Regulation of the Reduced Nicotinamide Adenine Dinucleotide Phosphate-Ferredoxin Reductase System in Clostridium kluyveri*

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SUMMARY

The mechanism of regulation of NADPH-ferredoxin reductase was studied in cell-free lysates of Clostridium kluyveri. The following activities, which are assumed to be linked to the enzyme, were investigated: ferredoxin reduction by NADPH, NADP+ reduction by reduced ferredoxin, transhydrogenation from NADPH to NAD+, and methyl viologen reduction by NADPH.

Ferredoxin reduction by NADPH is controlled by the oxidation-reduction state of the NAD+-NADH couple. NAD+ is an obligatory activator (Michaelis activation constant, $K_a = 0.9 \times 10^{-4}$ M), which increases $V_{max}$, while the $K_m$ of the substrate NADPH ($K_m = 2.25 \times 10^{-5}$ M) remains unaffected. β-NAD+ analogues can substitute to varying degrees for β-NAD+, while α-NAD+ and NMN or AMP analogues are totally inactive. NADH is an inhibitor, competitive to NAD+ rather than to NADPH.

NADP+ reduction by reduced ferredoxin is subject to product inhibition; NADPH is competitive to NADP+ ($K_a = 1.52 \times 10^{-4}$ M).

The transhydrogenation from NADPH to NAD+ is stimulated by oxidized and inhibited by reduced ferredoxin; ferredoxin is not involved in the electron flow.

Methyl viologen reduction by NADPH is not controlled by either NAD+ or NADH.

* This project was supported by grants from the Deutsche Forschungsgemeinschaft, Bad Godesberg, through the Sonderforschungsbereich “Molekulare Grundlagen der Entwicklung,” Freiburg. It is Paper I in the series “Regulation of Ferredoxin Reduction in Clostridia.” Paper II is Reference 10.

MATERIALS AND METHODS

Chemicals and Enzymes

Reagent grade chemicals were used throughout. Gases were obtained from Messer-Griesheim GmbH, Düsseldorf: Argon extrem rein: Ar >99.999 volume % (O₂, <1 vpm); hydrogen extrem rein: H₂ >99.999 volume % (O₂, <0.1 vpm). Enzymes, coenzymes, and substrates were purchased from Boehringer, Mannheim, except nicotinic amide hypoxanthine dinucleotide, acetylpyridine adenine dinucleotide, pyridine-3-aldehyde hypoxanthine dinucleotide, and pyridine-3-aldehyde hypoxanthine dinucleotide, which were from P-L Biochemicals. Methyl viologen was supplied by Serva, Heidelberg.

Regenerating Systems

NADPH Regenerating System

Disodium Glucose 6-phosphate, 40 mM; glucose 6-phosphate dehydrogenase, 1 unit; NADP+, 0.5 mM.

NAD+ Regenerating System

Sodium pyruvate, 20 mM; sodium glyoxylate, 5 mM; lactate dehydrogenase (pig muscle), 2 units; NAD+, as indicated in the tables or figures. Glyoxylate was routinely added to block pyridine nucleotides can be regarded as physiological electron donors to ferredoxin.

The activities catalyzing ferredoxin reduction by NADPH and NADH are tentatively named NADPH-ferredoxin reductase and NADH-ferredoxin reductase in order to indicate that the direction in vivo of electron flow is opposite to that mediated by the ferredoxin-NADP+ reductase of plants.

The clostridial reductases are regulatory enzymes (1, 2). In the present investigation the mechanism of regulation of the NADPH-ferredoxin reductase has been studied in cell-free lysates of C. kluyveri. The study was extended to the ferredoxin-NADP+ reductase, the NADPH-NAD+ transhydrogenase, and the NADPH-methyl viologen diaphorase activities, which were also shown in the lysates and which, by analogy with the reductase activities (7-9), might be associated with the clostridial reductase.

The regulation of the NADH-ferredoxin reductase, which appears to be a separate enzyme, is the subject of the accompanying paper (10).
ferredoxin reduction by pyruvate via the endogenous pyruvate dehydrogenase activity of the lysates (3).

**NADH Regenerating System I**

Galactose, 20 mM; galactose dehydrogenase, 0.1 unit; NADH, 0.1 mM.

