***Escherichia coli* Molybdoenzymes Can Be Activated by Protein FA from Several Gram-negative Bacteria**

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Six Gram-negative bacteria (*Klebsiella pneumoniae*, *Erwinia chrysanthemi*, *Proteus vulgaris*, *Serratia marescens*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa*) were shown to contain an FA-type protein capable of activating aponitrate reductase, apotrimethylamine *N*-oxide reductase and apoformate dehydrogenase of *Escherichia coli*. Protein FA activity was highest in *Erwinia chrysanthemi* and lowest in *Pseudomonas aeruginosa*. All the species also contained the low- M_r (≤ 1500) heat-resistant material previously reported to be necessary for the protein-FA-dependent activation of *E. coli* *chlB* nitrate reductase.

INTRODUCTION

Respiratory nitrate reductase (EC 1.7.99.4) is found in many Gram-positive and Gram-negative bacteria (Pichinoty *et al.*, 1969), and the enzyme has been characterized in several different genera, e.g. *Micrococcus* (Forget, 1971; Rosso *et al.*, 1983), *Klebsiella* (Van't Riet & Planta, 1975), *Escherichia coli* (Enoch & Lester, 1975; MacGregor, 1975), *Pseudomonas* (Radcliffe & Nicholas, 1970), *Proteus* (Oltman *et al.*, 1976) and *Bacillus* (Kiszkiss & Downey, 1972). However, assembly of the enzyme has been studied mainly in *E. coli*, partly because of the advanced knowledge of the genetics of this bacterium.

The isolation of chlorate-resistant (*chl*) mutants of *E. coli* by Puig *et al.* (1967) provided a means for studying the assembly of active respiratory nitrate reductase and other molybdoenzyme complexes since, by incubating a mixture of inactive extracts of *chl* mutants, such as *chlA* and *chlB*, nitrate reductase activity is obtained (Azoulay & Puig, 1968). This *in vitro* complementation was shown to depend on the contribution, by each extract, of activities that are defective in the other extract. In this way, components involved in the biosynthesis of active nitrate reductase, namely aponitrate reductase subunits, proteins FA and PA, the molybdenum cofactor and low- M_r substances distinct from the molybdenum cofactor, have been identified, (Rivière *et al.*, 1975; Grillet & Giordano, 1983; Rolfe & Onodera, 1972; Glaser & DeMoss, 1971; Johnson & Rajagopalan, 1987; Boxer *et al.*, 1987). In the case of complementation between extracts of *chlA* and *chlB* mutants, the *chlA* extract provides protein FA which is lacking in *chlB* mutants and the *chlB* extract provides protein PA which is absent from the *chlA* extract.

The addition of purified protein FA to extracts of *chlB* mutants leads to the activation of nitrate reductase (Rivière *et al.*, 1975). Recently, we have described a quantitative assay for protein FA activity and have shown that this activity is constitutively expressed in *E. coli* (Low *et al.*, 1988). Since protein FA is actively expressed in all classes of *chl* mutants except *chlB* it is probably the product of the *chlB* gene.

We report here the *in vitro* activation of nitrate reductase in an *E. coli* *chlB* mutant by extracts of six Gram-negative bacteria. All of them contained protein FA activity.

Table 1. Nitrate reductase activities in crude extracts of bacteria

Crude extracts were prepared from cells grown anaerobically with nitrate as described in Methods.

Strain	Source	Relevant genotype	Nitrate reductase activity [$\mu\text{mol NO}_3^-$ reduced min^{-1} (mg protein) $^{-1}$]
<i>Escherichia coli</i> PA601	This laboratory	Wild-type	0.9
<i>Escherichia coli</i> AP24		<i>chlB</i>	≤ 0.001
<i>Escherichia coli</i> AP15		<i>chlA</i>	≤ 0.001
<i>Klebsiella pneumoniae</i> 52.145	Institut Pasteur, Paris, France		1.79
<i>Serratia marcescens</i> 5389			0.97
<i>Proteus vulgaris</i> 5860			1.14
<i>Pseudomonas aeruginosa</i> PAO25	A. Lazdunski*	Wild-type	0.3
<i>Erwinia chrysanthemi</i> 3739	J. P. Chambost*		0.920
<i>Salmonella typhimurium</i> LT2			1.12
<i>Salmonella typhimurium</i> LCB1	M. Chippaux	<i>chlA</i>	≤ 0.003
<i>Salmonella typhimurium</i> LCB69		<i>chlB</i>	≤ 0.001
<i>Salmonella typhimurium</i> LCB8		<i>chlE</i>	≤ 0.006

