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Effect of NADH dehydrogenase-disruption and over-expression on respiration-related metabolism in *Corynebacterium glutamicum* KY9714

Abstract The function of type II NADH dehydrogenase (NDH-2) in Gram-positive *Corynebacterium glutamicum* was investigated by preparing strains with *ndh*, the NDH-2 gene, disrupted and over-expressed. Although disruption showed no growth defects on glucose minimum medium, the growth rate of the over-expressed strain was lower compared with its parent, *C. glutamicum* KY9714. *Ndh*-disruption and over-expression did not lead to a large change in the respiratory chain and energetics, including the cytochrome components and the H^+/O ratio. However, in the strain that lacked NDH-2, membrane L-lactate oxidase activity increased, while NDH-2 over-expression led to decreased L-lactate and malate oxidase activities. In addition, relatively high cytoplasmic lactate dehydrogenase (LDH) activity was always present as was malate dehydrogenase, irrespective of NDH-2 level. Furthermore, L-lactate or malate-dependent NADH oxidase activity could be reproduced by reconstitution with the membranes and the cytoplasmic fraction isolated from the disruptant. These results suggest that coupling of LDH and the membrane L-lactate oxidase system, together with the malate-dependent NADH oxidase system, operates to oxidize NADH when the NDH-2 function is defective in *C. glutamicum*.

Introduction

NADH dehydrogenase is the main entry site for reducing equivalents from the central metabolism to the respiratory chain in all organisms that have an aerobic or anaerobic electron-transport system. There are two types of NADH

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dehydrogenase: proton-translocating NADH dehydrogenase complex I (NADH dehydrogenase I; NDH-1) and non-energy-generating NADH-quinone oxidoreductase (NADH dehydrogenase II; NDH-2). NDH-1 is a multi-subunit enzyme bearing FMN and several iron–sulfur clusters as the prosthetic groups, whereas NDH-2 is a single subunit protein containing FAD as a prosthetic group. In some bacteria, an additional sodium-translocating NADH dehydrogenase has been shown to be present. The mitochondria of higher eukaryotes have only NDH-1, with the exception of plants and fungi. In fungal mitochondria, e.g. *Neurospora crassa*, both NDH-1 and NDH-2 are present (Videira and Duarte 2002), while *Saccharomyces cerevisiae* has only the latter (de Vries and Grivell 1988). Prokaryotes such as *Escherichia coli* (Jaworowski et al. 1981; Matsushita et al. 1987; Leif et al. 1995) and *Azotobacter vinelandii* (Bertsova et al. 1998, 2001) have been shown to contain both NDH-1 and NDH-2, whereas only NDH-1 is found in *Paracoccus denitrificans* (Yagi 1986; Yagi et al. 2001) and NDH-2 in *Bacillus subtilis* (Bergsma et al. 1981).

The amino acid-producing coryneform bacterium, *Corynebacterium glutamicum*, is widely used to produce L-glutamate and L-lysine on an industrial scale. Although the central metabolism of this bacterium has been thoroughly studied, few reports have investigated the composition and function of the respiratory chain (for a review, see Bott and Niebisch 2003). According to previous studies, the respiratory chain branches into two main terminal oxidases at menaquinone (MK): a cyanide-sensitive terminal oxidase and a resistant one (Matsushita et al. 1998). The former pathway consists of cytochrome bc_1c and cytochrome aa_3 oxidase (Niebisch and Bott 2001; Sakamoto et al. 2001; Sone et al. 2001) which seem to generate a proton-motive force across the membrane, whereas the latter has cytochrome *bd* (Kusumoto et al. 2000) or an cyanide-resistant bypass oxidase (Matsushita et al. 1998) which generates a minor proton-motive force, or is not coupled with energy generation. As for the primary dehydrogenase, part of the respiratory chain and NADH-, NADPH-, lactate-, malate-, and succinate-