**Culture of Clostridia**

*C. kluyveri* was grown in 50-liter plastic tanks at 35° on the ethanol-acetate-bicarbonate medium described earlier (4) with the following modifications: sulfate concentration was lowered to 0.01 mM and ethanol deaminated with 5% methyl ethyl ketone was used. Sodium dithionite was added (70 mg per liter of medium) just before inoculation with 2.5 liters of an actively growing culture. The bacteria were harvested in a continuous flow centrifuge (model Junior 15,000, Christ Osterode, Germany, at 10,000 rpm) after having produced about 500 ml of H₂ per liter of culture. The yield averaged 500 mg of wet cells per liter. The cells were stored at -15° under argon and used for the preparation of cell-free extracts within 2 months. *Clostridium pasteurianum* was grown on synthetic media with (NH₄)₂SO₄ as nitrogen source as described by Lovenberg, Buchanan, and Rabinowitz (11).

**Cell-free Protein Preparations**

**Crude Lysate**

In 10-ml Servall plastic centrifuge tubes 3 g of frozen cells were added to 6 ml of 10 mM Tris-HCl buffer, pH 7.5, containing 10 mM mercaptoethanol. The tubes were closed with rubber stoppers, evacuated, and filled with hydrogen. A homogeneous cell suspension was obtained by stirring with a magnetic paddle at 0°. Then 1 ml of a lysozyme solution (5 mg of lysozyme and 0.5 mg of deoxyribonuclease per ml of water) was injected into a syringe. The mixture was heated at 90° for 30 min with occasional stirring. After centrifuging at 35,000 × g for 40 min a dark supernatant (crude cell lysate) was obtained containing about 40 mg of protein per ml. Protein was determined with the biuret method.

**Sephadex G-25 Lysate**

Crude lysate (3 ml) was passed through an anaerobically prepared Sephadex G-25 column (Whatman, 1-cm diameter, 15 cm) to yield 6 ml of Sephadex G-25 lysate.

**Dowex 2-Acetate-Charcoal Lysate**

Crude lysate (3 ml) was passed through a small column (Whatman, 1-cm diameter, 3 cm), filled with a mixture of 2 g of Dowex 2-acetate and 100 mg of HCl- and EDTA-previously treated charcoal, to yield 3 ml of Dowex charcoal lysate. Anaerobic conditions were maintained throughout.

**DEAE-cellulose Lysate**

Crude lysate (3 ml) was passed anaerobically through a small DEAE-cellulose column (Whatman, 1-cm diameter, 3 cm) to yield 3 ml of DEAE-cellulose lysate.

The crude lysates were stable for about 1 week, while all other lysates had to be prepared immediately before use.

**Ferredoxins—** *C. kluyveri* and *C. pasteurianum* ferredoxins were prepared as described by Mortenson (12).

**Enzyme Assays**

Assays were carried out at 37° in 22-ml Thunberg tubes or in 14-ml Warburg vessels with continuous shaking or in 3-ml anaerobic cuvettes. Anaerobic conditions were secured by repeated evacuation and refilling with the desired gas. Detailed conditions are described in legends to tables and figures.

Hydrogenase was measured by determining H₂ production from reduced ferredoxin which was generated with sodium dithionite. NADPH-ferredoxin reductase was assayed by coupling with hydrogenase. Electron flow from NADPH to oxidized ferredoxin was determined by measuring H₂ formation, and electron flow from reduced ferredoxin to NADP⁺ was measured by following either H₂ consumption or NADPH formation. NADPH.NAD⁺ transhydrogenation was measured by determining NADH enzymatically after the reaction was stopped by addition of KOH. Methyl viologen reduction by NADPH was measured by coupling with hydrogenase or optically by following color formation at 578 nm.

**Analytical Procedures**

**Hydrogen Determination**

Gas chromatography—Hydrogen formation was measured gas chromatographically (13) in experiments carried out in Thunberg tubes. The inlet was closed with a rubber tubing so that gas samples could be taken with a syringe. Two milliliters of the gas phase were taken with a gas-tight syringe and injected into the gas chromatograph. Hydrogen was determined quantitatively, relating the peak heights to a standard curve.

Manometry—Hydrogen formation or consumption was measured in a Warburg apparatus with 0.1 ml of 1 N KOH in the center well. For H₂ formation gas chromatographic and volumetric determinations led to identical results.

