THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 31, Issue of August 2, pp. 27991–27995, 2002

Printed in U.S.A.

Kinetic Regulation of the Mitochondrial Glycerol-3-phosphate Dehydrogenase by the External NADH Dehydrogenase in *Saccharomyces cerevisiae**

Received for publication, April 26, 2002
Published, JBC Papers in Press, May 24, 2002, DOI 10.1074/jbc.M204079200

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In the yeast *Saccharomyces cerevisiae*, the two most important systems for conveying excess cytosolic NADH to the mitochondrial respiratory chain are external NADH dehydrogenase (Nde1p/Nde2p) and the glycerol-3-phosphate dehydrogenase shuttle. In the latter system, NADH is oxidized to NAD+ and dihydroxyacetone phosphate is reduced to glycerol 3-phosphate by the cytosolic Gpd1p; glycerol 3-phosphate gives two electrons to the respiratory chain via mitochondrial glycerol-3-phosphate dehydrogenase (Gut2p)-regenerating dihydroxyacetone phosphate. Both Nde1p/Nde2p and Gut2p are located in the inner mitochondrial membrane with catalytic sites facing the intermembranal space. In this study, we showed kinetic interactions between these two enzymes. First, deletion of either one of the external dehydrogenases caused an increase in the efficiency of the remaining enzyme. Second, the activation of NADH dehydrogenase inhibited the Gut2p in such a manner that, at a saturating concentration of NADH, glycerol 3-phosphate is not used as respiratory substrate. This effect was not a consequence of a direct action of NADH on Gut2p activity because both NADH dehydrogenase and its substrate were needed for Gut2p inhibition. This kinetic regulation of the activity of an enzyme as a function of the rate of another having a similar physiological function may be allowed by their association into the same supramolecular complex in the inner membrane. The physiological consequences of this regulation are discussed.

The yeast *Saccharomyces cerevisiae* lacks transhydrogenase activity (1, 2), and the redox couple NAD^+/NADH cannot pass the mitochondrial membrane. Hence, systems for NADH turnover in mitochondria as well as in the cytosol are required during both aerobic and anaerobic conditions. The reason being is that several processes result in production of NADH, *i.e.* several processes are contrary to ethanol fermentation not redox-neutral. Synthesis of 1 mol of glycerol, the second major by-product of glucose-fermenting cells of *S. cerevisiae*, results in consumption of 1 mol of NADH, while other by-products like acetate lead to the production of cytosolic NADH. However, the largest part of excess cytosolic NADH formation is connected to biomass production (3, 4). Synthesis of proteins, nucleic acids, and even the highly reduced lipids are associated with the assimilatory NADH production. In particular, NADH is generated in the biosynthetic pathways of amino acid synthesis (3, 4). Anaerobically, the only means by which *S. cerevisiae* can reoxidize surplus production of NADH is by glycerol production (2, 5). Aerobically, several systems for conveying excess cytosolic NADH to the mitochondrial electron transport chain exist in *S. cerevisiae* (6). The two most important systems in this respect seem to be the external NADH dehydrogenase (Nde1p/Nde2p) (7, 8) and the glycerol 3-phosphate shuttle (9). The Nde1p/Nde2p localized in the inner mitochondrial membrane with the catalytic sites projecting toward the intermembrane space are proved to directly oxidize cytosolic NADH (7, 8). The glycerol 3-phosphate shuttle system, involving the FAD-dependent Gut2p (10), which is situated in the inner membrane of the mitochondria with the catalytic site projecting toward the cytosol, is also shown to be active in maintaining a cytosolic redox balance (9). In this system the cofactor, NADH, is oxidized to NAD^+ by the cytosolic glycerol-3-phosphate dehydrogenase (Gpd1p) when catalyzing the reduction of dihydroxyacetone phosphate (DHAP)[1] to glycerol 3-phosphate (G3P). Subsequently G3P delivers its electrons to the respiratory chain via the Gut2p, and DHAP will reappear.