Table 1 Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Source
E. coli DH5α	*SupE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan (1983)
C. glutamicum strains		
KY9714	Lysozyme-sensitive strain (*ltsA* mutation)	Hirasawa et al. (2000)
ndh Disruptant	KY9714 *ndh*::Km	This work
ndh Over-expressed	KY9714 carrying pCS-CGndh plasmid	This work
Plasmids		
pGEM-T-easy	Cloning vector, *lacZ*, Ap^r	Promega
pUC4K	Tn903, Ap^r, Km^r	Oka et al. (1981)
pTndh	pGEM-T-easy containing a 1.9-kb fragment of the *C. glutamicum ndh* gene	This work
pTndh-Km	pTndh carrying the Km cassette isolated from pUC4K	This work
pT-CGndh	pGEM-T-easy harboring a 2.0-kb fragment of the *C. glutamicum ndh* gene	This work
pCS299P	Cloning vector, *repCG*, Km^r	Yokoi et al. (2000)
pCS-CGndh	pCS299P carrying a 2.0-kb fragment of the *C. glutamicum ndh* gene	This work

oxidizing activities have been found (Matsushita et al. 1998; Molenaar et al. 1998, 2000). Recently, disruption of the *ndh* gene encoding NDH-2 was performed in *C. glutamicum* ATCC13032, which did not cause any serious effects on growth and metabolism (Molenaar et al. 2000). Moreover, NDH-2 in *C. glutamicum* was purified and shown to exhibit NADPH-oxidizing activity in addition to NADH dehydrogenase activity (Matsushita et al. 2001).

In this paper, the role of NDH-2 in *C. glutamicum* was investigated by examining *ndh*-deficient and over-expressing mutant strains of *C. glutamicum* KY9714, since this enzyme conceivably works in close relation to both the central metabolism and energy generation. The results indicate that the *ndh* deficiency leads to an increase in membrane L-lactate oxidase activity and its over-expression leads to the opposite.

Materials and methods

Bacterial strains, plasmids, and growth condition

The bacterial strains and plasmids used are listed in Table 1. *E. coli* DH5α (Hanahan 1983) was grown aerobically on Luria–Bertani (LB) medium at 37°C, with ampicillin (100 μg/ml) or kanamycin (Km; 25 μg/ml) when indicated. *C. glutamicum* KY9714, a lysozyme-sensitive strain (Hirasawa et al. 2000), was kindly supplied by Kyowa Hakko Co. (Tokyo, Japan) and used as the type strain in this study. The cultivation of *C. glutamicum* and its derivatives was performed at 30°C under 200 rpm shaking. The bacterium was cultured on 5 ml of LB overnight; and 1 ml of the overnight culture was transferred to 100 ml of glucose minimum medium in a 500-ml Erlenmeyer flask and grown to the late exponential phase. The glucose minimum medium consisted of (per liter): 10 g glucose, 1 g KH_2PO_4, 3 g K_2HPO_4, 3 g NH_4Cl, 2 g urea, 0.5 g $MgSO_4 \cdot 7H_2O$, 10 mg $FeSO_4 \cdot 7H_2O$, 1 mg $MnSO_4 \cdot 7H_2O$, 30 mg biotin, 1 mg thiamin-HCl, 20 mg cysteine-HCl, 0.5 g casamino acid, and 1 ml metal mixture. The metal mixture contained (per liter): 990 mg $FeSO_4 \cdot 7H_2O$, 880 mg $ZnSO_4 \cdot 7H_2O$, 393 mg $CuSO_4 \cdot 5H_2O$, 72 mg $MnCl_2 \cdot 4H_2O$, 88 mg $Na_2B_4O_7 \cdot 10H_2O$, and 37 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$.

General molecular biology techniques

Restriction endonuclease and modified enzymes were purchased from Toyobo and Promega and used as instructed by the manufacturers. Chromosomal DNA from *C. glutamicum* was isolated according to the method of Eikmanns et al. (1994). Plasmids from *E. coli* were isolated using the Qiaprep spin miniprep kit (Qiagen), whereas plasmids from *C. glutamicum* were isolated using the Qiaprep spin miniprep kit with prior incubation in P1 buffer containing 20 mg/ml lysozyme at 37°C for 30 min. The DNA was purified from agarose gel using the Qiaquick gel extraction kit (Qiagen). *E. coli* was transformed using the $CaCl_2$ method as described by Cohen et al. (1972) or electroporation as described by Dower et al. (1988), whereas *C. glutamicum* was carried out by electroporation in a 0.1-cm cuvette using 50 μl of competent cells with parameters set at 25 μF, 600 Ω, and 2.0 kV to yield a pulse duration of 9–10 ms.