**NADH and NADPH Determination**

NADH and NADPH levels under the conditions of oxidized ferredoxin reduction by NADPH in Thunberg tubes were measured enzymatically. The reactions were stopped by injection of 2 N KOH to a final concentration of 0.1 M. After heating for 90 sec at 80° the mixture was cooled and kept at room temperature for no longer than 1 hour. Just before analysis the solution was brought to pH 8 with 2 N KH₂PO₄; 0.5-ml aliquots were added to 2.5 ml of 0.1 M triethanolamine buffer, pH 7.6; NADH was assayed with fructose-1,6-diphosphate, aldolase, triose phosphate isomerase, and glycerol 1-phosphate dehydrogenase (14), and NADPH by further addition of α-ketoglutarate, ammonium chloride, and glutamate dehydrogenase (15). With this method (16) recoveries of 85 to 90% were obtained; the loss was due to a very stable and highly active NAD(P)H oxidase present in the lysates.

**RESULTS**

**NADPH-Ferredoxin Reductase**

**Determination of Ferredoxin Reduction**—Ferredoxin reduction can be measured with hydrogenase by following H₂ formation. If the mechanism and regulation of ferredoxin reduction are to be investigated with hydrogenase as indicator enzyme, hydrogenase must meet two requirements: it must be in excess of the enzyme studied, and it must not be a regulatory enzyme.

The NADPH-ferredoxin reductase of *C. kluyveri* could be
measured in cell-free lysates by following \( \text{H}_2 \) formation, as the endogenous hydrogenase of the lysates was sufficiently more active than the reductase (250 units per g of protein with respect to 100 units per g of protein) and as it was not regulated by the effectors \( \text{NAD}^+ \) and \( \text{NADH} \) of the reductase.

\[
\text{NADPH} \rightleftharpoons e^- \quad \text{Fd} \rightleftharpoons e^- \quad \text{H}_2
\]

where Fd is ferredoxin.

\( \text{NAD}^+ \) and \( \text{NADH} \) as Antagonistic Effectors—The reduction of ferredoxin by NADPH was reported to be activated by \( \text{NAD}^+ \) and to be inhibited by \( \text{NADH} \) (1). The mechanism of regulation remained unknown. Three modes of action can be envisaged: (a) \( \text{NAD}^+ \) stimulates and \( \text{NADH} \) inhibits the active enzyme; (b) \( \text{NADH} \) inhibits the active enzyme and \( \text{NAD}^+ \) protects the enzyme from \( \text{NADH} \) inhibition; (c) \( \text{NAD}^+ \) activates the inactive enzyme and \( \text{NADH} \) counteracts this activation. For these three mechanisms a regulatory binding site for \( \text{NAD}^+ \) must be postulated, while \( \text{NADH} \) could bind at this regulatory site or the substrate site for NADPH or ferredoxin.

Mechanism of \( \text{NAD}^+ \) Activation—Since in cell-free lysates \( \text{NAD}^+ \) was rapidly reduced to \( \text{NADH} \) by NADPH (as a result of the transhydrogenase activity described below), an \( \text{NAD}^+ \)-regenerating system was used for the study of the mechanism of \( \text{NAD}^+ \) activation. Lysates freed from pyridine nucleotides by treatment with charcoal and an anion exchange resin did not reduce ferredoxin with NADPH unless \( \text{NAD}^+ \) was included in the incubation mixture. Ferredoxin reduction was clearly a function of \( \text{NAD}^+ \) concentration (Fig. 1); from Lineweaver-Burk plots a Michaelis activation constant \( K_a \) of \( 0.9 \times 10^{-4} \text{ M} \) was obtained for \( \text{NAD}^+ \). The \( K_a \) was independent of NADPH concentration. Activation by \( \text{NAD}^+ \) altered the \( V_{\text{max}} \) of the reaction, but had no effect on the \( K_m \) for the substrate NADPH, which was found to be \( 2.25 \times 10^{-5} \text{ M} \) (Fig. 2). These findings indicate that \( \text{NAD}^+ \) is an obligatory activator in accord with the third mechanism described above.

Specificity of \( \text{NAD}^+ \) Activation—Activation by \( \text{NAD}^+ \) could not be mimicked by NMN or AMP, the two moieties of \( \text{NAD}^+ \) or by their analogues, either alone or in various combinations (Table I). The AMP analogues appeared to bind to the enzyme, as they inhibited activation by \( \beta\text{-NAD}^+ \). \( \alpha\text{-NAD}^+ \) and ADP were the best inhibitors.

