Purification and Properties of Nitrogenase in Ethylene Glycol at Sub-Zero Temperatures

By Alexander D. TSOPANAKIS,* Robert C. BRAY* and Barry E. SMITH†

*School of Molecular Sciences and †A.R.C. Unit of Nitrogen Fixation, University of Sussex, Falmer, Brighton BN1 9QJ, U.K.

(Received 4 May 1979)

Both the protein components Kp1 and Kp2 of nitrogenase from *Klebsiella pneumoniae* were found to be stable in aq. 50% (v/v) ethylene glycol at +30°C or below. At –20°C in this medium their sensitivities to O₂ were diminished somewhat. Though purification could be carried out at –20°C, the product had the same specific activity and was obtained in the same yield as when the purification was carried out by standard procedures. This suggests that such procedures yield enzyme undamaged in the course of the purification by O₂, thermal denaturation or proteolytic digestion.

Nitrogenase is the enzyme that catalyses the reduction of N₂ to NH₃. It consists of two components, the Fe protein and the Mo-Fe protein (Zumft & Mortenson, 1975; Eady & Smith, 1979), both of which are irreversibly damaged by brief exposure to O₂. The specific activities of nitrogenase from different organisms and of different preparations from the same organism vary (Zumft & Mortenson, 1975; Eady *et al.*, 1972; Smith *et al.*, 1976); particularly this latter variation could be due to O₂ damage and resulting contamination by inactive enzyme species. Thus it seemed conceivable that even the best preparations of the enzyme might contain molecules that had been damaged in some way. To investigate this hypothesis we purified nitrogenase from *Klebsiella pneumoniae* by using an ethylene glycol/water system at sub-zero temperatures. Such methods have been used to advantage with unstable nuclear enzymes (C. Tsopanakis *et al.*, 1978) as well as for isolation of short-lived enzyme intermediates (Hastings *et al.*, 1975; Balny *et al.*, 1976; Debey *et al.*, 1976). Special apparatus and procedures were developed in our laboratory (A. D. Tsopanakis *et al.*, 1978c), with use of in principle the methodology of Douzou and his co-workers (Douzou, 1977; Douzou & Balny, 1978).

Materials and Methods

Materials

Sephacrose CL-6B and Sephadex G-200 were obtained from Pharmacia (London W.5, U.K.), and DEAE-cellulose (DE-52) was from Whatman

Abbreviations used: pH* represents 'apparent pH' of buffers in aqueous/organic solvent media; Kp1 and Kp2 respectively denote the Mo-Fe protein and the Fe protein components of nitrogenase from *Klebsiella pneumoniae*; Mops, 4-morpholinopropanesulphonic acid.

(Maidstone, Kent, U.K.). Solvents and chemicals were the best available grade (from BDH Biochemicals, Poole, Dorset, U.K.).

Apparatus

The apparatus for low-temperature chromatography was described previously (A. D. Tsopanakis *et al.*, 1978c), and consisted of a low-temperature cabinet (–20±3°C), with a bath circulating cooling liquid (–20°C) through the jackets of the columns. For anaerobic work, copper lines piped pure N₂ gas into the cabinet. Adjustable glass-jacketed chromatography columns were used.

Values of pH*

Values of pH* (T°C), which is the 'apparent pH' at T°C, in the aqueous/organic solvent system (Hui Bon Hoa & Douzou, 1973), were estimated by using a calibration over the range 0 to +50°C for the appropriate solvent/buffer system, followed by extrapolation to the desired sub-zero temperature (A. D. Tsopanakis *et al.*, 1978a).

