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FUNCTION OF REDUCED PYRIDINE NUCLEOTIDE–FERREDOXIN OXIDOREDUCTASES IN SACCHAROLYTIC *CLOSTRIDIA*

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SUMMARY

The physiological function of the clostridial NADH- and NADPH–ferredoxin oxidoreductases was investigated with *Clostridium pasteurianum* and *Clostridium butyricum*.

The NADH–ferredoxin oxidoreductases are concluded to be catabolic enzymes required for the reduction of ferredoxin by NADH. The conclusion is based on the finding that during the entire growth phase the fermentation of glucose can be formally represented by the weighted sum of Eqns 1 and 2,

$$\text{Glucose} + 2\ H_2O \rightarrow 1\ \text{butyrate}^- + 2\ HCO_3^- + 3\ H^+ + 2\ H_2 \quad (1)$$

$$\text{Glucose} + 4\ H_2O \rightarrow 2\ \text{acetate}^- + 2\ HCO_3^- + 4\ H^+ + 4\ H_2 \quad (2)$$

and that in these redox processes NADH rather than NADPH is specifically formed during glyceraldehyde phosphate dehydrogenation. This NADH can be consumed by substrate reduction in Process 1 only, while it must be reoxidized in Process 2 by the ferredoxin-dependent proton reduction to hydrogen which involves the NADH–ferredoxin oxidoreductases.

The kinetic and regulatory properties of these enzymes are in line with their catabolic role: they are found with high specific activities typical for other catabolic enzymes; essentially they catalyze electron flow from NADH to ferredoxin only because the back reaction is very effectively inhibited by low concentrations of NADH. These enzymes have a key role in the coupling of the two partial processes and in regulating the overall thermodynamic efficiency of the fermentations.

The NADPH–ferredoxin oxidoreductases are concluded to participate in anabolism; they are required for the regeneration of NADPH. The conclusion is based on the finding that in the two clostridia all catabolic oxidations–reductions are specific for NAD(H) and that the usual NADPH-producing processes such as the glucose 6-phosphate dehydrogenase or malate enzyme reactions are absent. The kinetic properties of the enzymes are in agreement with their anabolic function: the NADPH–ferredoxin oxidoreductases are found with sufficient specific activities; they preferentially catalyze electron transfer from reduced ferredoxin to $NADP^+$.

INTRODUCTION

The reduction of ferredoxin by NADH[1,2] and by NADPH[3,4] was first demonstrated in cell-free extracts of the non-saccharolytic *Clostridium kluyveri*. This orga-

nism grows on ethanol, acetate and bicarbonate as sole carbon and energy sources and produces 2 moles of H_2 per mole ATP formed in the fermentation[5]. Pyruvate, the usual ferredoxin reductant in chemotrophs, is obviously not available as a catabolic intermediate in *C. kluyveri*. As the dehydrogenating steps in the ethanol–acetate fermentation are specific for pyridine nucleotides[6], NADH or NADPH were regarded as the physiological electron donors to ferredoxin[4]. Based on circumstantial evidence NADPH was initially favored.

Ferredoxin reduction by NADH was later observed also in the saccharolytic *Clostridium pasteurianum* and *Clostridium butyricum*[7] and in the ethanol-fermenting S-organism isolated from *Methanobacillus omelianskii* cultures[8]. Ferredoxin reduction by NADPH has been described in the aerobic bacterium *Azotobacter vinelandii*[9]. However, no evidence has so far been presented showing whether ferredoxin reduction with NADH or NADPH, or the reverse processes, are operative *in vivo*.

The present investigation is an attempt to clarify the physiological role of the NADH- and the NADPH–ferredoxin oxidoreductases in the clostridia. Such a study was necessary as an *in vivo* reduction of ferredoxin ($E_0' = -400$ mV) by reduced pyridine nucleotides ($E_0' = -320$ mV) is thermodynamically highly unfavorable and has therefore not been generally accepted. The study was carried out with *C. pasteurianum* and *C. butyricum*, which were known to contain the two oxidoreductases[7,10]. The results presented indicate that in the saccharolytic organisms the NADH–ferredoxin oxidoreductases are catabolic enzymes whose physiological function is the reduction of ferredoxin by NADH, and that the NADPH–ferredoxin oxidoreductases are anabolic enzymes whose role is the regeneration of NADPH.

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 vol.% (O_2 <1 vpm*); hydrogen, extrem rein: H_2 >99.999 vol.% (O_2 <1 vpm). Enzymes, coenzymes and substrates were purchased from Boehringer, Mannheim.

