Biochimica et Biophysica Acta, 682 (1982) 59–66
Elsevier Biomedical Press

BBA 41168

REDUCTION OF OXYGEN BY HYDROGEN IN CELLS OF ANAEROBICALLY GROWN *PROTEUS MIRABILIS*

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(Received March 25th, 1982)

Key words: Electron transport; Knallgas reaction; Hydrogenase; Oxygen reduction; (P. mirabilis)

Anaerobically grown cells of the enterobacteria *Proteus mirabilis, Escherichia coli, Salmonella typhimurium* and *Enterobacter cloacae* catalyse the reduction of oxygen by hydrogen (The Knallgas reaction). We have studied this reaction in detail in *P. mirabilis*. Oxygen at concentrations above approx. 20 μM inactivates the catalytic pathway for this reaction in a reversible way. The site of inactivation is located in the part of the pathway shared with the reduction of fumarate by hydrogen, possibly hydrogenase. Oxygen exerts its effect via the oxidation state of an unknown component in the bacteria, such that electron transfer from H_2 is blocked when this component is oxidised. We suggest that this component is identical to the regulating factor controlling hydrogen production via the formate-hydrogenlyase reaction (Krab, K., Oltmann, L.F. and Stouthamer, A.H. (1982) Biochim. Biophys. Acta 679, 51–59).

Introduction

When the enterobacterium *Proteus mirabilis* is grown anaerobically in the absence of high concentrations of alternative extracellular electron acceptors (such as nitrate), an extensive network of electron-transfer pathways is synthesised. This network includes the enzyme hydrogenase, that is involved both in the production of hydrogen from formate in the formate-hydrogenlyase reaction, and in the oxidation of hydrogen [1]. In spite of the anaerobic growth conditions, the organism also possesses the ability to reduce oxygen with the oxidases cytochrome a_1, cytochrome *d* [2,3] and cytochrome *o* [3]. This situation is analogous to that in the related *Escherichia coli*, when grown under the same conditions [4–6].

Because of the simultaneous presence of hydrogenase and oxidases, it may be expected that both *P. mirabilis and E. coli* (and perhaps other enterobacteria as well) are able to catalyse the Knallgas (oxyhydrogen) reaction:

$$2H_2 + O_2 \rightarrow 2H_2O \quad (1)$$

However, hydrogenases are notoriously susceptible to oxygen [7–12]. Also, we have shown that electron transfer towards hydrogenase in anaerobically grown *P. mirabilis* cells is under redox control [13], and the same may be true for electron transfer from hydrogenase.

If demonstrable, the Knallgas reaction provides and excellent system to test such effects (both redox and inhibitory) of oxygen. Inhibitory effects of oxygen on hydrogenase are one of the main problems that have to be faced in the development of a biophotolytic system for the production of hydrogen from water [7,14].

In this paper we show that *P. mirabilis* and *E. coli* do catalyse the Knallgas reaction, as do *Salmonella typhimurium* and *Enterobacter cloacae* but *Klebsiella aerogenes, K. edwardsii* and *K. pneu-*

monia do not. In *P. mirabilis* (and in *E. coli*) the system is inactivated reversibly by oxygen at concentrations above approx. 20 μM, presumably via a redox effect on a regulator of the pathway of electron transfer shared with reduction of fumarate by H_2.

Materials and Methods

The enterobacteria *P. mirabilis* (strain S 503), *E. coli, S. typhimurium* (S 785), *Ent. cloacae* (S 478), *K. aerogenes* (S 45), *K. edwardsii* (S 13) and *K. pneumoniae* (S 8) were each grown anaerobically under N_2 in pH-regulated batch cultures at pH 7 and 37°C in a medium containing 0.5% glucose, 0.5% NaCl, 0.5% yeast extract (Difco) and 0.8% nutrient broth (Oxoid No. 2). 1-l or 10-l batches were grown using a Microferm fermentor (New Brunswick Scientific Co.), cells were harvested at an absorbance of 1.0 (which for *P. mirabilis* corresponds to late linear growth phase, cf. Ref. 13), washed once in 25 mM Tris-HCl buffer, pH 7.2, and then stored in the same buffer at −80°C.

For determination of total $H_2 + O_2$ uptake the Warburg method was used [15]. No distinction was made between H_2 and O_2 because of the nearly identical flask constants of the two gases.