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METHODS

Growth of bacteria and preparation of subcellular fractions. The bacteria used, listed in Table 1, were grown in the medium of Giordano *et al.* (1978), which contain glucose, yeast extract (Difco), Bacto-peptone (Difco) (2 g l $^{-1}$ of each) and 1 μM -sodium molybdate. Nitrate reductase was induced with 1 g KNO $_3$ l $^{-1}$. Sodium molybdate in the medium was replaced by 10 mM-sodium tungstate as indicated. For anaerobic growth, closed vessels were filled almost to the top with medium and incubated without shaking. For aerobic growth, cultures were incubated in 2 litre Fernbach flasks containing 500 ml of medium with shaking. Cultures were grown at 32 °C and harvested at the mid- to late-exponential phase. The bacteria were washed twice by centrifugation with 50 mM-Tris/HCl, pH 7.6, containing 1 mM-benzamidine.HCl and subsequently used immediately or stored at -80 °C.

Bacteria were ruptured in a French pressure cell at 69 MPa and cell debris was removed by centrifugation at 18000 g for 25 min to give the crude extract. Since the latter was very concentrated (20 mg protein ml $^{-1}$) it was necessary to centrifuge it twice at 120000 g for 90 min to obtain a soluble fraction free of membrane vesicles. All operations were done at 4 °C. Heat-treated extracts were prepared as described previously (Boxer *et al.*, 1987). Samples (1 ml; 30 mg protein) of the crude extract were boiled for 8 min under nitrogen. Suspensions were centrifuged at 18000 g for 15 min to yield a clear supernatant which was used immediately.

Activation of nitrate reductase. This was done according to Azoulay *et al.* (1969) by incubating crude extracts of *E. coli chlB* with a source of protein FA (soluble fraction or purified FA). Incubations were done under N $_2$ at 32 °C for the times indicated and the reaction stopped by cooling to 0 °C. Soluble fractions prepared for testing protein FA activity were made from cells grown aerobically without nitrate. One unit of protein FA activity produces a nitrate reductase activity of 1 nmol NO $_3^-$ reduced min $^{-1}$ per min of incubation period in the presence of excess *chlB* soluble fraction under the conditions described (Low *et al.*, 1988).

Heat treatment of protein FA. Samples (5 μl) of partially purified protein FA (approximately 700 protein FA units ml $^{-1}$) were boiled in a water-bath for 5–200 s; 50 μl (1 mg protein) of *chlB* strain (AP24) soluble fraction was then added and the mixture made up to 100 μl with 50 mM-Tris/HCl, pH 7.6. Nitrate reductase was assayed after incubation at 32 °C for 10 min.

Trypsin treatment of protein FA. Samples (5 μl) of partially purified protein FA (as above) were incubated with 10 μl trypsin (1 $\mu\text{g ml}^{-1}$; bovine pancreas type III, Sigma) at 30 °C for 30–200 s. Trypsin was inactivated by the addition of 10 μl soya-bean trypsin inhibitor (10 mg ml $^{-1}$; type II-S, Sigma). To this mixture 50 μl of the soluble fraction of *chlB* was added, the volume made up to 100 μl with buffer and the mixture incubated at 30 °C for 10 min before nitrate reductase was assayed. Controls were done by incubating trypsin with its inhibitor prior to protein FA addition, followed by the *chlB* fraction.

Enzyme assays. Nitrate reductase activity was measured by the spectrophotometric method of Jones & Garland (1977), based on the oxidation of reduced benzyl viologen, coupled to the reduction of nitrate to nitrite. Trimethylamine *N*-oxide reductase activity was measured with 10 mM-trimethylamine *N*-oxide instead of nitrate. Benzyl-viologen-linked formate dehydrogenase activity was assayed spectrophotometrically by following the reduction of benzyl viologen coupled to the oxidation of formate as described by Enoch & Lester (1975).

