CO_2 REDUCTASE FROM *CLOSTRIDIUM PASTEURIANUM*: MOLYBDENUM DEPENDENCE OF SYNTHESIS AND INACTIVATION BY CYANIDE*

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1. Introduction

The reduction of CO_2 to formate is an important reaction in the metabolism of many clostridia. It is the initial step both in the total synthesis of acetate from CO_2 in *Cl. thermoaceticum* and *Cl. acidi urici* [1,2] and in one-carbon unit synthesis from CO_2 in *Cl. pasteurianum* [3,4].

For many years a reduction of CO_2 to formate could not be demonstrated in vitro. All known formate dehydrogenases only catalyzed the oxidation of formate to CO_2 rather than the reverse reaction. It was not until 1970 that a direct CO_2 reduction to formate was discovered in cell free lysates of *Cl. pasteurianum* [3]. The enzyme catalyzing the reaction was tentatively named CO_2 reductase. In the meantime 'CO_2 reductases' have also been found in *Cl. thermoaceticum* [5] and *Cl. acidi urici* [6].

In the present investigation evidence is presented indicating that the CO_2 reductase from *Cl. pasteurianum* most probably is a molybdoenzyme: It is synthesized only in the presence of molybdenum and is inactivated by low concentrations of cyanide, as are other molybdoenzymes. A dependence of CO_2 reductase synthesis on selenium, as has been reported for the formate dehydrogenase from *Escherichia coli* [7–11], is definitely ruled out.

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2. Materials and methods

All chemicals were reagent grade from E. Merck, Darmstadt, Germany. Methyl viologen was supplied by Serva, Heidelberg, Germany.

Cl. pasteurianum ATCC 6013 and *Escherichia coli* B 25 were grown at 30°C on sterilised standard medium: glucose, 20 g; NaCl, 100 mg; NH_4Cl, 1 g; $MgSO_4 \times 7\ H_2O$, 200 mg; $FeSO_4 \times H_2O$, 50 mg; $K_2HPO_4 \times 3\ H_2O$, 20.5 g; KH_2PO_4, 1.4 g; biotin, 0.01 mg; *p*-aminobenzoic acid, 0.1 mg; glas redistilled water to 1 litre. Sodium molybdate (10^{-9} M to 10^{-4} M), sodium selenite (10^{-6} M), and sodium tungstate (10^{-3} M) were added where indicated. A 10% inoculum was used in all experiments.

Cell-free lysates of *Cl. pasteurianum* were prepared by incubating 3 g of frozen cells (wet weight) in 9 ml H_2O with 6 mg of lysozyme and 1 mg of DNAase under H_2 at 35°C for 30 min and by then centrifuging at 35 000 *g* for 30 min. Five to twenty µl of the supernatant were used in the inactivation studies.

The activity of the CO_2-reductase from *Cl. pasteurianum* and of the formate dehydrogenase from *E. coli* was determined by measuring the rate of methyl viologen reduction with formate as electron donor, a reaction which has been shown to be catalyzed by both enzymes [3, 11]. The reduction was followed photometrically at 30°C at 578 nm ($\epsilon_{578} = 9700$ cm^2/mmole). Protein was determined by the biuret method [12]. Detailed procedures are given in the legends to tables and figures.

