NADH, A PHYSIOLOGICAL ELECTRON DONOR IN CLOSTRIDIAL NITROGEN FIXATION*

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1. Introduction

Clostridial nitrogenase catalyzes the ATP dependent reduction of N_2 to NH_3 with reduced ferredoxin as electron donor [1]. In cell-free extracts of N_2-grown *Clostridium pasteurianum* several systems have been used to regenerate the reduced ferredoxin required in the nitrogenase reaction: pyruvate and pyruvate-ferredoxin oxidoreductase [2], hydrogen and hydrogenase [3] as well as formate and CO_2- reductase [4]. Therefore pyruvate, hydrogen and formate are generally regarded as the physiological reductants in clostridial nitrogen fixation [5,6]. NADH has also been reported to reduce ferredoxin via a NADH–ferredoxin oxidoreductase in several clostridia, which were however NH_3-grown and thus devoid of the nitrogenase system [7–10]. Since the NADH-ferredoxin oxidoreductase has not been demonstrated in N_2-grown clostridia as yet, NADH does not appear to be accepted as an electron donor for clostridial N_2-reduction, although this was indicated by preliminary evidence [3] and theoretical considerations [11].

In this communication it is shown that NADH-ferredoxin oxidoreductase is present also in cell-free extracts of N_2-grown *Cl. pasteurianum* and that it can be coupled with nitrogenase to catalyze acetylene reduction to ethylene (the usual test reaction [12]) with NADH. Evidence is discussed indicating that only pyruvate and NADH rather than hydrogen or formate function as physiological reductants in clostridial nitrogen fixation.

2. Methods

Cl. pasteurianum ATCC 6013 was grown on 50 l standard medium [13] with NH_3 or N_2 as nitrogen source. The bacteria were harvested when the growth medium had reached a pH of 5.3 and stored at minus 20°C. Cell-free crude lysates were prepared by incubating 10 g of frozen cells (wet weight) in 20 ml water with 15 mg lysozyme and 1 mg of DNase at pH 7 under H_2 at 35°C for 30 min and by then centrifuging at 20 000 *g* for 30 min at 20°C. Nucleotide free lysates were made by anaerobically stirring 10 ml of the crude lysate with 5 g (wet weight) Dowex-acetate 2 × 8, 100–200 mesh at 20°C for 30 min. Ferredoxin free lysates were obtained by anaerobically passing 10 ml of the crude lysate through a small column filled with DEAE-cellulose (1 cm diameter and 1 cm high) previously equilibrated with 100 mM Tris–acetate, pH 7.5, containing 25 mM mercaptoethanol and 5 mM dithioerythritol. Ferredoxin was purified as described by Mortenson [14].

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All assays were carried out at 35°C in 7 ml test tubes closed with a rubber stopper. Anaerobic conditions were obtained by repeatedly evacuating and refilling the test tubes with the desired gas. The gas phase and the liquid phase were equilibrated by continuous shaking. Nitrogen fixation was studied using the acetylene to ethylene reduction test [12]. Ethylene [15] and hydrogen [16] were measured by gas chromatography.

3. Results

Cell-free lysates of N_2 grown *Cl. pasteurianum* actively catalyzed the reduction of acetylene to ethylene with NADH as electron donor. The reaction was strictly dependent on acetyl-CoA, ferredoxin and ATP (fig. 1). When regenerating systems (RS) for NADH (galactose, NADH and galactose dehydrogenase), for acetyl-CoA (acetyl phosphate, CoA and phosphotransacetylase) and for ATP (acetyl phosphate, ATP and acetate kinase) were used in order to maintain constant concentrations of NADH, acetyl-CoA and ATP the reaction proceeded linearily with time up to 25 min and the rate of the reaction was proportional with protein from 3 to 12 mg/ml.