Table 2. Nitrate reductase activating ability of extracts of different bacteria

Nitrate reductase activities (expressed as $\mu\text{mol NO}_3^-$ reduced min^{-1}) were measured, after 30 min incubation at 32 °C under N_2 , in a mixture (1 ml) containing an excess of *E. coli chlB* crude extract (20 mg protein) and a limiting concentration of either soluble fraction (4 mg protein) or partially purified activating extract from the bacteria listed.

Strain	Nitrate reductase*	Column eluate	
		Activating activity†	Protein‡
<i>E. coli</i>	120	130	5.1
<i>Salmonella typhimurium</i>	48	50	3.4
<i>Proteus vulgaris</i>	80	120	3.5
<i>Erwinia chrysanthemi</i>	160	170	2.9
<i>Pseudomonas aeruginosa</i>	0	7	2.2
<i>Klebsiella pneumoniae</i>	90	80	4.9
<i>Serratia marcescens</i>	100	80	3.0

* Nitrate reductase activating activity of 100 mg soluble-extract protein of *E. coli chlB*.

† Total nitrate reductase activating activity recovered after gel filtration of 100 mg soluble fraction from the strain indicated.

‡ Total protein (mg) of active fractions after gel filtration on Sephadex G-75.

RESULTS

Nitrate reductase activity in Gram-negative bacteria

The presence of *E. coli* protein FA can be demonstrated by its ability to activate aponitrate reductase in extracts of *chlB* mutants. At zero-time such assay mixtures should contain little or no nitrate reductase activity. Therefore, to look for protein FA activity in different bacteria we chose species that were likely to have nitrate reductase activity only when grown anaerobically in the presence of nitrate and not when grown aerobically in the absence of nitrate (Table 1).

All strains used had benzyl-viologen-linked nitrate reductase activity when grown anaerobically with nitrate. *Klebsiella pneumoniae*, the most active strain, had six times the activity of *Pseudomonas aeruginosa*, the least active strain. Under aerobic conditions, nitrate reductase activity in all the bacteria tested was less than $0.06 \mu\text{mol nitrate reduced min}^{-1} (\text{mg protein})^{-1}$.

Protein FA activity in soluble fractions of bacteria

Soluble fractions of all bacterial strains tested activated aponitrate reductase in crude extracts of *E. coli chlB* (Table 2). Mixtures containing active nitrate reductase were centrifuged and supernatants passed through a 1.5 m column of Biogel A. Analysis of column fractions with antiserum developed against *E. coli* nitrate reductase showed that the activated enzyme originated from *E. coli* (Giordano *et al.*, 1980) (results not shown).

The activation process (Fig. 1) reached a plateau at an activity of $4.8 \mu\text{mol NO}_3^-$ reduced $\text{min}^{-1} \text{ml}^{-1}$. Activation rates depended on the source of the soluble fraction added to the *E. coli* extract. Extracts of *Erwinia chrysanthemi* and *Pseudomonas aeruginosa* were the most and least active, respectively (Fig. 1). Extracts of some of the bacteria, and particularly *Salmonella typhimurium*, when used in low concentration, showed a lag period before reaching their maximum activation rates (results not shown). With *Serratia marcescens* a lower plateau was attained.

These results indicate that one or several compounds in the soluble fraction of the bacteria tested can activate aponitrate reductase in *E. coli chlB*. They are thus similar to protein FA of *E. coli*.

Similarity to protein FA of the activating compound from different bacteria

M_r . As a first step towards characterizing the activator(s) in soluble fractions of the different bacteria, we estimated its apparent M_r by gel filtration. This procedure also gave a partial purification. Fractionation of soluble fractions of *E. coli* or its *chlA* mutant on Sephadex G-75

Fig. 1. Kinetics of nitrate reductase activation by the soluble fractions from different bacteria. *E. coli chlB* was grown anaerobically in nitrate-containing medium. The other bacteria were grown aerobically without nitrate. The activation assay mixtures (1 ml) contained *E. coli chlB* crude extract (20 mg protein) and the soluble fraction from another bacterium (*P. aeruginosa*, 10 mg protein; others 2.5 mg). $\square$, *Pseudomonas aeruginosa*; $\blacktriangledown$, *Salmonella typhimurium*; ∇ , *Serratia marcescens*; $\blacktriangle$, *Erwinia chrysanthemi*; $\circ$, *Klebsiella pneumoniae*; $\bullet$, *E. coli*; $\triangle$, *Proteus vulgaris*.

gave high yields of protein FA activity. In previous studies protein FA was purified about 20-fold (Rivière *et al.*, 1975; Low *et al.*, 1988). The apparent M_r of protein FA estimated by this method was about 18000 (Fig. 2) which is half that reported by Rivière *et al.* (1975).