It is believed that the Nde1p/Nde2p is used preferentially over the Gut2p system when cells grow fast and a high ATP-producing capacity per unit of time is needed (9, 11). On the other hand, the Gut2p shuttle system is more efficient in producing ATP, *i.e.* the phosphate to oxygen ratio is higher and is believed to be important when cells have a reduced growth rate (9, 11). It has been shown that the Gut2p system becomes less important when increasing the dilution rate in ethanol-limited chemostat cultures of *S. cerevisiae* (9, 11), and additionally it is less important when maintenance requirements are increased by lowering external pH values (9).

Furthermore, results from a study on glucose-limited chemostat cultures of *S. cerevisiae* showed that when deleting Nde1p/Nde2p higher yields of glycerol were obtained when increasing the dilution rate compared with the wild type and the *gut2*Δ mutant (12). It seems as if the two systems are able to, at least

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‖ Supported by Commission of the European Union via Contract BIO4-CT98-0562, by Swedish National Energy Administration Grant P1009-5, and by Swedish National Board for Industrial and Technical Development Grant P10765-1.

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[1] The abbreviations used are: DHAP, dihydroxyacetone phosphate; G3P, glycerol 3-phosphate.

TABLE I
S. cerevisiae strains used in this study All strains were derived from W303–1A

Strain	Description
wt	*ade*2–1, *his*3–11,112*trp*1–1a, *ura*3–1, can100
gpd1Δ	*ade*2–1, *his*3–11, *ura*3–1, can100, *gpd*1Δ::TRP1
gut2Δ	*ade*2–1, *his*3–11, 112*trp*1–1a, can100, *gut*2Δ::URA3
gpd1Δgut2Δ	*ade*2–1, *his*3–11, can100, *gpd*1Δ::TRP1, *gut*2Δ::URA3
nde1Δnde2Δ	*ade*2–1, *his*3–11, 112*trp*1–1a, can100, *nde*1Δ::URA3, *nde*2Δ::LEU2

TABLE II
Respiratory rates, $K_{0.5}$, and efficiency of isolated mitochondria from wildtype, Gpd1Δ,Gut2Δ, Gpd1ΔGut2Δ, and Nde1ΔNde2Δ strains using NADH or glycerol 3-phosphate as a substrate

Strain	NADH			G3P		
	V_{max}	$K_{0.5}$	Efficiency[a]	V_{max}	$K_{0.5}$	Efficiency[a]
	natom O/min mg of protein	*μM*		*natom O/min mg of protein*	*mM*	
wt	456 ± 145	72 ± 13	6.3 ± 2.3	153 ± 66	2.1 ± 0.5	0.073 ± 0.036
gpd1Δ	362 ± 103	55 ± 6	6.6 ± 2.0	141 ± 21	2.4 ± 0.2	0.059 ± 0.010
gut2Δ	355 ± 47	20 ± 2	17.8 ± 3.0	0		
gpd1Δgut2Δ	393 ± 50	24 ± 6	16.4 ± 4.6	0		
nde1Δnde2Δ	0			138 ± 19	0.8 ± 0.2	0.173 ± 0.063

[a] Efficiency is expressed as the ratio between V_{max} (natom of oxygen/min mg of protein) and $K_{0.5}$ (μM).

TABLE III
Enzyme activities, $K_{0.5}$ and efficiency of the external NADH dehydrogenase (Nde1/2p) and the mitochondrial glycerol-3-phosphate dehydrogenase (Gut2p) of isolated mitochondria from wild type, Gut2Δ, and Nde1ΔNde2Δ strains

Strain	Nde1/2p			Gut2p		
	V_{max}	$K_{0.5}$	Efficiency[a]	V_{max}	$K_{0.5}$	Efficiency[a]
	nmol of NADH/min mg of protein	*μM*		*nmol of G3P/min mg of protein*	*mM*	
wt	1150 ± 214	272 ± 91	4.2 ± 1.6	2396 ± 138	34 ± 4	0.070 ± 0.009
gut2Δ	1203 ± 263	115 ± 54	10.5 ± 3.4			
nde1Δnde2Δ				2858 ± 144	23 ± 4	0.124 ± 0.022

[a] Efficiency is expressed as the ratio between V_{max} (nmol of substrate/min mg of protein) and $K_{0.5}$ (μM).

in part, substitute for each other, but the capacity of the Gut2p system is not enough to completely replace Nde1p/Nde2p activity during the growth conditions used. Deleting Nde1p and Nde2p together with Gut2p lead to a more severely affected phenotype, *i.e.* even more glycerol but no ethanol was formed with the increasing dilution rate (12).