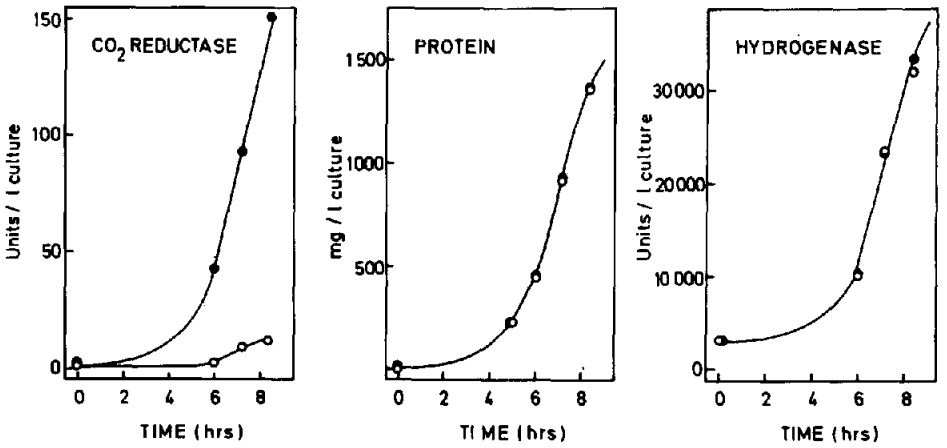

Fig. 1. Effect of molybdenum on the rate of CO_2 reductase synthesis, of growth, and of hydrogenase synthesis of *Cl. pasteurianum*. (○) = standard medium; (●) = standard medium + Na_2MoO_4 (10^{-6} M); 10 ml aliquots were taken from a growing 250 ml culture after the culture had produced 200 ml, 400 ml and 600 ml hydrogen. After centrifugation (10 min at 0°C and 8000 *g*) the supernatant was discarded and the cell pellet resuspended in 1, 2 or 3 ml Tris acetate buffer pH 8.0, 50 mM, containing 25 mM mercaptoethanol. The suspension was incubated for 30 min at 30°C under hydrogen in order to exhaust the cells from endogenous substrates. Aliquots of the suspension were then immediately assayed for enzyme activities and proteins. *CO_2 reductase*: Tris acetate pH 8.5, 100 mM; mercaptoethanol, 50 mM; methyl viologen, 5 mM; formate, 10 mM; H_2O to 1 ml; gas phase, argon; anaerobic cuvettes; start with 10 or 20 µl suspension. *Protein* was determined after precipitation with trichloroacetic acid by the biuret method [12]. *Hydrogenase*: Tris acetate pH 8.5, 100 mM; mercaptoethanol, 50 mM; methyl viologen, 5 mM; H_2O to 1 ml; gas-phase, hydrogen; anaerobic cuvettes; start with 1 or 2 µl suspension. 1 Unit = 1 µmole methyl viologen reduced by formate per minute. The data represent mean values of 5 experiments.

3 . Results

3.1. *Dependence of CO_2 reductase synthesis on molybdenum in growing cultures*

Formate dehydrogenase formation in *E. coli* is known to be dependent on both molybdenum and selenium [7–11]. Therefore it was of interest to determine the effect of these trace elements on CO_2 reductase synthesis in *Cl. pasteurianum*. This organism was grown on standard media deficient in molybdenum or selenium and on standard media supplemented with either of these elements. The rate of synthesis of CO_2 reductase was measured and related to the rate of growth and of hydrogenase formation. Hydrogenase synthesis was chosen as a control as this enzyme does not contain either molybdenum or selenium [13].

3.1.1. Molybdenum

The rate of CO_2 reductase synthesis was drastically different when *Cl. pasteurianum* was grown on molybdenum deficient or on molybdenum supplemented media (fig. 1). In the presence of sodium molybdate enzyme synthesis was increased over ten-fold. Concentrations as low as 10^{-9} M were sufficient for this effect. Tungstate did not substitute for molybdate even at levels as high as 10^{-3}. The low rate of CO_2 reductase formation observed when *Cl. pasteurianum* was grown on molybdenum deficient media is probably due to impurities in the reagents and water used to prepare the media. It is known that it is practically impossible to obtain media free of molybdenum [7,9].

The rate of growth (overall increase of bacterial protein) and of hydrogenase formation was not altered by the molybdenum content of the media (fig. 1). These controls indicated that molybdenum had a specific effect on CO_2 reductase formation only rather than an unspecific effect on overall protein synthesis.

3.1.2. Selenium

The rate of CO_2 reductase synthesis and of growth of *Cl. pasteurianum* were not altered when selenite (10^{-6} M) was added to the growth media. The specific activity of the enzyme remained unchanged (table 1).

Table 1
Effect of selenium on the specific activity of the CO_2 reductase from *Cl. pasteurianum* and of the formate dehydrogenase from *E. coli* in growing cultures.

	+Se	−Se
	U/g protein	
CO_2 reductase (*Cl. pasteurianum*)	110	110
Formate dehydrogenase (*E. coli*)	210	10

+ Se = standard medium + $Na_2MoO_4(10^{-6}$ M) + Na_2SeO_3 (10^{-6} M);
− Se = standard medium + $Na_2MoO_4(10^{-6})$.
The specific activities, which were determined as described in the legends to fig. 1, were constant over the whole growth period. 1 Unit = 1 μmole methyl viologen reduced by formate per minute. The data represent mean values of 5 experiments.

When *E. coli* was grown anaerobically on the same medium a drastic effect on the specific activity of the formate dehydrogenase was observed (table 1), indicating that the standard medium was essentially free of selenium. 10^{-9}M selenite has been shown to be sufficient to obtain maximal activities of the formate dehydrogenase in *E. coli* [7]. It therefore must be concluded that CO_2 reductase formation in *Cl. pasteurianum* is independent of selenium.

