it tells us nothing of the nature of the alteration. However we can consider several possibilities. The mutation could cause an alteration of a structural protein whose presence and integrity are necessary for several enzymatic activities and for some morphological unit of the cytoplasmic membrane. It is also possible that this mutation affects an enzyme system which

assembles the constituent elements of the unit. In any case, it seems that the mutation has not caused modifications of the structures of the reductases themselves.

If this model of a structural protein and of an enzyme catalyzing the assembly of particle elements is correct, we should be able to find many genes controlling the chlorate-resistant phenotype.

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