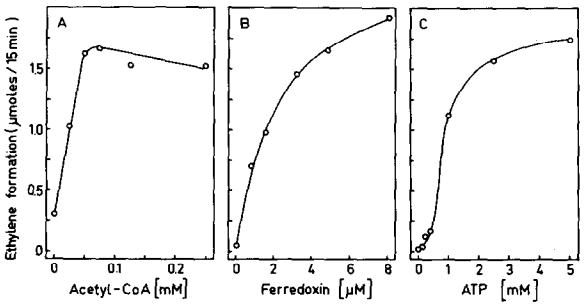

Fig. 1. Acetylene reduction to ethylene with NADH in cell-free lysates of *Clostridium pasteurianum*. Dependence on acetyl-CoA (A), ferredoxin (B) and ATP (C). Assay: 25 mM Tris–acetate, pH 7.5; 5 mM dithioerythritol; 5 mM magnesium acetate; acetyl-CoA regenerating system consisting of acetyl phosphate K, 50 mM Li, 0.25 mM coenzyme A or as indicated, and endogenous phosphotransacetylase of the lysates; ferredoxin 10 μM or as indicated; ATP regenerating system consisting of acetyl phosphate K, 50 mM Li, 5 mM ATP or as indicated, and endogenous acetate kinase of the lysates; NADH regenerating system consisting of 50 mM galactose, 0.5 mM NADH and galactose dehydrogenase (EC 1.1.1.48) 1 U; nucleotide free lysate in A and C, 10 mg protein: ferredoxin free lysate in B, 16 mg protein: H_2O to 1 ml; gas phase: argon 80%–acetylene 20%; start with lysate; stop with 0.5 ml 1 N KOH; ethylene detection: gas chromatography.

Table 1
Acetylene reduction to ethylene and proton reduction to hydrogen by NADH in cell-free lysates of *Clostridium pasteurianum*. Different cofactor requirements

Electron donor	Omissions	H_2	$H_2C = CH_2$
		μmoles / 15 min	
NADH-RS	None	5.8	1.73
	– ATP-RS	6.0	0.06
	– Acetyl-CoA-RS	0.3	0.20
	– Ferredoxin	0.1	0.05

Assay: Basic system see fig. 1. Nucleotide-free lysate 10 mg protein or ferredoxin-free lysate 16 mg protein, where appropriate. The ATP regenerating system (RS) consisted of ATP, acetyl phosphate and the endogenous acetate kinase of the lysate; when the ATP-RS was omitted, ATP was missing. The acetyl-CoA-RS consisted of acetyl phosphate, coenzyme A and the endogenous phosphotransacetylase of the lysate; when the acetyl-CoA-RS was omitted, coenzyme A was missing or in the case of H_2-formation alternatively acetyl phosphate (indicating that acetyl-CoA rather than CoA alone is required for ferredoxin reduction with NADH).

The lysates also catalyzed the reduction of protons to H_2 with NADH. This reaction was dependent on ferredoxin and acetyl-CoA, while ATP was not required (table 1), indicating that hydrogen was formed via the hydrogenase reaction rather than via the ATP-dependent nitrogenase reaction [4]. The observed dependence of ethylene formation on acetyl-CoA, ferredoxin and ATP and of hydrogen formation on only acetyl-CoA and ferredoxin clearly indicates that acetyl-CoA is required for ferredoxin reduction with NADH while ATP is required for acetylene reduction with reduced ferredoxin. These results then show that the acetyl-CoA activated NADH-ferredoxin oxidoreductase previously demonstrated only in NH_3-grown *Cl. pasteurianum* [9,10] is also present in N_2-grown cells and that it can be coupled via ferredoxin with the ATP-requiring nitrogenase [1,17] to catalyze acetylene reduction with NADH.

In order to evaluate the in vitro efficiency of

Table 2
Comparison of various electron donors in acetylene reduction to ethylene in cell-free lysates of *Clostridium pasteurianum*

Electron donor	$H_2C = CH_2$ μmoles/ 15 min
Pyruvate	5.52
NADH-RS	1.73
Hydrogen	3.86
Dithionite	2.82
Formate	1.55

Assay: 25 mM Tris–acetate, pH 7.5; 5 mM dithioerythritol; acetyl phosphate K, 50 mM Li; 5 mM ATP; 5 mM magnesium acetate; electron donors: 50 mM sodium pyruvate; 30 μmoles hydrogen injected into the gas phase; 10 mM sodium dithionite; NADH regenerating system (RS) see fig. 1; 25 mM sodium formate; crude lysate 10 mg protein; water to 1 ml; gas phase: argon 80% acetylene 20%; anaerobic vessels; start with lysate; stop with 0.5 ml 1 N KOH; ethylene detection: gas chromatography; when the NADH-RS was used as electron donor 0.25 mM coenzyme A was added.

NADH as electron donor in nitrogen fixation the rate of acetylene reduction with the other known electron donors was determined in cell-free lysates of *Cl. pasteurianum* and compared with the rate observed in the presence of NADH (table 2). Pyruvate via pyruvate–ferredoxin oxidoreductase and hydrogen via hydrogenase were most potent; dithionite, which directly reduces nitrogenase, was more efficient than NADH via NADH-ferredoxin oxidoreductase or formate via CO_2 reductase. It is important to note that the efficiency of all the electron donors tested was in the same order of magnitude differing only by a factor of 3.

