INFLUENCE OF TUNGSTATE ON THE FORMATION AND ACTIVITIES OF FOUR REDUCTASES IN *PROTEUS MIRABILIS*

Identification of two new molybdo-enzymes: chlorate reductase and tetrathionate reductase

L. F. OLTMANN, V. P. CLAASSEN, P. KASTELEIN, W. N. M. REIJNDERS and A. H. STOUTHAMER
Department of Microbiology, Biological Laboratory, Free University, De Boelelaan 1087, 1007 MC Amsterdam, The Netherlands

Received 16 July 1979

1. Introduction

Proteus mirabilis can form four reductases, which function as terminal oxidases of anaerobic respiratory chains. They are nitrate reductase (reduction of nitrate and chlorate), chlorate reductase (reduction of only chlorate), tetrathionate reductase (reduction of tetrathionate, trithionate and thiosulfate) and fumarate reductase (reduction of fumarate) [1–3]. The reduction of chlorate by either nitrate reductase or chlorate reductase can be distinguished since only the latter one is functioning in the presence of 1 mM azide [1,4].

Normally, none of these reductases is formed during aerobic growth. During anaerobic growth in the presence of nitrate, only nitrate reductase is formed, the formation of the other three reductases is repressed. During anaerobic growth without nitrate, chlorate reductase, tetrathionate reductase and fumarate reductase are formed, whilst the formation of nitrate reductase is repressed [3,5].

It is widely accepted that nitrate reductase, like a number of other oxidoreductases, needs the incorporation of a molybdenum-cofactor for its activity [6–9]. Therefore, during growth in the presence of tungstate in a 1000-fold excess with respect to molybdate, bacteria cannot form active nitrate reductase since tungstate ousts molybdate during the assemblage procedure in that case [10,11].

Up till now it is unknown whether a molybdenum-cofactor is concerned with the activities of chlorate reductase, tetrathionate reductase and fumarate reductase. Here, chlorate reductase and tetrathionate reductase, but not fumarate reductase, are identified as molybdo-enzymes. In addition the interference of tungstate with the regulation of the formation of the four reductases is shown.

2. Materials and methods

P. mirabilis was grown in complete medium consisting of 0.8% Nutrient Broth (Oxoid no. 2), 0.5% Yeast Extract (Oxoid L21), 0.5% glucose and 0.5% NaCl, if necessary supplemented with 0.35% KNO_3.

Cell-free extracts were prepared by sonification (Branson Sonifer B 12) of cell suspensions (~200 mg/ml, wet wt) during two periods of 90 s, interrupted for cooling of the sample and followed by a moderate centrifugation step (8000 × *g* during 5 min) for the removal of unbroken cells.

The activities of nitrate reductase, chlorate reductase, tetrathionate reductase and fumarate reductase were measured manometrically as described before [5].

Protein was measured by the Lowry method [12]. The molybdenum content of protein sample was measured by flameless atomic absorption spectrophotometry employing a Pye Unicam SP 1900 equipped with a graphite cell.

3. Results

3.1. *Influence of tungstate on reductase activities*

P. mirabilis was grown anaerobically in batch cultures on complex medium either with and without nitrate and various amounts of tungstate. Cell-free extracts, prepared from those cultures, were tested on specific reductase activities. The results, summarized in table 1, indicate a decimation of the reduction rates of all substrates except fumarate in cells grown in the presence of 10^{-3} M Na_2WoO_4. It strongly suggests that besides nitrate reductase also chlorate reductase and tetrathionate reductase, but not fumarate reductase are molybdo-enzymes. This supposition could be confirmed by direct measurements of molybdenum by atomic absorption spectrophotometry. In preparations of both chlorate reductase and tetrathionate reductase, purified as in [13,14], $\geqslant$0.75 atoms molybdenum/molecule reductase were found.

Another conclusion could be drawn from the data shown in table 1. The usual regulation pattern, formation of nitrate reductase and repression of the formation of the other three reductases during anaerobic growth in the presence of nitrate, appears to be disturbed by the addition of tungstate to the growth medium. In parallel with decreasing nitrate reductase activity under the influence of increasing amounts of tungstate, more fumarate reductase eludes from repression. How far the regulation of the formation of the other reductases is altered too, cannot be screened of course by direct determination of their activities. They should be reactivated first (see section 3.2).

