Purification and partial characterization of a pyruvate oxidoreductase from the photosynthetic bacterium *Rhodospirillum rubrum* grown under nitrogen-fixing conditions

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A pyruvate oxidoreductase with the capacity to support pyruvate-dependent nitrogenase activity *in vitro* has been purified from the photosynthetic bacterium *Rhodospirillum rubrum*. The enzyme requires CoA for activity and is irreversibly inactivated by oxygen. The molecular properties and K_m values for the substrates have been studied. In supporting nitrogenase activity addition of ferredoxin is required. Overall the enzyme is similar to the *nif*-specific pyruvate:flavodoxin oxidoreductase purified from *Klebsiella pneumoniae*.

INTRODUCTION

Biological nitrogen fixation is catalysed by nitrogenase which consists of two proteins, the Fe-protein (dinitrogenase reductase) and the MoFe-protein (dinitrogenase). In the nitrogenase reaction electrons are transferred from the Fe-protein to the MoFe-protein, and in this process 2 ATP are hydrolysed per electron transferred. In most studies on nitrogenase *in vitro*, dithionite has been used as the electron donor to the Fe-protein.

The physiological pathway of electron transfer to nitrogenase has been biochemically and genetically established in one diazotroph, *Klebsiella pneumoniae*, only. In this organism electrons are transferred from pyruvate to the Fe-protein by two proteins, pyruvate:flavodoxin oxidoreductase and flavodoxin, encoded by the nitrogen-fixation-specific genes *nifJ* and *nifF* respectively [1–8]. Flavodoxin, which is the direct electron donor to the Fe-protein, contains FMN as cofactor and its molecular mass has been calculated from the *nifF* sequence to be 19.1 kDa, a value that is in good agreement with those obtained in studies on the flavodoxin [3,6,9]. The pyruvate:flavodoxin oxidoreductase from *K. pneumoniae* is a dimer of two identical polypeptides with a molecular mass of around 120 kDa [4–6,9]. This enzyme has been shown to contain iron–sulphur clusters, with a total of eight [5] or 16 [6] atoms of iron/mol of enzyme. The DNA sequence of *nifJ* exhibits two iron–sulphur cluster-binding motifs [9]. The pyruvate:flavodoxin oxidoreductase, which is inactivated by oxygen, requires coenzyme A and thiamin pyrophosphate [5,6].

Electron transport to nitrogenase in other diazotrophs has not been as well demonstrated. Pyruvate has been shown to function as an electron source for nitrogenase activity in *Clostridium pasteurianum* [10,11], and the activity of a pyruvate:ferredoxin oxidoreductase has been reported [12]. This enzyme has also been purified from other *Clostridia*; however, these studies were not on nitrogen fixation [13,14]. In the obligate aerobes *Azotobacter vinelandii* and *Azotobacter chroococcum*, flavodoxin(s) has been demonstrated to function in nitrogen fixation [8,15–18]. Studies on *nifF* mutants of *A. vinelandii* show that, although not essential for nitrogen fixation, the *nifF* gene product is required for maximal activity [19]. The enzyme reducing flavodoxin in these organisms has not been identified, but the involvement of the respiratory chain and the protonmotive force has been suggested [20,21].

The photosynthetic bacteria represent another group of diazotrophs with respect to energy metabolism. Although the idea that electrons could be supplied to nitrogenase directly from photosynthesis is attractive, the redox potential of the primary acceptor

in the photosynthetic reaction centre is too positive to be effective in reducing nitrogenase. However, the involvement of light-generated membrane potential in the transport of electrons to nitrogenase in *Rhodobacter sphaeroides* has been proposed [21].