Regenerating systems

NADH regenerating system. Galactose, 20 mM; galactose dehydrogenase, 1 unit; NADH, 0.4 mM (for *C. pasteurianum*) or 2.5 mM (for *C. butyricum*).

NAD^+ regenerating system. Sodium pyruvate, 20 mM; lactate dehydrogenase (pig muscle), 5 units, NAD^+, 5 mM.

NADPH regenerating system. Trisodium DL-isocitrate, 20 mM; isocitrate dehydrogenase (pig heart), 0.5 unit; magnesium acetate, 2.5 mM; $NADP^+$, 1.25 mM.

Acetyl-CoA regenerating system. Acetyl phosphate (potassium, lithium), 20 mM; phosphotransacetylase, 5 units; CoA, 1 mM.

Reduced-ferredoxin regenerating systems. (I) Hydrogen, 1 atm; ferredoxin, 0.5 mg protein; hydrogenase of the lysates. (II) Sodium pyruvate, 20 mM; CoA, 0.5 mM; ferredoxin, 0.5 mg protein; thiaminepyrophosphate, 1 mM; magnesium

* vpm = volumes/million.

acetate, 2 mM; sodium arsenate, 5 mM; phosphotransacetylase, 2 units; pyruvate dehydrogenase of the lysates.

Culture of Clostridia

C. pasteurianum (ATCC 6013) and *C. butyricum* (ATCC 19398) were grown in 50-l plastic tanks at 35 °C on a synthetic medium with $(NH_4)_2SO_4$ as the nitrogen source as described by Lovenberg *et al.*[11]. A washing flask filled with 4 M KOH to trap the CO_2 evolved, together with a gas-flow meter to measure the H_2 produced, were attached to the tanks. Sodium dithionite was added (50 mg per l of medium, previously gassed with nitrogen) just before inoculation with 4 l of an actively growing culture. H_2 production, which can be continuously monitored and which is proportional to growth[5], was used as a measure of the development of the cultures. The bacteria were harvested in a continuous-flow centrifuge (Model Junior 15000, Christ Osterode, Germany, at 10000 rev./min) after having produced about 2.5 l (*C. pasteurianum*) and 2 l (*C. butyricum*) of H_2 per l of culture. The cells were stored at −15 °C.

Protein preparations

The preparation of crude lysate, of Dowex 2 (acetate)–charcoal lysate (free of all nucleotides) and of Sephadex G-25 lysate (free of ions and other low molecular weight compounds) have been described previously[4]. Ferredoxin was prepared by a slight modification of the procedure described by Mortenson[12]; it was obtained in homogeneous form as indicated by disc gel electrophoresis at pH 8.0, 9.0 and 10.3.

Enzyme assays

Assays were carried out at 37 °C in 22-ml Thunberg tubes or in 14-ml Warburg vessels with continuous shaking or in 1.5 ml anaerobic cuvettes of 1 cm lightpath. Anaerobic conditions were obtained by repeated evacuation and refilling with the desired gas.

NAD(P)H–ferredoxin oxidoreductases

The oxidoreductases were assayed by coupling with hydrogenase or with pyruvate dehydrogenase. Electron flow from NADH or NADPH to ferredoxin was determined by measuring hydrogen formation; from reduced ferredoxin (regenerated from hydrogen) to NAD^+ by following hydrogen consumption; from reduced ferredoxin (regenerated from hydrogen or pyruvate) to $NADP^+$ by following NADPH formation photometrically.

Ferredoxin reduction by NADH. Tris–acetate (pH 7.5), 100 mM; mercaptoethanol, 25 mM; FAD, 10 μM; ferredoxin, 0.3 mg protein; acetyl-CoA regenerating system; NADH regenerating system; Dowex 2(acetate)–charcoal lysate, 1.5 mg protein; water to 1 ml; gas phase, argon; Thunberg tubes, started with the galactose of the NADH regenerating system; H_2 detection, gas chromatography.

NAD^+ reduction by reduced ferredoxin. Tris–acetate (pH 7.5), 100 mM; mercaptoethanol, 25 mM; FAD, 10 μM; ferredoxin, 0.3 mg protein; NAD^+ regenerating system: reduced-ferredoxin regenerating System I; Dowex 2(acetate)–charcoal lysate, 6 mg protein for *C. pasteurianum* and 9 mg for *C. butyricum*; water to 1 ml; gas phase, hydrogen; Warburg vessels; started with NAD^+; H_2 determination, manometry.

Ferredoxin reduction by NADPH. Tris–acetate (pH 7.5), 100 mM; mercaptoethanol, 25 mM; FAD, 10 μM; ferredoxin, 0.3 mg protein; NADPH regenerating system; crude lysate, 12 mg protein for *C. pasteurianum* and 8 mg for *C. butyricum*; NAD^+, 5 mM (only for *C. butyricum*); gas phase, argon; Thunberg tubes, started with the isocitrate of the NADPH regenerating system; hydrogen detection, gas chromatography.