H_2 and O_2 were separately determined by polarography using Clark electrodes (Yellow Springs Instruments Co.). For determination of H_2 the electrode was polarised at −0.4 V [16,17], and before use treated according to the method of Janz [18] to improve performance of the Ag/AgCl cathode.Polarographic determinations were done in an open system consisting of a continuously refreshed gas phase (H_2, N_2, air or a mixture of air and H_2) in contact with a stirred liquid phase. O_2 was measured in both phases, H_2 in the liquid phase only. The vessel used was a thermostatically controlled multi-purpose vessel designed and build at the workshops of the Biological Laboratory of the Vrije Universiteit [19].

Protein was determined according to the method of Lowry et al. [20].

Catalase was obtained from Boehringer.

Results

When cells of *P. mirabilis* incubated anaerobically under hydrogen atmosphere are pulsed with a small amount of hydrogen peroxide (which is converted to oxygen by catalase present in the cells) gas uptake is observed (Fig. 1, trace a). This uptake proceeds to a final level at which all oxygen is taken up, together with twice its amount of hydrogen. Controls show that there is no H_2 uptake without addition of H_2O_2 (Fig. 1, trace b) and that gas uptake is much slower in the absence of H_2 (Fig. 1, trace d; trace c is a control with catalase instead of cells that shows the total amount of oxygen liberated from the H_2O_2). The experiment shows that these cells do catalyse the Knallgas reaction.

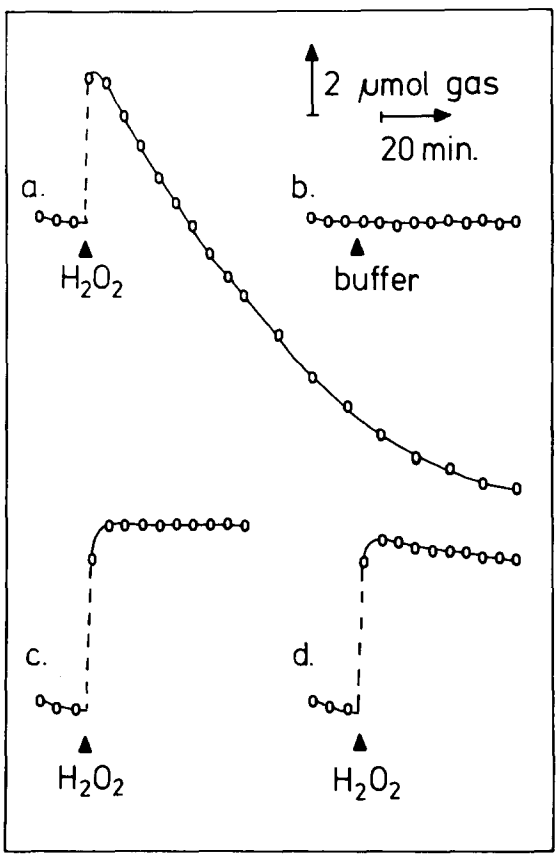

Fig. 1. Uptake of hydrogen and oxygen in the Knallgas reaction. (a) *P. mirabilis* cells (11.4 mg protein) were incubated in Warburg flasks at 37°C under H_2 in 300 mM sodium and potassium phosphate buffer, pH 6.5. The reaction was started by addition of 9.8 μmol H_2O_2 as indicated, after which the incubation volume was 3 ml. (b) As a, but with addition of buffer instead of H_2O_2. (c) As a, but with catalase instead of cells. (d) As a, but under N_2 instead of H_2.

Preincubation of the cells under air, hydrogen or nitrogen did not affect the rate of gas uptake, which may just indicate that any effects of aerobic preincubation are reversed during the final incubation under H_2 necessary for the experiment.

To resolve possible effects of oxygen on a shorter time scale, the experiments of Fig. 2 were carried out. Cells were incubated under a hydrogen atmosphere and pulsed with H_2O_2; O_2 and H_2 concentrations in solution were measured polarographically. Fig. 2A shows that after a small pulse of H_2O_2, the oxygen formed is consumed so rapidly that the oxygen concentration remains below 5 μM. The uptake of 129 nmol H_2 in response to addition of 122 nmol H_2O_2 shows that this rapid O_2 consumption is due to the Knallgas reaction. From the H_2 trace a rate of 0.96 μmol H_2/min per mg protein may be calculated, which is considerably faster than the rates measured manometrically (approx. 0.01 μmol H_2/min per mg protein, cf. Fig. 1).