Fractionation of the soluble fractions from cells of the different bacteria gave similar activity profiles consistent with a similar M_r for the active substances (Fig. 2). The yields ranged from 70 to over 100% of the activity applied to this column with 18 to 43-fold purification (Table 2). For *Proteus vulgaris*, the total amount of *chlB* nitrate reductase activating activity recovered from the column was significantly greater than that measured in the original supernatant, suggesting that this extract contains an inhibitor of the activation process. With the exception of *Pseudomonas aeruginosa* there was a less than 3.5-fold variation in protein FA specific activity among the six species tested (Table 2).

Sensitivity to heat or trypsin treatment. Partially purified protein FA of *E. coli* is very sensitive to heat. Its ability to activate nitrate reductase in *chlB* was destroyed by boiling for 25 to 30 s. Protein FA is also very sensitive to the action of trypsin: exposure to 0.1 mg ml^{-1} for 10 s caused 98% inactivation. The partially purified activators obtained from the six other species of bacteria were similarly sensitive to heat and to trypsin treatment suggesting that they too are proteins.

Activation of other molybdoenzymes by the nitrate reductase activator. Giordano *et al.* (1984) showed that protein FA of *E. coli* was able to activate precursors of trimethylamine *N*-oxide reductase (EC 1.6.6.9), (benzyl-viologen-dependent), formate dehydrogenase (EC 1.2.-.-) and nitrate reductase. Partially purified activators of the other bacterial species could activate these enzymes to levels similar to, or higher than, that found with protein FA of *E. coli* (Table 3).

Activation of *E. coli chlB* nitrate reductase as a function of activator concentration. The rate of nitrate reductase activation increased linearly as a function of the concentration of partially purified FA or other activator (Fig. 3). Activation rates varied depending on the source of the activator. For *Pseudomonas aeruginosa* relatively large amounts of the activator preparation were necessary to attain the activation plateau. Experiments with soluble fractions instead of

Fig. 2. Fractionation of protein FA activity in extracts of *E. coli* and *Salmonella typhimurium*. Soluble fractions (100 mg protein) obtained from wild-type cells of *E. coli* and *S. typhimurium* (a) or from various *chl* mutants of *S. typhimurium* (b) were applied to a Sephadex G-75 Superfine column (2.5 × 80 cm), equilibrated and eluted with 20 mM-Tris/HCl, pH 7.6, 0.25 M-NaCl, 1 mM-benzamidine at 4 °C. Samples of the fractions (0.2 ml) were incubated for 30 min with the crude extract of *E. coli chlB* (20 mg in 1 ml total volume). Protein: *E. coli* wild-type (Δ); *S. typhimurium* wild-type (▼); *S. typhimurium chlA* (▽); *S. typhimurium chlB* (▲). Nitrate reductase activity: *E. coli* wild-type (●); *S. typhimurium* wild-type (■); *S. typhimurium chlA* (○); *S. typhimurium chlB* (□).

Table 3. Activation of molybdoenzymes by protein FA from different bacteria

Complementation was done as described in Methods. Mixtures (0.5 ml) containing 0.2 mg partially purified protein FA and 4 mg crude extract of *E. coli chlB* grown under inducing conditions (Giordano *et al.*, 1984) were incubated for 90 min at 32 °C. Trimethylamine *N*-oxide reductase and nitrate reductase activities are expressed as nmol TMAO or NO₃⁻ reduced min⁻¹ ml⁻¹. Benzyl-viologen-dependent formate dehydrogenase activity is expressed as nmol formate oxidized min⁻¹ ml⁻¹.

