Effect of Nitrogenase Components from Mutant and Wild-Type Strains of *Azotobacter* on the Dilution Effect of Nitrogenase

By G. J. SORGER

Department of Biology, McMaster University, Hamilton, Ont., Canada

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Nitrogenase activity exhibits a dilution effect. Evidence is presented that the reason for the dilution effect is that one of the component proteins of nitrogenase is limiting in preparations of this enzyme. The limiting component appears to be the non-haem-iron-containing protein (also called fraction II, iron protein, azoferredoxin), which is equivalent to the enhancement factor for nitrogenase activity present in extracts of nitrogenaseless mutant 22R1. A mathematical function of specific activity is described that is useful in describing nitrogenase. It takes into account the dilution effect and the exponential nature of the relationship between nitrogenase activity and enzyme protein concentration.

Numerous investigators have observed that nitrogenase exhibits a dilution effect, i.e. that at low extract concentration in the reaction mixture there is no detectable enzyme activity (Mortenson, 1964; Bulen, LeComte, Burns & Hinkson, 1965; Dilworth, 1966; Hardy & Knight, 1967; Sorger & Trofimenkoff, 1970). The reason for this effect has not previously been explored.

An enhancement factor (component EF+), present in extracts of a nitrogenaseless mutant (22R1) of *Azotobacter*, overcame the dilution effect in mixtures of extracts of strain 22R1 and the wild-type strain (OP) (Sorger & Trofimenkoff, 1970). Formation of the enhancement factor was repressed by ammonia, but the factor was present in extracts of de-repressed 22R1 cells whether these had been de-repressed in a normal or in a molybdate-deficient medium (Sorger & Trofimenkoff, 1970).

Nitrogenase from all nitrogen-fixing organisms thus far examined seems to be composed of at least two components, which can be separated by anaerobic DEAE-cellulose chromatography. One of these components, fraction I, contains non-haem iron and molybdenum; the other, fraction II, contains only non-haem iron (Bulen & LeComte, 1966; Mortenson, 1966; Kelly, Klucas & Burris, 1967; Detroy, Witz, Parejko & Wilson, 1968; Klucas, Koch, Russell & Evans, 1968; Kelly, 1969).

Because of the considerations discussed above, component EF+ was predicted to be fraction II (Sorger & Trofimenkoff, 1970). The experiments described below explore the reason for the dilution effect by examining the identity of the enhancement

factor that overcomes it. The results establish that component EF+ and fraction II are very similar, if not identical, and they indicate that the reason for the dilution effect is probably the presence of a relatively limited concentration of active fraction II in cell-free preparations of normal cells.

MATERIALS AND METHODS

Strains. *Azotobacter vinelandii* OP (Bush & Wilson, 1959) was used as the wild-type strain. Strain 22R1 is a nitrogenaseless mutant derived from strain OP (Sorger & Trofimenkoff, 1970).

Growth of cells. Cells were usually cultured in Burk's nitrogen-free medium (Wilson & Knight, 1952) containing 20 g of glucose/l as carbon source and 5 g of casamino acids (casein hydrolysate, vitamin-free; Difco Laboratories, Detroit, Mich., U.S.A.)/l in a 16-litre Microferm Laboratory Fermenter (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.) with moderate agitation and aeration at $27 \pm 2^\circ\text{C}$ until they were in the exponential phase of growth. The cells were then harvested at maximum speed in a Sharples Super Centrifuge (Sharples Co. Inc., Warminster, Pa., U.S.A.) and resuspended in twice the original culture volume of Burk's nitrogen-free medium (strain OP) or the same volume of Burk's nitrogen-free medium (mutant 22R1), containing 20 g of glucose/l as carbon source. The cultures were then incubated under the same conditions of agitation, aeration and temperature for 13-15 h, after which the cells were harvested in the same manner as before and stored at -18°C until used.

Dr W. Kurz of the Prairie Regional Laboratory of the National Research Council of Canada, Saskatoon, Sask., Canada, kindly provided a large batch of wild-type cells, grown in a 125-litre fermenter in nitrogen-free medium containing mannitol as carbon source. These cells were also stored at -18°C .