No enzyme similar to the pyruvate:flavodoxin oxidoreductase of *K. pneumoniae* has been identified in phototrophs, although pyruvate-dependent nitrogenase activity in crude extracts of *Rhodospirillum rubrum* has been demonstrated [22]. The activity was about 10 % of that with dithionite as reductant and required CoA. Addition of ferredoxin greatly enhanced the activity. Also in this study the capacity of other carbon metabolites to substitute for pyruvate was examined. 2-Oxoglutarate was almost as effective as pyruvate (80 %), whereas the activity was only 20 % with oxaloacetate. Formate, succinate and malate were ineffective [22].

Our present knowledge about the component that could transfer electrons directly to the Fe-protein is, however, somewhat more detailed. Two soluble ferredoxins have been purified and characterized from *Rsp. rubrum* [23] and both were shown to support nitrogenase activity *in vitro* [24]. However, in this study the ferredoxins were reduced using illuminated chloroplasts [24]. Ferredoxin I, which is only present in light-grown cells, was more effective. Four ferredoxins have been identified in another phototroph, *Rhodobacter capsulatus* [25–28]. Two of these, ferredoxin I and II, have been shown to support nitrogenase activity *in vitro*, but only ferredoxin I is repressed by ammonium ions [25,26,28]. This ferredoxin is encoded by the *fdxN* gene, which has been localized within a *nif* coding region [27]. A flavodoxin has been purified from *Rsp. rubrum*, but only from cells grown in high-ammonia medium with no Fe added, i.e. conditions under which nitrogenase is repressed [29].

In this paper we report on the purification and partial characterization of a pyruvate oxidoreductase from *Rsp. rubrum* grown under nitrogen-fixing conditions. The enzyme catalyses reduction of Methyl Viologen and supports nitrogenase activity in the presence of added ferredoxin.

MATERIALS AND METHODS

Materials

Q-Sepharose FF, Sephacryl S-300 HR, Red Sepharose CL-6B and gel-filtration standards were from Pharmacia-LKB. Sodium pyruvate, dithiothreitol, ATP, CoA, Tris, Hepes, phenylmethanesulphonyl fluoride and 2-oxoglutarate were from Boehringer-Mannheim. Reactive Red 120-agarose, creatine phosphate, creatine phosphokinase, thiamin pyrophosphate and oxaloacetate

were from Sigma. SDS, acrylamide, bisacrylamide and molecular-mass markers for SDS/PAGE were from Bio-Rad. All other chemicals were of analytical grade available commercially.

Growth of *Rsp. rubrum*

Rsp. rubrum (A.T.C.C. 1170) was grown photoheterotrophically in the medium of Ormerod *et al.* [30] with the omission of fixed nitrogen, under an atmosphere of N₂/CO₂ (19:1, v/v). Cells were harvested by filtration using a Pellicon cell (Millipore). The cells were frozen and stored in liquid nitrogen until used.

Anaerobic techniques

Because of the oxygen sensitivity of pyruvate oxidoreductase and nitrogenase, all purification steps were performed under anaerobic conditions. Buffers and vials were evacuated and flushed with argon that had been passed over a heated catalyst (BASF R3-11). Transfers of solutions containing the enzymes were done with syringes that had been flushed with anaerobic buffer.

Preparation of cell extract

Cell extracts were produced by thawing 100 g of cells in 250 ml of 0.1 M-Tris/HCl, pH 7.8, containing 1 mM-pyruvate, 1 mM-MgCl₂, 1 mM-thiamin pyrophosphate, 0.5 mM-dithionite and 2 mM-dithiothreitol. Cells were broken in a Ribi cell fractionator at 138 MPa, phenylmethanesulphonyl fluoride (to 75 µg/ml) was added and unbroken cells were removed by centrifugation at 20000 g for 25 min. The supernatant was further centrifuged at 110000 g for 90 min, and the supernatant used for purification of pyruvate oxidoreductase or for preparation of nitrogenase.

