FERREDOXIN MEDIATED HYDROGEN FORMATION FROM NADPH IN A CELL-FREE SYSTEM OF *CLOSTRIDIUM KLUYVERI*

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1. Introduction

Hydrogen evolution in *Clostridium kluyveri* was shown to be stoichiometrically ($2H_2$ per 1 ATP) coupled to ATP generation [1]. The mechanism of hydrogen formation, however, remained unknown. Fredricks and Stadtman [2] found that hydrogen uptake was catalyzed by a ferredoxin (Fd) dependent protein system with NAD^+ as electron acceptor. The process was apparently irreversible [3]. This seemed to agree with thermodynamic considerations ($NAD^+/NADH$, $E'_0 = -320$ mV; Fd_{ox}/Fd_{red}, $E'_0 = -420$ mV), even more so as only a few low potential electron donor systems (pyruvate/acetyl CoA + CO_2 [4], $E'_0 = -508$ mV; acetaldehyde/acetate [5], $E'_0 = -605$ mV; formate/CO_2 [6]; $E'_0 = -420$ mV; H_2/H^+ [2], $E'_0 = -420$ mV) were known to physiologically reduce bacterial ferredoxin [4,7,8]. *Clostridium kluyveri* requires ferredoxin for both anabolic [9] and catabolic key reactions [1,2]; however, none of the ferredoxin reducing processes have been shown to occur in the organism.

This communication reports the formation of molecular hydrogen from NADPH in a ferredoxin-dependent reaction. The system is effectively regulated by the $NAD^+/NADH$ redox couple. $NAD^+ > NADH$ is a positive, $NADH > NAD^+$ a negative effector in hydrogen evolution from NADPH.

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2. Methods

Enzymes, coenzymes and substrates were obtained from Boehringer Mannheim, methyl viologen from Serva Heidelberg.

Preparation of cell-free extracts: ethanol-acetate-bicarbonate grown frozen cells were incubated with lysozyme (EC.3.2.1.17) at 37° under H_2 for 30 min and centrifuged at 6,000 × *g*. DEAE cellulose treated lysates were made according to Mortenson et al. [10]. Strictly anaerobic conditions were maintained throughout the preparations.

Regenerating systems: NADH regenerating system (NADH-RS): galactose, 20 mM; galactose dehydrogenase (EC.1.1.1.48), 3.5 U; NAD, 0.5 mM. NADPH regenerating system (NADPH-RS): glucose-6-phosphate, 16 mM; glucose-6-phosphate dehydrogenase (EC.1.1.1.49), 3.5 U; NADP, 0.5 mM. NAD regenerating system (NAD-RS): fructose-1,6-diphosphate, 2 mM; aldolase (EC.4.1.2.7), 0.5 U; triose phosphate isomerase (EC.5.3.1.1), 2.5 U; glycerol-1-phosphate dehydrogenase (EC.1.1.1.8), 0.7 U; NAD, 0.5 mM. Assays were carried out in a total volume of 2.5 ml in 17.5 ml Thunberg tubes at 37°.

Detection of hydrogen: H_2 was quantitated by gas chromatography relating the peak heights to a standard curve (Molecular sieve 5A, E. Merck AG, Darmstadt; fractometer F7, Perkin and Elmer, Überlingen); sample column: length 4 m, inner diameter 2 mm, material steel; reference column: none; temperatures: injection port 50°, column 50°; carrier gas: argon; detection: TDC; gas samples: 2 ml of the 15 ml gas phase were injected with a gas-tight syringe.

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Preparation of ferredoxin: *Clostridium kluyveri* ferredoxin was obtained by the method of Mortenson et al. [10]. The DEAE cellulose fraction eluted with 1 M Tris buffer pH 8 was used. *Clostridium pasteurianum* ferredoxin was made according to Mortenson's procedure [11].

3. Results

When cell lysates were incubated at 37° under strictly anaerobic conditions together with NAD^+ and a NADPH regenerating system molecular hydrogen was rapidly evolved (table 1). No other low molecular weight substances, including ATP, were necessary for the reaction since exhaustively dialyzed extracts showed the same activity as crudes. Hydrogen evolution was proportional to protein concentration up to 10 mg. Ferredoxin was an essential component of the hydrogen-forming system (table 1). DEAE cellulose-treated lysates were inactive but could be restored to full activity by the addition of either *Clostridium kluyveri* ferredoxin, *Clostridium pasteurianum* ferredoxin or methyl viologen, which usually substitutes for ferredoxins.

Lysates supplemented with a NADPH regenerating system had no proton-reducing activity when NAD^+ was omitted from the incubation mixture. This finding together with the observation that NAD was being reduced in the system suggested at first that NADH and not NADPH was the electron donor. Surprisingly lysates fortified with a NADH-regenerating system in the presence or absence of $NADP^+$ did not produce hydrogen (table 2). Nor was hydrogen evolution observed when lysates were incubated with both a NADH- and a NADPH-regenerating system (table 2). The highest rate of H_2 formation was obtained when NAD^+ was kept predominantly in its oxidized and NADPH in its reduced form. The stimulatory effect of the NAD^+-regenerating system was up to 5 times higher than that of NAD^+ alone.

The significance of the presented results was ensured by the appropriate controls, namely cross-omission of all the single substrates and enzymes of the regenerating systems.