Enzyme assays, analysis and choice of solvent system

Nitrogenase activity was assayed by using the acetylene reduction technique (Eady *et al.*, 1972); 1 unit of activity corresponds to 1 nmol of ethylene produced/min. Protein estimations by the Lowry method, solubility tests and choice of solvent were as described previously (C. Tsopanakis *et al.*, 1978). In preliminary tests, ethylene glycol, propylene glycol and methanol were tested as possible solvents, and the aq. 50% (v/v) ethylene glycol system was chosen for use at –20°C, as having no effect on enzyme activity and since its dielectric constant (80.7) is very near to that of water. E.p.r. studies were carried out as described by Smith *et al.* (1973).

Purification procedure

Nitrogenase was purified either by the method of Eady *et al.* (1972) or by modifications of this method involving low-temperature procedures carried out in the presence of ethylene glycol, as described below. Maintenance and growth of organism was as described by Eady *et al.* (1972). All purification steps were in 25 mM-Mops/NaOH buffer, pH* (+20°C) 7.4, containing 1 mM-dithionite and 1 mM-dithiothreitol. When ethylene glycol was used, its concentration was 50% (v/v) and the temperature was generally -20°C. An atmosphere of N₂ was generally employed.

Preparation of crude extracts and chromatography, in the presence of ethylene glycol

K. pneumoniae cells were allowed to thaw at +4°C, ethylene glycol/buffer was added immediately and the vessel was put into a bath at -20°C. The cells were disrupted in a French pressure cell precooled to -27°C and the extracts were kept at -20°C. Centrifugation in an MS-25 centrifuge (Fisons MSE, Crawley, Sussex, U.K.) was performed at -18°C, in a 6 × 100 ml head, at 35 000g for 2 h. Other details were as described by Eady *et al.* (1972).

The nitrogenase components (Kp1 and Kp2) were separated by column chromatography in ethylene glycol at -20°C on DEAE-cellulose (DE-52). Component Kp1 was purified further, by similar low-temperature chromatography, but at pH* (+20°C) 8.7 and with a linear gradient of MgCl₂ from 30 to 90 mM (Smith *et al.*, 1976). For the further purification of component Kp2, we were unable to adapt to low-temperature use the Sephadex G-200 gel-filtration procedure of Eady *et al.* (1972). Instead, in some cases, we employed Sepharose CL-6B.

Removal of ethylene glycol from the final samples of component Kp1 or Kp2 could be accomplished by adsorbing the proteins on small DEAE-cellulose (DE-52) columns at +4°C, and then eluting with 90 mM-MgCl₂ in the usual buffer, without glycol. Ultrafiltration could not be employed because of the high viscosities.

Results and Discussion

Enzyme prepared by standard procedures was stable in 50% (v/v) ethylene glycol at temperatures of +30°C and below. Thus anaerobic stability studies of nitrogenase (20 μM-component Kp1 plus 20 μM-component Kp2) in 50% (v/v) ethylene glycol [25 mM-Tris/HCl, pH* (+30°C) 7.4] at -20°C and at +20°C both showed that the enzyme retained 100% activity when assayed in an aqueous medium after up to 8 h incubation. The same enzyme also retained 100% activity after shaking for 30 min at +30°C in the glycol mixture. Nitrogenase assays could be carried out in the presence of ethylene glycol; however, as reported by A. D. Tsopanakakis *et al.* (1978b), a

substantial inhibition was found with concentrations of glycol greater than 10% (v/v), and inhibition approached 100% with 35% (v/v) ethylene glycol assays run for 10 min. The incubation experiment described above shows that such effects must be reversible. Further work will be required to understand the effect of glycol on the nitrogenase reaction.