Purification of pyruvate oxidoreductase

The cell-free extract was loaded on a column (5 cm × 6.5 cm) of Q-Sepharose FF equilibrated in 0.15 M-NaCl in the buffer used throughout the purification (25 mM-Tris/HCl, pH 7.5, containing 1 mM-MgCl₂, 1 mM-thiamin pyrophosphate, 2 mM-dithiothreitol and 0.25 mM-dithionite). This and all other purification steps were run at 4 °C. After the column had been washed with two bed volumes of equilibration buffer, the enzyme was eluted with 0.25 M-NaCl in buffer. The active fractions were pooled and concentrated by ultrafiltration in a Diaflo cell equipped with a PM-10 membrane (Amicon).

The concentrated pool was subjected to gel filtration on a column (2.4 cm × 70 cm) of Sephacryl S-300 HR in 0.17 M-NaCl in buffer. Fractions containing activity were pooled and chromatographed on a column (5 cm × 6.5 cm) of Red Sepharose CL-6B equilibrated in 0.2 M-NaCl in buffer. The enzyme did not bind to this gel and the active eluate was applied directly to a column (2.4 cm × 6.5 cm) of Q-Sepharose FF equilibrated with 0.2 M-NaCl in buffer. After the column had been washed with two bed volumes of this buffer, the enzyme was eluted in a linear gradient of 0.2–0.3 M-NaCl in buffer (200 + 200 ml). The pooled active fractions were frozen and stored as pellets in liquid nitrogen.

Enzyme assays

Throughout the purification procedure, enzyme activity was monitored spectrophotometrically at 600 nm, as the pyruvate-dependent reduction of Methyl Viologen [6]. The reaction mixture contained 50 mM-Tris/HCl, pH 7.5, 2.5 mM-dithiothreitol, 0.1 mM-coenzyme A, 50 µM-thiamin pyrophosphate, 1 mM-MgCl₂, 5 mM-sodium pyruvate and 1 mM-Methyl Viologen in a final volume of 2 ml. The reaction was run in cuvettes closed with a Suba Seal stopper. Before addition of the enzyme, cuvettes containing the reaction mixture were evacuated and gassed with

argon. The reaction was run at 30 °C. One unit of activity is defined as 1 µmol of Methyl Viologen reduced/min, using ϵ_{600} 13 mM⁻¹·cm⁻¹ for Methyl Viologen [6].

The assay for pyruvate-dependent acetylene reduction was run in 12.5 ml injection vials containing, in a total volume of 0.8 ml, 50 mM-Hepes, pH 7.3, 5 mM-ATP, 10 mM-MgCl₂, 30 mM-creatine phosphate, 2 units of creatine phosphokinase, 0.5 mM-thiamin pyrophosphate and 0.5 mM-CoA. This mixture was made anaerobic before the addition of nitrogenase (50 µg), a mixture of Fe-protein and MoFe-protein, and ferredoxin (15 µg). The vials were incubated for 10 min to consume dithionite carried over from the nitrogenase preparation. Then pyruvate oxidoreductase (417 µg), specific activity 6.5 units/mg, was added. After another 10 min of incubation, pyruvate (5 mM) and acetylene were added. The acetylene assay was run for 20 min and the ethylene produced was determined by gas chromatography [31]. Nitrogenase used in this assay was prepared from nitrogen-starved cells as described before [32] with the Fe-protein in its active form. The preparation of ferredoxin contained both ferredoxin I and II from *Rsp. rubrum*.

Analytical procedures

SDS/PAGE was performed as described by Laemmli [33] with 10.2 % (w/v) polyacrylamide and 2.6 % cross-linker. Molecular-mass markers were myosin (200000 Da), β-galactosidase (116250 Da), phosphorylase b (97400 Da), BSA (66200 Da) and ovalbumin (45000 Da). Iron was determined according to Fish [32] and protein concentrations were measured by the method of Bradford [34] using the Bio-Rad Protein Assay kit with BSA as standard.