4. Discussion

In order to be able to decide whether NADH is also a reductant for nitrogen fixation in vivo it has to be known whether the NADH-ferredoxin oxidoreductase mediates in the living cell the reduction of ferredoxin with NADH or the reverse reaction, the reduction of NAD with reduced ferredoxin. Both reactions are readily catalyzed by the enzyme in vitro [10]. The function of the NADH-ferredoxin oxidoreductase can be deduced from fermentation balances and from the coenzyme specificities of the oxidation–reduction processes of the fermentation. The catabolism–breakdown of glucose via pyruvate to acetyl-CoA, CO_2 and H_2 following the Embden–Meyerhof pathway and conversion of the acetyl-CoA to butyrate or acetate – is very similar on either NH_3 or N_2 as nitrogen source and can be approximated by eqs. 1 and 2 taking into account the amount of N_2 fixed [18].

$$\text{Glucose} + 2.6\ H_2O = 0.6\ \text{Acetate}^- + 0.7\ \text{Butyrate}^- + 2\ HCO_3^- + 3.3\ H^+ + 2.6\ H_2 \quad (1)$$

$$\text{Glucose} + 2.6\ H_2O + 0.1\ N_2 = 0.6\ \text{Acetate} + 0.7\ \text{Butyrate}^- + 2\ HCO_3^- + 3.3\ H^+ + 2.3\ H_2 + 0.2\ NH_3 \quad (2)$$

The similarity is indicated by the observation that nearly equal amounts of the major products acetate and butyrate were formed [10,18] and that the major ferredoxin-dependent enzymes, hydrogenase, pyruvate-ferredoxin oxidoreductase and NADH-ferredoxin oxidoreductase, showed essentially identical specific activities (μmoles/min/mg soluble protein) and half-saturation concentrations $[S]_{0.5V}$ (unpublished). In the redox process there are two dehydrogenating steps: the glyceraldehyde phosphate dehydrogenase reaction yielding 2 NADH/glucose and the pyruvate-ferredoxin oxidoreductase reaction yielding 2 reduced ferredoxin/glucose. Only 1.4 of the 2 NADH are reoxidized by the reduction of 1.4 acetyl-CoA to finally 0.7 butyrate; the remaining 0.6 NADH are reoxidized by the reduction of 0.6 ferredoxin. Thus 2.6 reduced ferredoxin/glucose are produced which are reoxidized in NH_3-cells by the reduction of 5.2 H^+ to 2.6 H_2 and in N_2-cells by the reduction of 4.6 H^+ and 0.1 N_2 to 2.3 H_2 and 0.2 NH_3. Since per 1 glucose 2 ferredoxins are reduced with pyruvate and 0.6 with NADH, approximately 25% of the electrons flowing to ferredoxin come from NADH. NADH is thus in vivo a quantitatively important reductant for all biosynthetic processes requiring reduced ferredoxin such as nitrogen fixation, CO_2-reduction to formate and NADPH-formation (fig. 2).

Finally it should be mentioned that at least in *Cl. pasteurianum* hydrogen and formate cannot be considered as physiological reductants of ferredoxin, since in vivo it is the function of the enzyme hydro-

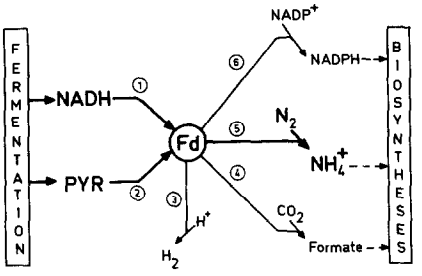

Fig. 2. Short scheme of the ferredoxin dependent metabolism of growing saccharolytic clostridia: NADH and pyruvate as the physiological electron donors in N_2-fixation. PYR, pyruvate; Fd, ferredoxin; (1) NADH-ferredoxin oxidoreductase; (2) pyruvate-ferredoxin oxidoreductase; (3) hydrogenase; (4) CO_2-reductase; (5) nitrogenase; (6) reduced ferredoxin-NADP oxidoreductase.

genase to catalyze hydrogen formation [10] and of the enzyme CO_2-reductase to mediate CO_2 reduction to formate [19]. In conclusion then, only pyruvate and NADH can be regarded as physiological electron donors to oxidized ferredoxin, while protons, nitrogen, CO_2 and $NADP^+$ are the physiological electron acceptors from reduced ferredoxin (fig. 2).

Acknowledgements

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