The two enzyme systems, the external NADH dehydrogenase and the G3P shuttle, obviously have a similar metabolic function, and hence concerted regulation can be envisaged. In a recent study by Grandier-Vazeille *et al.* (13) it is found that five known intermembrane space-facing dehydrogenases (Nde1p, Nde2p, Gut2p, Dld1p, and Cyb2p) are associated in a complex. They suggest a possible channeling effect of substrates and products as a result of the close physical contact between the enzymes. Moreover, this observation opens up the possibility to explain regulatory mechanisms in a completely new way. Is it possible that close physical interactions between enzymes provide a mode of regulating enzyme activities?

The aim of this study was to analyze the functional relationship between the two cytosolic NADH re-oxidation systems, Nde1p/Nde2p and Gut2p.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions—The *S. cerevisiae* strains used in this study were all derived from W303–1A (Table I). The cells were cultivated as batch cultures in a defined medium, yeast nitrogen base, Difco), using 5 g/liter glucose as the carbon and energy source. The cells were harvested subsequent to glucose depletion after the diauxic shift during respiratory growth on ethanol.

Preparation of Spheroplasts and Mitochondria—Spheroplasts were prepared according to the enzymatic procedure described previously (14). Spheroplasts were suspended at 1 mg/ml in buffer containing 1 M sorbitol, 0.5 mM EGTA, 2 mM $MgSO_4$, 1.7 mM NaCl, 10 mM KH_2PO_4, and 1% bovine serum albumin, pH 6.8, (medium 1) at 28 °C and permeabilized by nystatin as described (15). Yeast mitochondria (*S. cerevisiae*) were prepared from spheroplasts as described (14) and suspended in a medium containing 0.65 M mannitol, 0.36 mM EGTA, 10 mM Tris maleate, 5 mM Tris phosphate, pH 6.8 (medium 2).

Respiration Assay—Oxygen consumption was measured at 28 °C in a 2-ml thermostatically controlled chamber equipped with a Clark oxygen electrode (Gilson) connected to a microcomputer giving an on-line display of rate values. Spheroplasts (1 mg/ml) were incubated in medium 1 and permeabilized by nystatin before assay. Mitochondria (0.5 mg/ml) were incubated in medium 2. Substrate supply was either various concentrations of NADH, glycerol-3-P, ethanol, or an NADH-regenerating system: 4 mM glucose-6-P, 2 mM NAD^+, and various amounts of glucose-6-P dehydrogenase (EC 1.1.1.49) from *Leuconostoc mesenteroides*, which is able to work with NAD^+ as a cofactor.

Enzymatic Activities and Measurement of DHAP—NADH-ferricyanide reductase and glycerol-3-P reductase were assayed in medium 2 on mitochondria in the presence of 0.2 μg/ml antimycin and 1 mM potassium cyanide and different concentrations of either NADH or glycerol-3-P. The reaction was started by addition of 1.5 mM ferricyanide, and its reduction was followed between 405 and 436 nm ($\epsilon = 0.21\ mM^{-1} \cdot cm^{-1}$). DHAP was measured spectrophotometrically in neutralized $HClO_4$/EDTA extracts as described by Bergmeyer (16).

Data Analysis—From plots of the initial respiratory rate velocity *versus* substrate concentration [NADH] or [G3P]) we can determine the value of V_{max} and K_m by fitting the data to the Michaelis-Menten equation using the non-linear least squares fit. It is well known that this treatment applies to numerous mechanisms more complex than the Michaelis-Menten mechanism and in which K_m and V_{max} have more complex definitions. This is particularly true when we consider a metabolic pathway in which the kinetic control distribution may vary as a function of the flux. However, when experimental data fit the following general equation $v = V_{max}[S]/(K_{0.5} + [S])$, the curve is a rectangular hyperbola for which asymptotes are $S = -K_{0.5}$ and $v = V_{max}$. Thus, for very low values of respiratory substrate concentration (very small compared with $K_{0.5}$), the initial rate is directly proportional to [S] and $v = V_{max}[S]/K_{0.5}$. In this condition, $V_{max}/K_{0.5}$ corresponds to a rate constant for the reaction (17). Moreover, for very small values of respiratory substrate concentrations, the flux control coefficient of the dehydrogenase over the respiratory rate is very high (near 1 for either NADH dehydrogenase or GUT2p), and we can assume that the $V_{max}/K_{0.5}$ ratio is, in such a complex mechanism, the most easy and accurate kinetic

