RECONSTITUTION OF ENZYMATICALLY ACTIVE PARTICLES FROM INACTIVE SOLUBLE ELEMENTS IN ESCHERICHIA COLI K 12

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In E. coli and Proteus vulgaris, nitrate-reductase activity has been localized in membrane-associated particles that are relatively homogeneous [1]. These particles have been separated from cell-free extracts by centrifugation in a linear sucrose gradient 20-80 % by weight.

Two pleotropic and genetically distinct groups of chlorate-resistant mutants have been reported in E. coli K 12 : chl A$^-$ and chl B$^-$ [2]. They are defective for several enzymatic activities specific to anaerobic respiration [3,4] ; that is, in becoming chlorate-resistant, they have lost nitrate-reductase, hydrogen lyase, and chlorate-reductase activities. This pleotropic effect can be explained by alterations either to the structure of the components of the particles on which these enzymes are localized, or to the mechanism of assembling these components.

Biochemical studies of mutants chl A and chl B in this laboratory, have resulted in the in vitro reconstitution of "structures" from soluble protein elements. This reconstitution brought about the simultaneous appearance of nitrate reductase activity

MATERIALS AND METHODS

Organisms : Escherichia coli K 12, PA 601 (Pasteur Institute) (n°356 of our collection) and its two ClO_3^- resistant mutants. The first mutant, 356_{15} is altered in gene chl A which maps in the gal-biotin region, and the second, 356_{24}, is altered in gene chl B which maps in the mtl region [4,2].

Cells are grown anaerobically at 32° C as previously described [3].

Suspended washed cells are broken in a French press and then centrifuged at 10,000 rpm for 15 minutes to eliminate cell debris. These crude extracts are recentrifuged at 170,000 x g for 90 minutes to sediment the particulate fraction. The "supernatant" referred to in this article is this 170,000 g supernatant.

Nitrate reductase activity was measured manometrically by rate of hydrogen consumption[5] The system contains H_2, hydrogenase, benzyl viologen, enzyme extract, and KNO_3. Enzyme activity is expressed as μmoles NO_3^- reduced per hour per mg protein. Cytochromes in the crude extracts were determined by the Fujita method [6]. Protein was assayed by the method of Lowry et al.[7].

RESULTS

Neither of the two mutants, chl A⁻ et chl B⁻, has nitrate reductase activity when cultivated anaerobically in the presence of nitrate. When the cell free extracts of these two mutants are mixed in equal proportions under certain, well defined conditions, nitrate reductase activity is reconstituted. The enzymatic activity thus obtained after one hour of incubation at 32° C is equal, on the average, to 11 units per mg of total protein (table I) and represents one tenth of the nitrate reductase activity measured in crude extracts of the wild type under the same conditions. This reconstitution, which we call complementation, is possible only with cell-free preparations incubated at 32° C in the absence of oxygen and in buffered medium (Tris 0.04 M pH 7.6). The "supernatant" extracts "complement" under the same conditions and sometimes give activities of the order of 19 units per mg of protein. There is no complementation if the two preparations are separated by a membrane impermeable to proteins. When the mutants are cultivated in the absence of inducer

TABLE I

In vitro reconstitution of nitrate-reductase activity from extracts of E. coli K 12 chl A and chl B. Incubation one hour at 32° C.

Anaerobic cultures	Cell-free preparations Chl A	Chl B	Nitrate reductase activity reconstituted (units)
Without KNO_3	Crude extract	Crude extract	3.6
With KNO_3	Crude extract	Crude extract	8-15
	Supernatant	Supernatant	7.5-19
	Particles	Particles	0
	Supernatant	Particles	0
	Particles	Supernatant	0

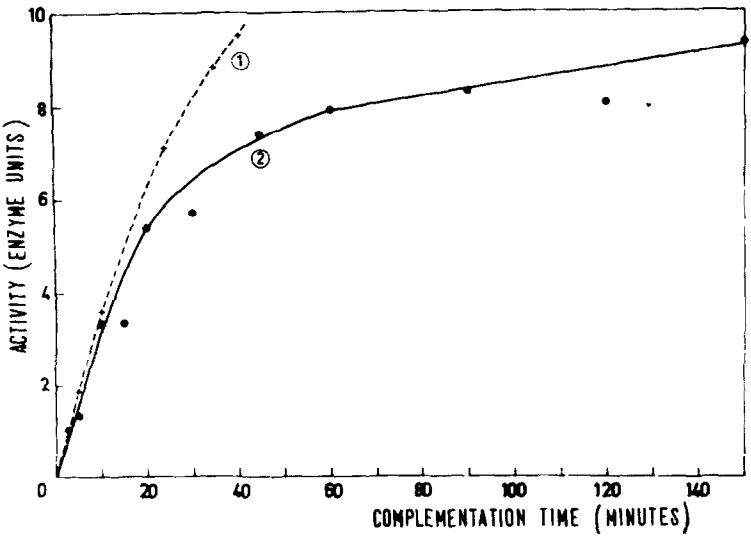

Fig. 1 Kinetics of the appearance of nitrate reductase activity. 1) crude extracts. 2) 170,000 g supernatants of mutants chl A and chl B. Each system contains 800 µg of protein from each of the two extracts, 0.04M Tris pH 7.6 ; incubation is carried out at 32° C, in a vacuum. The complementation is stopped by introducing air into the systems and chilling to 0° C. Enzymatic activity is then measured and expressed in nitrate reductase units.

complementation of their extracts gives a lower nitrate reductase activity that probably corresponds to the baseline activity found in the wild strain when it is cultivated in the absence of nitrate.