Apparently, activation required the binding of an intact dinucleotide with \( \beta \) configuration, independent of minor changes in the adenine or nicotinamide moieties (Table II). Adenine analogues were of comparable activity; however, major differences were observed with the nicotinamide analogues. Replacement of the oxaamide group by a thioamide or an acetyl function was well tolerated by the enzyme, while replacement by an aldehyde group resulted in low activity.

Mechanism of \( \text{NADH} \) Inhibition—The inhibitory effect of \( \text{NADH} \) could be due to competition of \( \text{NADH} \) either with the substrate NADPH or with the activator \( \text{NAD}^+ \). Ferredoxin and \( \text{NAD}^+ \) were simultaneously reduced by NADPH (Fig. 3). Thus the inhibitor \( \text{NADH} \) of ferredoxin reduction was continuously generated. The observation that the velocities of both ferredoxin and \( \text{NAD}^+ \) reduction were the same at different saturating NADPH concentrations indicated that \( \text{NADH} \) did not compete for the NADPH substrate site. Plotting the data obtained from Fig. 3, A and B, according to Hunter and Downs (17) clearly shows that \( \text{NADH} \) is competitive to \( \text{NAD}^+ \); the \( K_i \) for \( \text{NADH} \) was calculated to be \( 0.25 \times 10^{-4} \text{ M} \) (Fig. 4). The validity of this result was substantiated by the finding that the \( K_a \) value for \( \text{NAD}^+ \) obtained from the Hunter Downs plot (\( K_a = 1 \times 10^{-4} \text{ M} \)) is in good agreement with that obtained from the Lineweaver-Burk plot (\( K_a = 0.9 \times 10^{-4} \text{ M} \), Fig. 1).

Effect of Ferredoxin—Addition of ferredoxin to cell-free lysates...
**Table I**

Specificity of NAD+ activation of ferredoxin reduction by NADPH

<table>
<thead>
<tr>
<th>Additions</th>
<th>Minus β-NAD+/RS</th>
<th>Plus β-NAD+/RS</th>
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</thead>
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<tr>
<td>NMN analogue</td>
<td>AMP analogue</td>
<td>µM/20 min</td>
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<tr>
<td>ATP</td>
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<td>AMP</td>
<td>0.5</td>
<td>31.4</td>
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<tr>
<td>cyclic AMP</td>
<td>0.4</td>
<td>64.8</td>
</tr>
<tr>
<td>ADP-ribose</td>
<td>0.5</td>
<td>50.3</td>
</tr>
<tr>
<td>NAm b</td>
<td>0.5</td>
<td>100.0</td>
</tr>
<tr>
<td>NAm</td>
<td>0.2</td>
<td>25.8</td>
</tr>
<tr>
<td>NAm</td>
<td>0.2</td>
<td>59.6</td>
</tr>
<tr>
<td>NAm</td>
<td>0.3</td>
<td>50.8</td>
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<td>NMN</td>
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<tr>
<td>NMN</td>
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<tr>
<td>AMP</td>
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<td>Acetyl CoA-RS</td>
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<td>α-NAD+</td>
<td>0.6</td>
<td>28.0</td>
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<tr>
<td>ITP</td>
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<td>50.3</td>
</tr>
<tr>
<td>GDP</td>
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<tr>
<td>GTP</td>
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<td>57.8</td>
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<td>GDP</td>
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<table>
<thead>
<tr>
<th>Additions</th>
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<tr>
<td>AMP analogue</td>
<td>2.0 mM</td>
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<tr>
<td>ADP</td>
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<tr>
<td>ADP</td>
<td>1.0 mM</td>
</tr>
<tr>
<td>cyclic AMP</td>
<td>1.0 mM</td>
</tr>
<tr>
<td>ADP-ribose</td>
<td>1.0 mM</td>
</tr>
<tr>
<td>NAm</td>
<td>1.0 mM</td>
</tr>
<tr>
<td>NAm</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>NAm</td>
<td>0.3 mM</td>
</tr>
<tr>
<td>NMN</td>
<td>0.4 mM</td>
</tr>
<tr>
<td>NMN</td>
<td>0.3 mM</td>
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<tr>
<td>AMP</td>
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</tr>
<tr>
<td>CoA</td>
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<tr>
<td>Acetyl CoA-RS</td>
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<tr>
<td>α-NAD+</td>
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</tr>
<tr>
<td>ITP</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>GDP</td>
<td>0.6 mM</td>
</tr>
<tr>
<td>GTP</td>
<td>0.3 mM</td>
</tr>
<tr>
<td>GDP</td>
<td>0.5 mM</td>
</tr>
</tbody>
</table>

a RS, regenerating system.

b NAm, nicotinic amide.