$NADP^+$ reduction by reduced ferredoxin. Tris–acetate (pH 7.5), 100 mM; mercaptoethanol, 25 mM; FAD, 10 μM (only for *C. butyricum*); ferredoxin, 0.3 mg protein; reduced-ferredoxin regenerating System I for *C. pasteurianum* and System II for *C. butyricum*; $NADP^+$, as required; crude lysate, 1.5 mg protein; water to 1 ml; gas phase, hydrogen; anaerobic cuvettes, started with protein; NADPH detection, photometry.

Glyceraldehyde phosphate dehydrogenase

The dehydrogenase was tested by measuring $NAD(P)^+$ reduction in the presence of arsenate. The substrate, glyceraldehyde phosphate, was formed from fructose 1,6-diphosphate by the action of aldolase. The detailed assay mixture is described in the legend to Fig. 1.

Acetyl-CoA reduction to butyrate

Acetyl-CoA reduction by reduced pyridine nucleotides was followed photometrically, using the acetyl-CoA regenerating system as the substrate. The detailed assay mixture is described in the legend to Fig. 2.

Analytical procedures

H_2 determination

Gas chromatography. Hydrogen formation was measured by gas chromatography[13] when the experiments were carried out in Thunberg tubes. The inlet was closed with a rubber tubing so that 2 ml of the gas phase could be taken with a gas-tight syringe and could then be injected into the gas chromatograph. Hydrogen was determined quantitatively, relating the peak heights to the standard curve.

Manometry. Hydrogen consumption was measured in a Warburg apparatus with 0.1 ml of 1 M KOH in the center well.

NADH and acetyl-CoA determination

NADH and acetyl-CoA levels under the conditions of oxidized ferredoxin reduction by NADH in Thunberg tubes were measured enzymatically. In the case of NADH the reaction was stopped by the injection of 2 M KOH to a final concentration of 0.1 M. After heating for 90 s at 80 °C the mixture was cooled and kept at room temperature for no longer than 1 h. Just before analysis the solution was brought to pH 8 with 1 M KH_2PO_4; NADH was then assayed in a coupled system with aldolase, triose phosphate isomerase and glycerol 1-phosphate dehydrogenase[14]. In the case of acetyl-CoA the reaction was stopped by the injection of 0.25 ml of 0.9 M $HClO_4$. After 30 min at 0 °C the mixture was centrifuged; the supernatant was neutralized with $KHCO_3$. The precipitate was removed. Acetyl-CoA was then estimated in a coupled assay with citrate synthase and malate dehydrogenase[15].

Determination of fermentation products

The bacteria were grown in 1-l volumetric flasks at 35 °C under continuous rapid stirring with a magnetic stirring bar. A bubble counter filled with 4 M KOH was attached directly to the flask in order to trap CO_2. The evolution of hydrogen

gas was measured volumetrically with the appropriate corrections made for temperature and pressure. The concentration of acetate and butyrate was assayed by gas chromatography[5] at the desired intervals.

RESULTS

NADH–ferredoxin oxidoreductase

Kinetic and regulatory properties

Cell-free lysates of *C. pasteurianum* and *C. butyricum* catalyzed both the reduction of ferredoxin with NADH and the back reaction, *i.e.* the reduction of NAD^+ with reduced ferredoxin. The specific activities were much higher for the flow of electrons from NADH to ferredoxin than for the reverse reaction (Table I). The activities of the forward reaction are comparable with the activities found for pyruvate dehydrogenase and glyceraldehyde phosphate dehydrogenase of the two bacteria (700–1100 μmoles/min per g soluble protein). They are therefore in the range typical of catabolic enzymes.

TABLE I

KINETIC AND REGULATORY PROPERTIES OF CLOSTRIDIAL NADH–FERREDOXIN OXIDOREDUCTASES

Organism	*NADH → ferredoxin*			*Ferredoxin → NAD^+*		
	Substrate NADH $[S]_{0.5\,V}$ *(mM)*	*Activator acetyl-CoA* $[A]_{0.5\,V}$ *(mM)*	*Spec. act.**	*Substrate NAD^+* $[S]_{0.5\,V}$ *(mM)*	*Inhibitor NADH* $[I]_{0.5\,V}$ *(mM)*	*Spec. act.**
C. pasteurianum	$\ll 0.3$	0.22**	500	0.37	< 0.02	150
C. butyricum	—	0.09**	250	0.33	< 0.02	60
C. kluyveri[2]	—	0.28**	60	—	< 0.02	40

* μmoles/min per g soluble protein.
** Obligatory activator.