To prepare competent *C. glutamicum* cells, 1 ml of overnight culture in LB was transferred to a 500-ml Erlenmeyer flask containing 100 ml of LB and cultivated at 30°C under 200 rpm rotation. Penicillin G was supplemented at 7 μg/ml when the cells were grown to 200 klett units. The cultivation was continued at the same conditions for another 2 h. The bacterial cells were harvested, washed three times with 15% ice-cold glycerol, and dissolved in 1.5–2.0 ml of 15% glycerol. The cell suspension was then used or stored at −80°C.

Over-expression of the *ndh* gene in *C. glutamicum*

A *C. glutamicum ndh* expression plasmid, pCS-CGndh, was constructed as follows. A genomic DNA fragment (2 kb) from KY9714 which included the whole *ndh* gene was PCR-amplified with primers C.ndh-N1 (5′-CTTGCCGTGCGC*GTCGAC*CAG-CAAACGCTA-3′) and C.ndh-C3/1 (5′-GCGGAATT-CACCTGC*GGTACC*TCACACGTC-3′), which were respectively positioned at −527 nt to −497 nt from the starting codon and at 84–114 nt after the termination codon. The resulting DNA fragment was cloned into a pGEM-T-easy vector, which was designated as pT-CGndh. To isolate the 2-kb fragment of the *ndh* gene, plasmid pT-CGndh was digested with *Sal*I and *Kpn*I, sites that were present within the C.ndh-N1 and C.ndh-C3/1 primers, respectively, as indicated by italics. The isolated fragment was then subcloned into plasmid pCS299P (Yokoi et al. 2000), resulting in the recombinant plasmid pCS-CGndh. Then, pCS-CGndh was electroporated into *C. glutamicum* KY9714, Km-resistant clones were isolated, and the presence of the plasmid was confirmed.

Inactivation of the chromosomal *ndh* gene in *C. glutamicum*

A 1.9-kb fragment of the *C. glutamicum ndh* gene was amplified using primers C.ndh-N (5′-CTGCTTGCCCTGCAGGTGCAC-CAGCAAACG-3′) and C.ndh-C2 (5′-CGAGCTGCGCGACAAC-CAGGAATTCAGCGG-3′), which were respectively positioned at −530 nt to −500 nt from the start codon and 34–4 nt before the termination codon. The amplified fragment was ligated into a pGEM-T-easy vector, resulting in the recombinant plasmid pTndh. The Km-resistant gene cassette isolated at the *Bam*HI sites from plasmid pUC4K was ligated into pTndh, which was digested at the *Bam*HI site 480 nt from the start codon. The resulting recombinant plasmid was digested with *Eco*RI using sites located at the cloning site in the T vector. The DNA fragment from the *C. glutamicum ndh* gene harboring the Km cassette was then electroporated into KY9714 and Km-resistant clones were obtained. PCR was performed to check whether the Km cassette was integrated into the *ndh* gene of these clones.

Preparation of membrane and cytoplasmic fractions

KY9714 cells were harvested at the late-log phase by centrifugation and washed twice with 20 mM potassium phosphate buffer (KPB), pH 7.5. The washed cells were resuspended in the same buffer at a concentration of 2.5 ml/g wet cells in the presence of 0.5 mg/ml lysozyme, and incubated at 30°C under 80 rpm rotation for 1 h. Then, the lysozyme-treated cells were disrupted by passing through a French press at 110 MPa and cell debris was removed by centrifugation at 6,000 rpm for 20 min at 4°C. The supernatant was then ultracentrifugated at 40,000 rpm for 90 min at 4°C, to separate the soluble fraction from the precipitate. Precipitated membrane was homogenized in 50 mM KPB (pH 7.5) at a concentration of 0.2 g/ml wet membrane and used as the membrane fraction. The soluble fraction was used as the cytoplasmic fraction.