**Table II**

Activation of ferredoxin reduction by NADPH with β-NAD+ analogues

<table>
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<tr>
<th>Additions</th>
<th>β-NAD+ analogues</th>
<th>H2</th>
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<tbody>
<tr>
<td>NAD+</td>
<td>100 µM</td>
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</tr>
<tr>
<td>NADH</td>
<td>111 µM</td>
<td></td>
</tr>
<tr>
<td>AP-AD+</td>
<td>94 µM</td>
<td></td>
</tr>
<tr>
<td>AP-HD+</td>
<td>113 µM</td>
<td></td>
</tr>
<tr>
<td>PA-AD+</td>
<td>22.5 µM</td>
<td></td>
</tr>
<tr>
<td>PA-HD+</td>
<td>19.8 µM</td>
<td></td>
</tr>
<tr>
<td>TNAD+</td>
<td>73 µM</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0 µM</td>
<td></td>
</tr>
</tbody>
</table>

* NAD+, nicotinamide hypoxanthine dinucleotide; AP-AD+, acetylpyridine adenine dinucleotide; AP-HD+, acetylpyridine hypoxanthine dinucleotide; PA-AD+, pyridine-3-aldehyde adenine dinucleotide; PA-HD+, pyridine-3-aldehyde hypoxanthine dinucleotide; TNAD+, thionicotinamide adenine dinucleotide.

**Figure 3.** The simultaneous reduction of oxidized ferredoxin and of NAD+ by NADPH in cell-free lysates. A, oxidized ferredoxin reduction as a function of time; B, NAD+ reduction as a function of time. Assay—Tris-HCl, pH 7.5, 100 mM; GSH, 2 mM; FAD, 12 µM; NADPH regenerating system; NAD+, 2.0 mM, where indicated; NAD+ regenerating system, where indicated; crude lysate, 4 mg of protein; water to 1 ml; gas phase, argon; Thunberg tubes, start with glucose-6-P of NADPH regenerating system; H2 detection, gas chromatography.

**Figure 4.** Hunter-Downs plot of the data of Fig. 3. The inhibitor concentration (I) and the activator concentration (A) were obtained from the NAD+ curve of Fig. 3B; the initial velocity (v0) was calculated from the slope of the NAD+ regenerating system curve, the velocity at time t (vt) was calculated from the slope of the NAD+ curve of Fig. 3A.

The type of ferredoxin used did not influence the regulatory properties of the enzyme; all regulatory effects could also be shown in ferredoxin-free (DEAE-cellulose-treated) lysates with ferredoxin from C. pasteurianum as substrate.

Based on the standard potentials of hydrogen and ferredoxin (both, £0 = -0.42 volt (18)), it must be expected that under an atmosphere of hydrogen and in the presence of hydrogenase ferredoxin should be half-reduced, while under argon it should be nearly completely oxidized. Therefore, the effect of reduced
ferredoxin could be studied by comparing the velocity of ferredoxin reduction by NADPH in the presence of NAD+ under hydrogen and under argon. No differences were observed, showing that reduced ferredoxin had no effect.

**Ferredoxin-NADP⁺ Reductase Activity**

**NADPH Inhibition**—Cell-free lysates catalyzed NADP⁺ reduction by hydrogen in a ferredoxin-dependent reaction. Pyruvate could substitute for hydrogen in the regeneration of reduced ferredoxin, as the lysates contained pyruvate dehydrogenase activity as well as hydrogenase. Ferredoxin oxidation was followed by measuring either hydrogen consumption or NADPH formation. The $K_m$ for NADP⁺ was found to be $1.82 \times 10^{-4} \text{ M}$. The reaction product NADPH was an inhibitor competitive to NADP⁺; the $K_i$ for NADPH was calculated to be $0.9 \times 10^{-4} \text{ M}$ (Fig. 5).