Ferredoxin reduction by NADH. This required acetyl-CoA as an obligatory activator[2]. Both the substrate NADH and the activator acetyl-CoA could not be used in substrate amounts but had to be added in the form of regenerating systems. This technique was necessitated by the highly active fatty acid-forming system of the lysates which reduced acetyl-CoA with NADH *via* butyryl-CoA to butyrate. Using the regenerating systems, constant levels of NADH and acetyl-CoA could be maintained. The steady-state levels of NADH and of acetyl-CoA were determined after alkalization and acidification, respectively, of the reaction mixture. The half-saturation concentration, $[A]_{0.5\,V}$, for the activator acetyl-CoA was found to be 0.22 mM for the enzyme of *C. pasteurianum* and 0.09 mM for that of *C. butyricum* (Table I). The half-saturation concentration, $[S]_{0.5\,V}$, for the substrate NADH was considerably below 0.3 mM; however, it could only be roughly estimated as no NADH regenerating system was available with a $[S]_{0.5\,V}$ for NAD^+ low enough

and a V high enough to completely override the endogenous fatty acid-forming system. The system of NADH–ferredoxin oxidoreductase *plus* hydrogenase was saturated with ferredoxin at a concentration of 50 μM.

NAD^+ reduction with reduced ferredoxin. This, when followed photometrically, ceased almost immediately after the start of the reaction. Apparently the product, NADH, was a very potent inhibitor of the process even at concentrations as low as 0.02 mM (Table I). If NADH was continuously removed from the reaction mixture by means of NAD^+ regenerating systems the reaction, when tested manometrically, was linear with time. The half-saturation concentration, $[S]_{0.5\ V}$, for NAD^+ was found to be 0.37 with *C. pasteurianum* and 0.33 mM with *C. butyricum* (Table I).

TABLE II

PARTIAL BALANCE OF PRODUCTS IN THE FERMENTATIONS OF GROWING *C. PASTEURIANUM* AND *C. BUTYRICUM*

Organism	*Growth time* (*h*)	*Product formation* (*mmoles/l*)			*Balance** $\frac{2(A+B)}{(H_2)}$
		Acetate (*A*)	*Butyrate* (*B*)	*Hydrogen* (H_2)	
C. pasteurianum	7.75	9.8	9.1	34.9	1.08
	10.25	19.9	19.3	74.4	1.05
	12.25	26.2	29.4	113.3	0.98
	8.00	5.7	7.7	27.9	0.96
	10.75	15.9	18.8	67.7	1.02
	12.75	22.7	29.8	106.5	0.99
C. butyricum	5.75	4.9	12.8	34.2	1.03
	6.80	8.6	19.0	56.2	0.98
	8.00	14.7	23.6	80.3	0.96
	7.50	6.0	14.7	41.5	1.00
	8.75	10.9	22.8	65.5	1.03
	10.00	14.3	31.4	90.7	1.01

* See Eqns 1 and 2.

Function

The function of the NADH–ferredoxin oxidoreductase can be deduced from fermentation balances and from the coenzyme specificity of the oxidation–reduction processes of the fermentation.

Fermentation balances. C. pasteurianum and *C. butyricum* are known to convert carbohydrates to acetate, butyrate, CO_2, and H_2 as major products[16–18]. The balance of acetate, butyrate and H_2 was determined during growth on glucose (Table II). It can be seen that 2 moles of H_2 are formed per mole each of acetate and butyrate. Thus the fermentations can be formally represented by the weighted sum of Eqns 1 and 2.

$$\text{Glucose} + 2\ H_2O \rightarrow 1\ \text{butyrate}^- + 2\ HCO_3^- + 3\ H^+ + 2\ H_2 \quad (1)$$

$$\text{Glucose} + 4\ H_2O \rightarrow 2\ \text{acetate}^- + 2\ HCO_3^- + 4\ H^+ + 4\ H_2 \quad (2)$$