E.p.r. spectra of nitrogenase were at most very slightly modified by the presence of ethylene glycol. The spectrum of component Kp2 was not changed at all, and that of component Kp1 was changed only slightly, e.g. the position of the *g*₂ feature was shifted by about 2 mT towards higher fields, by ethylene

Fig. 1. Anaerobic chromatography of nitrogenase on DEAE-cellulose (DE-52) in 50% (v/v) ethylene glycol at -20°C

Crude extract (100 ml), diluted twofold by 25 mM-Mops/NaOH buffer, pH* (20°C) 7.4 [pH* (-20°C) 7.9], in 50% (v/v) ethylene glycol containing 1 mM-dithionite and dithiothreitol, was applied anaerobically to a DEAE-cellulose column (4.5 cm × 15 cm) equilibrated with the same buffer. A stepwise procedure was used to elute the proteins at -20°C, with a flow rate of 100 ml/h; Kp1 fraction was eluted at 0.18 M-NaCl in the above buffer (500 ml total effluent volume of 0–0.18 M-NaCl). Kp2 fraction was then eluted with 250 ml of 0.055 M-MgCl₂ in the same buffer. Enzyme activities in samples of the fractions were measured by acetylene assays at +30°C and are expressed in nmol of ethylene produced/min; specific activities are in nmol/min per mg of protein. ○, Kp1 activity; ●, Kp2 activity; △, Kp1 specific activity; ▲, Kp2 specific activity.

glycol. Such effects resemble and are no doubt closely related to those of pH variations on the e.p.r. spectrum of component Kp1 (Smith *et al.*, 1973).

Encouraged by the small effects of the solvent on nitrogenase, we carried out purification of the enzyme at -20°C in 50% (v/v) ethylene glycol, comparing the fractions so obtained with those from standard purifications without glycol. Flow rates in the columns in the low-temperature chromatographic steps were adequate. In Fig. 1 an elution profile for a DE-52 column is illustrated. Separation was similar to that in the aqueous method. However, there were some shifts of the peaks; component Kp1 was eluted at 0.18 M-NaCl and component Kp2 at 0.055 M-MgCl₂ (0.23 M and 0.09 M in the aqueous method respectively).

Component Kp1 from the 50%-(v/v)-glycol method at -20°C had a final specific activity of 1182 units/mg with a yield of 6160 units/g of cells; a similar preparation from the same batch of cells, in water at $+20^{\circ}\text{C}$, gave a specific activity of 975 units/mg with a yield of 7470 units/g of cells. In separate experiments, anaerobic purification at -20°C in 50% (v/v) glycol but in the absence of Na₂S₂O₄ also gave comparable results. For component Kp2 direct comparison of the high-temperature and low-temperature purifications was only made up to the second stage (DEAE-cellulose chromatography). Again the purification temperature had little effect on the protein. In 50% (v/v) glycol at -20°C a specific activity of 450 units/mg and a yield of 6013 units/g of cells were obtained, whereas at $+20^{\circ}\text{C}$ in water a similar preparation gave

380 units/mg and 6300 units/g of cells. Results were repeatable for the same batch of cells; higher or lower specific activities and/or yields were occasionally obtained, but the above numbers are from comparative studies on the same batch of cells. One preparation of component Kp2 in water at $+20^{\circ}\text{C}$ gave a final specific activity of 1756 units/mg and a yield of 9000 units/g, from a particularly active batch of cells, but such activities were unusual. Anaerobic purification of component Kp1 at -20°C in 50% (v/v) glycol in the absence of Na₂S₂O₄ resulted in decreased specific activity and yields.

Sensitivity to O₂ for both proteins was found to be diminished in 50% (v/v) glycol at -20°C . However, even under these conditions, reactivity towards O₂ was too high to permit nitrogenase to be manipulated in its presence. In Table 1, half-lives in air of components Kp1 and Kp2 are shown. The proteins with or without dithionite were shaken with air. Results are compared with lifetimes for dithionite solutions, as a 'model' reaction and to check the effectiveness of dithionite as a protecting agent. (Relative values for dithionite are of more interest than absolute ones, since the Methyl Viologen used as indicator could well catalyse the reaction with O₂.)