TABLE IV
Relative respiratory rates (%) of isolated mitochondria from wild type and Gut2Δ strains with different mixtures of NADH and glycerol 3-phosphate as a substrate

The respiratory rate using 5 mM NADH as substrate is set to 100 % in the wild type strain.

Strain	Respiratory rates (%)		
	NADH[a]	G3P[b]	NADH + G3P
wt	100 ± 18	28 ± 5	94 ± 7[c]
gut2Δ	73 ± 24		65 ± 2[d]

[a] 5 mM NADH
[b] 5 mM glycerol 3-phosphate.
[c] Four different mixtures of NADH and glycerol 3-phosphate were used (5 mM NADH + 5 mM G3P, 5 mM NADH + 2.5 mM G3P, 2.5 mM NADH + 5 mM G3P, 1 mM NADH + 9 mM G3P), and the values ranged from 83 to 103%.
[d] Mixture of 5 mM NADH and 5 mM glycerol 3-phosphate.

parameter of the dehydrogenases. By analogy with a well defined enzyme parameter, *i.e.* k_{cat}/K_m, we called $V_{max}/K_{0.5}$ enzyme efficiency.

RESULTS

Kinetic Parameters of Respiration—Mitochondria were isolated from a wild type strain (W303–1), and different mutant strains (*Gpd1*Δ, *Gut2*Δ, *Gpd1*Δ*Gut2*Δ, *Nde1*Δ*Nde2*Δ) were grown in defined medium with a low concentration of glucose as the carbon and energy source. The mitochondria were isolated after the diauxic shift during growth on ethanol to guarantee respiratory metabolism. The dependence of the oxidative phosphorylation rate (respiration with saturated concentrations of ADP; state 3) on different concentrations of one of the substrates NADH or glycerol 3-phosphate was determined.

Kinetic parameters (V_{max} and $K_{0.5}$) were obtained from the experimental data by the best curve fitting according to the Michaelis-Menten equation (see "Materials and Methods"). Table II shows that when NADH was used as substrate the V_{max} was not significantly different between the tested mitochondria except for mitochondria from the double mutant of the external NADH dehydrogenase (*Nde1*Δ*Nde2*Δ), which does not use external NADH as a respiratory substrate. In contrast, the $K_{0.5}$ for NADH is largely decreased in mitochondria isolated from the mutant strains in which the gene encoding the mitochondrial glycerol-3-phosphate dehydrogenase as well as the genes encoding the cytosolic and mitochondrial glycerol-3-phosphate dehydrogenases (*Gut2*Δ and *Gpd1*Δ*Gut2*Δ, respectively) have been deleted compared with the $K_{0.5}$ of mitochondria isolated from the wild type strain and from a mutant strain deleted of the gene encoding the cytosolic glycerol-3-phosphate dehydrogenase (*Gpd1*Δ). The consequence of the observed changes in the kinetic parameters result in a three-time increase in efficiency (V_{max} to $K_{0.5}$ ratio) of the external NADH oxidation system in mitochondria from strains lacking the *GUT2* gene. The maximal respiratory rate when using the glycerol 3-phosphate as a substrate was also similar in mitochondria from the different strains tested, except for those lacking the Gut2p (Table II). However, in comparison to NADH respiration the V_{max} was significantly lower. Again there was a large decrease in $K_{0.5}$, and consequently the efficiency was enhanced when the alternative cytosolic NADH oxidation system (Nde1p/Nde2p) was absent.

