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Regulation of the Biosynthesis of NADH–Rubredoxin Oxidoreductase in *Clostridium acetobutylicum*

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Abstract. The effects of the pH of the culture medium on the biosynthesis of NADH–rubredoxin oxidoreductase by *Clostridium acetobutylicum*, strain ATCC 824, have been studied. Between pH 6.8 and pH 4.2, the rate of enzyme formation fluctuated in the proportions of 1–50 respectively. The induction of the rubredoxin reductase synthesis, normally observed at pH 4.3, was stopped immediately after the addition of rifampicin. It is suggested that NADH–rubredoxin reductase could play a role in some deacidification mechanism in relation to proton transport.

Acetone–butanol fermentation has been reported in *Clostridium acetobutylicum*, strain ATCC 824, which can convert glucose into solvents at pH below or close to the pKa of the butyric and acetic acids via aldehyde and alcohol dehydrogenases [3, 5]. Moreover, an NADH-dependent ferredoxin oxidoreductase was shown in Clostridia to be synthetized at a constant rate in order to regulate the metabolism by directing the electrons either to the NAD(P)H-dependent reductases or to the hydrogenase [8]. In contrast, the role of the NADH–rubredoxin oxidoreductase isolated from *C. acetobutylicum* [9] remains unclear, since no specific function has been proposed yet for rubredoxin in anaerobic bacteria. Recently, a chemically defined medium for culturing *C. acetobutylicum* was described [5] that allowed the detection of reductase activities under given conditions [4]. We report here that the biosynthesis of the rubredoxin reductase is regulated by the pH of the culture medium.

Materials and Methods

Organism and culture maintenance. *Clostridium acetobutylicum*, strain ATCC 824, was maintained in Reinforced Clostridia Media (Oxoid Ltd., Basingstoke, Hampshire, England) at 35°C for five days followed by storage at 4°C. For inoculum preparation, cultures were transferred to Reinforced Clostridia Media. After being heat shocked at 80°C for 45 min, the cultures were incubated at 35°C under anaerobic conditions.

Media and test conditions. Growth was accomplished on a chemically defined medium with the following composition (per 1 liter distilled water): glucose, 40 g; KH_2PO_4, 0.5 g; $K_2HPO_4 \cdot 3H_2O$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $MnSO_4 \cdot 1H_2O$, 0.01 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; NaCl, 0.01 g; ammonium acetate, 2.2 g; *p*-amino-benzoic acid, 1 mg; biotin, 0.01 mg.

Cells were cultivated for enzymatic and growth yield studies in a 2-l BIOLAFITTE fermentor (Maison Lafitte, France) that contained 1.5 l of medium. The volume of the inoculum formed 10% of the total volume. The pH of the cultures was maintained at desired values by means of an automatic titrator, with 2 *N* NaOH used as the titrant. Preliminary transfers were made after 24 h of culture. The temperature inside the fermentor was adjusted and maintained at 32°C.

Growth and fermentation analysis. Cell growth was estimated by measuring the optical density at 600 nm with a Beckman model 25 recording spectrophotometer. Cell concentration was estimated by cell dry weight measurement by use of a predetermined correlation between optical density at 600 nm and cell dry weight.

Analyses were made on supernatants of 10-ml samples previously centrifuged at 20,000 *g* for 10 min. The concentrations of solvents (ethanol, acetone, and butanol) and acids (acetate and butyrate) were determined by injecting acidified supernatants into an Intersmat IGC 121 FL gas chromatograph (Courtry, France) equipped with a flame ionization detector. The glass column was 2.10 m long, with an internal diameter of 2 mm, and was packed with Porapak Q (100/120 mesh). The analysis of products was carried out under the following conditions. Column temperatures: initial, 165°C; final, 225°C. Injector temperature, 230°C; detector temperature, 230°C; N_2 (carrier gas) flow rate, 13 ml/min; H_2 flow rate, 20 ml/min; air flow rate, 140 ml/min.