RESULTS AND DISCUSSION

Using the procedure described we have purified a pyruvate oxidoreductase from *Rsp. rubrum*, which supports pyruvate-dependent nitrogenase activity. This is the first purification of such an enzyme from a nitrogen-fixing bacterium since the demonstration and purification of the enzymes involved in electron transport to nitrogenase in *K. pneumoniae*. The results of a representative purification are shown in Table 1. The enzyme was purified 30-fold and with a recovery of 7%. The preparation was 90–95% pure. However, some variation between different batches was observed. Some comments on the individual steps should be made. Although the gel-filtration step does lead to a loss in activity and yield, a number of contaminating proteins were removed. Addition of glycerol to the buffer, which has been reported to stabilize the enzyme from *K. pneumoniae* [5], did not increase the stability of the enzyme from *Rsp. rubrum*. Addition of pyruvate was also ineffective. It is not likely that the loss of activity is due to removal of thiamin pyrophosphate from the enzyme since the buffer contains this cofactor at a concentration of 1 mM. The enzyme from *Rsp. rubrum* behaves differently from pyruvate:flavodoxin oxidoreductase from *K. pneumoniae* [6] and pyruvate oxidoreductase from *Clostridium thermoaceticum* [6] in that it does not bind to the dye–ligand affinity gel, not even when the affinity gel from the same manufacturer as in [6] was used or under conditions other than those used in the purification procedure. However, the step did remove contaminating proteins.

The molecular mass for the purified pyruvate oxidoreductase from *Rsp. rubrum* was determined by gel-filtration on Sephacryl S-300 to be 252 kDa and, for the subunits, a molecular mass of 114 kDa was estimated by SDS/PAGE. Both values are in good agreement with previous reports [1–6,9], indicating that the enzyme is a dimer of two identical subunits. The iron content was determined to be 13 ± 4 atoms/mol of dimer (eight determin-

Table 1. Purification of pyruvate oxidoreductase from *Rsp. rubrum*

Cells (100 g) grown in N₂ medium were used. One unit is defined as 1 μ mol of Methyl Viologen reduced/min.

Purification step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
Cell extract	212	1983	4240	0.5	1	100
Q-Sepharose	2.8	1639	70.7	23.2	47	83
Sephacryl S-300	19.5	427	35.7	12.0	24	22
Red Sepharose + Q-Sepharose	41	132	9	15	30	7

Table 2. Requirements for pyruvate-dependent nitrogenase activity

The reaction was run at 30 °C for 20 min as described in the Materials and methods section.

Assay mixture	Nitrogenase activity (nmol of ethylene produced)
Complete	53
– Oxidoreductase	2
– Ferredoxin	11
– Coenzyme A	1
– Pyruvate	2
– Pyruvate + 6 mM-dithionite	124

ations), the variations being between different preparations. The enzyme from the purification shown in Table 1 contained 9.7 atoms of Fe/mol of dimer. The amount of thiamin pyrophosphate bound to the enzyme was not determined, since the buffer was supplemented with this cofactor.

The Methyl Viologen-reducing activity of the enzyme was strictly dependent on the addition of pyruvate and CoA. The apparent K_m values for these substrates were determined to be 179 μ M and 9 μ M respectively. 2-Oxoglutarate and oxaloacetate could not substitute for pyruvate as electron donor. This observation is in contrast with a previous study on cell extracts of *Rsp. rubrum* where activity with these dicarboxylic acids was obtained [22]. Since ferredoxin is not only present under diazotrophic conditions, it is possible that there are other enzymes in *Rsp. rubrum* that can reduce ferredoxin, with these dicarboxylic acids as electron donors. The purified enzyme was inactivated by oxygen; activity was totally lost on exposure of the enzyme to air for 5 min and could not be recovered by subsequent anaerobic treatment. The inhibitory effect of dithionite observed previously [6] was also exhibited by the enzyme from *Rsp. rubrum*.