Extraction of nitrogenase and preparation of fractions I and II. Cells were ground with a mortar and pestle with an approximately equal volume of levigated alumina (Beckman Instruments Inc., Spinco Division, Palo Alto, Calif., U.S.A.). The paste was suspended evenly by further grinding in 3 vol. of 50 mM-tris-HCl buffer, pH 7.0, and centrifuged at 3000g for 20 min in a refrigerated Servall RC2B centrifuge (Ivan Sorvall Inc., Norwalk, Conn., U.S.A.). The supernatant was used as crude extract, and usually contained 28–35 mg of protein/ml. The crude extract was next centrifuged at 129000g for 40 min at 15°C in a Spinco model L2 ultracentrifuge. The resulting supernatant was mixed with 10 μ g each of deoxyribonuclease and ribonuclease/ml and dialysed under a constant stream of N₂ for 3 h against two changes of 50 mM-tris-HCl buffer, pH 7.0, containing approx. 20 mg of Na₂S₂O₄/l. This partially purified extract contained 12–18 mg of protein/ml.

If the preparation was to be used to isolate fractions I and II, the high-speed supernatant was dialysed in the same fashion against 25 mM-tris-HCl buffer, pH 7.2, containing 5 mM-MgCl₂, 1 mM-2-mercaptoethanol and 20 mg of Na₂S₂O₄/l (TMM buffer). Between 240 and 600 mg of protein of this preparation was layered anaerobically on a DEAE-cellulose column (2.5 cm $\times$ 10 cm) previously made anaerobic by flushing with TMM buffer under a constant stream of purified N₂. The eluent was monitored periodically with benzyl viologen to ensure that it was anaerobic. The column and the adsorbed protein were first washed with 400 ml of TMM buffer, after which fraction I was eluted in 10–20 ml of TMM buffer containing 0.17 M-NaCl. The column was then washed further with 150–200 ml of the same buffer. Finally fraction II was eluted in 10–20 ml of TMM buffer containing 0.34 M-NaCl. All fractions were collected under liquid paraffin and under a constant stream of purified N₂.

The column was regenerated by washing extensively with TMM buffer containing 1 M-NaCl, and subsequently with TMM buffer alone.

Fractions I and II were dialysed anaerobically for 3 h against two changes of 50 mM-tris-HCl buffer, pH 7.2, containing approx. 40 mg of Na₂S₂O₄/l. They were either used immediately or stored in liquid N₂. Fractions I and II contained 7–10 and 2.5–3.3 mg of protein/ml respectively.

Assay of nitrogenase activity. The assay was carried out as described by Sorger (1968, 1969). The reaction mixture, containing all components except reductant, was flushed with gas and equilibrated for 0.75 h. The reaction was started by adding dithionite. One unit of nitrogenase is defined as a difference of 1 nmol of ammonia formed/min between a reaction mixture incubated under N₂ and its otherwise identical control incubated under argon.

Determination of protein. Protein concentration was measured by the biuret method (Dawson, Elliott, Elliott & Jones, 1959), with bovine serum albumin as standard.

Chemicals. Benzyl viologen was purchased from Mann Research Laboratories, New York, N.Y., U.S.A.; bovine serum albumin, ATP, creatine phosphokinase, creatine phosphate and tris base were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.; Nessler's reagent was from the Paragon Co., New York, N.Y., U.S.A.; gases of certified grade were from Canadian Liquid Air Ltd., Hamilton, Ont., Canada; 2-mercaptoethanol was pur-

chased from Eastman Organic Chemicals, Rochester, N.Y., U.S.A.; all inorganic reagents were of analytical grade and were from Fisher Scientific Co., Fairlawn, N.J., U.S.A., or from Baker Chemical Co., Phillipsburg, N.J., U.S.A. Ribonuclease and deoxyribonuclease were from the Worthington Biochemical Corp., Freehold, N.J., U.S.A.; DEAE-cellulose (0.91 mequiv./g) was purchased from C. Schleicher and Schuell Co., Keene, N.H., U.S.A.

