PARTICIPATION OF CYTOCHROME *b* TO THE IN-VITRO RECONSTITUTION OF THE MEMBRANE-BOUND FORMATE–NITRATE REDUCTASE OF *ESCHERICHIA COLI* K 12 AND THE POSSIBLE ROLE OF SULFHYDRYL GROUPS AND TEMPERATURE IN THE RECONSTITUTION PROCESS

E. AZOULAY, C. RIVIÈRE, G. GIORDANO and J. POMMIER
Structure et Fonction des Biomembranes, C.N.R.S. - E.R. 143 - U.E.R. de Luminy, 13288 Marseille Cédex 2

and

M. DENIS and G. DUCET
Physiologie Cellulaire, U.E.R. de Luminy, 13288 Marseille Cédex 2, France

Received 23 April 1977

1. Introduction

From all the papers dealing with the chlorate-resistant mutants of *Escherichia coli* K12, it could be concluded that the in vitro reconstitution of the membrane-bound formate–nitrate reductase complex, first described by Azoulay et al. [1] then by MacGregor and Schnaitman [2], was permitted (i) by the weakness of the membranes of these mutants which release in a soluble state some of their components upon cell-breakage treatments and (ii) by the presence in their cytoplasm of some precursors of nitrate-reductase [3–6]. In these works, it was shown that the reconstitution process called 'complementation' evolved through a sequence of increasingly complex organizational stages which could be a soluble nitrate reductase of 250 000 daltons, a lipid-protein complex (estimated 3–4 × 10^6 daltons), non-vesiculated particles of buoyant density 1.12, and particles of buoyant density 1.18, consisting of membrane vesicles. These successive molecular forms all exhibited a nitrate reductase activity that could only accept reduced dyes (Benzylviologen) as electron donor, except the last form (1.18 dalton particles) which could also accept the electrons of formate [7]. Taking in account the incorporation of a *b*-type cytochrome into the 1.18 dalton particles [1] and their insensitivity to sulfhydryl group reagents, specially NEM and PCMB [8] that were very active on the other forms of reconstituted nitrate reductase activity, the object of the present work was to obtain information on the molecular organization process giving membrane vesicles exhibiting the whole formate–nitrate reductase pathway.

Abbreviations: NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoate; BVH, reduced benzylviologen

2. Materials and methods

E. coli K12 PA 601, chlorate-sensitive, and its chlorate-resistant mutants *chlA* [9] and *chlB* [10] were grown and harvested at the late exponential phase of growth, washed and starved as indicated previously [1,4].

Soluble and particulate fractions [3,4], protein F_A [4], complementation studies with mixtures of extract *chlB* and protein F_A [4], collection and purification of reconstituted particles [4] were prepared or carried out as described in previous publications from this laboratory.

Cytochrome spectrophotometry was carried out using the rapid scan spectrometry method of Denis

Table 1
Effects of various inhibitors on the reconstitution of nitrate reductase and the formation of particles by complementation

Inhibitor	(mM)	Particulate protein mg%	inhibition	Reconstituted enzyme units %	inhibition
Control		3.0	0	104	0
2-Mercaptoethanol	0.3	nd	nd	92	12
Dithiothreitol	0.3	nd	nd	80	23
Glutathion-H_2	3.0	2.85	5	13	87
	0.3	2.94	2	60	42
Cysteine	5.0	nd	nd	8	92
NEM	3.0	0.66	78	19	82
	0.3	1.2	60	35	66
PCMB	0.1	0.6	80	14	87
	0.01	1.4	47	50	52

nd = non-determined

Inhibitors were added to 1 ml reaction mixtures (30 mg protein) at the final concentration mentioned in the table, after which the mixtures were incubated for complementation under standard conditions [4], then dialyzed against 0.04 M Tris–HCl buffer (pH 7.6) for 1 h before being assayed for reconstituted nitrate reductase activity. After centrifugation at 220 000 × *g* for 2 h, pellets were assayed for protein.

and Ducet [13]. The tool of this method is a rapid scan spectrometer, completed by a fast signal analyzer and followed by a computerized data treatment.

Nitrate reductase activities [11], F_A activity [4] and protein determinations [12] were measured as previously described.