Preparation of cell-free extracts. The pellets of the previous centrifugation were suspended in 100 m*M* Tris–acetate buffer (pH 7.0). To these suspensions was added lysozyme (7.7 mg/ml) at a concentration based on the cell determination (0.47 mg/mg dry cells). The final volume was adjusted with the buffer to 10 ml. Each sample was gently stirred at 32°C during 30 min. The

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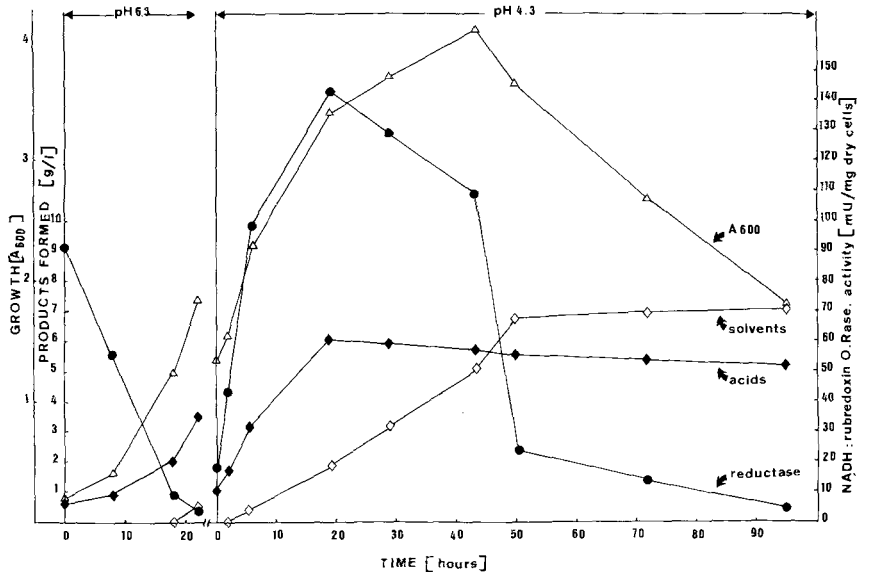

Fig. 1. Fermentation time course and NADH–rubredoxin reductase activity level of *Clostridium acetobutylicum* cultured successively at two different pHs. Cell treatment and enzyme assays were performed as described in *Materials and Methods*.

supernatants were collected from the cell lysates by centrifugation at 60,000 *g* for 15 min.

Enzyme assay. NADH-dependent rubredoxin reductase activity was tested spectrophotometrically in 1-ml optical cuvettes with a 1-cm light path and with metmyoglobin as electron acceptor [7]. The assay system contained 100 m*M* Tris–acetate buffer (pH 7.0), 0.18 m*M* NADH, 0.185 m*M* metmyoglobin (from horse heart), 0.4 μM rubredoxin, and enzyme sample. Change in absorbance at 581 nm was recorded before and after the addition of rubredoxin, and the difference between the two slopes was taken as a measure of enzyme activity. Usually, no or negligible activity was recorded prior to the addition of rubredoxin. E_{581} for the metmyoglobin was 11,900 $M^{-1}cm^{-1}$ [2]. Enzyme unit is defined as the amount of enzyme needed to form 1 μmol of product per milligram of dry cells under the assay conditions.

Results

Effect of pH. If the ATCC 824 strain of *C. acetobutylicum* is able to convert glucose into solvents at pH 4.8 or below, it can also grow at higher pH up to 7.0. In the latter case, the cells no longer produce solvents [6]. We found that other strains of *C. acetobutylicum*, such as the strains NRRL 592 and NCIB 619, can give a good production of solvents at pH 5.5–6.0, but they cannot ferment glucose at pH below 5.0. We did not detect any NAD(P)H-dependent rubredoxin reductase activity in these strains, although they do also contain rubredoxin.

All these observations led us to follow the influence of pH of the culture medium on the biosynthesis rate of the rubredoxin reductase. Table 1 shows a progressive decrease of the reductase activity level when the culture medium was maintained at increasing pH values. At pH 6.8, the enzyme level was only about 2% of that observed from a culture the pH of which was not controlled.