Enzyme activity assays

NADH dehydrogenase activity was determined spectrophotometri-cally at 25°C by measuring the change in the absorbance of NADH at 340 nm. The reaction mixture contained an appropriate amount of enzyme, 0.2 mM NADH, 2 mM KCN, 50 μM ubiquinone-1 (Q_1; kindly supplied by Eizai Co., Tokyo, Japan) dissolved in dimethyl sulfoxide, and 50 mM KPB (pH 6.5) in a total volume of 1 ml. The activity unit was defined as 1 μmol/min NADH oxidized, which was calculated using a millimolar extinction coefficient of 6.81. NAD(P)H oxidase activity was measured spectrophotometrically at 25°C by following the decrease in NAD(P)H at 340 nm. The reaction mixture (1 ml) contained an appropriate amount of enzyme, 0.2 mM NAD(P)H, and 50 mM KPB (pH 7.5) or Na acetate buffer (pH 5.5) for NADH or NADPH, respectively. To see the full enzyme activity, 20 μM FAD was added to the reaction mixture when indicated. The amount of enzyme oxidizing 1 μmol/min NAD(P)H was defined as 1 unit, using a millimolar extinction coefficient of 6.2 or 6.3 for NADH or NADPH, respectively. *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenyl-enediamine (TMPD) oxidase activity was spectrophotometrically determined at 25°C by measuring the increase in the absorbance of TMPD at 520 nm. The reaction mixture (1 ml) contained an appropriate amount of enzyme, 2 mM TMPD, 1 mM EDTA, and 30 mM Mops–NaOH buffer, pH 7.0. The amount of enzyme oxidizing 1 μmol/min TMPD was defined as 1 unit. A millimolar extinction coefficient of 6.1 was used for the calculation. Cytoplas-mic lactate or malate dehydrogenase (LDH or MDH) activity was determined spectrophotometrically at 25°C by following the decrease in the absorbance of NADH at 340 nm. The reaction mixture contained an appropriate amount of enzyme, 0.2 mM NADH, 10 mM pyruvate or oxaloacetate, and 50 mM KPB (pH 7.0 or pH 7.5, for the LDH or MDH activity assay, respectively) in a total volume of 1 ml. Cytoplasmic isocitrate dehydrogenase activity was spectrophotometrically determined at 25°C by measuring the increase in the absorbance of NADPH at 340 nm. The reaction mixture (1 ml) was composed of an appropriate amount of enzyme, 0.2 mM NADP, 0.1 mM $MgCl_2$, 10 mM isocitrate, and 50 mM Tris-HCl pH 7.5. The activity unit was defined as 1 μmol/min NADP reduced, using a millimolar extinction coefficient of 6.3. Membrane-associated succinate, D-lactate, L-lactate and malate dehydrogenase activities were measured spectrophotometrically at 25°C by following the change in the absorbance of 2,6-dichlorophenolindo-phenol (DCIP) at 600 nm. The reaction mixture contained an appropriate amount of enzyme, 0.2 mM DCIP, 0.4 mM phenazine methosulfate (PMS), 1 mM NaN_3, 10 mM substrate (succinate, D-lactate, L-lactate, or malate), and 50 mM KPB, pH 7.0, in a total volume of 1 ml. The amount of enzyme reducing 1 μmol/min DCIP was defined as 1 unit, using a millimolar extinction coefficient of 14.7. To measure D-lactate, L-lactate, malate, and succinate oxidase activities, an O_2 uptake assay was performed using a Clark-type oxygen electrode at 25°C. The reaction mixture (600 μl) was composed of an appropriate amount of enzyme, 10 mM substrate (succinate, D-lactate, L-lactate, or malate), and 50 mM KPB, pH 7.0. The amount of enzyme reducing 1 natom/min O was defined as 1 unit, considering the amount of O dissolved in buffer as 298.8 nmol. The generation of superoxide (O_2^-) was spectrophotometrically determined at 25°C by following the decrease in the absorbance of cytochrome *c* at 550 nm. The reaction mixture (1 ml) contained an appropriate amount of membrane solution, 1 mM EDTA, 40 μM horse heart cytochrome *c*, 50 mM Tris-HCl, pH 7.5, and either 0.2 mM NAD(P)H, or 10 mM substrate (succinate, malate, D-lactate, or L-lactate). The enzyme amount reducing 1 μmol/min cytochrome *c* was defined as 1 unit, using a molecular extinction coefficient of 19.0 mM^{-1}. Superoxide dismutase (SOD) activity was measured as the activity inhibiting the reduction of cytochrome *c*, which was achieved by O_2^- generated with xanthine oxidase. The reaction mixture contained an appropriate amount of enzyme, 1 mM EDTA, 0.2 mM xanthine, 0.034 units of xanthine oxidase (Sigma, Germany), 30 μM cytochrome *c*, and 50 mM KPB, pH 7.0, in a total volume of 1 ml. One unit was defined as the amount of enzyme that inhibited the cytochrome *c* reduction by 50%, using a molecular extinction coefficient of 19.0 mM^{-1}.