Source of protein FA	Enzyme activity		
	Nitrate reductase	Trimethylamine <i>N</i> -oxide reductase	Formate dehydrogenase
No protein FA added	≤ 1	≤ 1	≤ 1
<i>E. coli</i>	880	320	124
<i>Klebsiella pneumoniae</i>	780	280	80
<i>Salmonella typhimurium</i>	860	320	68
<i>Proteus vulgaris</i>	920	520	144
<i>Erwinia chrysanthemi</i>	980	800	132
<i>Pseudomonas aeruginosa</i>	720	280	86
<i>Serratia marcescens</i>	280	140	60

partially purified preparations gave similar results. There was a linear relationship between the amount of *E. coli chlB* extract and activation of nitrate reductase. Activation stopped upon depletion of aponitrate reductase or any other required compounds in the *E. coli chlB* extracts (observed with crude as well as partially purified activator). These results show that a compound similar to protein FA of *E. coli* is present in soluble fractions of the various bacteria.

Fig. 3. Rate of nitrate reductase activation as a function of source and concentration of protein FA. Protein FA preparations from the soluble fraction of bacteria grown aerobically as described in Fig. 1 were purified by gel filtration on Sephadex G-75 Superfine. The assay mixtures contained 1 ml crude extract (20 mg protein) of *E. coli* *chlB* and various amounts of protein FA. Nitrate reductase activity was measured after 15 min of incubation. The rate of nitrate reductase activation is expressed in protein FA units as described in Methods. ▲, *Erwinia chrysanthemi*; ●, *E. coli*; ○, *Klebsiella pneumoniae*; ▼, *Salmonella typhimurium*; □, *Pseudomonas aeruginosa*; △, *Proteus vulgaris*.

Nitrate reductase activation by crude extracts of *chl* mutants of *Salmonella typhimurium*

Mutants of *S. typhimurium*, termed *chlA*, *chlB* and *chlE* by analogy with the *E. coli* genome (Casse *et al.*, 1972; Casse, 1973) were examined for protein FA activity (Fig. 2). All possessed a protein of about 18 kDa able to activate nitrate reductase of *E. coli* *chlB* with the exception of the *S. typhimurium* *chlB* strain. This confirms the existence of protein FA in *S. typhimurium* and shows that protein FA activity is absent only from *chlB* strains as has been shown for *E. coli* (Low *et al.*, 1988).

Presence of low- M_r factors necessary for activation

In *E. coli* one or more thermostable compounds of low M_r (≤ 1500), together with protein FA, are required for activation of *chlB* nitrate reductase (Boxer *et al.*, 1987), as shown by the inability of protein FA to activate *E. coli* *chlB* extract that has been depleted of low- M_r material by passage through a Sephadex G-25 column. Activation ability is restored by addition of a deproteinized (heat-treated) extract from the soluble fraction of the wild-type or *chl* strains. The proteins FA of the different bacteria studied here were also active in this system and gave comparable levels of activation (data not shown).

To see whether the various bacteria studied also contained the low- M_r material essential for *E. coli* *chlB* nitrate reductase activation, desalted *E. coli* *chlB* soluble fractions were incubated with *E. coli* protein FA, together with heated, clarified extract prepared from one of the other bacteria. All the bacteria tested contained the active fraction (Table 4). In all cases the active compound(s) had an apparent $M_r \leq 1500$ on a Sephadex G-50 column. Moreover, as for *E. coli*, the compound could be distinguished from the molybdenum cofactor by the fact that heated extracts of cells grown in the presence of tungstate, an antagonist of molybdate, were also active (Saracino *et al.*, 1986; Giordano *et al.*, 1987; Amy & Rajagopalan, 1979; Scott *et al.*, 1979).

Activation of nitrate reductase in extracts of *E. coli* *chlB* grown in the presence of tungstate

Heated, clarified extracts of *E. coli* contain the molybdenum cofactor which can, together with protein FA, activate nitrate reductase in extracts of *chlB* grown anaerobically in tungstate-containing medium (Saracino *et al.*, 1986). Protein FA preparations obtained from the different species were tested in this system and were found to be active, although *Pseudomonas aeruginosa* protein FA had a notably weaker activity (result not shown).

Table 4. Activation of *E. coli chlB* nitrate reductase by heat-treated fractions from different bacteria

Assay mixtures (0.5 ml) for nitrate reductase activation contained 0.2 mg partially purified protein FA from *E. coli*, 0.3 ml of one of the heat-treated fractions and either 4 mg protein from *E. coli chlB* soluble extract excluded by Sephadex G-25 (*chlB*; G-25) or 4 mg crude-extract protein from *E. coli chlB* grown in the presence of 10 mM-tungstate (*chlB*; tungstate). Heat-treated fractions of bacteria were prepared as described in Methods. Incubations were for 90 min at 32 °C. Nitrate reductase activities are expressed as percentages of the activity (860 nmol NO₃⁻ reduced min⁻¹ ml⁻¹) in an assay mixture containing 0.2 mg protein FA and 4 mg *E. coli chlB* crude extract.