After addition of a larger amount of H_2O_2, however, a completely different picture emerges. The oxygen concentration during the pulse reaches 48 μM. This is not exclusively due to the faster evolution of O_2 by catalase (the rate being proportional to the H_2O_2 concentration [21]) but as the figure shows, also to limited O_2 consumption in the Knallgas reaction: only 62 nmol H_2 are taken up fast. The remainder of the O_2 is taken up at a rate not much faster than the rate of endogenous respiration (not shown). After anaerobiosis a subsequent small H_2O_2 pulse again leads to fast O_2 and H_2 uptake via the Knallgas reaction. Hence, in this experiment the catalytic system needed for this reaction is inactive during the latter part of the large pulse (at relatively high oxygen concentration), but active again after anaerobiosis.

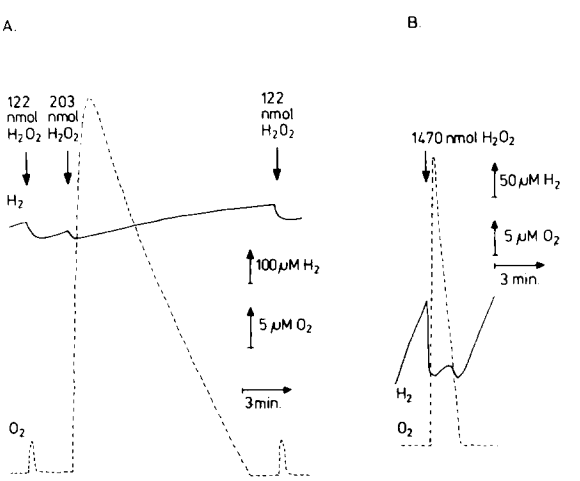

Fig. 2. Reversible inactivation of H_2 and O_2 uptake. (A) Cells (0.214 mg protein) were incubated anaerobically in the presence of 660 μM H_2, at 25°C in 300 mM sodium and potassium phosphate buffer (pH 6.5) in a total volume of 2 ml. The Knallgas reaction was inititated by additions of H_2O_2 as indicated. (B) as A, but with 8.56 mg protein of cells, and at 530 μM H_2. (_____) H_2, (- - - - -) O_2.

Fig. 2B shows that in some cases reactivation occurs already during the last stage of the large H_2O_2 pulse. At the high protein concentration used, there is a clear second phase of rapid uptake of H_2 (and O_2), showing that anaerobiosis is not a prerequisite for reactivation. (The upward trend of the H_2 traces in these experiments is due to diffusion of H_2 from the gas phase into the solution, characteristic of an open system).

To exclude the possibility that hydrogen peroxide itself is involved in any way in the putative Knallgas reaction, experiments were carried out in which the reaction was started by addition of cells to a buffer containing H_2 and O_2. Fig. 3A, trace a, shows that when *P. mirabilis* cells from an anaerobic suspension are added to start the reaction between 730 μM H_2 and 18.5 μM O_2, all oxygen is consumed rapidly with the simultaneous uptake of H_2. The rate of O_2 uptake is 0.54 μmol/min per mg protein and of H_2 uptake 1.11 μmol/min per mg protein, giving an H_2/O_2 ratio close to 2. This rate agrees with that measured in the experiment of Fig. 2A.

When the initial oxygen concentration is 42.9 μM (trace b) the rate of uptake of H_2 and O_2 decreases during the experiment, reflecting that inactivation of the Knallgas pathway does not occur instantaneously. Trace c is a control that shows the rate of oxygen uptake (at 26.7 μM O_2, no H_2) due to endogenous respiration by the cells.

Exactly the same result is obtained with anaerobically grown *E. coli* instead of *P. mirabilis* (not shown). Also, in *Ent. cloacae* and *S. typhimurium* the Knallgas reaction was found, but rates were too low to study the effect of oxygen. In *K. aerogenes, K. pneumoniae* and *K. edwardsii* the reaction was absent.