Spectral characterization

Reduced minus oxidized-difference spectra were recorded in liquid nitrogen with a Hitachi model 557 dual-wavelength spectropho-tometer. The membrane solution was adjusted to a protein concen-tration of 10 mg/ml with 50 mM KPB, pH 7.5, and put into cuvettes of 2 mm light pass. The sample cuvette was treated by adding sodium hydrosulfite, whereas the reference cuvette was oxidized by the addition of ferricyanide.

Measurement of the H^+/O ratio

The pH change in the presence of resting cells induced by an oxygen pulse was anaerobically measured by using a radiometer pH meter (pHm 84) connected to a radiometer pH electrode (GK 2410 B) and a radiometer chart recorder (REC 61; Servograph). The experiment was performed in a closed vessel continuously flushed with water-saturated argon gas at 25°C. The cells were grown on glucose minimum medium up to the late exponential phase, when the culture was harvested and washed twice with 0.15 M KCl. The washed cells were resuspended in 0.15 M KCl at a concentration of 1 g wet weight per 10 ml. The reaction mixture (3 ml) contained 1 ml of the cell suspension, 0.3 ml of 1 M KSCN (100 mM final), and distilled water. After the vessel reached anaerobiosis, oxygen was supplied by injecting 20 μl of air-saturated 0.15 M KCl (9.96 natom of O), in which the resulting respiration caused transient acidification of the reaction. At the end of the reaction, 3 μl of 10 mM HCl (30 nmol H^+) was injected into the mixture to estimate the amount of translocated H^+.
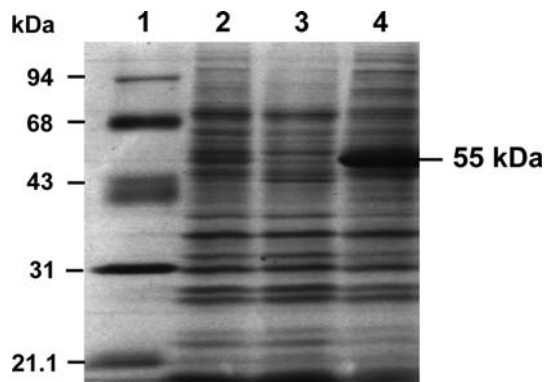
Fig. 1 SDS-PAGE of membranes isolated from KY9714, *ndh* disrupted, and *ndh* over-expressed strains. Membranes were prepared from cells cultivated on glucose minimum medium up to the late-log phase. The membrane suspensions (25 µg of protein each) were subjected to 12.5% SDS-PAGE. *Lane 1* Protein standard marker, *lane 2* KY9714, *lane 3 ndh* disrupted *lane 4 ndh* over-expressed

SDS-PAGE analysis

SDS-PAGE was performed according to the method of Laemmli (1970), followed by staining with Coomassie brilliant blue R250. The protein standard markers consisted of phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and lysozyme (14.4 kDa).

Protein determination

The concentration of protein was determined by a modified Lowry method (Dulley and Grieve 1975). Bovine serum albumin was used as a standard.

Results

Disruption and over-expression of NDH-2 in *C. glutamicum*

To understand the function of NDH-2 and its relation to energy metabolism in *C. glutamicum*, strains with *ndh* disrupted and over-expressed were constructed. Ndh disruption was performed by homologous recombination with a 1.9-kb *ndh* fragment having a Km cassette in the center. The double cross-over mutation was confirmed by PCR (data not shown) and the mutant was used as the disruptant for further experiments. *Ndh* over-expression was performed by transformation with pCS-CGndh in which the entire *ndh* gene including the promoter region (2 kb) was ligated in a pCS299P shuttle vector. Colonies isolated on LB plates containing Km were examined by measuring NADH dehydrogenase activity with the membranes; and the strain having a higher activity than the wild strain was selected as the over-expressed strain for further experiments.

Disruption and over-expression in these mutants were confirmed by SDS-PAGE (Fig. 1). When compared with KY9714, the over-expressed strain harboring pCS-CGndh

Table 2 Several dehydrogenase activities in the membrane of KY9714, *ndh* disrupted, and *ndh* over-expressed strains grown on glucose minimum medium. NADH dehydrogenase activity was measured by following the reduction of Q_1, while other activities were measured with PMS and DCIP, as described in the Materials and methods