Source of heat-treated extract	Nitrate reductase activity (%) after incubation with:	
	<i>chlB</i> ; G-25	<i>chlB</i> ; tungstate
No extract added	2	3
<i>E. coli</i>	78	80
<i>E. coli</i> *	3	2
<i>Salmonella typhimurium</i>	49	48
<i>Proteus vulgaris</i>	62	36
<i>Erwinia chrysanthemi</i>	64	55
<i>Pseudomonas aeruginosa</i>	27	38
<i>Klebsiella pneumoniae</i>	71	48.5
<i>Serratia marcescens</i>	Not determined	61

* No protein FA added.

In parallel with the above experiment, heat-treated extracts of the six species were tested for activity in a system containing protein FA from *E. coli* plus an extract of *E. coli chlB* grown in tungstate-containing medium. All extracts gave similar activation of nitrate reductase suggesting that they possessed active molybdenum cofactor (Table 4). This was confirmed by complementation of the aponitrate reductase in extracts of induced *Neurospora crassa nit-1* (Ketchum *et al.*, 1970). The identity of the active substance in heat-treated extracts with active molybdenum cofactor was further supported by analysis of Sephadex G-50 filtrates for activity in both the *N. crassa nit-1* and tungstate-grown *E. coli chlB* complementation assays. The elution profiles of the activities were indistinguishable from one another and were similar to that reported previously for *E. coli* (Saracino *et al.*, 1986). Clearly, molybdenum cofactor activity is present in heat-treated extracts of all the bacteria examined.

DISCUSSION

All the species examined contained protein FA similar to that of *E. coli*, molybdenum cofactor activity and the small, heat-stable material necessary for activation of *E. coli chlB* nitrate reductase.

Nitrate reductase activation by *in vitro* complementation of extracts of different *chl* mutants was first studied by Azoulay & Puig (1968). This work led to the discovery that proteins FA and PA were required for activation of molybdoenzymes (Rivière *et al.*, 1975; Grillet & Giordano, 1983). More recently, Boxer *et al.* (1987) showed that one or more thermoresistant compounds, distinct from the molybdenum cofactor, were required for activation of nitrate reductase.

Using aerobically grown cells as a source of protein FA in the activation system described previously (Rivière *et al.*, 1975; Low *et al.*, 1988), we have shown that a protein FA also exists in other Gram-negative bacteria, and that activation of molybdoenzymes in these bacteria requires protein FA along with the molybdenum cofactor and a thermoresistant compound(s). The molybdenum cofactor from the bacteria examined also functioned in the *in vitro* activation of an *E. coli* molybdoenzyme.

Comparison of the genetic maps of *E. coli* and *Salmonella typhimurium* reveals that the genes involved in chlorate resistance (*chlA*, *chlB*, *chlE*) are in the same relative positions (Bachmann,

1983; Sanderson & Roth, 1983). Until now, there was no indication that *chl* genes localized in the same position in these two organisms encoded proteins with the same functions. Our results show a correlation in *S. typhimurium* between protein FA activity and the *chlB* locus, as has been shown for *E. coli*. Since protein FA in *E. coli* is almost certainly the product of the *chlB* gene (Low *et al.*, 1988) the same presumably applies to *S. typhimurium*.

Rivière *et al.* (1975) reported that, after several purification steps, protein FA of *E. coli* had an M_r of 35000. Their purified protein tended to polymerize. The one purification step we used (gel filtration) did not lead to polymerization and yielded a value of 18000 for the M_r . We assume that the value reported earlier was that of the dimeric form.

The rate of nitrate reductase activation by partially purified protein FA is directly related to the protein FA activity present (Low *et al.*, 1988). However, since all the parameters of activation are not yet known, the possibility that the active fraction contains compounds other than protein FA which play a role in activation cannot be eliminated. In this respect, it should be noted that nitrate reductase of *Klebsiella pneumoniae* differs significantly from that of *E. coli* in the configuration of its subunits in the membrane (Abraham *et al.*, 1981).

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