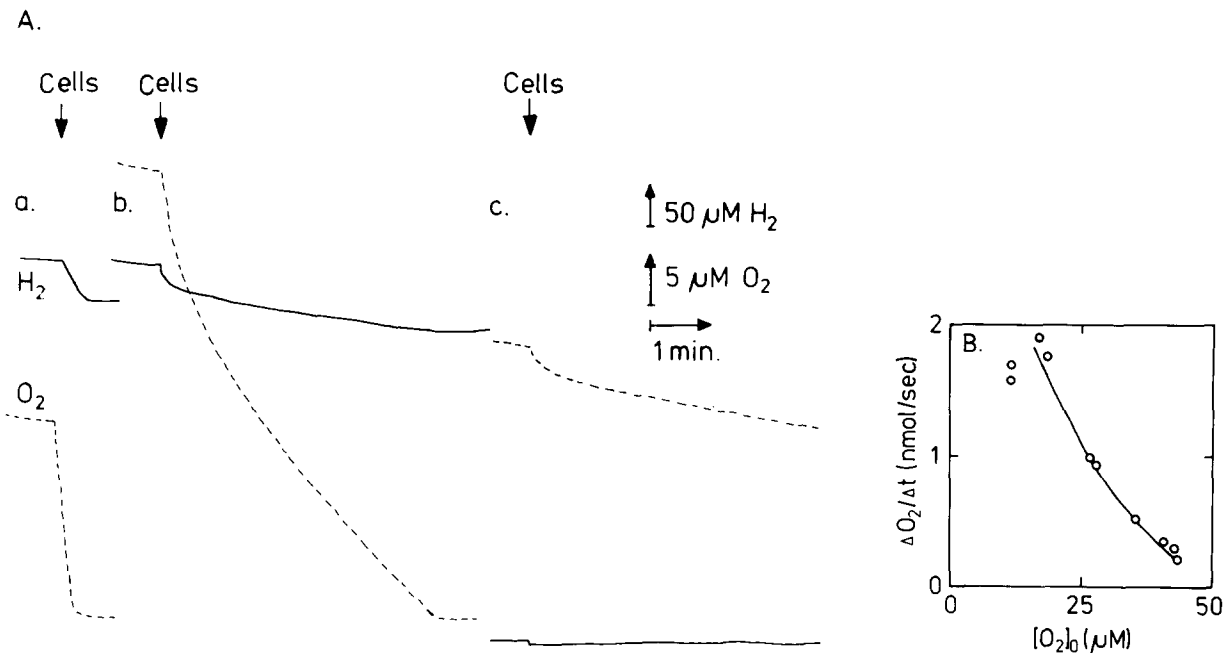

Fig. 3. The effect of oxygen on the Knallgas reaction in *P. mirabilis*. (A) Uptake of hydrogen and oxygen. The reaction was started by addition of cells (0.214 mg protein in anaerobic suspension) to 300 mM sodium and potassium phosphate buffer (pH 6.5) containing H_2 and O_2. The temperature was 25°C and the volume 2 ml. Initial concentrations were 730 μM H_2 and 18.5 μM O_2 (a); 690 μM H_2 and 42.9 μM O_2 (b); 0 μM H_2 and 26.7 μM O_2 (c). (———) H_2, (— — —) O_2. The H_2 traces are zero-shifted with respect to each other. (B) Inactivation as a function of inital O_2 concentration. The initial amount of O_2 present when cells are added as in A, divided by the time required to reach anaerobiosis is plotted against the initial oxygen concentration $[O_2]_0$.

Because of the inactivation during the reaction observed at higher O_2 concentrations, it is hard to calculate the rate of the Knallgas reaction directly from traces as in Fig. 3A. However, we can use the average rate of O_2 uptake defined as the initial amount of oxygen present (ΔO_2) divided by the time needed to reach anaerobiosis (Δt) as a semi-quantitative measure of inactivation. Fig. 3B is a plot of ($\Delta O_2/\Delta t$) against the inital oxygen concentration, and it clearly shows that inactivation increases with O_2 concentration (the decrease in $\Delta O_2/\Delta t$) at the lowest oxygen concentration is due to the slowness of the O_2 electrode, which leads to an overestimation of Δt). According to this figure the maximum average rate of the reaction is about 0.54 μmol O_2/min per mg protein. However, at the lowest O_2 concentrations, possible higher rates are obscured by the slowness of the O_2 electrode. At an inital O_2 concentration of 11.8 μM the rate of H_2 uptake (measured with the somewhat faster H_2 electrode) was as high as 1.86 μmol H_2/min per mg protein (trace not shown).