Table 1
Dependence of hydrogen formation on ferredoxin.

Protein	Additions	$\mu l\ H_2$/60 min
Crude lysate	none	1.0
	NADPH-RS	10.5
DEAE lysate	none	<0.2
	NADPH-RS	1.2
	Fd_{kl}	<0.2
	Fd_{kl} + NADPH-RS	11.4
	Fd_{past} + NADPH-RS	11.5
	MV + NADPH-RS	14.9

Basic system: Tris buffer pH 8.0, 70 mM; glutathione red., 2 mM; NAD^+, 0.5 mM; FAD, 12 μM; crude cell lysate or DEAE cellulose-treated cell lysate, 5 mg protein; H_2O to 2.5 ml; gas atmosphere: argon extremely pure (1 ppm O_2).

Additions: NADPH-regenerating system (NADPH-RS); partially purified *Clostridium kluyveri* ferredoxin (Fd_{kl}), 1 mg; *Clostridium pasteurianum* ferredoxin (Fd_{past}), 0.3 mg; methyl viologen (MV), 10 mM.

(for further details see methods)

Table 2
Dependence of hydrogen formation on NADPH as electron donor. Regulatory effects of the redox state of the NAD^+/NADH couple.

Protein	Additions	$\mu l\ H_2$/60 min
DEAE lysate	none	<0.2
	NADH-RS	<0.2
	NADPH-RS	1.7
	NADH-RS + NADPH-RS	1.0
	NADH-RS + $NADP^+$	<0.2
	NADPH-RS + NAD^+	8.1
	NADPH-RS + NAD^+-RS	42.6

Basic system: Tris buffer pH 8.0, 70 mM; glutathione red., 2 mM; FAD, 12 μM; partially purified *Clostridium kluyveri* ferredoxin, 1 mg; DEAE cellulose-treated lysate, 5 mg protein; H_2O to 2.5 ml; gas atmosphere: argon extremely pure (1 ppm O_2).

Additions: NADPH-regenerating system (NADPH-RS); NADH-regenerating system (NADH-RS); $NADP^+$, 0.5 mM; NAD^+, 0.5 mM; NAD^+-regenerating system (NAD^+-RS).

(for further details see methods)

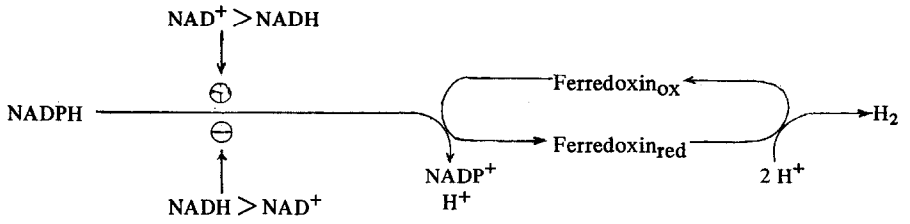

Fig. 1. Schematic representation of hydrogen formation from NADPH and its control by the NAD^+/NADH system in *Clostridium kluyveri*. (The arrows indicate the physiological directions of electron flow; they are not meant to represent an irreversible system.)

4. Discussion

Evidence has been presented that hydrogen is produced in a ferredoxin-dependent reaction from NADPH. NADPH can be formed in the ethanol-acetate-bicarbonate fermentation of *Clostridium kluyveri* (overall eqs. 1–3) [1] during the conversion of acetaldehyde to acetyl CoA ($\Delta G'_0 = -5.12$ kcal/mole). This step is catalyzed by one of the two aldehyde dehydrogenases [12], the NADP-specific type, whose function is now readily explained.

The participation of NADPH and not NADH in the reduction of protons can very well be understood in view of the energy metabolism of *Clostridium kluyveri* (eqs. 1–3).

$$CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + H^+ + 2\,H_2 \quad (1)$$

$$CH_3CH_2OH + CH_3COO^- \rightarrow CH_3CH_2CH_2COO^- + H_2O \quad (2)$$

$$2\,CH_3CH_2OH + CH_3COO^- \rightarrow CH_3(CH_2)_4COO^- + 2\,H_2O \quad (3)$$

The NAD^+-dependent dehydrogenation of ethanol to acetaldehyde (first partial reaction in eqs. 1–3, $\Delta G'_0 = +5.41$ kcal/mole) is only feasible with a high potential NAD^+/NADH couple; this excludes NADH as a potential electron donor to ferredoxin in *Clostridium kluyveri*. Consequently, in the absence of other ferredoxin reductants, the $NADP^+$/NADPH couple is the most likely to have the low potential required for hydrogen formation.

The finding that hydrogen can be formed in a ferredoxin-mediated reaction from NADPH adds this reduced pyridine nucleotide to the list of physiological reductants of ferredoxin. Reduction of this low potential electron carrier by NADPH should be important not only for hydrogen evolution in anaerobic catabolism but also for key reactions in anabolism as pyruvate synthesis from acetyl CoA and CO_2 and NH_4^+ formation from molecular nitrogen.

The formation of hydrogen from NADPH in cell-free extracts of *Clostridium kluyveri* is effectively controlled by the redox state of the NAD^+/NADH couple (table 2). A high potential $NAD^+ >$ NADH strongly activates hydrogen evolution while a low potential NADH > NAD^+ inhibits it (fig. 1).

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