3. Results and discussion

3.1. *Effects of the sulfhydryl group reagents*

These compounds were surveyed with respect to their effects on the complementation process itself. Table 1 shows that NEM and PCMB were very strong inhibitors of the nitrate reductase reconstitution, as also cysteine and reduced glutathion. In these experiments, the reagents were the more efficient as they were earlier introduced into the complementation mixture, which probably means that they block the first steps of the reconstitution process. In order to eliminate any effect of these compounds on the reconstituted activity itself, the incubated reaction mixtures were all dialyzed against 0.04 M Tris–HCl buffer (pH 7.6) for 1 h before being assayed for reconstituted nitrate reductase activity. As shown in table 1, NEM and PCMB inhibited both enzyme reconstitution and formation of particles, whereas reduced glutathion had very little effect on the formation of particles.

The effects of the sulfhydryl group reagents on the complementation process permit us to suppose that –SH groups might play a role in the reconstitution by being exposed during the first phase of the phenomenon, since non-sedimentable nitrate reductase is sensitive to the reagents of sulfhydryl groups when the particulate form of the enzyme is not. Such free –SH groups should possibly be required for the association processes between proteins and phospholipids that are involved in complementation [5], as earlier suggested by Estrugo et al. [14] for the assembly of proteins and lipids in the membranes of *Micrococcus lysodeikticus*.

3.2. *Low temperature difference spectroscopy of 1.18 dalton particle-bound b-type cytochrome*

Until now, the precise nature of the *b*-type cytochrome which incorporates into the 1.18 dalton particles remained unknown. This problem has been

approached by carrying out analysis of low temperature spectra.

Low temperature difference spectra of intact wild-type cells (fig.1) show that anaerobic growth in the presence of nitrate gives results (fig.1b,c) similar to those recorded by Haddock et al. [15] under the same conditions: the addition of nitrate to cells previously reduced by dithionite lowers α peak and shows the presence of two maximum absorptions at 556 nm and 558 nm. This agrees with the presence of a *b*-type cytochrome, specifically oxidized by its substrate nitrate, known as cytochrome $b_{556}^{NO_3^-}$ since the works of Ruiz-Herrera and DeMoss [16] and Haddock et al. [15]. Aerobically grown cells have a lower cytochromic content with two peaks at 556 nm and 562 nm (fig.1a); these cytochromes are not oxidized by nitrate, which also corroborates the data of Haddock et al. [15].

Difference spectra obtained with membrane particles of both mutants *chlA* and *chlB* exhibit the peak at 556 nm but lack the 562 nm peak observed in intact cells, which is not surprising since cytochrome b_{562} has been suggested to be a soluble protein by Fujita [17]. Figure 2 shows difference spectra

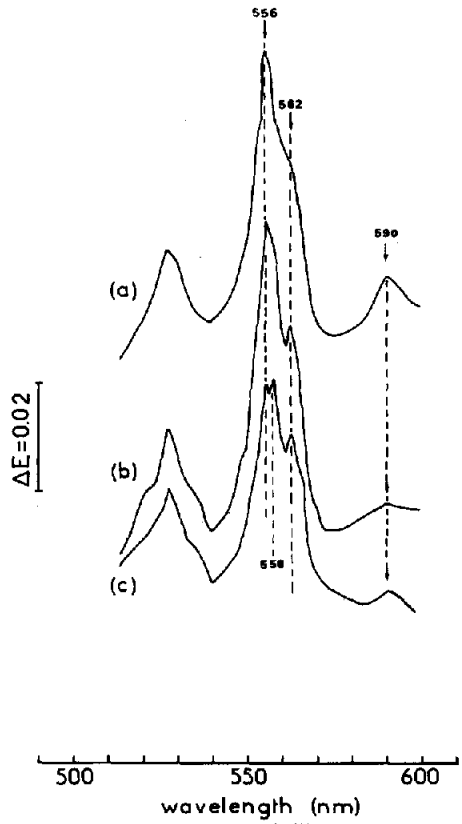

Fig.1. Difference spectra of intact wild-type cells grown under aerobiosis or anaerobiosis plus nitrate. Cell suspensions were prepared by diluting harvested and washed cells in 40 mM Tris–HCl buffer (pH 7.6). Samples (0.4 ml) were oxidized with $FeCN_6K_4$ then reduced with $Na_2S_2O_4$ and, where indicated, reoxidized by the addition of KNO_3 before immersion into liquid N_2 and the recording of difference spectra at 77°K. The growth conditions used, the type of difference spectrum recorded and the final dry weight in the cuvettes, for each trace, were: (a) aerobically grown, ferricyanide-oxidized versus dithionite-reduced, 25 mg dry wt; (b) anaerobically grown in the presence of nitrate, ferricyanide-oxidized then dithionite-reduced versus nitrate-reoxidized, 28 mg dry wt.