Figure 1 shows that nitrate reductase activity increases as a function of incubation time. We therefore define the complementation rate as the increase in nitrate reductase activity in units per minute per mg of total protein in the mixture. These rates are markedly higher in extracts made from cells grown in the presence of KNO_3 (Table 2). When the 170,000 g supernatants are used, the complementation rate is 0.47 units per minute at the beginning and diminishes progressively with time. Although the complementation rate in crude extracts is lower at the beginning, it slows down less abruptly. The total nitrate reductase activity synthesized from crude extracts is also lower (Table I).

- Particle formation by complementation .

Nitrate reductase activity is significant only in membranous structures. This is shown by unsuccesful attempts to solubilize native particles[8] Moreover, the

TABLE II

Complementation rates of cell-free preparations of mutants chl A⁻ and chl B⁻ of E. coli K 12.

Mutants cultivated anaerobically	Cell-free preparation	Complementation rate	
		In the first minutes of incubation	After 45 minutes of incubation
With KNO_3	- Crude extracts	0.32	0.21
	- 170,000 g supernatants	0.47	0.10
Without KNO_3	- crude extracts	0.06	0.10

restoration of nitrate reductase activity by complementation of "supernatants" of mutants chl A⁻ and chl B⁻ is accompanied by de novo formation of particles which, after 15 minutes incubation, can be harvested by centrifugation at 170,000 g for 15 minutes. The new pellet is made up of large particles that are visible in the light microscope. The particles are transparent, and have nitrate reductase activity. When the incubation is allowed to continue beyond 15 minutes, the large particles rapidly disappear with concomitant appearance of much smaller particles that are not visible in the light microscope.

The newly formed particles are washed and resuspended in Tris buffer 0.04M pH 7.6, then layered on a linear sucrose gradient 20 to 80 %, and centrifuged 5 hours at 39,000 rpm^4. A major fraction of these particles behave like native particles. Another, lighter fraction, also having nitrate reductase activity, corresponds to the above described large particles. The supernatant of the complementation mixture also contains nitrate reductase activity. This activity would correspond to a soluble nitrate reductase which does not exist in the E. coli K 12 wild type. It should be emphasized that 1) after 2 hours of complementation there is no appreciable increase in nitrate reductase activity, 2) in the course of complementation, 15 to 20 % of the soluble protein passes into a particulate state, 3) the newly formed particles, like the native particles, contain 0.25 mμmoles of a b_1 type cytochrome per mg of protein, and 4) complementation of supernatants of extracts of the two mutant cultivated anaerobically, but in the absence of nitrate,

also results in particle formation. The percentage of protein that becomes particulate in this case is identical to that obtained with supernatants of the same cells cultivated in the presence of nitrate.

- Properties of complementation.

Certain experimental conditions are required for the simultaneous appearance of nitrate reductase activity and reconstitution of particles. For maximum efficiency, equal quantities of the mutant extracts (chl A^- and chl B^-) are mixed in a Thunberg tube and incubated in a vacuum or under an atmosphere of hydrogen or nitrogen. The reaction depends on the conditions of preparing the cell-free extracts, and in particular, on the nature, molarity, and pH of the buffer used ; 0.04 M phosphate pH 7.2 and 0.04 M Tris pH 7.6 give the best results. A 2.5-fold increase of their molarities causes an activity loss of the order of 80 %. There is no complementation if the extracts are preheated at 100° C for 10 minutes. The same is true if only one of the two is heated . The conservation of extracts is very difficult. If the extracts are left in contact with air, they must be used within 20 hours, whereas if they are kept in a vacuum, they remain active up to 3 days. After this interval, there is an activity loss of 87 %. Freezing also destroys complementation activity. The maximum rate of complementation occurs in a narrow range of pH 7 - 7.6. At 0° C and at 60° C there is no reaction. Among the strong inhibitors of the reaction are divalent ions, oxygen, and sulfhydryl groups, especially cysteine. Nitrate is the only activator found until now.

The nitrate reductase activity in formed particles is insensitive to ClO_3^- ion, and inhibited by NaN_3 and KCN. Like the native nitrate reductase, it is thermoresistant. Heating at 62° C for 35 minutes results in 50 % inactivation. The K_m for NO_3^- (3.1×10^{-4}M) is lower than that of the native enzyme ($K_m = 7.8 \times 10^{-4}$M). These formed particles catalyse ClO_3^- reduction under the same conditions as those used for nitrate reduction. The K_m for chlorate is 2.5×10^{-3} M.

DISCUSSION

The complementation described differs from such other phenomena as the assembling of "multienzyme complexes" that bear other enzyme activities [9,10]. In the mixture of cell-free preparations of the two mutant types studied, there are in a soluble state, all the factors that exist in the particulate state in the wild type.

These results suggest that this reconstitution is under the control of a protein factor f_a which is the product of the chl B gene and which is altered in the mutation to chlorate resistance.

The appearance of nitrate reductase activity concurrent with an aggregation of several elements opens a vast field of investigation of the nature and functioning of the active site(s) of particulate enzymes.

These pleotropic mutants of *E. coli* K 12 are important because they provide material containing, in a loluble state, all the elements necessary for the synthesis of particles that are, in fact, an integral part of the cytoplasmic membrane.

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