The capacity of the purified pyruvate oxidoreductase from *Rsp. rubrum* to support pyruvate-dependent nitrogenase activity *in vitro* was investigated (Table 2). Nitrogenase used in this experiment was a crude preparation containing both the Fe-protein and the MoFe-protein and no attempt was made to remove ferredoxin(s), which offers an explanation for the activity obtained in the absence of added ferredoxin. The results in Table 2 clearly show that the activity with pyruvate is dependent on added pyruvate oxidoreductase and that the activity of this enzyme in this 'physiological' assay is also dependent on added coenzyme A. The ferredoxin preparation used contained both ferredoxins, as shown by SDS/PAGE; thus it is not possible to conclude which of them, or both, is active in this assay. The activity shown in Table 2 is lower than that obtained in the Methyl Viologen assay, which to some extent could be due to nitrogenase being limiting compared with the amount of oxidoreductase added. Furthermore, the enzyme solutions used con-

tained high NaCl concentrations owing to the way in which they were isolated, which could inhibit the interaction of the enzymes in this complex reaction mixture. It has also been reported that the activity in this assay shows a non-linear response to varying the concentrations of the enzymes [6]. Using purified ferredoxin and nitrogenase proteins as well as optimizing the concentration of all components in the assay would therefore probably give a higher pyruvate-dependent activity as compared with that obtained with dithionite. The results, however, clearly show that pyruvate oxidoreductase can function in the physiological electron transport to nitrogenase in *Rsp. rubrum*.

In *K. pneumoniae*, pyruvate:flavodoxin oxidoreductase and flavodoxin, encoded by *nifJ* and *nifF* respectively, are only expressed under *nif* derepressing conditions and are specific for the transport of electrons to nitrogenase [1,2]. We have examined crude extracts of *Rsp. rubrum* grown in the presence of high ammonia (*nif* repressed) and under photoautotrophic, nitrogen-fixing conditions. In both cases pyruvate oxidoreductase activity, measured as Methyl Viologen reduction, was found. However, the specific activities were 50 and 10 % respectively of the activity obtained in extracts from nitrogen-fixing cells. The activity in both extracts was dependent on added coenzyme A and inactivated by exposure to air. These results indicate that the pyruvate oxidoreductase in *Rsp. rubrum* may have some additional metabolic function(s) and that its regulation is not only dependent on the nitrogen status of the cell but also on the supply of carbon.

This study has provided evidence showing that electron transfer from pyruvate to nitrogenase in *Rsp. rubrum* is mediated by a pyruvate oxidoreductase similar to the *nifJ* gene product functional in *K. pneumoniae*. However, whether this is the pathway common to all nitrogen-fixing purple non-sulphur phototrophs remains to be demonstrated, especially since a gene homologous with *nifJ* has not been found in *R. capsulatus* [35]. The other component of the transport in *K. pneumoniae*, flavodoxin, is probably replaced by ferredoxin in phototrophs, as suggested by this investigation and other studies on *Rsp. rubrum* [22,24] and *R. capsulatus* [25–28]. Furthermore, no gene homologous to *nifF* has been identified in either of these bacteria (J. Jansson & S. Nordlund, unpublished work; [35]).

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REFERENCES

1. Roberts, G. P., MacNeil, T., MacNeil, D. & Brill, W. J. (1978) *J. Bacteriol.* **136**, 267–279
2. Hill, S. & Kavanagh, E. P. (1980) *J. Bacteriol.* **141**, 470–475
3. Nieva-Gomez, D., Roberts, G. P., Klevickis, S. & Brill, W. J. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2555–2558
4. Bogusz, D., Houmard, J. & Aubert, J.-P. (1981) *Eur. J. Biochem.* **120**, 421–426