Coenzyme specificity of the oxidation–reduction processes of the fermentation. There are two electron-donating steps in the glucose fermentation of *C. pasteurianum* and *C. butyricum*: the conversion of glyceraldehyde phosphate to 1,3-diphosphoglycerate and of pyruvate to acetyl-CoA and bicarbonate. The glyceraldehyde phosphate dehydrogenase was specific for NAD^+ rather than for $NADP^+$ (Fig. 1A). The $[S]_{0.5\,v}$ for NAD^+ was found to be 0.39 mM with *C. pasteurianum* and 0.23 mM with *C. butyricum* (Fig. 1B). The pyruvate dehydrogenase is specific for ferredoxin[19]. Correspondingly, there are two electron-consuming processes in the fermentation: the reduction of protons to hydrogen and of acetyl-CoA to butyrate. The hydrogenase is specific for ferredoxin[20]. Acetyl-CoA reduction to butyrate could be shown to proceed only with NADH (Fig. 2A). The $[S]_{0.5\,v}$ for NADH was found to be 0.05 mM with *C. pasteurianum* and 0.04 mM with *C. butyricum* (Fig. 2B). Thus the catabolic oxidoreduction processes of the fermentation are specific either for NAD^+ or ferredoxin; $NADP^+$ is not involved.

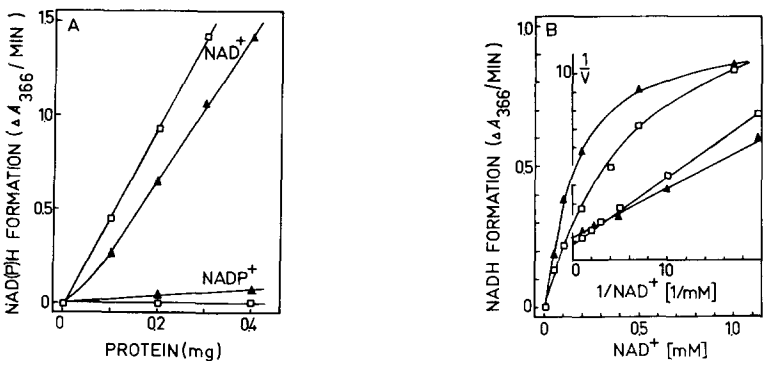

Fig. 1. Glyceraldehyde phosphate dehydrogenase of *C. pasteurianum* (□) and of *C. butyricum* (▲). A, specificity of glyceraldehyde phosphate dehydrogenation for NAD^+. B, $[S]_{0.5\,v}$ of NAD^+ in glyceraldehyde phosphate dehydrogenation. Assays: (A) Tris–acetate (pH 7.5), 100 mM; mercaptoethanol, 25 mM; fructose 1,6-diphosphate, 20 mM; fructose 1,6-diphosphate aldolase, 2 units; sodium arsenate, 10 mM; NAD^+, 1 mM, where indicated; $NADP^+$, 2 mM, where indicated; Dowex 2(acetate)–charcoal lysate, as indicated; water to 1 ml. (B) as in A; NAD^+, as indicated; Dowex 2(acetate)–charcoal lysate, 0.3 mg protein. Gas phase, argon; anaerobic cuvettes, started with protein; NAD(P)H detection, photometry.

Conclusion

As has been described above, the glucose catabolism of *C. pasteurianum* and of *C. butyricum* can be regarded as a system of two coupled processes (Eqns 1 and 2). In this fermentation the two dehydrogenating steps catalyzed by glyceraldehyde phosphate dehydrogenase and pyruvate dehydrogenase yield NADH and reduced ferredoxin, respectively. In Eqn 1, the oxidized electron carriers can be regenerated by proton reduction to hydrogen and by acetyl-CoA reduction to butyrate. The stoichiometry of the redox process is such that the electrons generated in the form of reduced ferredoxin during pyruvate oxidation equal those required for proton reduction to hydrogen, and that the electrons yielded in the form of NADH during glyceraldehyde phosphate oxidation equal those required for acetyl-CoA reduction to butyrate (Fig. 3). In Eqn 2, however, NADH can be reoxidized only *via* the ferredoxin-depen-

