AMPEROMETRIC ASSAY OF ACTIVITY AND pH-OPTIMUM OF N_2O REDUCTASE OF *Paracoccus denitrificans*

Igor KUČERA, Pavla BOUBLÍKOVÁ and Vladimír DADÁK

Department of Biochemistry, Purkyně University, 611 37 Brno

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The paper reports on a novel experimental procedure of amperometric assay of the activity of N_2O reductase from the bacteria *Paracoccus denitrificans* by a Clark-type element. A suspension of baker's yeast is used to ensure the necessary anaerobic conditions and the reaction itself is triggered by the addition of *P. denitrificans* cells to the medium. The physiological pH-optimum of the N_2O reductase of intact cells as determined by this assay is 8·0.

The nitrogen cycle in nature involves denitrification processes catalyzed by microorganisms during which nitrates are reduced to gaseous products (mostly nitrogen). Nitrite and nitrous oxide have been identified as intermediary products on the pathway of successive nitrate reduction[1,2]. The denitrification processes are of major economic importance: it has been noted, *e.g.*[3], that they may cause an approximately 30% loss of nitrogen applied to soil in the form of nitrate fertilizers.

Biochemical studies on the individual processes of the denitrification pathway require the activities of the corresponding enzymes to be measured. Whereas the assays of nitrate reductase and nitrite reductase can be carried out without major problems since sensitive analytical methods of nitrate and nitrite determination are available, the measurement of the activity of N_2O reductase of intact cells requires the use of gas chromatography or mass spectrometry[4-6]. A fact important[7] from the viewpoint of development of a continuous assay for nitrous oxide uptake is the finding that its content can be recorded amperometrically by a Clark-type element with a silver electrode. This method has been used[8] for the determination of nitrous oxide in *Paracoccus denitrificans*.

The present study critically examines the amperometric method of activity determination of bacterial N_2O reductase and a suitable experimental procedure is designed. The determination of the pH-optimum of N_2O reductase of intact *Paracoccus denitrificans* cells is presented by using this method.

EXPERIMENTAL

Material and Methods

The activity of N_2O reductase was measured by a Clark-type element (Instrument Development Workshops, Czechoslovak Academy of Sciences, Prague) with a silver cathode 8 mm in diameter and a conical anode electrolytically coated with AgCl. The teflon membrane used by other authors[8] was replaced by a polypropylene (Mikroten) membrane. The space between the membrane and the electrode was filled up by the electrolyte composed of 0·1 mol l^{-1} KCl and 1 mol . l^{-1} KOH, the voltage at the cathode was −1·3 V (*cf.*[8]). The measurement was carried out in a glass vessel jacketed at 30°C with electromagnetic stirring of the sample. The vessel contained 2·5 ml of 0·1 mol l^{-1} phosphate (NaH_2PO_4) whose pH had been adjusted by sodium hydroxide. The current was recorded in a polarograph (Model OH-105, Radelkis, Hungary). Nitrous oxide was added as an aqueous solution saturated at 0°C (concentration 58 mmol l^{-1}, *cf.*[8]).

Paracoccus denitrificans (NCIB 8944) was cultivated anaerobically as described elsewhere[9] except that the medium contained 50 mmol l^{-1} succinate instead of glucose. Nitrate served as a terminal electron acceptor. Baker's yeast was resuspended several times in 0·1 mol l^{-1} phosphate buffer (pH 7·3) and centrifuged before the assay.

All the chemicals used were of analytical grade of purity and were purchased from Lachema with the exception of N,N,N′,N′-tetramethyl-*p*-phenylenediamine (TMPD), which was from Fluka (Switzerland).

RESULTS AND DISCUSSION

Since oxygen is also reduced at the voltage of −1·3 V at the silver electrode, which is required for electrochemical reduction of nitrous oxide to nitrogen, the assay of the concentration of nitrous oxide must be carried out under anaerobic conditions. These conditions were achieved in experiments[8] with *P. denitrificans* by removing oxygen from the medium by the oxidase activity of the cells. After the electrode response had stabilized nitrous oxide was added and the current decrease corresponding to its uptake was recorded.

The described assay of N_2O reductase is considerably disadvantageous in the respect that the reaction is triggered by the addition of nitrous oxide to the anaerobic mixture. The response corresponding to the initial concentration of nitrous oxide cannot stabilize under these conditions because of enzymatic reduction of nitrous oxide; hence, the exact relation between the concentration of the latter and the recorded current which is necessary for the activity calculation is not known. This drawback can be curtailed by using a smaller number of cells; this, however, unbearably prolongs the period necessary for the completion of the initial oxygen uptake.