RESULTS

Nitrogenase exhibits a dilution effect that cannot be overcome by adding bovine serum albumin (Fig. 1). This dilution effect can be overcome by a factor (component EF+) present in extracts of de-repressed cells of the nitrogenaseless mutant 22R1 (Fig. 2). More component EF+ is needed in the nitrogenase reaction mixture to obtain maximum activity with a dilute extract of normal cells than with a more concentrated extract of normal cells. The above pattern might be expected if component EF+ were a component of nitrogenase, and if its concentration were limiting in wild-type extracts.

When extracts of de-repressed cells of mutant 22R1 were partially purified, by the procedure described in the Materials and Methods section, component EF+ activity was not lost (Fig. 2), indicating that component EF+ is not likely to be diffusible on dialysis or to be a nucleic acid. Since the formation of component EF+ had already been shown to be repressed by ammonia, and since component EF+ was found in extracts of de-repressed cells of mutant 22R1 under molybdenum-sufficient or molybdenum-deficient conditions, component EF+ was predicted to be fraction II (Sorger & Trofimenkoff, 1970).

The results in Table 1 show that extracts of

Fig. 1. Effect of the concentration of wild-type crude-extract protein in the reaction mixture on nitrogenase activity in the presence (○) and in the absence (□) of bovine serum albumin (50 mg/ml).

de-repressed cells of mutant 22R1 contain active fraction II but not active fraction I.

Fraction II prepared from wild-type cells overcomes the dilution effect of nitrogenase (Fig. 3). Fraction I, however, does not overcome the dilution effect of nitrogenase (Fig. 4), though there is a

suggestion of a slight stimulation of nitrogenase activity by fraction I.

Fraction II and fraction I, both from wild-type cells, complement each other *in vitro* (Fig. 5), as does an extract of cells of mutant 22R1 and fraction I (Table 1).

The marked lability of fractions I and II makes it difficult to make a precise analysis of the kinetics at this point.

DISCUSSION

Two examples of a dilution effect are described by Dixon & Webb (1964). In one of these examples the dilution effect was due to impurities in the reaction mixture that were inhibitory to enzyme activity. The effect could be overcome by increasing the concentration of the enzyme, or of any other protein that could remove the impurities. In this case the slope of the curve relating enzyme protein concentration in the reaction mixture and activity

Fig. 2. Effect of the concentration of partially purified wild-type extract protein in the reaction mixture on nitrogenase activity in the presence of 6.8 mg of partially purified mutant 22R1 extract protein (■), of 5.4 mg of partially purified mutant 22R1 extract protein (□) and of no addition (Δ).

Fig. 3. Effect of the concentration of wild-type crude-extract protein in the reaction mixture on nitrogenase activity in the presence of 3.3 mg of wild-type fraction II.

Table 1. Reconstitution of active nitrogenase from pairwise combinations of fractions I and II from Azotobacter vinelandii wild-type and mutant 22R1 cells

Partially purified extract (PPE), fraction I and fraction II were prepared and nitrogenase activity was assayed as described in the Materials and Methods section.

Components in assay mixture							Nitrogenase activity (units)
Preparation	Protein (mg)	Strain	+	Preparation	Protein (mg)	Strain	
PPE	6.9	OP					24.0
Fraction I	10.1	OP					0.0
Fraction II	4.0	OP					1.7
Fraction I	5.0	OP	+	Fraction II	2.0	OP	68.0
Fraction I	5.0	OP	+	PPE	5.5	22R1	77.3
Fraction II	2.0	OP	+	PPE	5.5	22R1	0.9
Fraction I	4.6	OP	+	Fraction II	2.4	22R1	83.3
Fraction II	2.2	OP	+	Fraction I	3.4	22R1	0.0

Fig. 4. Effect of the concentration of partially purified wild-type extract protein on nitrogenase activity in the presence of 2.4 mg of wild-type fraction I protein (○), of 1.9 mg of mutant 22R1 fraction I protein (□) and of no addition (Δ).