Table 1 shows that at -20°C in 50% (v/v) ethylene glycol component Kp1 was significantly stabilized towards O₂. A similar conclusion seems indicated by our low-temperature purification in the absence of Na₂S₂O₄. The results with component Kp2 are

Table 1. *Reaction of nitrogenase and dithionite with atmospheric O₂*

Solutions were placed in serum vials, open to the air, with shaking (100 strokes/min, 4 cm stroke). At intervals, samples of component Kp1 or Kp2 were withdrawn for nitrogenase activity measurements (see the Materials and Methods section). Reaction of dithionite alone with O₂ was followed by using Methyl Viologen as internal indicator (see the text). The buffer was 25 mM-Mops/NaOH, pH* ($+20^{\circ}\text{C}$) 7.8. Dithionite, when present, was 2 mM. Errors indicate the range in duplicates.

Temperature ($^{\circ}\text{C}$)	Medium	Sample	Half-life ($t_{\frac{1}{2}}$) (min)
+22	Aqueous	Kp1	9.0*
+22	Aqueous	Kp2	$0.5 \pm 0.1^*$
+22	Aqueous	Na ₂ S ₂ O ₄	$1.2 \pm 0.2^{\dagger}$
+22	50% (v/v) glycol	Kp1	10
+22	50% (v/v) glycol	Kp1+Na ₂ S ₂ O ₄	15.0
+22	50% (v/v) glycol	Kp2	0.4 ± 0.1
+22	50% (v/v) glycol	Kp2+Na ₂ S ₂ O ₄	0.4 ± 0.1
+22	50% (v/v) glycol	Na ₂ S ₂ O ₄	$3.0 \pm 0.2^{\dagger}$
-20	50% (v/v) glycol	Kp1	16
-20	50% (v/v) glycol	Kp1+Na ₂ S ₂ O ₄	20.0 ‡
-20	50% (v/v) glycol	Kp2	0.6 ± 0.1
-20	50% (v/v) glycol	Kp2+Na ₂ S ₂ O ₄	$2.5 \pm 0.2^{\dagger}$
-20	50% (v/v) glycol	Na ₂ S ₂ O ₄	$9.5 \pm 0.5^{\dagger}$

* Values taken from Eady *et al.* (1972).

† Times for dithionite are those for approx. 90% decolorization of Methyl Viologen indicator, judged by eye.

‡ A different batch of enzyme was used for these experiments.

not so clear-cut. They are complicated by increased solubility of O_2 at $-20^\circ C$ and its slower reaction with $Na_2S_2O_4$ at this temperature. However, component Kp2 plus $Na_2S_2O_4$ was significantly more stable at $-20^\circ C$ than at $+20^\circ C$ in 50% (v/v) glycol solution.

Our results suggest that, since decreasing sensitivity of the proteins to O_2 did not improve the purification of either protein, satisfactory anaerobiosis is normally achieved in the standard room-temperature purification procedure and the resultant proteins are undamaged by O_2 . Furthermore, since at $-20^\circ C$ many reactions are stopped, and since in the presence of 50% (v/v) glycol most enzymic reactions are strongly inhibited (Douzou, 1974), we may also conclude that in the standard purification the nitrogenase proteins have not suffered significant proteolysis or thermal degradation.

Nevertheless component Kp1, free of contaminating polypeptides, has been prepared with specific activities of 1200 and 2150 nmol of C_2H_4 /min per mg with higher metal contents in the latter preparation (Eady *et al.*, 1972; Smith *et al.*, 1976), and as noted above we have occasionally observed component-Kp2 specific activities 50% higher than those usually obtained.

These observations all suggest that factors importantly related to nitrogenase protein specific activities are being affected before cell breakage. This conclusion is supported by our current knowledge of the complexity of the genetics of nitrogenase protein in synthesis (Kennedy & Dixon, 1977; MacNeil *et al.*, 1978; Roberts *et al.*, 1978). We conclude that the observed variations in specific activities are due to contamination of the proteins with inactive or only partially active species that are probably precursors of the active proteins.

This work was supported by the Medical Research Council and the Agricultural Research Council. We thank Mr. K. Baker for growing the cells.

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