These disadvantages are eliminated in our procedure employing washed baker's yeast, which does not show any interfering N_2O reductase activity, for the removal of oxygen from the medium. A typical record of the measurement is shown in Fig. 1.

As obvious from Fig. 1, nitrous oxide was added to the medium after oxygen had completely been taken up by the yeast and after the sensitivity of the instrument

had been increased. The reaction was started by the addition of cells. The specific activity of N_2O reductase can be calculated from the recorded time profile of the decrease of current response and from the knowledge of dry weight of the cells used; the activity determined in the experiment shown in Fig. 1 was 2·8 μcat/g cell dry weight. The ratio of the sensitivity of the amperometric assay of oxygen to that of the N_2O assay (the electrode is about 5-times more sensitive to oxygen than to nitrous oxide) can also be determined from Fig. 1. We found in independent experiments that the quantity of baker's yeast used did not affect the pH of the medium during the experiment (8 min).

The procedure described in this study has been employed for the determination of the pH-profile of N_2O reductase of intact *P. denitrificans* cells (Fig. 2). An endogeneous physiological substrate and also an artificial donor used in cytochrome c assays (mixture of TMPD and ascorbate) served as electron donors during the assay.

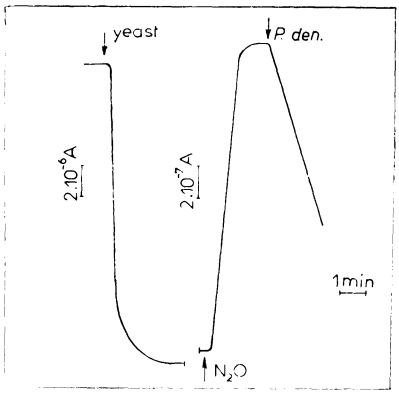

FIG. 1

Amperometric assay of N_2O reductase activity. The procedure of the measurement is described in the text. Additions marked: suspension of baker's yeast (dry weight 15 mg), nitrous oxide (290 nmol), suspension of *P. denitrificans* cells (dry weight 0·5 mg). The initial oxygen concentration of the medium was 0·25 mmol l^{-1}, of nitrous oxide 0·116 mmol l^{-1}

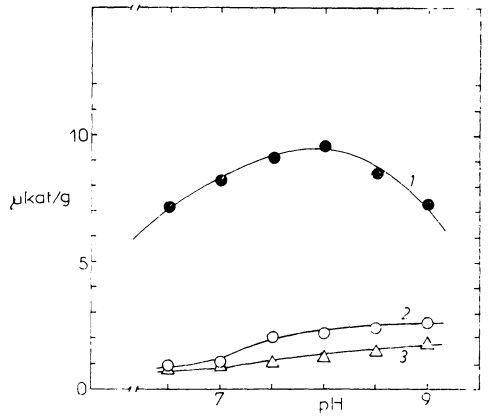

FIG. 2

Specific activity of N_2O reductase in intact *P. denitrificans* cells (μcat g^{-1}) as function of pH of the medium. The procedure of the measurement is described in the text. *P. denitrificans* cells which had been preincubated with antimycin A (1 μg antimycin/mg cell dry weight) were used for the measurement of the N_2O reductase activity in the presence of a mixture of 0·2 mmol l^{-1} TMPD and 10 mmol l^{-1} ascorbate. *1* N_2O reductase in the presence of TMPD and ascorbate, *2* endogenous N_2O reductase, *3* oxidases

Whereas the plot of the rate of nitrous oxide reduction *versus* pH showed a marked maximum at pH 8·0 in the presence of TMPD and ascorbate, an insignificant maximum was observed when working with succinate as a natural electron donor; the absolute rate values were about four times lower. These differences in behavior can be accounted for by a limited flow of redox equivalents into the respiration pathway of *P. denitrificans* cells[9]. The analogy in the pH-profiles of N_2O reductase activity and respiratory activity shows that the rate of the dehydrogenase reaction increases with the increasing pH and that this effect most likely masks the dependence of the rate of nitrous oxide reduction on pH. The found pH-optimum of 8·0 is in accordance with the data reported in paper[10] for partly purified *P. denitrificans* N_2O reductase when reduced benzylviologen was used as electron donor. This accordance indicates that pH 8·0 is the real physiological optimum of *P. denitrificans* N_2O reductase.

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