BBA Report

BBA 41209

Purification of the nitrogenase proteins from *Clostridium pasteurianum*

MAN-YIN W. TSO, TORBJØRN LJONES and R.H. BURRIS

Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisc. 53706 (U.S.A.)

(Received March 10th, 1972)

SUMMARY

A method is described for the purification of the nitrogenase proteins from *Clostridium pasteurianum* by two polyethylene glycol precipitations and chromatography on columns of DEAE-cellulose, Sephadex G-100 and Sephadex G-200. The Mo–Fe protein and the Fe protein have been purified 70–80-fold from the crude extract, and they appear essentially pure when tested by anaerobic polyacrylamide gel electrophoresis.

The nitrogenase complex from *Clostridium pasteurianum* can be resolved into two protein components, here designated as the Mo–Fe protein and the Fe protein. Purification of these proteins by the method described here avoids some of the difficulties encountered with other methods. Protamine sulfate precipitation[1,2] is somewhat inconsistent because protamine sulfate is a variable product. A DEAE-cellulose column[3] may be disturbed by the evolution of H_2 when a crude extract is placed on it. The scheme we report here is reproducible and can be scaled up easily.

C. pasteurianum W5 was grown, harvested, dried and stored as described earlier[3]. The extracts were handled under strictly anaerobic conditions throughout the purification. Cell-free extracts were prepared under H_2 by autolysis of dried cells for 1 h at 30°C in anaerobic 20 mM Tris–HCl buffer, pH 7.4 (15 ml buffer per g of dry cells). The crude extract obtained after centrifugation (27 000 × *g* for 30 min) contained 15 to 20 mg of protein per ml. Protein content was measured by the micro-biuret method of Goa[4]. The pH of the extracts (about 1100 ml from 100 g of dry cells) was adjusted to 6.0 with 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES). Solid polyethylene glycol 6000 (Union Carbide Corp.) was placed in a suction flask

Abbreviation: MES, 2-(*N*-morpholino)ethanesulfonic acid.

connected with 10 mm inside diameter rubber tubing to another suction flask containing the clostridial extract. After the flasks were evacuated and filled with H_2, the solid polyethylene glycol was added slowly to a final concentration of 10% by weight, while the temperature was kept near 15°C. The solution was centrifuged at 27 000 × *g* for 20 min, and the precipitate (nucleic acids and larger macromolecules) was discarded. More polyethylene glycol was added to the supernatant to bring it to 30%. Both the Mo–Fe and Fe proteins precipitated and were collected by centrifugation at 27 000 × *g* for 1.5 h. The supernatant contains the ferredoxin.

The sedimented material was resolubilized, either directly or after storage in liquid N_2, in 200 ml of anaerobic 20 mM Tris–HCl buffer, pH 7.4, containing 2 mg deoxyribonuclease-1, 4 mg ribonuclease-A (both from Sigma Chemical Co.), and 400 mg of $MgCl_2$. A small amount of insoluble material was discarded after centrifugation (10 min at 15 000 × *g*). The dark brown supernatant was introduced onto a 3.5 cm diameter × 12 cm long column of DEAE-cellulose (Whatman DE-52), which had been equilibrated anaerobically at room temperature with 0.15 M NaCl in 20 mM Tris–HCl buffer, pH 7.4. The flow rate was approximately 150 ml/h. Approximately 150 ml of the equilibrating buffer were used to elute a brown band containing most of the hydrogenase activity and to wash the column until the bottom few cm turned white. The Mo–Fe protein was eluted with 0.25 M NaCl in the same buffer as a distinct dark brown band which was collected in a volume of 30 to 40 ml, and the column was washed with another 30 ml. 0.4 M NaCl in the same buffer with 1 mM sodium dithionite then was used to elute another brown band containing the Fe protein. It was concentrated on-line with the Amicon column eluate concentrator attached directly to the DEAE-cellulose column; a total volume of 10 ml was collected. Total time for the chromatography on DEAE-cellulose was about 4 h. H_2 evolution by the polyethylene glycol fraction was suppressed compared to evolution by the crude extract and did not interfere with the chromatography. Immediately after elution from the DEAE-cellulose column, the Fe protein fraction was loaded onto an anaerobic 2.5 cm × 85 cm column of Sephadex G-100 equilibrated with 50 mM Tris–HCl buffer, pH 8.0, containing 1 mM dithionite. The column was run at 15°C with a flow rate of 40 ml/h, and three colored bands of protein separated. The first brown band, eluted at the void volume, contained Mo–Fe protein. The second brown band (elution volume 180 ml) contained the Fe protein which was concentrated directly on-line with the Amicon column eluate concentrator to the range of 10 to 20 mg protein/ml. It was stored in liquid N_2. The red band of the paramagnetic protein[5] appeared third.