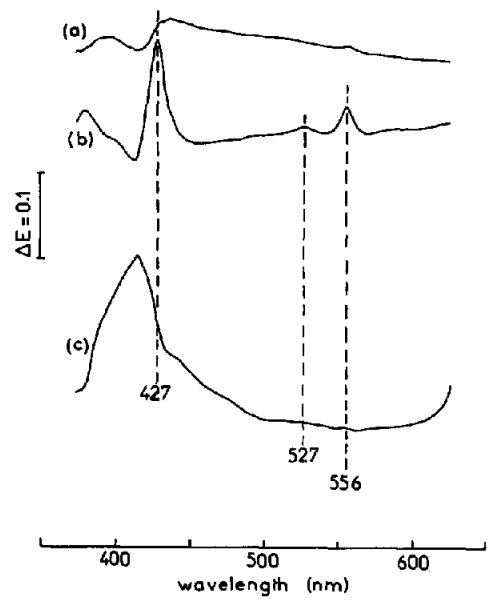

Fig.2. Difference spectra of reconstituted particles. Reconstituted particles obtained from a F_A + *chlB* complementation mixture were fractionated by sucrose density centrifugation according to a procedure described previously [4]. Samples (10 mg protein/0.4 ml) of each particulate type were oxidized with $FeCN_6K_4$ then reduced with formate and, where indicated, reoxidized by the addition of KNO_3 before immersion into liquid N_2 and the recording of difference spectra at 77°K. The particulate types used and the kind of spectrum recorded were: (a) 1.12 dalton particles, ferricyanide-oxidized versus formate-reduced; (b) 1.18 dalton particles, ferricyanide-oxidized versus formate-reduced; (c) 1.18 dalton particles, ferricyanide-oxidized then formate-reduced versus nitrate-reoxidized.

recorded at 77°K of reconstituted particles obtained from complementation between extract *chlB* and assembly factor F_A. For 1.18 dalton particles, the spectrum shows peaks at 527 nm and 556 nm (fig.2b) which disappear upon reoxidation by nitrate (fig.2c). In contrast, the light 1.12 dalton particles do not exhibit the characteristic maximum absorption at 556 nm (fig.2a), even with high protein concentration, and regardless of whether the reducing agent was, formate or dithionite.

The following conclusions can be drawn from these data:

(i) Reconstituted 1.18 dalton particles, exclusive of the 1.12 dalton type, are able to couple the oxidation of formate to the reduction of nitrate via a cytochrome b_{556}.
(ii) This cytochrome which can be reoxidized by nitrate, is to be referred to as cytochrome $b_{556}^{NO_3^-}$ according to the definition of Ruiz-Herrera and DeMoss [16].
(iii) The 1.18 dalton particles are unlikely to contain any other cytochrome *b*, since the other two cytochromes b_{556} and b_{558} are not oxidizable by nitrate.

It can be added that the spectra of 1.18 dalton particles are completely similar to those obtained with the total particulate fraction before fractionation by sucrose density-gradient centrifugation, which corroborates the very small influence, if any, of the 1.12 dalton particles on the properties of the reconstituted particles.

3.3. *Effects of temperature on the formation of enzymatically active membrane vesicles*

Reconstitution of nitrate reductase and formation of membrane particles by complementation depend upon temperature as seen before by Azoulay et al. [1] who observed that this reaction, currently operated at 32°C, could not be performed at 0°C and was inhibited by 80% at 50°C.

In order to know the effects of temperature on the reconstitution process giving enzymatically active membrane vesicles (1.18 dalton particles), a series of complementations was carried out at various temperatures (22°C, 32°C and 42°C). It was observed that the total reconstituted nitrate reductase amounts were not significantly different at the three temperatures: 928 units, 912 units and 1040 units, respectively. But after ultracentrifugation of the reaction mixtures to separate the non-sedimentable and particulate fractions, it appeared that both percentages of particulate nitrate reductase and particulate protein increased with temperature: 17%, 21% and 34% on the one hand, and 7%, 10% and 16% on the other; correlatively the specific activities of both types of nitrate reductase, non-sedimentable and particulate, remained unchanged. The particles obtained at the three temperatures were then subjected to fractionation by centrifugation on sucrose density gradient (20–60%, w/v) and the various resulting peaks analyzed for nitrate reductase activity and protein.