Fig. 5. Effect of the concentration of wild-type fraction II protein in the reaction mixture on nitrogenase activity in the presence of 4.2 mg of wild-type fraction I protein.

was constant beyond the dilution effect. This example would not seem to be analogous to the situation described above: (a) because the dilution effect can be overcome by fraction II protein, but

not by an extract of repressed cells (G. J. Sorger, unpublished work), by fraction I protein, or by bovine serum albumin; (b) because the curves relating activity to concentration of wild-type extract protein in the reaction mixture are sigmoidal beyond the dilution effect. It is not possible from the data to exclude the possibility that a specific fraction II poison, present in the reaction mixture, is responsible for the dilution effect.

In the second example given by Dixon & Webb (1964) the concentration of a dissociatable cofactor or activator present in the enzyme preparation fell to subsaturating values when the enzyme was diluted in the reaction mixture. The relationship between enzyme concentration and activity was exponential, giving rise to what appeared to be a dilution effect in a non-exponential plot. In this case a plot of activity versus some exponential function of the enzyme protein concentration should give a linear relationship, which might be expected to go through the origin unless the cofactor or activator were present in subsaturating amounts in the original enzyme preparation. Careful examination of the shape of the plot of activity versus low normal extract concentration has revealed that it is not a straight line but a curve which does not have a sharp break as I had previously thought (Sorger 1968, 1969). If one plots nitrogenase activity versus the square of the concentration of crude or partially purified extract protein (by using the results in Fig. 1 or Fig. 2, for example) a linear relationship is obtained, but the linear portion does not extrapolate to the origin. The fact that the dilution effect can be overcome by partially purified and dialysed fraction II suggests that, if an activator is responsible for the foregoing observations, then it is associated with fraction II and is not likely to be a small freely dissociatable molecule.

Yates (1970) has reported that the nitrogenase activity of crude dialysed extracts or partially purified preparations of *Azotobacter chroococcum* are stimulated by a diffusible factor, by ferredoxin, by azotoflavin, by a 'ferridin-like protein', by cytochrome c_4 or by NADH dehydrogenase. One cannot account for the activity of component EF+ or of fraction II by postulating that it is due to contamination by any one or a combination of the above factors. The diffusible factor did not stimulate crude undialysed preparations, whereas fraction II and component EF+, which are non-diffusible, did. Although crude component EF+ may contain all of the stimulatory proteins Yates (1970) observed, fraction II probably does not. Cytochromes c_4 and c_5 are reported to be eluted from a DEAE-cellulose column at a lower ionic strength than is fraction I (Bulen & LeComte, 1966; Kelly *et al.* 1967). Azotoflavin, *Azotobacter* ferredoxin and 'ferridin-like protein' adsorb more tenaciously on

DEAE-cellulose than does fraction II (Benemann, Yoch, Valentine & Arnon, 1969; Yoch, Benemann, Valentine & Arnon, 1969). The stimulatory NADH dehydrogenase was purified (Yates, 1970) by standing in the presence of deoxycholate overnight at 4°C, followed by ammonium sulphate precipitation and further dialysis overnight at 4°C. Fraction II is so labile that it would probably not withstand such treatment.

The similarity between the effects of component EF+ and fraction II seems to indicate that most of the effect of component EF+ is due to active fraction II. The possibility that other proteins present in crude preparations of cells of mutant 22R1 can also stimulate nitrogenase activity can obviously not be ruled out.

One explanation that is consistent with the results and that would take into account the well-known lability of fraction II (Kelly *et al.* 1967) is the following: fractions I and II are easily dissociatable, and fraction II is present in limiting concentrations in wild-type extracts, perhaps owing to partial inactivation during handling.

The dilution effect of nitrogenase makes the usual definition of specific activity (e.g. initial reaction velocity/mg of enzyme protein) an inaccurate way of describing nitrogenase. A more useful term would be the following function of specific activity [f(Sp. Act.)]:

$$[f(\text{Sp. Act.})] = \frac{A}{p^2 - x^2}$$

Where A is nitrogenase activity in standard units, p^2 is the square of the amount of extract protein in the reaction mixture and x^2 is the square of the amount of extract protein in the reaction mixture at which the dilution effect ends. This point can be obtained by extrapolation of the squared plot to the horizontal axis.

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