5. Shah, V. K., Stacey, G. & Brill, W. J. (1983) *J. Biol. Chem.* **258**, 12064–12068
6. Wahl, R. C. & Orme-Johnson, W. H. (1987) *J. Biol. Chem.* **262**, 10489–10496
7. Deistung, J., Cannon, F. C., Cannon, M. C., Hill, S. & Thorneley, R. N. F. (1985) *Biochem. J.* **231**, 743–753
8. Deistung, J. & Thorneley, R. N. F. (1986) *Biochem. J.* **239**, 69–75
9. Arnold, W., Rump, A., Klipp, W., Piefer, U. B. & Pühler, A. (1988) *J. Mol. Biol.* **203**, 715–738
10. Carnahan, J. E., Mortenson, L. E., Mower, H. F. & Castle, J. E. (1960) *Biochim. Biophys. Acta* **44**, 520–535
11. Mortenson, L. E. (1964) *Proc. Natl. Acad. Sci. U.S.A.* **52**, 272–289
12. Sauer, F. D., Bush, R. S. & Stevenson, I. L. (1976) *Biochim. Biophys. Acta* **445**, 518–520
13. Uyeda, K. & Rabinowitz, J. C. (1971) *J. Biol. Chem.* **246**, 3111–3119
14. Meinecke, B., Bertram, J. & Gottschalk, G. (1989) *Arch. Microbiol.* **152**, 244–250
15. Beneman, J. R., Yoch, D. C., Valentine, R. C. & Arnon, D. I. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **64**, 1079–1086
16. Beneman, J. R., Yoch, D. C., Valentine, R. C. & Arnon, D. I. (1971) *Biochim. Biophys. Acta* **226**, 205–212
17. Yates, M. G. (1972) *FEBS Lett.* **27**, 63–67
18. Klugkist, J., Voorberg, J., Haaker, H. & Veeger, C. (1986) *Eur. J. Biochem.* **155**, 33–40
19. Bennett, L. T., Jacobson, M. R. & Dean, D. R. (1988) *J. Biol. Chem.* **263**, 1364–1369
20. Klugkist, J., Haaker, H. & Veeger, C. (1986) *Eur. J. Biochem.* **155**, 41–46
21. Veeger, C., Haaker, H. & Laane, C. (1981) in *Current Perspectives in Nitrogen Fixation* (Gibson, A. H. and Newton, W. E., eds.), pp. 101–104, Australian Academy of Sciences, Canberra
22. Ludden, P. W. & Burris, R. H. (1981) *Arch. Microbiol.* **130**, 155–158
23. Yoch, D. C., Arnon, D. I. & Sweeney, W. V. (1975) *J. Biol. Chem.* **250**, 8330–8336
24. Yoch, D. C. & Arnon, D. I. (1975) *J. Bacteriol.* **121**, 743–745
25. Hallenbeck, P. C., Jouanneau, Y. & Vignais, P. M. (1982) *Biochim. Biophys. Acta* **681**, 168–176
26. Yakunin, A. F. & Gogotov, I. N. (1983) *Biochim. Biophys. Acta* **725**, 298–308
27. Schatt, E., Jouanneau, Y. & Vignais, P. M. (1989) *J. Bacteriol.* **171**, 6218–6226
28. Jouanneau, Y., Grabau, C., Schatt, E., Duport, C., Meyer, C. & Vignais, P. M. (1990) in *Nitrogen Fixation: Achievements and Objectives* (Gresshoff, P. G. M., Roth, L. E., Stacey, G. & Newton, W. E., eds.), p. 158, Chapman and Hall, New York
29. Cusanovich, M. A. & Edmondson, D. E. (1971) *Biochem. Biophys. Res. Commun.* **45**, 327–336
30. Ormerod, J. G., Ormerod, K. S. & Gest, H. (1961) *Arch. Biochem. Biophys.* **94**, 449–463
31. Nordlund, S. & Noren, A. (1984) *Biochim. Biophys. Acta* **791**, 21–27
32. Fish, W. W. (1988) *Methods Enzymol.* **158**, 357–364
33. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
34. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
35. Klipp, W. (1990) in *Nitrogen Fixation: Achievements and Objectives* (Gresshoff, P. G. M., Roth, L. E., Stacey, G. & Newton, W. E., eds.), pp. 467–474, Chapman and Hall, New York

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