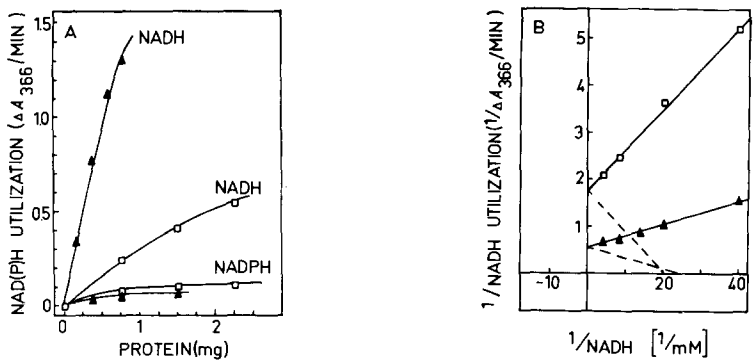

Fig. 2. Acetyl-CoA reduction by reduced pyridine nucleotides in *C. pasteurianum* (□) and in *C. butyricum* (▲). (A) Specificity of acetyl-CoA reduction for NADH. (B) $[S]_{0.5\ V}$ of NADH in acetyl-CoA reduction. Assays: (A) Tris–acetate (pH 7.5), 100 mM; mercaptoethanol, 25 mM; acetyl-CoA regenerating system; Dowex 2(acetate)–charcoal lysate, as indicated; water to 1 ml. Additions: NADH, or NADPH, *C. pasteurianum*, 0.125 mM, *C. butyricum*, 0.25 mM. (B) as in A; NADH, as indicated; Dowex 2(acetate)–charcoal lysate, *C. pasteurianum*, 1.5 mg protein, *C. butyricum*, 0.75 mg protein. Gas phase, argon; anaerobic cuvettes, started with the acetyl phosphate of the acetyl-CoA regenerating system; NAD(P)H detection, photometry.

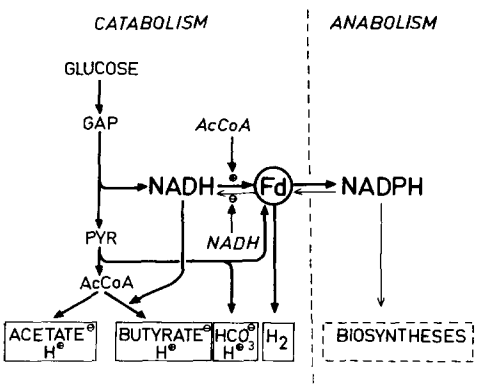

Fig. 3. Function of the NADH–ferredoxin oxidoreductase and of the NADPH–ferredoxin oxidoreductase in the metabolism of saccharolytic clostridia. GAP, glyceraldehyde phosphate; PYR, pyruvate; AcCoA, acetyl-CoA; Fd, ferredoxin.

dent reduction of protons to hydrogen; this clearly indicates that the NADH–ferredoxin oxidoreductase is essential in these fermentations for mediating electron transfer from NADH to ferredoxin. The kinetic and regulatory properties of the reductase are in line with this function: the reduction of ferredoxin by NADH appears to be catalyzed unidirectionally, because the reverse reaction is very effectively inhibited by low NADH concentrations.

NADPH–ferredoxin oxidoreductase

Kinetic and regulatory properties

Cell-free lysates of *C. pasteurianum* and *C. butyricum* also catalyzed both the reduction of ferredoxin with NADPH, and the reduction of $NADP^+$ with reduced

ferredoxin. The specific activities were higher for the flow of electrons from reduced ferredoxin to $NADP^+$ than for the reverse reaction (Table III). The difference was especially prominent with *C. pasteurianum*. This might be taken to indicate that the NADPH–ferredoxin oxidoreductases of the saccharolytic clostridia catalyze the reduction of $NADP^+$ under physiological conditions. The activities observed were in the range typical for anabolic enzymes.

TABLE III

KINETIC AND REGULATORY PROPERTIES OF CLOSTRIDIAL NADPH–FERREDOXIN OXIDOREDUCTASES

Organism	*NADPH → ferredoxin*			*Ferredoxin → $NADP^+$*		
	Substrate NADPH $[S]_{0.5\ V}$ *(mM)*	*Activator NAD^+* $[A]_{0.5\ V}$ *(mM)*	*Spec. act.**	*Substrate $NADP^+$* $[S]_{0.5\ V}$ *(mM)*	*Inhibitor NADPH* K_i *(mM)*	*Spec. act.**
C. pasteurianum	—	n.r.**	9	0.03	0.25§	140
C. butyricum	—	<0.5***	37	0.17	—	55
C. kluyveri[4]	0.02	0.09***	100	0.15	0.13§§	40

* μmoles/min per g soluble protein.
** n.r., not required as activator.
*** Obligatory activator.
§ Non-competitive inhibitor.
§§ Competitive inhibitor.

Ferredoxin reduction by NADPH. This could be demonstrated in crude and – with a drastic loss of activity – also in nucleotide-free lysates of both *C. pasteurianum* and *C. butyricum*. With *C. butyricum* the reaction required NAD^+ as an obligatory activator: NADH was not inhibitory. With *C. pasteurianum* none of the common nucleotides had any effect. As the activities were strongly reduced in nucleotide-free lysates, exact $[S]_{0.5\ V}$ values for the substrate NADPH or $[A]_{0.5\ V}$ values for the activator NAD^+ could not be determined.