When an experiment as in Fig. 3A, trace a, was carried out with cells that were preincubated aerobically, the rate of O_2 uptake decreased by 95%. During the reaction there was no further inactivation, and the effect of aerobic preincubation was at least partly reversible.

Now that the experiments seem to establish that there is an inactivating effect of oxygen on the Knallgas reaction, it is worthwhile to find out which part of the catalytic pathway is involved in this sensitivity for oxygen. To this purpose we make use of a probe reaction, the reduction of fumarate by H_2 to succinate:

$$\text{fumarate} + H_2 \rightarrow \text{succinate} \qquad (2)$$

This reaction shares at least hydrogenase in its catalytic pathway with the Knallgas reaction.

Fig. 4, trace a, shows that when *P. mirabilis* cells that are incubated anaerobically in the presence of H_2 are given fumarate, H_2 is taken up at a rate of 0.67 μmol/min per mg protein.

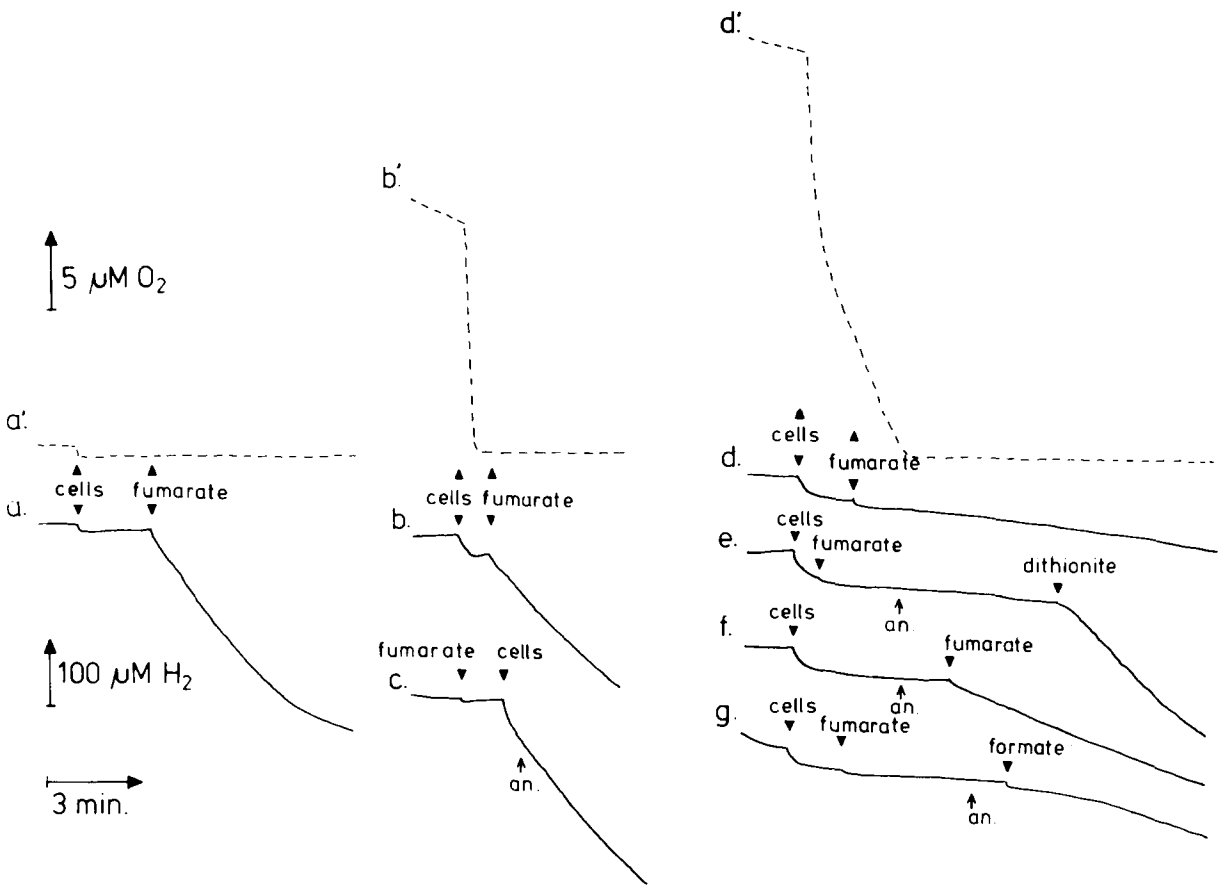

Fig. 4. The effect of oxygen on reduction of fumarate by H_2. The reactions were carried out as described in the legend to Fig. 3A. Additions: cells, 0.214 mg protein in anaerobic suspension; fumarate, 2.5 μmol sodium fumarate; formate, 6.25 μmol sodium formate; dithionite, a few grains of sodium dithionite dissolved in water; an. indicates point of anaerobiosis. Traces a', b' and d' give O_2 changes occurring during the corresponding H_2 traces a, b and d, respectively. (———) H_2, (- - - - -) O_2. Initial concentrations: a+a', 780 μM H_2, anaerobic; b+b', 770 μM H_2, 14.4 μM O_2; c, 770 μM H_2, 15.4 μM O_2; d+d', 710 μM H_2, 25.2 μM O_2; e, 710 μM H_2, 33.9 μM O_2; f, 710 μM H_2, 28.4 μM O_2; g, 710 μM H_2, 33.1 μM O_2.

Traces b (H_2) and b' (O_2) show that this rate is roughly the same as that obtained just after the Knallgas reaction has been carried out at low (14.4 μM) inital oxygen concentration. Also, when fumarate is added aerobically, before the Knallgas reaction is started at low inital oxygen concentration, there is no discernable effect on the rate of H_2 uptake due to fumarate reduction (trace c).

