HYDROGEN FORMATION FROM NADH IN CELL-FREE EXTRACTS OF *CLOSTRIDIUM KLUYVERI** Acetyl coenzyme A requirement and ferredoxin dependence

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1. Introduction

Recently cell-free extracts of *Clostridium kluyveri* were found to produce hydrogen from NADPH [1,2]. Proton reduction by NADPH was ferredoxin-dependent and effectively regulated by the NAD^+/NADH redox couple, NAD^+ being an activator and NADH an inhibitor.

In this communication it will be shown that cell-free lysates of the organism also catalyze the formation of hydrogen from NADH. The NADH system is dependent on ferredoxin and has a strict requirement for acetyl CoA. The reduction of ferredoxin is not effected via an acetyl CoA/acetaldehyde shuttle as has been proposed [3]. Acetyl CoA appears to have a regulatory function in ferredoxin reduction by NADH.

2. Methods

Enzymes, coenzymes and substrates were obtained from Boehringer Mannheim GmbH, Mannheim.

The preparation of cell-free extracts and of ferredoxin, the detection of hydrogen by gas-chromatography and the determination of NADH levels have been described [1,2]. Assays were carried out in a total volume of 1 ml in 17.5 ml Thunberg tubes at 37° with shaking. Strictly anaerobic conditions were maintained throughout the experiments.

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Regenerating systems (RS): NADH-RS: galactose, 40 mM; NADH, 2.5 mM; galactose dehydrogenase (EC 1.1.1.48), 0.5 U; NADPH-RS: glucose-6-phosphate Na_2, 40 mM; $NADP^+$, 0.5 mM; glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 0.5 U;NAD^+-RS: fructose-1,6-diphosphate Na_3, 20 mM; NAD^+, 0.5 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.

3. Results

3.1. *Hydrogen formation from NADH*

Extracts freed from low-molecular weight compounds by sephadex G 25 chromatography (G 25 lysate) catalyzed hydrogen formation from NADH only in the presence of either acetyl phosphate plus CoA or acetaldehyde plus CoA (fig. 1, table 1). These observations and the finding that lysates contain high activities of phosphotransacetylase (EC 2.3.1.8) and acetaldehyde dehydrogenase (EC 1.2.1.10) are taken to indicate that acetyl CoA rather than its precursors is required for the activation of hydrogen production. However, addition of acetyl CoA (2 mM) to the system remained without effect (table 1). Gas chromatographic analysis [4] of the reaction mixture showed that acetyl CoA was quantitatively transformed by NADH to ethanol, butyrate and caproate. From the initial rate of NADH disappearance (fig. 2) it can be estimated that between 0.5 and 1 μmole of acetyl CoA was reduced per min in the assay. Therefore both an acetyl CoA- and an NADH-regenerating system

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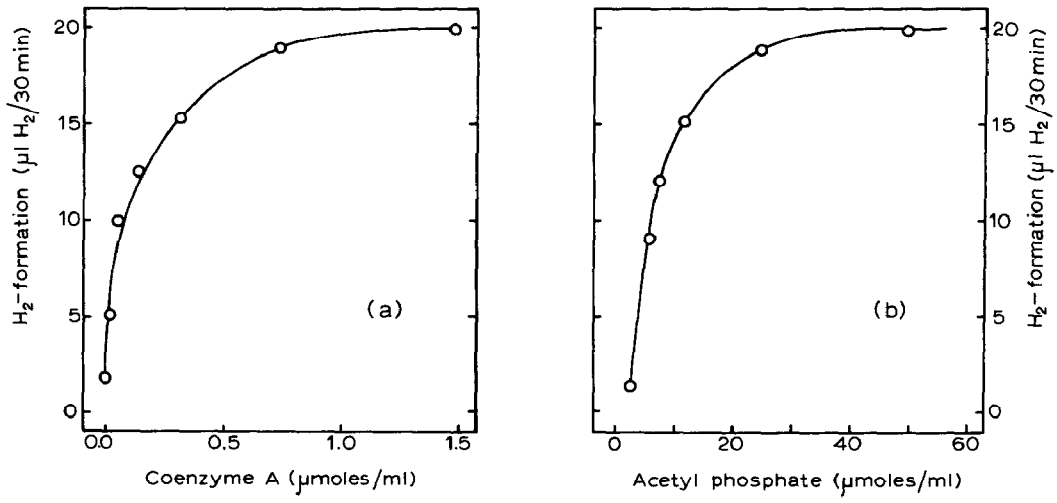

Fig. 1. Hydrogen formation from NADH. (A) as a function of coenzyme A; (B) as a function of acetyl phosphate. Complete: Tris HCl pH 7.4, 75 mM; glutathione red, 2 mM; FAD, 12 µM; phosphotransacetylase (EC 2.3.1.8) 1 U; NADH-RS; sephadex G 25 treated lysate, 2.5 mg protein; water to 1 ml; gas phase: argon. (A) coenzyme A varied with acetyl phosphate K, Li 25 mM; (B) acetyl phosphate K, Li varied with coenzyme A 0.75 mM.