The role of pH on the biosynthesis rate of the rubredoxin reductase was demonstrated by culturing *C. acetobutylicum* successively at two different pHs after harvest and resuspension of the cells between the two steps in order to remove all the fermentation products from the medium. Figure 1 shows a decrease of the rubredoxin reductase specific activity level during fermentation initiated by an inoculum without pH control and maintained at pH 6.3 during 22 h. There was no net biosynthesis of the enzyme, although the growth and the acid production reached high rates.

After harvest and resuspension of the cells in the same culture medium maintained at pH 4.3, Fig. 1 shows a very fast increase of the specific activity level during the first 19 h. In view of the growth rate, the reductase biosynthesis was induced until the highest level of acid production was reached. At this point of the fermentation, the biosynthesis of the enzyme stopped, which explains the decrease of the specific activity, since cell growth was not completely achieved.

Effect of rifampicin. In order to demonstrate the pH-dependent induction of the rubredoxin reductase biosynthesis, cells were cultured both in the presence and in the absence of a compound, such as rifampicin, known to stop protein synthesis by inhibiting the bacterial RNA polymerase [11].

The results shown in Table 2 clearly show a 100% repression of the enzyme formation after the addition of rifampicin (50 μg/ml of culture) to Hun-

Table 1. Influence of pH on the rubredoxin reductase activity of *Clostridium acetobutylicum*[a]

Cultures	1[b]	2	3	4	5	6	7	8	9
pH Control	4.2	4.3	4.5	4.8	5.2	5.7	6.0	6.3	6.8
NADH–Rb OR ase (mU/mg dry cells)	181	144	110	66	31	20	9	5	4

[a] All the samples were taken from the cultures during the late exponential phase and tested as described in *Materials and Methods*.
[b] The pH of the culture 1 was not controlled.

Table 2. Effect of rifampicin on the NADH–rubredoxin oxidoreductase activity of *Clostridium acetobutylicum*

Sample	Time of fermentation (h)	Addition	NADH:Rb ORase activity[a] (mU/mg dry cells) Harvest time	Next harvest time
1	0	None	18	45
		Rif	18	17
2	2	None	44	102
		Rif	42	39
3	6	None	99	149
		Rif	96	79
4	19	None	144	—
		Rif	139	—

[a] Enzyme assays were carried out by taking cell samples containing or not containing rifampicin from the fermentor at the harvest time and from the Hungate tubes at the end of the interval between two harvests.

gate tubes containing 10 ml of cell culture taken at different steps of the fermentation course from the fermentor maintained at pH 4.3. Samples containing rifampicin exhibited at any time of the fermentation the same as or slightly lower reductase activity than that of free rifampicin samples tested prior to a given induction period. These observations demonstrate that change of pH of the culture medium from 6.3 to 4.3 leads to a net biosynthesis of the NADH–rubredoxin oxidoreductase.

Discussion

While rubredoxin has been extensively studied for its structural properties [10], no role has been found in anaerobic bacteria for this protein and for the electron transport chain in which it takes part. Since variations of the biosynthesis rate of the NADH–rubredoxin oxidoreductase as a function of pH have been observed for the first time in Clostridia, hypotheses about its role have been made possible. First it must be pointed out that the strain ATCC 824 of *C. acetobutylicum* is the only one to contain both rubredoxin and rubredoxin reductase, since the last one has been detected neither in the strains NRLL 592 and NCIB 619, nor in other Clostridia of the butyric group [7]. The strain ATCC 824 is also the only one to be able to convert acids into solvents at a pH close to or below the pKa of acids (4.8). The conversion of one molecule of glucose to ethanol and butanol, which occurs through NADH-dependent aldehyde and alcohol dehydrogenases, supplies only two ATPs to the cell versus three ATPs for the production of butyrate and acetone and four ATPs for the production of acetate. The role of rubredoxin reductase could be to oxidize NADH provided for in part by the ferredoxin–NAD^+ reductase to struggle against the acidity of the medium. As has been suggested for the L-glutamate decarboxylase [1], the rubredoxin reductase could participate in a proton extrusion mechanism that would allow the cell to shift its metabolism from an "acid type" to a "solvent type" with minimal energy cost. Such an interpretation, if confirmed by further studies, could contribute to a better understanding of the mechanism of acetone–butanol fermentation.

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