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Fractionation and Storage of Nitrogenase from *Azotobacter vinelandii*

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The nitrogenase system of *Azotobacter vinelandii* was fractionated by Bulen & LeComte (1966) by chromatography on DEAE-cellulose and stepwise elution with NaCl. Fraction 1 contained iron and molybdenum, and fraction 2 contained iron. Work with these fractions, including further purification, has been hampered by their extreme sensitivity to oxygen and loss of activity under most storage conditions tested (Bulen & LeComte, 1966).

In this communication we report a simplified and comparatively rapid method for the fractionation of nitrogenase from *A. vinelandii*, and some observations on the stability of the fractions under various storage conditions and activity with various substrates.

Experimental. A crude extract of *A. vinelandii*, strain OP, that had been grown on Burk's nitrogen-free medium (Newton, Wilson & Burris, 1953) was prepared by passing a cell paste containing 70mg. dry wt. of cells/ml. through a French pressure cell at 16000lb./in.^{2}. Whole cells and debris were removed by centrifuging at 117000*g* for 60min. The supernatant was decanted, protein determined by the Biuret method (Gornall, Bardawill & David, 1949) and concentration adjusted to 30mg./ml. by the addition of 25mM-tris–HCl buffer, pH7·4. The remainder of the purification was carried out under an aerobic conditions; all buffers were flushed with high-purity nitrogen and contained 0·1mg. of $Na_2S_2O_4$/ml. A protamine sulphate fractionation of 4g. of crude-extract protein was performed by a procedure very similar to that described by Bulen & LeComte (1966). The nitrogenase from this step, about 20ml. containing 36mg. of protein/ml., was applied to the top of a 17cm. × 3cm. DEAE-cellulose column (Whatman microgranular DE32) that had been equilibrated with 25mM-tris–HCl buffer, pH7·4. The column was maintained at about 14° by circulating cold water through thin-walled plastic tubing coiled around it. The extract was washed into the column with 5ml. of the equilibrating buffer and then eluted at a flow rate of about 60ml./hr. with successive 50ml. volumes of 0·15M-NaCl, 35mM-$MgCl_2$, 60mM-$MgCl_2$ and 90mM-$MgCl_2$, each in 25mM-tris–HCl buffer, pH7·4. Fractions were collected from the column through a hypodermic needle into rubber-capped 20ml. serum bottles kept anaerobic by flushing with high-purity nitrogen.

The 0·15M-NaCl eluted a red fraction, which was identified spectroscopically as cytochrome, probably cytochrome c_5 (Neumann & Burris, 1959), 35mM-$MgCl_2$ eluted a very dark-brown fraction, 60mM-$MgCl_2$ eluted a light-brown fraction and 90mM-$MgCl_2$ eluted a light-red-brown fraction. About 80% of each fraction was eluted in about 10ml. and there was only slight trailing of each fraction.

The assay system for nitrogen fixation or azide, acetylene or cyanide reduction contained 10μmoles of $MgCl_2$, 5μmoles of ATP, 40μmoles of creatine phosphate, 0·2mg. of creatine kinase, 20μmoles of $Na_2S_2O_4$, 25μmoles of tris–HCl buffer, pH7·4, and water to give a total volume of 1ml. In most assays the reaction was started by the addition of the extract.

Nitrogen fixation was carried out in 20ml. serum bottles, and the ammonia formed was estimated by Nessler's reagent after micro-diffusion (Dilworth, Subramanian, Munson & Burris, 1965). The reduction of azide was determined under conditions similar to those described by Schöllhorn & Burris (1967).

Reduction of cyanide or acetylene was performed in 5ml. serum vials. Cyanide was used at a final concentration of 2mM and acetylene at approx. 0·05atm. Reactions were stopped by the addition of 0·1ml. of 25% (w/v) trichloroacetic acid, and analysis of the gas phase was made by gas chromatography (Kelly, Postgate & Richards, 1967).

Results. Assays of the fractions eluted from the column showed that the cytochrome fraction alone had no activity with any of the substrates. The fraction eluted by 35mM-$MgCl_2$, fraction 1, did not reduce azide or fix nitrogen, but small amounts of ethylene and methane were produced from acetylene and cyanide respectively. The fraction eluted by 90mM-$MgCl_2$, fraction 2, had a low level of activity towards all four substrates. In one particular experiment it had a specific activity of about 30mμmoles of nitrogen fixed/mg. of protein/min., and when fractions 1 and 2 were combined there was a considerable enhancement of activity. For nitrogen fixation a specific activity of 186 was observed (based on the protein of fraction 2 only). The enhancement of activity by combination of fractions 1 and 2 was: for nitrogen, 6·2-fold (186 versus 30); azide, 5·8-fold; acetylene, 5·5-fold;

cyanide 3·4-fold. Addition of the cytochrome fraction to fractions 1 plus 2 caused only a slight increase in nitrogen fixation or azide reduction and had no observed effect on acetylene or cyanide reduction. The fraction eluted from the column by 60 mM-$MgCl_2$ behaved essentially like fraction 1.

