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

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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 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 the use of in principle the methodology of Douzou and his co-workers (Douzou, 1977; Douzou & Balny, 1978).

Materials and Methods

Materials

Sepharose CL-6B and Sephadex G-200 were obtained from Pharmacia (London W.5, U.K.), and DEAE-cellulose (DE-52) was from Whatman (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 25mm-Mops/NaOH buffer, pH* (+20°C) 7.4, containing 1mm-dithionite and 1mm-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 6×100ml head, at 35000g for 2h. 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 90mm (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 90mm-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 µg-component Kp1 plus 20 µg-component Kp2) in 50% (v/v) ethylene glycol [25mm-Tris/HCl, pH* (+30°C) 7.4] at −20°C and at +30°C both showed that the enzyme retained 100% activity when assayed in an aqueous medium after up to 8h incubation. The same enzyme also retained 100% activity after shaking for 30min 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. Tsopanakis 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 10min. 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 g₂ feature was shifted by about 2mT towards higher fields, by ethylene.
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°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°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°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>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Medium</th>
<th>Sample</th>
<th>Half-life (t₅)(min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+22</td>
<td>Aqueous</td>
<td>Kp1</td>
<td>9.0+</td>
</tr>
<tr>
<td>+22</td>
<td>Aqueous</td>
<td>Kp2</td>
<td>0.5±0.1*</td>
</tr>
<tr>
<td>+22</td>
<td>Aqueous</td>
<td>Na₂S₂O₄</td>
<td>1.2±0.2†</td>
</tr>
<tr>
<td>+22</td>
<td>50% (v/v) glycol</td>
<td>Kp1</td>
<td>10</td>
</tr>
<tr>
<td>+22</td>
<td>50% (v/v) glycol</td>
<td>Kp1+Na₂S₂O₄</td>
<td>15.0</td>
</tr>
<tr>
<td>+22</td>
<td>50% (v/v) glycol</td>
<td>Kp2</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>+22</td>
<td>50% (v/v) glycol</td>
<td>Kp2+Na₂S₂O₄</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>+22</td>
<td>50% (v/v) glycol</td>
<td>Na₂S₂O₄</td>
<td>3.0±0.2†</td>
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<tr>
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<td>Kp1</td>
<td>16</td>
</tr>
<tr>
<td>−20</td>
<td>50% (v/v) glycol</td>
<td>Kp1+Na₂S₂O₄</td>
<td>20.0†</td>
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<tr>
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<td>Kp2</td>
<td>0.6±0.1</td>
</tr>
<tr>
<td>−20</td>
<td>50% (v/v) glycol</td>
<td>Kp2+Na₂S₂O₄</td>
<td>2.5±0.2±</td>
</tr>
<tr>
<td>−20</td>
<td>50% (v/v) glycol</td>
<td>Na₂S₂O₄</td>
<td>9.5±0.5†</td>
</tr>
</tbody>
</table>

* 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.

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not so clear-cut. They are complicated by increased solubility of O₂ at -20°C and its slower reaction with Na₂S₂O₄ at this temperature. However, component Kp2 plus Na₂S₂O₄ was significantly more stable at -20°C than at +20°C in 50% (v/v) glycol solution.

Our results suggest that, since decreasing sensitivity of the proteins to O₂ 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 O₂. Furthermore, since at -20°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 C₂H₄/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.

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References