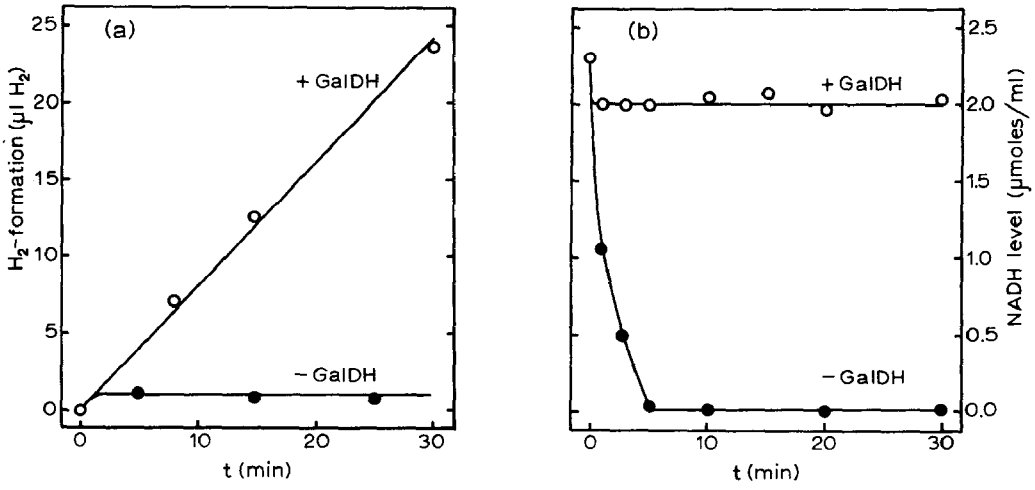

Fig. 2. Hydrogen formation from NADH in the presence of acetylcoenzyme A. (A) H_2-formation as a function of time; (B) levels of NADH as a function of time. Complete: Tris HCl pH 7.4, 75 mM; glutathione red, 2 mM; FAD 12 µM; coenzyme A, 0.75 mM; acetyl phosphate K, Li, 25 mM; phosphotransacetylase (EC 2.3.1.8), 1 U; NADH-RS; sephadex G 25 treated lysate, 2.5 mg protein; water to 1 ml; gas phase: argon.

Table 1
Acetyl coenzyme A requirement and ferredoxin dependence of hydrogen formation from NADH.

Protein	Additions		μ 1 H_2/30 min
	Electron donor	Cofactors	
G 25 lysate	NADH-RS	none	0
		AcP	2.9
		CoA	0
		AcP + CoA	64.4
		AcH	< 0.2
		AcH + CoA	68.4
		AcCoA	< 0.5
		ATP	< 0.3
	NADPH-RS	none	1.1
		AcP + CoA	5.3
		NAD^+	10.4
		NAD^+-RS	106.4
		AcP + CoA + NAD^+	102.4
	AcH	none	0
		CoA	< 0.2
		NAD^+	< 0.5
		CoA + NAD^+	26.8
	Pyr*	none	8.3
		CoA	50.8
		CoA + NAD^+	35.2
	$S_2O_4^=$	none	120.4
DEAE	NADH-RS	none	0
		Fd_{kl}	< 0.1
		AcP + CoA	0
		AcP + CoA + Fd_{kl}	59.2
	Pyr*	CoA	0
		CoA + Fd_{kl}	35.4
	$S_2O_4^=$	none	2.7
		Fd_{kl}	107.6

Basic system: Tris HCl pH 7.4, 75 mM; glutathione red, 2 mM; FAD, 12 μM; phosphotransacetylase (EC 2.3.1.8), 1 U; sephadex G 25 or DEAE cellulose-treated lysate, 5 mg protein; water to 1 ml; gas phase: Ar. Additions: Acetyl phosphate K, Li, 25 mM (AcP); coenzyme A, 0.75 mM (CoA); acetyl CoA, 2 mM (AcCoA); acetaldehyde, 50 mM (AcH); NAD^+, 0.5 mM; ATP, 2 mM; pyruvate Na, 30 mM (Pyr*); dithionite Na_2, 20 mM ($S_2O_4^=$); *Clostridium kluyveri* ferredoxin 0.9 mg protein (Fd_{kl}); regenerating systems (RS, see methods).
* Assays with pyruvate contained routinely thiamine pyrophosphate, 0.1 mM and $MnCl_2$, 1 mM.

were needed to maintain the acetyl CoA- and NADH-concentrations required for hydrogen formation.