Table 1
Specific reduction rates in cell-free extracts from *P. mirabilis* anaerobically grown either with or without nitrate in the presence of various amounts of tungstate

Substrate in assay	Nitrate during growth	Specific reduction rate[a]		
		Concentration of Na_2WoO_4		
		0	10^{-4} M	10^{-3} M
Nitrate	+	42	9	3
Nitrate	–	1	0	0
Chlorate	+	44	8	2
Chlorate	–	28	3	5
Thiosulfate	+	0	0	0
Thiosulfate	–	35	3	5
Fumarate	+	0	23	40
Fumarate	–	65	70	56

[a] Measured as μmol substrate reduced .h^{-1} .mg protein^{-1}

3.2. *Influence of tungstate on the formation of reductase proteins*

As shown in table 1, *P. mirabilis* grown in the presence of 10^{-3} M Na_2WoO_4 hardly exhibits nitrate-, chlorate- and tetrathionate reductase activities. However, a considerable reactivation of these enzymes could be achieved by the addition of Na_2MoO_4 (final conc. 10^{-3} M) to such cultures. Recently, similar reactivation experiments were described [11]. The reactivation reached its maximum effect within 10 min and seemed to be independant of protein synthesis, since previous addition of chloramphenicol (final conc. 10 μg/ml) did not interfere at all with the reactivation. The reactivation succeeded equally well directly in the culture and in suspensions of harvested cells, as long as the cells were intact. However there was a very strong influence of the state of growth of the cells on the levels of the ultimately measured enzyme activities. Employing preparations from different batch-cultures, the measured reductase activities after reactivation varied with a factor 10, with optimal values for cells which were harvested in the exponential growth phase. In order to get significant and reproducible data it appeared to be necessary to standardize the cells at their maximum growth rate by means of the pH-auxostat principle (see [15]). Such continuous cultures, with a working volume of 1 l, were grown anaerobically on complex medium + 10^{-3} M Na_2WoO_4, with or without nitrate, until a steady state at a cell density of ~2 g/l (wet wt) was achieved. At that moment the supply of fresh medium was cut off, and the growth in the culture was stopped by addition of chloramphenicol (10 μg/ml). After 10 min incubation time, half of the culture was harvested, the rest was supplied with Na_2MoO_4 (10^{-3} M) and harvested after a second incubation time of 10 min. Both samples were worked up into cell-free extracts and analysed for specific reductase activities. The results are summarized in table 2. The most striking result in cells grown without nitrate is the high nitrate reductase activity after reactivation (compare with table 1). Obviously, in the

Table 2
Specific reductase activities measured in cell-free extracts of *P. mirabilis*, after reactivation by the addition of molybdate

Enzyme	Nitrate during growth	Specific activities	
		Before reactivation	After reactivation
Nitrate reductase[a]	+	0	61
Nitrate reductase	–	0	48
Chlorate reductase[b]	+	0	0
Chlorate reductase	–	0	14
Tetrathionate reductase[c]	+	0	0
Tetrathionate reductase	–	0	3
Fumarate reductase	+	58	27
Fumarate reductase	–	100	102

[a] Measured as μmol KNO_3 reduced .h^{-1} .mg $protein^{-1}$
[b] Measured as μmol $KClO_3$ reduced .h^{-1} .mg $protein^{-1}$ in the presence of 1 mM NaN_3
[c] Measured as μmol $Na_2S_2O_3$ reduced .h^{-1} .mg $protein^{-1}$

Cells were grown anaerobically in the presence of tungstate, either with or without nitrate

presence of tungstate the bacteria need no nitrate any more for derepression of the synthesis of (inactive) nitrate reductase. The specific activities of chlorate reductase and especially tetrathionate reductase after reactivation did not come up to expectation. It could mean that less of these enzymes had been formed during growth in the presence of tungstate or that reactivation of these two enzymes passes less successfully.

In cells grown in the presence of tungstate and nitrate there is after reactivation a very high nitrate reductase activity, but no active chlorate reductase and tetrathionate reductase at all. Again, it is no inevitable proof for the entire absence of the latter two enzymes. Fumarate reductase was present in cells grown with and in cells grown without nitrate, although less in the former.

Treatment with molybdate always resulted in a further lowering of the specific fumarate reductase activity in nitrate containing cultures, which probably indicates a very rapid inactivation of this enzyme in the presence of an operating nitrate reductase.