A second fractionation with polyethylene glycol was done at 15°C on the Mo–Fe protein fraction from the DEAE-cellulose column. The protein concentration was about 15 mg/ml, and the pH was adjusted to 6.0 by adding anaerobic 0.1 M MES. 5% by weight polyethylene glycol was added and the sediment was discarded after centrifugation (15 min at 20 000 × *g*). More polyethylene glycol was added to the supernatant to a final concentration of 14%; the precipitate was sedimented by

centrifugation for 30 min at 20 000 × *g*, and contained 90% of the Mo–Fe protein activity from the DEAE-cellulose fraction. It was resolubilized in 20 ml of anaerobic 50 mM Tris–HCl buffer, pH 8.0, and then was chromatographed on an anaerobic 5.0 cm × 85 cm column of Sephadex G-200 equilibrated with the same buffer at 4°C (flow rate 100 ml/h). The fraction containing the purified Mo–Fe protein (elution volume 850 ml) was concentrated to 10–20 mg protein/ml by ultrafiltration and stored in liquid N_2.

The purities of Mo–Fe and Fe proteins at different stages of purification were analyzed (Fig. 1) by anaerobic polyacrylamide gel electrophoresis. There was only one

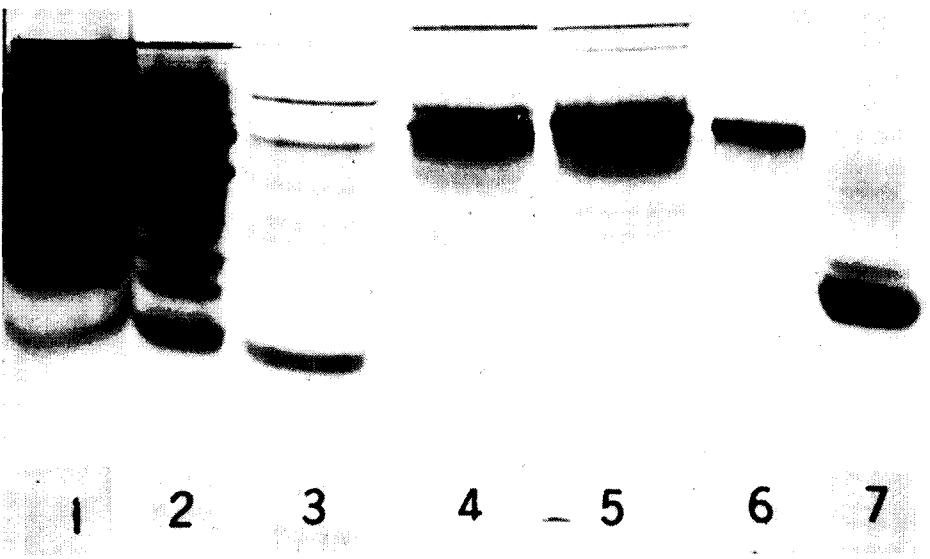

Fig. 1. Anaerobic polyacrylamide gel electrophoregrams. Samples were run at pH 8.9 in 65 mM Tris–borate buffer. The gels were stained with amido black. 1, crude extract, 1 mg protein; 2, 750 μg of 10 to 30% polyethylene glycol fraction; 3, 100 μg of Fe protein after DEAE-cellulose column; 4, 100 μg of Mo–Fe protein after DEAE-cellulose column; 5, 150 μg of 5 to 14% polyethylene glycol fraction of Mo–Fe protein; 6, 100 μg of purified Mo–Fe protein. 7, 100 μg of purified Fe protein.

band in the Mo–Fe protein. A very minor band in addition to the Fe protein band could be seen if the gel was heavily overloaded with Fe protein (Fig. 1, Gel 7). An experiment with polyacrylamide gel electrophoresis in the presence of dodecyl sulfate[6] confirmed the high degree of purity of both proteins (impurities were distinctly less than in crystalline, commercially available proteins used as standards for comparison).

Enzymic assays were performed to evaluate "active-site purity" as distinguished from protein purity. Because there are no known enzymic functions associated with the individual proteins, the optimal specific acitvity of one component can be determined only when it is mixed (titrated) with various amounts of the

complementary protein. Purified Fe protein and Mo–Fe protein were recombined and assayed for acetylene reduction[3]. To determine the maximal specific activity of each protein, assays were performed with different levels of the other protein (Figs 2 and 3). The highest specific activities for acetylene reduction were 3100 nmoles/min per mg Fe protein and 2500 nmoles/min per mg Mo–Fe protein. The same preparations also were assayed for N_2 fixation[3] (ammonia was determined with the indophenol method[7]) with four different combinations of the two proteins. The relative rates were about 4 moles acetylene reduced to 1 mole N_2 reduced. Neither the data from Fig. 2 nor Fig. 3 recognizes or corrects for the possibility that a portion of the Mo–Fe or Fe protein may be enzymically inactive despite meeting the criteria of protein purity. In relation to "active site purity" we only cite the fact that the optimal activities of both the Mo–Fe and the Fe proteins were higher than any previously reported in the literature.