Enzyme	Specific activity (units/mg)		
	KY9714	*ndh* Disrupted	*ndh* Over-expressed
Membrane-bound dehydrogenase			
NADH	8.53±2.64 (4)	0.03±0.004 (4)	77.0±15.6 (4)
Succinate	0.73±0.11 (5)	0.72±0.02 (5)	0.61±0.07 (5)
Malate	0.45±0.09 (2)	0.38±0.08 (2)	0.10±0.03 (4)
L-Lactate	0.14±0.04 (4)	0.30±0.05 (4)	0.04±0.01 (4)
D-Lactate	0.17±0.02 (4)	0.16±0.04 (4)	0.08±0.01 (4)
Cytoplasmic dehydrogenase			
Malate	1.98±0.43 (5)	1.37±0.20 (5)	1.27±0.44 (4)
Lactate	0.72±0.27 (3)	0.91±0.33 (3)	0.53±0.13 (2)
Isocitrate	0.52±0.08 (5)	0.38±0.04 (5)	0.39±0.07 (5)

showed a higher level of the 55-kDa band corresponding to NDH-2 polypeptide, whereas the absence of this band was observed in the disruptant. NADH-Q_1 reductase activities from KY9714, *ndh* disrupted, and over-expressed strains cultivated on glucose minimum medium were compared (Table 2). NADH dehydrogenase activity was ten-fold higher in the *ndh* over-expressed strain than in the parental strain, while no activity was detected in the disruptant. The growth rate of the disruptant was not much altered compared with KY9714, while that of the over-expressed strain was reduced (data not shown).

Effect of *ndh* disruption and over-expression on the respiratory chain in *C. glutamicum*

To know the effect of *ndh* disruption and over-expression on the respiratory chain, several oxidase activities and primary dehydrogenase activities were measured with the membrane fractions (Tables 2, 3). As shown in Table 3, the disruptant had no NADH and NADPH oxidase activities, whereas the *ndh* over-expressed strain showed two- to three-fold and eight-fold higher NADH oxidase and NADPH oxidase activities than KY9714, respectively. The *ndh* over-expressed strain showed lower succinate, malate, L-lactate, and D-lactate oxidase activities than KY9714 and the disruptant. Although succinate, malate, and D-lactate oxidase activities were similar between KY9714 and the disruptant, L-lactate oxidase activity in the disruptant was three-fold higher than in KY9714. Changes in these activities corresponded to those of the respective primary dehydrogenases (Table 2), whereas TMPD oxidase activity and cytochrome *c* oxidase activity did not differ among the three strains (Table 3). In contrast to such changes in respiratory activity, cytochrome components of the respiratory chain were not altered in either the disrupted or the over-expressed strains (data not shown), where cytochrome *c* (549 nm), multiple copies of cytochrome *b* (ca. 562.5 nm), and cytochrome aa_3

Table 3 Several oxidase activities in the membranes of KY9714, *ndh* disrupted, and *ndh* over-expressed strains grown on glucose minimum medium

Oxidase	Specific activity (units/mg)		
	KY9714	*ndh* Disrupted	*ndh* Over-expressed
NADH	0.260±0.05 (4)	0 (4)	0.600±0.22 (4)
NADPH	0.012±0.001 (2)	0 (2)	0.10±0.05 (2)
TMPD	1.28±0.04 (2)	1.45±0.02 (2)	1.04±0.57 (2)
Succinate	0.022±0.001 (2)	0.024±0.003 (2)	0.012±0.003 (2)
Malate	0.058±0.007 (2)	0.076±0.021 (2)	0.008±0.00 (2)
L-Lactate	0.010±0.00 (2)	0.029±0.006 (3)	0.003±0.00 (2)
D-Lactate	0.011±0.002 (3)	0.013±0.002 (3)	0.003±0.001 (3)

Table 5 SOD activity and O_2^- generation in KY9714, *ndh* disrupted, and *ndh* over-expressed strains grown on glucose minimum medium

	Specific activity (units/mg)		
	KY9714	*ndh* disrupted	*ndh* over-expressed
SOD activity	18.3±0.09 (2)	37.5±9.18 (2)	25.5±1.11 (2)
O_2^- generation			
NADH	0.045±0.012 (2)	0 (2)	0.19±0.01 (2)
NADPH	0.009	0	0.054
Succinate	0.023±0.012 (2)	0.013±0.003 (2)	0.010±0.00 (2)
Malate	0.012	0.015	0.002
L-lactate	0.011±0.001 (2)	0.021±0.002 (2)	0.002±0.001 (2)
D-lactate	0.006	0.008	0