Quite different phenomena are observed at higher inital O_2 concentrations (traces d–g). In the experiment of traces d (H_2) and d' (O_2) the Knallgas reaction is started by addition of cells to 25.5 μM O_2 and 710 μM H_2. After a first phase in which O_2 is taken up rapidly together with H_2 (cf. Fig. 3A, trace b) fumarate is added during the second (aerobic) phase in which O_2 is taken up relatively slowly. Now there is no uptake of H_2 due to fumarate reduction, not even shortly after anaerobiosis. Only approx. 10 min after anaerobiosis a slow uptake of H_2 starts (trace d). In trace e it is shown that this inactivation may be overcome with dithionite: addition of this reductant starts the reduction of fumarate by hydrogen.

In trace f it is shown that addition of fumarate after anaerobiosis immediately results in H_2 uptake, albeit at a relatively low rate. It seems that it is the combined effect of fumarate and oxygen

that causes the extreme sluggishness of H_2 uptake in trace d; which suggests that we are dealing with a redox effect (both oxygen and fumarate being oxidants). The rate of H_2 uptake increases when fumarate is added later after anaerobiosis (not shown).

Trace g finally shows that also addition of formate partly abolishes inactivation of fumarate reduction, and thus leads to H_2 uptake.

Together, these findings indicate that there is a redox-dependent activation/inactivation of fumarate reduction by H_2, which is likely to be the same effect as that found on the Knallgas reaction.

Discussion

Our experiments show that when *P. mirabilis* (or *E. coli*) is grown anaerobically on a rich broth medium in the absence of added electron acceptors, an electron-transfer pathway is present that catalyses the reduction of oxygen by hydrogen (the Knallgas reaction). This reaction is known to occur in hydrogen-oxidising bacteria, where it yields energy [4,22].

The reaction takes place at relatively low oxygen concentrations (below approx. 20 μM); at higher oxygen concentrations it is inhibited. This inhibition is the result of a slow inactivation of the catalytic pathway. Inactivation is reversed after anaerobiosis, but sometimes activation already is observed when the O_2 concentration falls to the low region, but is not yet zero (Fig. 2, trace b). Also, when inactivation is the result of aerobic preincubation of cells in the absence of H_2, no further inactivation is seen during the course of the Knallgas reaction.

Hence, inactivation depends on the concentration of oxygen, and not exclusively on the duration of aerobic exposure.