Figure 3 shows that:

(i) At 22°C, light particles (buoyant density equal to 1.12) were predominant (70% of the total particulate fraction) over the mid-range 1.18 dalton particles.
(ii) At 32°C, the regular profile, already described [4], was obtained.
(iii) At 42°C, a profile characterized by a very low proportion of 1.18 dalton particles and a very high proportion of heavy 1.23 dalton particles which exhibited a nitrate reductase activity, contrary to the particles of the same density obtained at lower temperatures, was recorded.

It is noteworthy that:

(i) The higher specific activities were obtained at 32°C, which could not be seen on the total particulate fraction, because of the presence of inactive particles.
(ii) The nitrate reductase of the 42°C heavy particles could only be coupled to reduced benzylviologen (BVH) as electron donor, exclusive of formate.

From this study and previous works it seems clear that complementation is a potentially powerful tool in investigations of the various precursors of the membrane-bound formate-nitrate reductase complex, as it is shown by the results on the incorporation of cytochrome $b_{556}^{NO_3^-}$, ATPase [3] and factor F_A [4] into the 1.18 dalton reconstituted particles.

It is noteworthy that:

(i) Only particles having a phospholipid content very similar to that of native particles [5] and the asymetric structure of typical membrane vesicles [18] can incorporate cytochrome

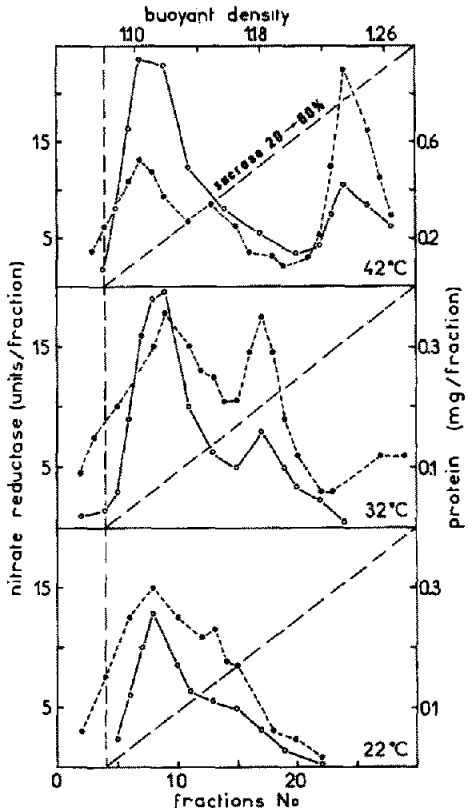

Fig.3. Sucrose density sedimentation profiles of particles reconstituted at various temperatures. Three 9.5 ml complementation mixtures were incubated for 2 h at 22°C, 32°C and 42°C, respectively. Reconstituted particles sedimented at 220 000 × *g* were resuspended in 4 ml 0.04 M Tris–HCl buffer (pH 7.6). Three ml of each particulate suspension were then layered onto a 26 ml sucrose density gradient (20–60%, w/v) and centrifuged at 24 000 rev./min for 14 h (rotor SW25). One ml fractions were collected from top to bottom and assayed for protein (dotted lines) and nitrate reductase activity (solid lines).

$b_{556}^{NO_3^-}$, thereby getting the ability to couple formate oxidation to nitrate reduction.

(ii) Cytochrome $b_{556}^{NO_3^-}$ incorporates into the membrane when BVH–nitrate reductase activity (due to the complex of subunits A and B) is already reconstituted in incomplete 1.12 dalton particles. According to MacGregor [6], the attachment of the membrane-bound BVH enzyme is unstable in the absence of subunit C (cytochrome *b*).