About 35–45% of the crude-extract nitrogenase activity was recovered from the column and enhancements by mixing of up to 20-fold for nitrogen fixation were observed. There was no reduction of any substrate if either ATP or $Na_2S_2O_4$ was omitted from the assay mixture.

When fractions 1 and 2 were frozen slowly and stored separately at −20°, all reducing activity was lost within 24hr. When fractions 1 and 2 were mixed and then frozen, although the nitrogen-fixing ability was apparently completely lost, 25% of the original cyanide-reducing activity remained. When fractions 1 and 2 and cytochrome in equal volumes were frozen together, 60% of the original nitrogen-fixing activity and 90% of the cyanide-reducing activity remained after 72hr.

Fractions 1 and 2 were also injected separately into liquid nitrogen contained in polypropylene bottles and kept completely immersed in a liquid-nitrogen storage container. Samples were thawed out anaerobically and tested for reducing activity. Under these conditions no loss of nitrogen fixation or azide, cyanide or acetylene reduction was observed after 14 days' storage.

The third storage procedure was as follows: Fractions 1 and 2 were stored separately, they were stored combined in 1:1 ratio and combined with the cytochrome fraction in a 1:1:1 ratio, and the cytochrome fraction was stored alone. The duplicate samples, in 20ml. serum bottles, well flushed with high-purity nitrogen and tightly stoppered, were placed in large jars, which also were flushed with nitrogen. Dithionite solution was placed in the bottom of the storage jars, which were sealed with rubber bungs and stored in running water at 10–12°. The fractions from one jar were assayed after 24hr. and those from the other jar after 72hr. Fractions 1 and 2, when recombined for assay, had about 75% of their original activity for nitrogen and azide reduction, about 65% of their acetylene-reducing activity and 100% of their cyanide-reducing activity after 72hr. Essentially similar results were observed with fractions 1 plus 2 plus cytochrome stored together. Although fractions 1 plus 2 stored together had some nitrogen fixation after 24hr., none was detected after 72hr., nor could fractions 1 plus 2 reduce acetylene or azide; cyanide was still reduced at 40% of the original rate after 72hr. The experiment was repeated with essentially the same results. When cytochrome was added back to fractions 1 plus 2 stored together nitrogen-fixing activity was restored to about 80% of the value of fractions 1 plus 2 plus cytochrome stored together, although cytochrome had no enhancing effect on fixation by fractions 1 and 2 stored separately, nor did it affect cyanide reduction by fractions 1 plus 2 stored together.

Restoration of nitrogen-fixing activity to fractions 1 and 2 stored together was also observed if mammalian cytochrome *c* or catalase was added to the assay mixture, but bovine serum albumin had no effect. Since it was possible that cyanide itself was reactivating 'inactive' fractions 1 plus 2, preincubation experiments were performed with fractions 1 plus 2 plus 0·1 mM-cyanide. No enhancement of nitrogen fixation was observed, although at this concentration cyanide does not inhibit nitrogen fixation (Lockshin & Burris, 1965).

Discussion. The simple and quick fractionation procedure described gives two fractions from the nitrogenase system of *A. vinelandii* that may be stored without preliminary dialysis at 12° for a few days, provided that strictly anaerobic handling procedures are used. For longer periods storage in liquid nitrogen seems more suitable. The reasonable stability of the two fractions at 12° makes it probable that further purification will be possible, although the high concentration of $MgCl_2$ may be a positive factor in stabilizing the fractions; NaCl has been reported to inhibit nitrogen fixation (Bulen & LeComte, 1966).

The loss of nitrogen-fixing activity when fractions 1 and 2 were stored together and the partial restoration by addition of the cytochrome fraction suggests that an inactive complex of the two, amenable to reactivation, exists. The observations that this inactivation did not occur when cyanide was the substrate and that the cytochrome fraction made no difference to cyanide reduction may mean that cyanide itself is able to reactivate the nitrogenase or that it can be reduced by a form of the nitrogenase that does not react with nitrogen, azide or acetylene. In either case, cyanide apparently has limitations as a model substrate for the purified nitrogenase system, and hypotheses about the active site for nitrogen fixation based on observations with crude extracts and a variety of cyanide analogues (Hardy & Jackson, 1967) must be interpreted with caution.

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