Ethanol, acetate, butyrate or caproate did not influence proton reduction by NADH. It cannot be excluded, however, that an acetyl CoA metabolite such as an acyl CoA intermediate of the endogenous fatty acid producing system was operative as the activator.

Besides acetyl CoA also ferredoxin was found to be an essential component of the NADH system (table 1).

3.2. *Hydrogen formation from NADPH*

Hydrogen evolution from NADPH, however, was not stimulated by acetyl CoA, but only by NAD^+. Optimal activation by NAD^+ was obtained only with an NAD^+-regenerating system (table 1) as NAD^+ is continuously reduced to the inhibitor NADH by an NADPH-dependent transhydrogenase activity of the extract [2]. Since acetyl CoA rapidly oxidized NADH in the lysates (fig. 2), the combination of NAD^+ acetyl phosphate and CoA constituted an endogenous NAD^+-regenerating system, with which maximal hydrogen formation from NADPH was also observed (table 1).

Finally, the failure to detect appreciable hydrogen production in the presence of acetyl phosphate plus CoA alone indicates that NAD^+ is an obligatory activator of the NADPH system.

3.3. *Hydrogen formation from acetaldehyde*

Acetaldehyde served as electron donor in hydrogen formation only in the presence of CoA and NAD^+ (table 1). Thus acetaldehyde, NAD^+, CoA and the acetaldehyde dehydrogenase (EC 1.2.1.10) of the extracts formed both the NADH- and the acetyl CoA-regenerating system required for proton reduction by NADH. These findings are taken as evidence that NADH and not acetaldehyde is the actual reductant of ferredoxin.

3.4. *Hydrogen formation from pyruvate*

Hydrogen formation from pyruvate was dependent on CoA and ferredoxin. Pyridine nucleotides were not necessary for activity (table 1); on the contrary a slight inhibition of H_2 production was observed in the presence of NAD^+, which competes with protons for the electrons of reduced ferredoxin.This system is therefore different from the H_2-forming process with acetaldehyde as electron donor in that ferredoxin is reduced by pyruvate directly rather than via NADH.

3.5. *Hydrogen formation from dithionite*

G 25 lysates catalyzed hydrogen formation from dithionite without any additions (table 1). Neither had acetyl CoA a stimulatory nor had NADH an inhibitory effect on the ferredoxin-dependent system. The hydrogenase therefore cannot be the regulatory site in hydrogen formation from pyridine nucleotides (fig. 3).

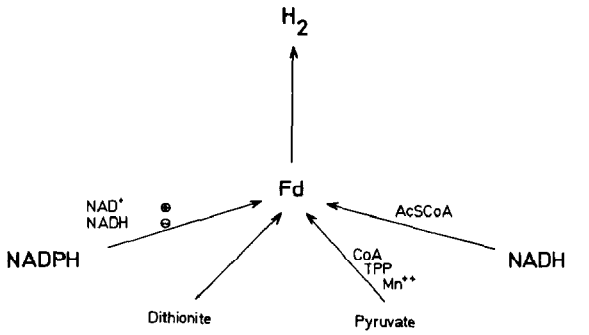

Fig. 3. Schematic representation of hydrogen-forming systems in cell-free extracts of *Clostridium kluyveri.*

4. Discussion

Hydrogen formation from NADH is observed only in the presence of acetyl CoA. Three modes of action can be considered for this compound. It may function (a) as an allosteric activator, (b) as an energy-rich intermediate whose group transfer potential is needed for proton reduction ($H^+ + NADH \rightleftharpoons H_2 + NAD^+$) ($\Delta G'_0 = +4.6$ kcal/mole) or (c) as a catalytic substrate if H_2-formation is effected via an acetyl CoA/acetaldehyde shuttle.

Gottschalk and Chowdhury have observed the synthesis of pyruvate from acetyl CoA and CO_2 with NAD(P)H as electron donor in a ferredoxin-mediated reaction [5]. Acetyl CoA reduction to acetaldehyde by the pyridine nucleotides followed by acetaldehyde reoxidation to acetyl CoA was proposed to be the mechanism of ferredoxin reduction [3]. The data of this investigation definitely exclude the acetaldehyde dehydrogenases of *Clostridium kluyveri* to function as a ferredoxin reductase [3].

It is at present not possible to distinguish between the other two modes of action of acetyl CoA. The observations that ATP had no activating effect in both the NADH (table 1) and the NADPH [2] system and that no high energy substrate was required in the NADPH system [1,2] make an allosteric rather than a stoichiometric mechanism appear more likely.

While the physiological role of the NADPH system may readily be envisaged [1,2], that of the NADH system appears to be less obvious and more complex.

References

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