Table 4 Endogenous respiration-dependent oxygen uptake rate and H^+/O ratio in intact cells of KY9714, *ndh* disrupted, and *ndh* over-expressed strains grown on glucose minimum medium

Strain	Oxidase activity (units/mg)	H^+/O ratio
KY9714	0.41±0.036 (2)	3.71±0.38 (8)
ndh disrupted	0.36±0.057 (2)	3.99±0.50 (11)
ndh over-expressed	0.15±0.043 (2)	4.34±0.55 (9)

(599 nm without CN, 589+599 nm with CN) were observed in the low-temperature redox difference spectrum with the membrane. One exception was that a cytochrome *d*-like component (ca. 630 nm) was observed only in the over-expressed strain. The respiratory chain has a cyanide-resistant bypass oxidase and thus the respiratory oxidase activities are insensitive to cyanide at high concentrations, except for cytochrome oxidase activity, which is almost completely inhibited with 1 mM cyanide. The cyanide sensitivity of cytochrome oxidase (TMPD oxidase) and the cyanide resistance of other respiratory oxidase activities, including NADH, succinate, malate, D-lactate, and L-lactate oxidase systems were not affected by *ndh* disruption and over-expression (data not shown).

Respiratory oxidase activity and energy coupling in the disrupted and over-expressed strains

Since *C. glutamicum* has a high endogenous respiratory activity in intact cells, the oxidase activity was compared among wild-type, disrupted and over-expressed strains (Table 4). Although the over-expressed strain exhibited a relatively low activity, the wild strain and disruptant had higher endogenous oxidase activity. To evaluate the efficiency of energy generation in the respiratory chain, the endogenous respiration-dependent H^+/O_2 ratio was measured by the oxygen pulse method (Table 4). The H^+/O_2 ratio was not much different among these strains, although there was a slight difference.

Generation of superoxide (O_2^-) in the respiratory chain

When O_2^- generation due to respiration was examined in the membrane of the KY9714, *ndh* disruptant, and over-expression strains, the *ndh* over-expression strain exhibited five- and six-fold higher NADH- and NADPH-dependent O_2^- generation, respectively, than that of KY9714, whereas decreased O_2^- generation was observed in the succinate-, malate-, D-lactate-, and L-lactate-dependent respiration in the over-expressed strain (Table 5). Higher O_2^- generation was observed with higher respiratory oxidase activity. However, although NADH oxidation may produce a high level of active oxygen species, the over-expressed strain had even lower SOD activity (Table 5).

NADH oxidation system in the disruptant

Molenaar et al. (2000) suggested that the coupling between cytoplasmic MDH and membrane-bound malate–quinone oxidoreductase (MQO) functions as a NADH re-oxidation system in the *ndh* disruptant instead of NDH-2. Since, in this study, membrane-bound L-lactate dehydrogenase (L-LDH) was shown to be increased in the disruptant (Table 2), cytoplasmic MDH and LDH activities were also measured in the wild, disrupted, and over-expressed strains, to confirm the presence of a possible NADH re-oxidation system coupled with malate and L-lactate oxidases in the membrane (Table 2). As shown, a relatively high level of both MDH and LDH activities was observed in KY9714, but these activities were not much changed by *ndh* disruption or over-expression. Furthermore, when the cytoplasmic fraction of the disruptant was mixed with the membrane fraction of the same strain, malate-dependent and L-lactate-dependent NADH oxidation activity was reconstituted (Table 5). Thus, reasonable NADH oxidation activity was reconstituted in the membrane of the NDH-2-deficient strain by adding the cytoplasmic fraction containing MDH or LDH. Although such a reconstitution cannot be examined in the over-expressed strain because of the presence of NDH-2,

Table 6 In vitro reconstitution of NADH oxidation activity using membrane and cytoplasmic fractions of the *ndh* disruptant. Membrane and cytoplasmic fractions were isolated from cells grown on glucose minimum medium up to the late-log phase. A 1-ml reaction mixture contained 50 mM KPB, pH 7.0, 330 µg of *ndh* disruptant membrane, 440 µg of cytoplasmic fraction, and 0.2 mM NADH. The reaction was started by the addition of 10 mM malate or L-lactate. Activity values in parentheses are the amount of enzyme used for the measurement of *Membrane + cytoplasm*

	MDH or LDH activity (units/mg)	NADH oxidation activity	
		Units/mg cytoplasm	Units/mg membrane
Malate system			
Membrane	0.44 (0.15)	–	–
Cytoplasm	1.37 (0.60)	–	–
Membrane + cytoplasm	–	0.060	0.081
L-Lactate system			
Membrane	0.33 (0.11)	–	–
Cytoplasm	0.91 (0.40)	–	–
Membrane + cytoplasm	–	0.013	0.021

the membranes of the strain had reduced levels of MQO and L-LDH, suggesting that malate-dependent or lactate-dependent NADH oxidation activity is very low and that NDH-2 plays a main role in oxidizing NADH in the over-expressed strain.