$NADP^+$ reduction by reduced ferredoxin. This could be followed photometrically. Reduced ferredoxin was regenerated in lysates of *C. butyricum* with pyruvate using the endogenous pyruvate dehydrogenase and in lysates of *C. pasteurianum* using the endogenous hydrogenase. The ferredoxin-linked enzyme systems were half-saturated with ferredoxin at a concentration of 5 μM. The half-saturation concentration for the substrate $NADP^+$ was determined as 0.03 mM with *C. pasteurianum* and 0.17 mM with *C. butyricum* (Table III). The reaction was time linear only to about 45 s, indicating that the product NADPH might be an inhibitor. Kinetic data obtained with the enzyme from *C. pasteurianum* revealed that NADPH indeed inhibits the reaction. The inhibition type was practically non-competitive. The K_i for NADPH was calculated[21] as 0.25 mM.

Function

Absence of glucose 6-phosphate dehydrogenase and malate enzyme. In organisms growing on glucose, NADPH is usually formed in the pentose phosphate pathway

by the action of glucose 6-phosphate dehydrogenase and of 6-phosphogluconate dehydrogenase. NADPH can also be generated *via* transhydrogenation by the combined action of NAD-dependent malate dehydrogenase and a NADP-specific malic enzyme. The key enzymes of these pathways, glucose 6-phosphate dehydrogenase and malic enzyme, respectively, could not be demonstrated in the two saccharolytic clostridia.

Conclusion

As was shown above, the catabolic oxidation–reduction reactions are specific for NAD^+. The NADPH–ferredoxin oxidoreductases can, therefore, be involved in anabolism only. Since the usual NADPH generating enzymes are absent, $NADP^+$ must be reduced with ferredoxin. The kinetic and regulatory properties of the reductase are in line with this function: the reduction of $NADP^+$ with ferredoxin is catalyzed in preference to the back reaction.

DISCUSSION

The evidence presented in this paper indicates that the function of the NADH–ferredoxin oxidoreductases of *C. pasteurianum* and of *C. butyricum* is to catalyze the reduction of ferredoxin by NADH, while the role of the NADPH–ferredoxin oxidoreductases is to mediate the reduction of $NADP^+$ by reduced ferredoxin.

Clostridial glucose fermentations

Fermentations may be divided into two groups: (1) the simple, unbranched processes such as the homolactic fermentation of lactobacilli or the ethanolic fermentation of yeast, and (2) the complex, branched processes such as the mixed acid fermentation of enterobacteria or the acetic/butyric acid fermentation of some clostridia (Fig. 3). While the equations required to describe the stoichiometry of the former processes are simple and clear, those necessary to represent the latter are rather complex and largely uninformative. *E.g.* the alcoholic fermentation of yeast can be accurately described by Eqn 3

$$\text{Glucose} + 2\,H_2O \rightarrow 2\ \text{ethanol} + 2\,HCO_3^- + 2H^+ \quad (3)$$

the acetic/butyric acid fermentations, as studied in this paper, however, can only be approximated for *C. pasteurianum* by Eqn 4 and for *C. butyricum* by Eqn 5

$$10\ \text{glucose} + 26\,H_2O \rightarrow 6\ \text{acetate}^- + 7\ \text{butyrate}^- + 20\,HCO_3^- + 33\,H^+ + 26\,H_2 \quad (4)$$

$$10\ \text{glucose} + 24\,H_2O \rightarrow 4\ \text{acetate}^- + 8\ \text{butyrate}^- + 20\,HCO_3^- + 32\,H^+ + 24\,H_2 \quad (5)$$

In order to improve the understanding of the mechanism and regulation of such complex fermentations it is suggested that they be regarded as systems of coupled partial processes, which can be represented by the weighted sum of simple equations such as Eqns 1 and 2. Thus, Eqn 4 is the sum of $7 \times$ Eqn 1 and $3 \times$ Eqn 2, while Eqn 5 is the sum of $8 \times$ Eqn 1 and $2 \times$ Eqn 2.

This concept has two apparent advantages: (1) Given the information, that glyceraldehyde phosphate dehydrogenation is specific for NAD^+, it is still very difficult to see from Eqns 4 or 5 that, in the overall fermentation, protons must be reduced to H_2 by NADH; however, this is immediately obvious from Eqn 2. (2) It is impossible to understand from Eqns 4 or 5 why the fermentation may be branched (Fig. 3), *i.e.* why the organisms apparently do not produce their metabolic energy from either acetate or butyrate production alone; however, a clue to this problem may come

from Eqns 1 and 2. The standard free energy change $\Delta G_0'$ of butyrate formation is -61.1 and that of acetate formation is -51.0 kcal per mole of glucose[22], while the energy yield is 3 and 4 moles of ATP per mole of glucose, respectively. If a $\Delta G_0'$ of -8.2 kcal/mole is used for the free enthalpy of hydrolysis of ATP, the thermodynamic efficiency of the two processes is quite different, 40% for the butyrate and 64.5% for the acetate fermentation. Though there is yet no theoretical derivation of the upper efficiency limit of metabolic processes in living organisms, efficiencies exceeding 50% have not been found in anaerobic organisms[22].

