Amperometric Method for Determining Nitrous Oxide in Denitrification and in Nitrogenase-catalyzed Nitrous Oxide Reduction

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Abstract. A conventional Clark-type O₂ probe was used to determine N₂O concentrations in suspensions. At a polarizing voltage of −0.95 V versus the reference Ag/AgCl electrode, the probe is almost half as sensitive for N₂O as for O₂, and the detection limit is less than 1 μM N₂O. The probe can also be used to determine NO for which the suitable polarizing voltage is −0.7 V. The method was successfully applied for continuously recording dissimilatory formation or utilization of N₂O by intact *Azospirillum brasilense* Sp 7, NO production by extracts from this bacterium, and N₂O reduction catalyzed by nitrogenase in intact *Klebsiella pneumoniae*. It is concluded that the probe is useful for measuring N₂O or NO contents in bacterial suspensions when the O₂ level is zero or kept constant during the assays.

Generally, the methods to measure nitrogen fixation and denitrification activities require sampling at intervals and subsequent analyses. They are often time consuming and expensive. An exception to the rule is the amperometric method to measure H₂ evolution formed by nitrogenase, which can be performed continuously [5]. Otherwise, there are few and only insensitive methods for determining the continuous formation of products or utilization of substrates in nitrogen fixation [1] or denitrification [4]. During the course of an investigation on nitrogen fixation and denitrification by *Azospirillum* [12, 16], we noticed that nitrous oxide could be determined quantitatively by means of a probe that was routinely used for measuring O₂ concentrations. The present communication describes the method of determining nitric oxide and nitrous oxide amperometrically. In addition, examples are presented where the method can successfully be applied for measuring denitrification and nitrogen fixation activities by microorganisms.

Materials and Methods

**Probe used.** A Clark-type probe commonly used for O₂ determinations served for the measurements in the present investigation. We used a type EO 12 probe from Wissenschaftlich-Technische Werkstätten (WTW GmbH, D-8120 Weilheim i. OB), containing a gold cathode, an Ag/AgCl anode as the reference, a KCl gel electrolyte of pH 13, and a Teflon membrane impermeable to H₂O and ions but permeable to O₂, NO, and N₂O. Any other Clark-type O₂ probe may be used, but the optimal polarizing voltage may differ slightly. In the current investigation, the electrolyte gel had a KCl concentration of 0.7 M, exhibiting an Ag/AgCl reference potential at the anode of +0.23 V versus the normal hydrogen electrode. Polarizing voltages of −0.95 V for N₂O determinations and of −0.70 V for NO measurements were applied between the cathode and anode by conventional 1.5-V batteries supplemented with a voltage divider. Thus, the effective polarizing voltages were −0.47 and −0.72 V versus the normal hydrogen electrode, respectively. Throughout the text, all the polarizing voltages are given versus the Ag/AgCl electrode (with a concentration of Cl⁻ = 0.7 M) and not versus the normal hydrogen electrode. The current signal from the cathode was amplified and recorded by a standard two-channel recorder (type 2210 from LKB, Bromma, Sweden; for the circuit arrangement of the amplifier see Oehme and Schuler [11], p. 70). The gas measurements were performed in a 1.8-ml jacketed cuvette, similar to the method described by Wang et al. [15]. The solution in the cuvette was stirred by a magnetic bar and the temperature was held at 30°C by circulating water. The cuvette allowed the insertion of a second probe for simultaneous O₂ determinations.

**Gas calibrations.** In order to calibrate the electrode, the concentrations of the gases O₂, NO, and N₂O had to be determined independently in gas chromatographs equipped with thermal conductivity detectors. A Varian model 920 with a molecular sieve 5Å column at 50°C and with N₂ as the carrier gas served for O₂ determinations, and a Perkin-Elmer Sigma 3B with a Porapak Q column (1.8 m x ½ inches) at 50°C and with He as carrier gas using a flow rate of 40 ml/min was used for NO and N₂O measurements. Under the latter conditions, NO (retention time,
Organisms and experimental conditions. Azospirillum brasilense Sp 7 (ATCC 29145) was grown in continuous culture anaerobically with nitrite as respiratory electron acceptor as described previously [16]. For the N₂O-formation assay, the cells were centrifuged (16,000 g, 10 min) and suspended in the malate medium [2] in which all combined nitrogen was omitted. All handlings were performed anaerobically. The cell suspension was supplemented with C₂H₂ (final concentration, 2 mM) and 1.8 ml cells were transferred into the cuvette, which had previously been flushed with argon. After the electrode had become stable (which was achieved within 10 min), the reaction was started by adding 50 μl of an anaerobic solution of NaNO₂ (final concentration in the cuvette = 0.5 mM NaNO₂), and N₂O formation and O₂ content were monitored by the Clark probes. For measuring N₂O utilization by Azospirillum brasilense Sp 7, the cells were grown anaerobically with N₂O as the respiratory electron acceptor under batch culture conditions [12]. The medium was supplemented with 20 mM NH₄Cl, which was a better nitrogen source than N₂ and, therefore, enhanced growth. When grown for 24 h, the cells were centrifuged, suspended in fresh medium, and 1.8 ml (optical density of 560 nm = 0.6) were transferred to the cuvette. All steps were also done under anaerobic conditions. The reaction was started by adding N₂O (final concentration, 0.1 mM), and the utilization of the gas was monitored as described above.

For NO formation, A. brasilense Sp 7 was used that had been grown anaerobically in continuous culture with nitrite to an optical density of 560 nm = 0.2 [16]. The cells were concentrated 50-fold by centrifugation, broken twice in a French press at 140,000 kPa, and centrifuged (16,000 g, 10 min). The supernatant was assayed for NO production in the cuvette, which contained: phenazine methosulfate, 0.4 mM; Na-ascorbate, 27 mM; phosphate buffer, pH 7.5, 50 mM; and supernatant with 1.8 mg protein in the cuvette. The reaction was started by adding nitrite (final concentration, 11 mM), and NO formation was recorded amperometrically as described above.

For NO formation, Klebsiella pneumoniae K11 was grown in Hino and Wilson's medium [3] under N₂-fixing conditions for 24 h. The cells were diluted to an optical density of 0.4 by fresh medium under anaerobic conditions, and directly assayed for N₂O reduction in the cuvette under argon. The reaction was started by adding N₂O (final concentration, 0.12 mM).

Results

The current signal of the probe for 10 μM O₂, NO, or N₂O dissolved in H₂O was dependent on the polarizing voltage (Fig. 1). When the polarizing potential applied to the cathode was −0.3 V with respect to the Ag/AgCl electrode, the probe measured O₂ as usual, but was insensitive to NO or N₂O. The sensitivity of the probe to O₂ decreased at polarizing voltages between −0.5 and −0.7 V, but then it responded to NO and N₂O. The sensitivity of the probe to O₂ decreased at polarizing voltages between −0.5 and −0.7 V, but then it responded to NO (Fig. 1). At the more negative potentials of −0.9 to −0.95 V, all three gases could be determined by the probe. The sensitivity for N₂O was nearly half as much as that for O₂ at −0.95 V, but was clearly detectable (Fig. 1). When the probe was preconditioned by incubation with N₂O overnight at a polarizing voltage of −0.95 V, the sensitivity increased approximately tenfold as compared with an untreated probe. Such an effect has also been described for the H₂ probe [5, 15]. In contrast to the H₂ probe, the stability of the N₂O probe did not change for several days, even when a different polarizing voltage was applied. The currents for zero N₂O, NO, or O₂ concentrations were negligible at all polarizing voltages up to −1.0 V (see Fig. 2), which could easily be compensated for by the am-