The reaction rates measured manometrically are two orders of magnitude lower than those measured polarographically. The reason for this discrepancy probably is the way the reaction is started in the manometric assay, i.e., by adding a massive pulse of H_2O_2 (initial concentration 3.3 mM). This may either result in effects of the H_2O_2 itself, or in the subsequent generation of a higher (inhibiting) steady-state O_2 concentration in solution than expected for a manometric system in equilibrium (from the solubility of oxygen and the volumes of gas and liquid phase in the flask, approx. 10 μM for this amount of H_2O_2). Such an explanation is in line with the results shown in Fig. 2, where it is shown that an increase in the amount of H_2O_2 added leads to inactivation of the Knallgas pathway.

Parallel to the Knallgas reaction, the reduction of fumarate by H_2 is inhibited at high oxygen concentrations. We cannot exclude the possibility that there may be a separate inhibiting effect on fumarate reduction, but because of the similarity with the effect of oxygen on the Knallgas reaction (cf. Fig. 4) we assume that this inhibition is an effect of the same cause. The site of inactivation then is located at the part of the pathway shared by the two reactions. This part includes hydrogenase, and perhaps other as yet unknown factors such as quinones or cytochromes.

Many hydrogenases, including those of *E. coli* [23] and *P. vulgaris* [8], are known to be O_2 sensitive. It is thought that this sensitivity is the result of interaction with oxygen radicals, expecially O_2^- [9–11]. Sometimes this radical is generated by the enzyme itself under reducing conditions [10,11]. The effect would presumably be on the iron-sulphur centers present in hydrogenase [24]. This mechanism seems to occur especially with isolated enzymes [7,10], but also in cells [11]. The membrane-bound hydrogenase from *Alcaligenes eutrophus* is inhibited (reversibly) by O_2 in a competitive way [12].

The results obtained with the probe reaction, reduction of fumarate by hydrogen, suggest another mechanism for the effect of oxygen. We have seen that fumarate, when added under aerobic conditions with an inhibited Knallgas reaction (i.e., during a Knallgas reaction started at high $[O_2]$; see Fig. 4, trace d), prevents reactivation of H_2 uptake a long time after anaerobiosis (cf. Fig. 4, trace f). This suggests that hydrogen uptake depends on the redox state of some regulatory component such that H_2 may be taken up only when this component is reduced. Oxygen (at high concentration) would keep the regulatory component oxidised; when the O_2 concentration is lowered, and especially after anaerobiosis the component is reduced (by endogenous substrate) and H_2 uptake is accelerated. Aerobic addition of fumarate would

delay reduction of the regulating component by competing for endogenous reducing equivalents (cf. Ref. 13), and hence postpone reactivation. The activating effects of the reductants dithionite and formate on H_2 uptake (Fig. 4, traces e and g) would also be via reduction of the regulator. Thus H_2 uptake in *P. mirabilis* (and presumably also in *E. coli*) is redox controlled.

We have shown before [13] that evolution of H_2 by means of the formate-hydrogenlyase reaction in *P. mirabilis* is subject to redox control. The question arises as to whether the same regulating factor is responsible. In both cases the regulating component has to be reduced to permit either production or uptake of H_2. A definite answer to this question in our opinion has to await identification of the redox element(s) involved, although some information may be obtained from a comparison of the concentration dependencies of the effects of oxygen on hydrogen uptake and production, respectively.

A similar inhibitory effect of O_2 on anaerobic electron transport to nitrate in *Pseudomonas aeruginosa* [25,26] and in *Paracoccus denitrificans* [27] has been interpreted in terms of oxidation of a controlling factor by oxygen [26,27].

It is interesting that the redox state of the regulator of H_2 uptake depends on the O_2 concentration in the range 10–35 μM. This means that oxidation of the regulator by oxygen occurs via an oxidase with relatively low affinity for O_2 (a high K_m). The kinetics of O_2 uptake in the Knallgas reaction itself (cf. Figs. 2–4), on the other hand, show that O_2 is used via a component with a high affinity for oxygen (no decrease of rate when $[O_2]$ falls from 10 μM to zero, in experiments without inactivation). Hence, oxygen exerts its control on the reaction via a different pathway.

This possibility for regulation may be one of the reasons for the co-existence of so many oxidases under anaerobic growth conditions.

Acknowledgements

The authors wish to thank K. Maurer for his skilful technical assistance, and H. Wong Fong Sang for advice regarding the use of the hydrogen electrode. This investigation was supported by a grant from the Netherlands Organisation for the Advancement of Pure Research (ZWO) under the auspices of the Netherlands Foundation for Chemical Research (SON).

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