This might explain why fermentations of carbohydrates which yield only CO_2, H_2 and acetate (*via* acetyl-CoA) have not been observed even though they are mechanistically feasible. An alternative explanation, however, might be that butyrate (fatty acid) formation from acetyl-CoA developed very early in evolution as a process necessary for all non-carbohydrate fermentations[22] and that it persisted even as a "waste of energy" when carbohydrate utilization became a major metabolic route.

Ferredoxin reduction by NADH

The problem of coupling the two processes (Eqns 1 and 2) is equivalent to that of controlling the relative flow at the acetyl-CoA branchpoint, which itself is stoichiometrically linked to the NADH branchpoint (Fig. 3). Since the NADH–ferredoxin oxidoreductase is both a regulatory enzyme and a constituent of one of the branchpoints, it must have an important role in coupling the two partial processes and in regulating the overall thermodynamic efficiency of the fermentations. The observation that the NADH branchpoint is under the control of the acetyl-CoA/CoA couple may also be physiologically significant.

Ferredoxin reduction by NADH is therefore a key step in the glucose catabolism of the two clostridia. This reaction has first been demonstrated in the non-saccharolytic *C. kluyveri*[1,2]; however, no information on its physiological role has been obtained. The similar kinetic and regulatory properties of the NADH–ferredoxin oxidoreductases of *C. kluyveri* and of the two saccharolytic clostridia (Table I) make it appear likely that in the ethanol–acetate fermentation of *C. kluyveri* also this enzyme is involved in the formation of hydrogen.

$NADP^+$ reduction by reduced ferredoxin

This is the only NADPH-generating process in the anabolism of *C. pasteurianum* and *C. butyricum*. An NADPH–ferredoxin oxidoreductase has been found also in *C. kluyveri*[3,4] (Table III). Ferredoxin reduction by NADPH was envisaged as its physiological function[4]. However, as the complicated ethanol–acetate fermentation of *C. kluyveri* is not as yet completely understood, $NADP^+$ reduction must also be considered. As in plants, the function of all three clostridial NADPH–ferredoxin oxidoreductases would then be to catalyze the generation of NADPH for anabolism. In certain aerobic bacteria ferredoxin reduction by NADPH might be of physiological importance. This is indicated by the finding that NADPH is a weak reductant in the ferredoxin-dependent nitrogen fixation catalyzed by cell-free extracts of *Azotobacter vinelandii*[9]. However, no evidence has yet been presented that NADPH is the electron donor *in vivo*.

Thermodynamic considerations

Based on a thermodynamic approach to cellular metabolism a reduction of

ferredoxin by reduced pyridine nucleotides has been held unlikely *in vivo*[23]. An electron flow from NADH ($E_0' = -320$ mV) to ferredoxin ($E_0' = -400$ mV) or to protons ($E_0' = -420$ mV) is indeed thermodynamically highly unfavorable if one assumes that (1) the intracellular pH is around 7 at a partial hydrogen pressure of close to 1 atm, that (2) the $NAD^+/NADH$ ratio is about 1, and that (3) NADH, ferredoxin and protons are freely diffusible reactants, *i.e.* form homogeneous cytoplasmic pools. However, the partial pressure of hydrogen during growth is 0.6 atm and the overall $NAD^+/NADH$ ratio has been determined as 3 (ref. 24, and unpublished data). However, the resulting changes in oxidation–reduction potentials are negligible ($E'[H^+/H_2] = -412$ mV; $E'[NAD^+/NADH] = -304$ mV). No information on the other assumptions is available.

As the reduction of ferredoxin and of protons by NADH does occur in the growing cells, either the intracellular pH cannot be 7 or the reactants cannot be freely diffusible and form homogeneous pools. If the pH were 4, then the standard oxidation–reduction potentials of the $NAD^+/NADH$ couple (E_0 [pH 4] $= -230$ mV) and of the H^+/H_2 couple (E_0 [pH 4] $= -240$ mV) would essentially be equal. If, in analogy to chloroplasts, the ferredoxin-linked electron transport reactions were membrane-associated, then compartments of not freely diffusible reactants and inhomogeneous pools would be formed. Therefore acidic pH and kinetic compartmentation (*e.g.* by membrane association) of the reactions, or both, have to be considered as important for the reduction of protons by NADH *via* ferredoxin.

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