**NAD⁺-NADP⁺ Transhydrogenase Activity**

Effect of Ferredoxin—In cell-free lysates NAD⁺ was rapidly reduced by NADPH (Figs. 3B and 6). The reaction was irreversible. The electron transfer could not have been mediated by ferredoxin, as oxidation of reduced ferredoxin by NAD⁺ is strictly inhibited by low concentrations of NADH (see the following paper (10)) and as the reaction proceeded in ferredoxin-free lysates (Fig. 6). Addition of ferredoxin enhanced the activity under argon ($= F_{d_{ox}}$), but not under hydrogen ($F_{d_{ox}}: F_{d_{red}} = 1$). It is therefore concluded that the NAD⁺-NADP⁺ transhydrogenase activity is stimulated by oxidized and inhibited by reduced ferredoxin.

**NADPH-Methyl Viologen Diaphorase Activity**

Crude and ferredoxin-free lysates mediated the reduction of methyl viologen by NADPH. The diaphorase activity was not regulated by NAD⁺ or NADH.

**DISCUSSION**

Similarities of the electron transport systems between ferredoxin and pyridine nucleotides in photosynthetic and hydrogen-metabolizing chemotrophic organisms have early been suggested (19, 20). These proposals have been substantiated mainly by the demonstration that the ferredoxins of phototrophs and chemotrophs are interchangeable (18, 21), and that the enzymes involved are flavoproteins containing FAD (22). Comparisons of the regulatory properties of the two systems were not presented.

The physiological direction of electron flow in plant photosynthesis is opposite to that in *C. kluyveri*. Therefore, regulation of the two systems must be different. For the photosynthetic electron transport enzyme the regulation of the physiological ferredoxin-NAD⁺ reductase activity is unknown; control mechanisms have mainly been studied with the transhydrogenase activity (8, 9), whose physiological role is not understood. This activity appears to be controlled by ferredoxin (9).

For the clostridial enzyme the regulation of the physiological NADPH-ferredoxin reductase activity was shown to be affected by oxidized and reduced NAD, while the reverse reaction was controlled by the oxidation-reduction state of the NAD⁺-NADP⁺

1 The NADPH-ferredoxin reductase of *C. kluyveri* is also a FAD enzyme (unpublished data).
NADPH couple (Fig. 7). The transhydrogenase activity was found to be modulated by oxidized and reduced ferredoxin.

The common regulation of the transhydrogenase activity by ferredoxin points to another similarity and a possible relatedness of the phototrophic and chemotrophic electron transport between ferredoxin and NADP.

A possible physiological role of the regulatory effect of NAD$^+$ and NADH on ferredoxin reduction by NADPH in C. kluyveri may be suggested in view of the energy metabolism of this organism (4):

$$\text{Ethanol} + \text{H}_2\text{O} \rightarrow$$ (1)

$$\text{acetyl}^- + \text{H}^+ + 2\text{H}_2 (\Delta G'_e = +1.5 \text{ kcal per reaction})$$

$$\text{Ethanol} + \text{acetate}^- \rightarrow$$ (2)

$$\text{butyrate}^- + \text{H}_2\text{O} (\Delta G'_e = -8.6 \text{ kcal per reaction})$$

$$2 \text{ ethanol} + \text{acetate}^- \rightarrow$$ (3)

$$\text{caproate}^- + 2\text{H}_2\text{O} (\Delta G'_e = -17.2 \text{ kcal per reaction})$$

NADPH is formed in this fermentation by the conversion of acetaldehyde to acetyl-CoA catalyzed by a NAD$^+$ and a NADP$^+$ specific aldehyde dehydrogenase (23). ATP synthesis is obligatorily linked to H$_2$ formation, i.e. ferredoxin reduction (Equation 1). Since this process is formally endergonic it must be driven by the exergonic NAD$^+$-mediated symproportionation of ethanol and acetate to butyrate or caproate (Equations 2 and 3) (24). The oxidation-reduction state of the NAD$^+$-NADH couple may therefore well be an indicator for the coupling of Process 1 to Processes 2 and 3.

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Regulation of the Reduced Nicotinamide Adenine Dinucleotide Phosphate-Ferrodoxin Reductase System in *Clostridium kluyveri*
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*J. Biol. Chem. 1971, 246:954-959.*

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