Kinetic Properties of the External NADH Dehydrogenase (Nde1p/Nde2p) and Glycerol-3-phosphate Dehydrogenase (Gut2p)—The question arose whether the change in substrate affinity of the respiratory chain (Table II) was due to changes in kinetic properties of the external NADH dehydrogenase and glycerol-3-phosphate dehydrogenase themselves. Indeed the K_m decreased and the efficiency of the enzymes, functionally isolated, improved when the alternative dehydrogenase activity was absent (Table III). Hence the change in substrate affinity of the respiratory chain was a direct consequence of a modification in the kinetic parameters of the dehydrogenases. Moreover, the comparison between Tables II and III shows that the $V_{max}/K_{0.5}$ ratio measured either by oxygen consumption *versus* substrate concentration or on the enzyme functionally isolated are similar, confirming that the $V_{max}/K_{0.5}$ ratio obtained from the relationships between respiratory rate and substrate concentration is a convenient estimation of the efficiency of the dehydrogenase itself. However, the efficiency of the external NADH dehydrogenase was two orders of magnitude higher than that of glycerol-3-phosphate dehydrogenase.

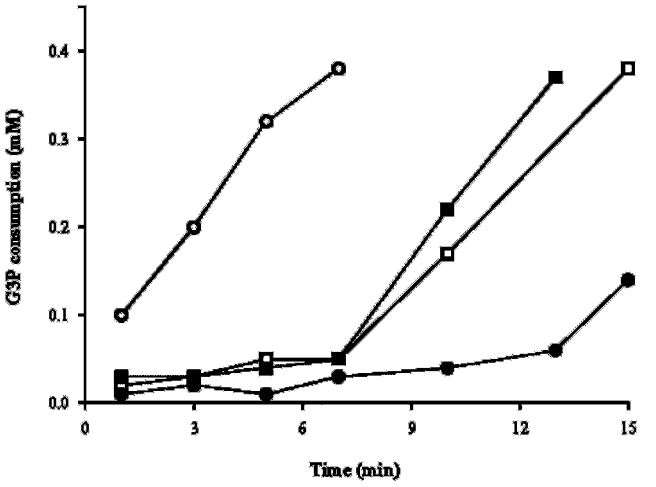

FIG. 1. **G3P consumption using 5 mM G3P (○), 5 mM NADH and 5 mM G3P (●), 2.5 mM NADH and 5 mM G3P (■), and 2.5 mM NADH, 5 mM G3P, and 1.3 mM NAD^+ (□).**

The Respiratory Rates and the Glycerol 3-Phosphate Consumption Using Mixtures of Glycerol 3-Phosphate and NADH as Substrates—The presence of glycerol 3-phosphate did not affect the respiratory rate supported by NADH, neither in the wild type nor in the *Gut2*Δ strain (Table IV). Subsequent experiments indicated that glycerol 3-phosphate was not consumed until NADH was depleted (Fig. 1) and that at a low concentration of NADH the activity of the glycerol-3-phopshate dehydrogenase was inhibited. However, as soon as NADH was consumed glycerol 3-phosphate utilization was initiated at a rate similar to the one observed in the control experiment in the absence of NADH (Fig. 1). Furthermore, addition of NAD^+ to a mixture of glycerol 3-phosphate and NADH did not influence the respiratory activity (Fig. 1).

The effect of NADH addition on glycerol 3-phosphate consumption may be due to a direct kinetic inhibition of Gut2p by NADH or to an indirect effect through the activation of the NADH dehydrogenase. Direct kinetic inhibition was not the case because oxygen consumption of either isolated mitochondria (not shown) or permeabilized spheroplasts of a *Nde1*Δ*Nde2*Δ strain with glycerol 3-phosphate as the respiratory substrate was not affected by the presence of NADH (Fig. 2). Consequently, the inhibition of the Gut2p by NADH addition requires an active external NADH dehydrogenase.