Discussion

To reveal the function of NDH-2 in *C. glutamicum*, we constructed *ndh* disrupted and over-expressed strains of *C. glutamicum* KY9714 and then examined the respiratory chain and related metabolic activities in cells grown on glucose minimum medium. We found that growth of the over-expressed strain was lower than wild-type KY9714 and the disruptant, suggesting that energy generation in this strain might be lower than the others. However, the H$+$/O$_2$ ratio showed that it was not much different among the strains. Since the over-expressed strain produced a higher level of O$_2^-$ which depended on NADH or NADPH respiration, the delay in growth for this strain might be due to the generation of O$_2^-$, although it seems that the overproduction of such a membrane-bound protein as NDH-2 may also have some negative effect on growth. Together with the other results described below, this suggests that NADH and NADPH oxidation is performed mainly by NDH-2 in the over-expressed strain.

Contrary to our expectation, the disruption and over-expression of NDH-2 did not affect the respiratory chain very much. These findings were also seen in a study by Molenaar et al. (2000), where *ndh* disruption did not lead to any severe growth defects. They observed that a growth defect could be seen only after a double mutation together with MQO, suggesting that coupling between MDH and MQO may lead to NADH re-oxidation instead of NDH-2. However, even the double-mutant strain grew slowly in glucose minimum medium. Thus, in this study, other metabolic enzyme activities seemingly related to NADH oxidation were examined. In the disruptant which lacked NDH-2-dependent oxidation of NADH, membrane-bound L-LDH activity was increased compared with KY9714, but succinate DH, MQO, and D-LDH activities were not. Whereas L-LDH and MQO activities were largely decreased in the over-expressed strain, succinate DH and D-LDH were not much altered. In addition, there was a reasonably high cytoplasmic LDH activity, similar to MDH activity, in the cytoplasm of the disruptant, but the activity was a little decreased in the over-expressed strain.

These results suggest that LDH–L-LDH coupling may operate as a NADH re-oxidation system in addition to a MDH–MQO system in *C. glutamicum* (Fig. 2), as speculated by Molenaar et al. (2000). Such coupling reactions were reproduced in this study by reconstitution of the cytoplasmic and membrane fractions of the disruptant (Table 6). Although the NADH oxidation activity was higher in the MDH–MQO system than in the LDH–L-LDH system, the large activity change in the LDH–L-LDH system of the disrupted and over-expressed

Fig. 2 Schematic representation of a hypothetical respiratory chain in *C. glutamicum*
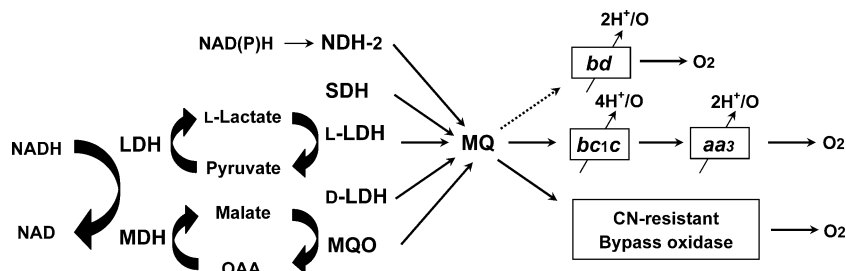
strains suggests that the LDH–L-LDH system is also working, like the MDH–MQO system, for NADH re-oxidation. The operation of NADH re-oxidation systems may also affect the way the tricarboxylic acid cycle operates. A high operation of such coupling systems for NADH oxidation might lead to a lower accumulation of oxaloacetate or pyruvate, which could cause a lower production of an important precursor for the anabolism of many amino acids, including glutamate. The opposite situation could be observed in the over-expressed strain, in which the coupling system may not be working. Thus, manipulation of *ndh* might be useful to control the central metabolism and also amino acid production.

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