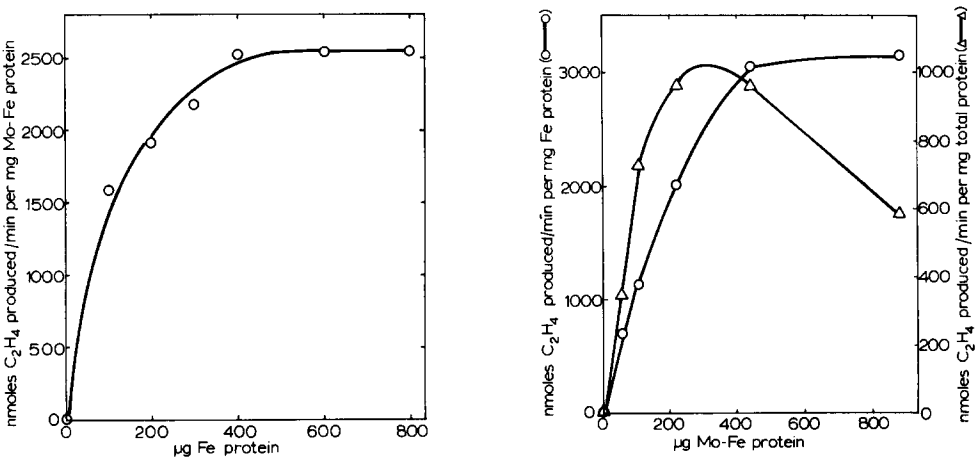

Fig. 2. Determination of the optimal specific activity for the Fe protein. 200 µg of Fe protein was added to each bottle, and the amount of Mo–Fe protein added is indicated in µg on the x axis; ○—○, nmoles of ethylene produced/min per mg of Fe protein; △—△, nmoles of ethylene produced/min per mg of total protein. Temperature was 30°C. The gas phase was 0.1 atm of C_2H_2 and 0.9 atm of Ar.

Fig. 3. Determination of the optimal specific activity for the Mo–Fe protein. 55 µg of Mo–Fe protein was added to each bottle and the amount of Fe protein added is indicated in µg on the x axis. Temperature was 30°C. The gas phase was 0.1 atm of C_2H_2 and 0.9 atm of Ar.

From 100 g of dried cells we obtained 180 mg of the Fe protein and 240 mg of the Mo–Fe protein. The crude extract contained 17 g protein with a specific activity of 40 nmoles acetylene reduced/min per mg protein. Calculations of total activity of crude extract and of recombined purified proteins showed that the overall yield of nitrogenase activity was 60%.

Data from Fig. 2 were used to calculate the combination ratio between the Fe

protein and the Mo–Fe protein as described by Vandecasteele and Burris[3]. Assuming molecular weights of 55 000 (ref. 8) and 170 000 (ref. 1) gives a ratio of 1.9 Fe protein molecules to 1 Mo–Fe molecule. The assumption that our unreported molecular weight determinations of 56 000 and 210 000 are correct gives a ratio of 2.3 Fe protein molecules to 1 Mo–Fe protein molecule. However, this may not be the true combination ratio, because as indicated the nitrogenase proteins are quite labile, and the preparations may contain substantial amounts of inactive enzyme proteins.

Information on the physical and chemical properties of the nitrogenase proteins will be published in another communication.

This investigation was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison; by Public Health Service Grant AI-00848 from the National Institute of Allergy and Infectious Diseases, and by National Science Foundation Grant GB-483. We thank M. Steighorst and W.J. Brill for aid with the anaerobic polyacrylamide gel electrophoresis.

REFERENCES

1 H. Dalton, J.A. Morris, M.A. Ward and L.E. Mortenson, *Biochemistry*, 10 (1971) 2066.
2 E. Moustafa and L.E. Mortenson, *Biochim. Biophys. Acta*, 172 (1969) 106.
3 J.-P. Vandecasteele and R.H. Burris, *J. Bacteriol.*, 101 (1970) 794.
4 J. Goa, *Scand. J. Clin. Lab. Invest.*, 5 (1953) 218.
5 R.W.F. Hardy, E. Knight, Jr, C.C. McDonald and A.J.D'Eustachio, in A. San Pietro, *Non-Heme Iron Proteins: Role in Energy Conversion*, Antioch Press, Yellow Springs, Ohio, 1965, p. 275.
6 A.K. Dunker and R.R. Rueckert, *J. Biol. Chem.*, 244 (1969) 5074.
7 S. Chaykin, *Anal. Biochem.*, 31 (1969) 375.
8 G. Nakos and L. Mortenson, *Biochemistry*, 10 (1971) 455.