4. Discussion

In the previous sections evidence was given for the identification of two new molybdo-enzymes, chlorate reductase and tetrathionate reductase. The presence of molybdenum in the former enzyme is not unexpected. It catalyzes a reaction which is also catalyzed by nitrate reductase, known for years to be a molybdo enzyme. Moreover the reduction of ClO_3^- to ClO_2^- has in common with all known reactions catalyzed by molybdo-proteins the collective action of protons and electrons on the oxidant. The model [16] for the action of molybdenum in enzymes is based on this type of coupled proton and electron transfer. Viewed in that light, the presence of molybdenum in tetrathionate reductase is more surprising. As described [2] this enzyme catalyzes the following reactions:

$$S_4O_6^{2-} + 2\,e \rightleftharpoons 2\,S_2O_3^{2-}$$

$$S_3O_6^{2-} + 2\,e \rightleftharpoons S_2O_3^{2-} + SO_3^{2-}$$

$$S_2O_3^{2-} + 2\,e \rightleftharpoons S^{2-} + SO_3^{2-}$$

Apart from pH-dependant proton association/dissociation, no protons seemed to be concerned with these reactions in the way formulated [16]. On the other hand, at the moment the reaction, mechanisms of the reduction of the concerning polythionates are not sufficiently understood to be sure that there is no involvement of protons at all.

The aspects of regulation described here, largely fit in with the conclusions [5] with regard to the formation of reductases in a chlorate resistant mutant of *P. mirabilis*. This mutant was supposed to be disturbed in the processing of molybdenum, and therefore, should be comparable to a certain extent with the wild-type strain grown in the presence of tungstate. In that mutant, fumarate reductase was also found to be the only active reductase, whereas analysis of the protein composition of cytoplasmic membrane preparations from the mutant indicated that during anaerobic growth either with or without nitrate the other three reductases were formed but inactive as well.

Based on these observations and data with respect to the formation of reductases in the presence of azide [17,18] it was proposed before that nitrate reductase represses its own biosynthesis unless it interacts with nitrate or azide (inhibitor of nitrate

reductase) or lacks a complete molybdenum-cofactor [5]. The present data on the formation of nitrate reductase in the presence of tungstate support this auto-regulation model. Furthermore it was concluded that the formation of the other three reductases is not repressed by nitrate directly, but only by the presence of a functioning electron transfer to nitrate. At least with regard to the formation of fumarate reductase, that conclusion is confirmed by the presented data here.

Acknowledgements

This work was supported in part by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Pure Research (ZWO).

References

[1] De Groot, G. N. and Stouthamer, A. H. (1969) Arch. Mikrobiol. 66, 220–233.
[2] Oltmann, L. F. and Stouthamer, A. H. (1975) Arch. Microbiol. 105, 135–142.
[3] Van der Beek, E. G., Oltmann, L. F. and Stouthamer, A. H. (1976) Arch. Microbiol. 110, 195–206.
[4] Pichinoty, F. (1965) Bull. Soc. Chem. Biol. 47, 1526–1528.
[5] Oltmann, L. F., Reijnders, W. N. M. and Stouthamer, A. H. (1976) Arch. Microbiol. 111, 37–43.
[6] Lam, Y. and Nicholas, D. J. D. (1969) Biochim. Biophys. Acta 178, 225–234.
[7] Lester, R. L. and DeMoss, J. A. (1971) J. Bacteriol. 105, 1006–1014.
[8] Der Vartanian, D. V. and Forget, P. (1975) Biochim. Biophys. Acta 379, 74–80.
[9] Bosma, H. J., Wever, R. and Van 't Riet, J. (1978) FEBS Lett. 90, 107–111.
[10] Enoch, H. G. and Lester, R. L. (1972) J. Bacteriol. 110, 1032–1040.
[11] Scott, R. H., Sperl, G. F. and DeMoss, J. A. (1979) J. Bacteriol. 137, 719–726.
[12] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[13] Oltmann, L. F., Schoenmaker, G. S. and Stouthamer, A. H. (1974) Arch. Microbiol. 98, 19–30.
[14] Oltmann, L. F., Reijnders, W. N. M. and Stouthamer, A. H. (1976) Arch. Microbiol. 111, 25.
[15] Oltmann, L. F., Schoenmaker, G. S., Reijnders, W. N. M. and Stouthamer, A. H. (1978) Biotechnol. Bioeng. XX, 921–925.
[16] Stiefel, E. I. (1973) Proc. Natl. Acad. Sci. USA 70, 988–992.
[17] De Groot, G. N. and Stouthamer, A. H. (1970) Biochim. Biophys. Acta 208, 414–427.
[18] De Groot, G. N. and Stouthamer, A. H. (1970) Arch. Mikrobiol. 74, 326–339.