SHORT COMMUNICATIONS

BBA 63449

Purification of the cold-labile component of the Azotobacter nitrogenase

Nitrogenase from *Clostridium pasteurianum*[1] and from *Azotobacter vinelandii*[2] has two components one of which is cold labile[3,4]. The cold-labile component of nitrogenase from *C. pasteurianum* can be separated from the Mo-containing component by gel filtration on Sephadex G-100[1]. Nitrogenase from Azotobacter passes through Sephadex G-50, G-100, G-150 and G-200 as one broad band containing both components, however. The two components of the Azotobacter nitrogenase may be partially separated by DEAE-cellulose chromatography[2,5,6] but the cold labile component always contains small but significant amounts of the Mo-containing component. Recently the cold-labile component of Clostridium nitrogenase was found to be stable at 60° whereas the Mo-containing subunit lost its activity as a result of the heat treatment[7]. It would be of value to be able to study the chemical, physical and biological properties of the cold-labile component if this could be obtained free of the Mo-containing component. This paper describes a procedure for the purification of the cold-labile component of *A. vinelandii* which results in the cold-labile component being completely free of the Mo-containing component.

Cell-free extracts of nitrogen-fixing cells of *A. vinelandii* strain O were prepared from cells grown on a nitrogen-free medium by the method of BULEN *et al.*[8]. All the steps in the purification procedure were performed under anaerobic conditions. In the first step 2% cetyltrimethylammonium bromide (CET) was used to precipitate successively nucleic acid and protein contaminants as well as the negatively charged nitrogenase. Commercial yeast RNA (a negatively charged polymer), purified by the method of RALPH AND BELLAMY[9], was used to dissociate the precipitated CET–nitrogenase complex, leaving the nitrogenase in solution. RNA from other sources, namely wheat germ and pea seed[10], was also used and functioned in the same manner.

The crude extract (400 ml) was subjected to CET fractionation and the precipitate obtained between 4.6 and 7.8% CET by weight of extract protein was collected and stirred for 10 min with 30 ml 25 mM Tris–HCl buffer at pH 7.5 containing 170 mg purified yeast RNA and dithiothreitol (0.6 mM). The mixture was centrifuged at 30 000 × *g* for 5 min to sediment the CET–RNA complex. The supernatant containing nitrogenase (CET fraction) could be stored at −70° for up to 4 weeks without significant loss of activity. The CET fraction (28 ml) was added to an anaerobic DEAE-cellulose column (2.5 cm × 12 cm) equilibrated with 25 mM Tris–HCl buffer at pH 7.5. The column was washed with 40 ml of the same buffer containing dithiothreitol (0.6 mM). Fractions were obtained by step-wise elution with NaCl and $MgCl_2$[5]. The Mo-containing component *plus* small amounts of the cold-labile component of the nitrogenase was eluted with 35 mM $MgCl_2$. The fraction eluted with 90 mM $MgCl_2$ (DEAE-cellulose fraction) contained most of the cold labile component of the nitrogenase. This fraction was heated at 60° for 15 min under H_2 and the precipitated

Abbreviation: CET, cetyltrimethylammonium bromide.

TABLE I

PURIFICATION OF THE COLD -LABILE COMPONENT OF AZOTOBACTER NITROGENASE

Assay conditions were as described in the text.

Fraction	*Vol. (ml)*	*Protein (mg/ml)*	*Mo (nmoles/ mg)*	*Specific activity (nmoles ethylene per min per mg)*	*Purity (-fold)*
Crude extract	400	25.4	1.1	20.2	1
CET fraction	28	22.0	2.1	160.5	7.9
DEAE-cellulose fraction	42	11.2	0.6	170.0	8.4
60° fraction	32	1.1	0	1181.2	58.4
Protamine sulphate fraction	28	0.6	0	2128.3	105.3

proteins centrifuged (5000 × *g* for 10 min). To the supernatant (60° fraction) 5% protamine sulphate per weight of treated protein was added, the mixture centrifuged and the supernatant which contained the cold-labile component (protamine sulphate fraction) was stored at room temperature under H_2 in a container which in turn was placed in a Buchner flask filled with H_2. During the last two steps of purification the protein was extremely O_2 sensitive and all manipulation was carried out under an atmosphere of purified H_2.

The cold-labile component of the nitrogenase was assayed by adding an aliquot (0.25 mg) to a crude extract which had been freed of the cold-labile component by the method described previously[11] (it took 3–5 weeks at 0–1° before the crude extract of *A. vinelandii* was free of the cold-labile component). No reduction of acetylene was observed when larger amounts of the purified cold-labile component (up to 1 mg) were used without the cold-inactivated crude extract. The use of excess partially purified Mo-containing component resulted in reaction rates similar to those obtained when cold-inactivated extract was used. The reaction mixture (2 ml) contained 50 μmoles potassium cacodylate, pH 7.0, 10 μmoles $MgCl_2$, 120 μmoles creatine phosphate, 0.5 mg creatine kinase, 10 μmoles ATP, 40 μmoles $Na_2S_2O_4$, cold labile fraction as required and the crude extract free from the cold-labile component (1.6 mg protein).

TABLE II

STABILITY OF THE COLD-LABILE COMPONENT OF AZOTOBACTER NITROGENASE

Reaction mixture (2 ml) contained 0.25 mg cold-labile component.

Treatment	*Activity (nmoles ethylene per min)*
As isolated	576
Shaken in air for 2 min	246
Shaken in air for 10 min	164
Shaken in air for 15 min	0
Stored at room temperature for 20 h	552
Stored at 0–1° for 20 h	143
Stored at 0–1° for 20 h with 10% ethanol	410

The gas phase was 0.85 atm argon and 0.15 atm acetylene. The rate of reaction was calculated from the amount of acetylene reduced to ethylene which was determined by gas chromatography[12].

The cold-labile component of the Azotobacter nitrogenase prepared by the method described here is purified 105-fold and is free of Mo (Table I). The protein is cold labile and the rate of cold inactivation is decreased when 10% ethanol is added (Table II) and in these respects the cold-labile component of Azotobacter behaves in the same way as that of Clostridium nitrogenase[3].

The cold-labile component of the Azotobacter nitrogenase is O_2 sensitive. When a sample of the purified protein was shaken in air for 2, 10 and 15 min it lost 57, 72 and 100% of its activity respectively (Table II). A comparison between O_2 sensitivity of the protein used in this work and that prepared by KELLY[6] from *Azotobacter chroococcum* reveals the enhanced sensitivity of the former, possibly the result of the higher purity of the protein used here. The protein used in this work has a specific activity of 2128 nmoles acetylene per min per mg whereas the protein previously prepared from *A. chroococcum*[6] had a specific activity of 540 nmoles acetylene per min per mg.

I wish to thank Miss P. Leach for technical assistance.

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Received December 22nd, 1969

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