It is therefore likely that the binding of cytochrome *b* to the reconstituted structures results in the stabilization of the complex formate–nitrate reductase within the membrane. The likely effects of the –SH group reagents on the association processes that are involved in complementation might possibly be put together with an earlier observation of Azoulay et al. [19] who found that cysteine blocked the biosynthesis of nitrate reductase in chlorate-sensitive cells grown anaerobically in the presence of nitrate. This kind of hypothesis could explain why non-sedimentable nitrate reductase, which seems to be an intermediate step of complementation, is inhibited by sulfhydryl group reagents, whereas membrane nitrate reductase, either native from wild-type strain or reconstituted, is not. The experimental conditions used to carry out the reconstitution must be regarded as imperfect, since some of the material aggregates into heavy (buoyant density above 1.24) inorganized and inactive particles. Among these conditions, temperature is of peculiar importance, since high temperature (42°C) promotes the formation of high-density, inorganized particles to the detriment of 1.18 dalton, well-organized particles and low temperature (22°C) permits only the formation of 1.12 dalton particles that are incomplete at both biochemical and structural levels. Temperature can therefore be regarded as an important parameter in the complementation process by promoting the transition of solubilized membranous elements to a particulate state consisting of well-organized membrane vesicles. But the existence of an optimal temperature (32°C) permit us to think that this transition must not be too fast for giving the 'best' reconstitutions from both biochemical and structural points of view. Other workers, such as Racker and Kandrach [20] also put emphasis on the importance of temperature in the reconstitution phenomena.

From the data available at the present time, we conclude that the reconstitution process should follow two successive steps:

(i) Formation of a non-sedimentable nitrate reductase, constituted of the subunits A and B and containing molybdenum, and capable to couple the reduction of nitrate with the oxidation of low-potential electron donor such as BVH, exclusive of formate. This soluble complex would associate through –SH changes with phospholipids resulting in a complex of high

molecular weight [5] then in low-density particles.

(ii) These particles would be the required state for further incorporation of subunit C (cytochrome $b_{556}^{NO_3^-}$) and subunits of formate dehydrogenase and complex membrane organization giving the 1.18 dalton particles.

Acknowledgements

This work was supported by grant No. 75-7-0055 from the Délégation Générale à la Recherche Scientifique et Technique.

References

[1] Azoulay, E., Puig, J. and Couchoud-Beaumont, P. (1969) Biochim. Biophys. Acta 171, 238–252.
[2] MacGregor, C. H. and Schnaitman, C. A. (1973) J. Bacteriol. 114, 1164–1176.
[3] Giordano, G., Rivière, C. and Azoulay, E. (1975) Biochim. Biophys. Acta 389, 203–218.
[4] Rivière, C., Giordano, G., Pommier, J. and Azoulay, E. (1975) Biochim. Biophys. Acta 389, 219–235.
[5] Azoulay, E. Pommier, J. and Rivière, C. (1975) Biochim. Biophys. Acta 389, 236–250.
[6] MacGregor, C. H. (1976) J. Bacteriol. 126, 122–131.
[7] Rivière, C. (1975) Doctoral thesis, CNRS A.O. 11728, p. 121, Université Aix-Marseille II, France.
[8] Azoulay, E., Couchoud-Beaumont, P. and Lebeault, J. M. (1972) Biochim. Biophys. Acta 256, 670–680.
[9] Puig, J., Azoulay, E., Gendre, J. and Richard, E. (1969) CR Acad. Sci. 268, 183–184.
[10] Casse, F. (1970) Biochem. Biophys. Res. Commun. 39, 429–436.
[11] Pichinoty, F. and Piéchaud, M. (1968) Ann. Inst. Pasteur 114, 77–98.
[12] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[13] Denis, M. and Ducet, G. (1975) Physiol. Vég. 13, 709–720.
[14] Estrugo, S. F., Larraga, V., Corrales, M. A., Duch, C. and Munoz, E. (1972) Biochim. Biophys. Acta 255, 960–973.
[15] Haddock, B. A., Downie, J. A. and Garland, P. B. (1976) Biochem. J. 154, 285–294.
[16] Ruiz-Herrera, J. and DeMoss, J. A. (1969) J. Bacteriol. 99, 720–729.
[17] Fujita, T. (1966) J. Biochem. 60, 329–334.
[18] Mutaftschiev, S., Olive, J. and Azoulay, E. (1976) CR Acad. Sci. 283, 825–828.
[19] Azoulay, E., Puig, J. and Martins Rosado de Souza, M. L. (1969) Ann. Inst. Pasteur 117, 474–485.
[20] Racker, E. and Kandrach, A. (1973) J. Biol. Chem. 248, 5841–5847.