3.2. *Inactivation by cyanide of CO_2 reductase activity in cell free lysates.*

Cyanide is known to inactivate many molybdoenzymes [14, 15]. Therefore the effect of this compound on the activity of the CO_2 reductase was determined.

Cyanide (10^{-4} M) rapidly inactivated the CO_2 reductase in cell-free lysates (fig. 2A). The inactivation process followed simple pseudo first order kinetics: The semi-logarithmic plot of $V_t/V_{t=0}$ versus time was linear (fig. 2B) and the slopes of the plots obtained at different cyanide concentrations were proportional to the cyanide concentration. Thus the second order rate constant for the inactivation process could be calculated:

$$k = -\frac{2.3 \log V_t/V_{t=0}}{(CN^-) \cdot t}$$

k was found to be equal to 10^2 M^{-1} sec^{-1} at 25°C and pH 9 and equal to 0.5 × 10 $M^{-1}sec^{-1}$ at pH 6.5.

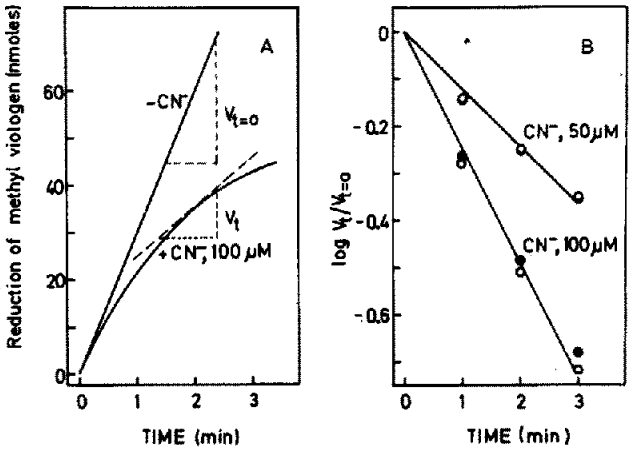

Fig. 2. Inactivation of the CO_2 reductase by cyanide. A. Kinetics of the reduction of methyl viologen by formate with and without cyanide. B. Semi log plot of the time course of the inactivation process. V_t = velocity of methyl viologen reduction at different times in the presence of cyanide. $V_{t=0}$ velocity of methyl viologen reduction in the absence of cyanide. *Assay*: Tris acetate, pH 9.0; methyl viologen, 5 mM; formate, 10 mM; mercaptoethanol, 50 mM; H_2O ad 1 ml; gas phase argon; potassium cyanide as indicated; anaerobic cuvettes; start with cell-free lysate of *Cl. pasteurianum* ((○) 5 μl, (●) 10 μl).

4. Discussion

In this paper evidence was presented that in growing cultures of *Cl. pasteurianum* the synthesis of an active CO_2 reductase is dependent on molybdenum. Dependence of the synthesis of an enzyme on molybdenum theoretically can be the result of three different independent effects: 1) the metal is essential for either transcription or translation, 2) the metal is required for the synthesis of a cofactor or 3) the metal is the cofactor proper. Possibility 1 was excluded, as no effect of molybdenum was observed on either the overall rate of protein synthesis or on the rate of synthesis of a particular enzyme, hydrogenase, known not to contain molybdenum. Possibility 2 seems unlikely as known cofactors as FAD, FMN, pyridine nucleotides or tetrahydrofolate e.g., are not formed in molybdenum dependent reactions. Thus possibility 3 appears highly probable; molybdenum functions directly as the cofactor of the CO_2 reductase. It should be pointed out that many molydoenzymes e.g., the nitrogenases and the nitrate reductases were first identified as molybdoenzymes by growth experi-

ments [14]. The best evidence that molybdenum is absolutely essential for the function of these two enzymes still comes from the finding that the synthesis of an active nitrogenase [16] and nitrate reductase [17] is specifically dependent on molybdenum.

The conclusion that the CO_2 reductase is a molybdoenzyme was further substantiated by the observed inactivation of the enzyme by low concentrations of cyanide. Cyanide is known to inactivate molybdoenzymes such as xanthine oxidases [15], aldehyde oxidases [18], sulfite oxidases [19], and nitrate reductases [20]. Only the molybdoenzyme nitrogenase, for which, however, cyanide can function as a substrate [21], appears not to be inactivated by cyanide [22].

Final evidence that the CO_2 reductase is a molybdoenzyme will have to await purification of the enzyme, which has already proved to be very difficult due to the extreme oxygen sensitivity of the CO_2 reductase.

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