Using the NADH-regenerating system (see "Materials and Methods"), different steady states of oxygen consumption can be obtained depending on the NADH concentration produced by the glucose-6-phosphate dehydrogenase. At a saturating concentration of glycerol 3-phosphate, the respiratory rate was modulated by adding different concentrations of NADH-producing enzyme (Fig. 3). For each experimental condition, NADH and glycerol 3-phosphate consumptions were determined. Fig. 3 shows that the higher the NADH dehydrogenase activity, the lower the glycerol 3-phosphate use. Moreover,

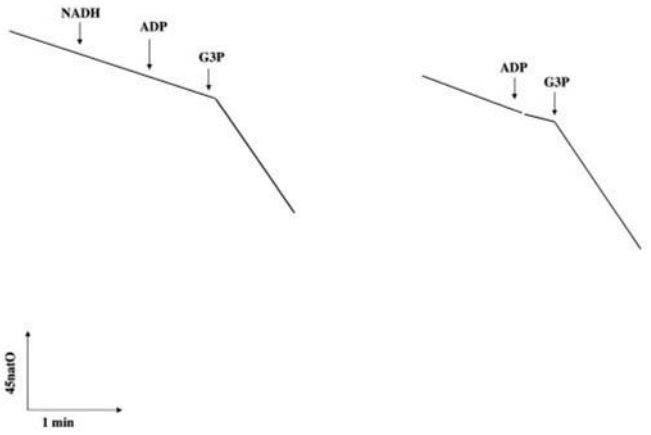

FIG. 2. **Respiratory rate of permeabilized spheroplasts from *Nde1*Δ*Nde2*Δ mutant strain with glycerol 3-phosphate as substrate.** Spheroplasts (1 mg of protein/ml) from *Nde1*Δ*Nde2*Δ strain were permeabilized by nystatin as described under "Materials and Methods." When added as indicated in the figure, NADH, ADP, and G3P concentrations were 2, 1, and 10 mM, respectively. This figure represents a typical experiment; similar results were obtained from two other preparations.

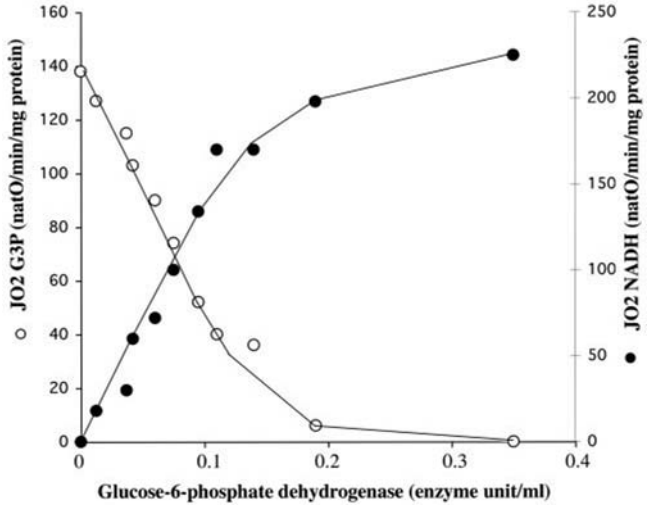

FIG. 3. **Respiratory rate on NADH and G3P of mitochondria isolated from wild type.** Isolated mitochondria (0.5 mg of protein/ml) were incubated in medium 2 (see "Materials and Methods") in the presence of 10 mM G3P, 4 mM glucose 6-phosphate, and 2 mM NAD^+. As indicated in the figure, addition of different amounts of glucose-6-phosphate dehydrogenase led to respiratory steady states. Respiratory rate and dihydroxyacetone formation were measured to determine the part of the respiration linked to NADH (●) and to G3P consumption (○). This figure represents a typical experiment; identical results were obtained in three parallel experiments.

when NADH concentration reached the $K_{0.5}$ value for NADH dehydrogenase, the respiratory activity linked to G3P utilization was inhibited by 50% (Fig. 3).

DISCUSSION

In *S. cerevisiae* there are several systems used for cytosolic oxidation of NADH, the most important ones probably being the external NADH dehydrogenase and the G3P shuttle (7, 9, 11, 12). Their respective role is still to a large extent an open question. Because the external NADH dehydrogenase and the G3P shuttle fulfil the same physiological function one would expect some kind of regulatory interactions between the two systems.

This study showed that indeed there are interactions between the two external inner membrane enzymes, Nde1p/Nde2p and Gut2p. Deletion of one of the external dehydrogenases caused an increased affinity of the remaining enzyme for its substrate and enhanced its efficiency. The increase in efficiency suggests a specific alteration of the remaining enzyme rather than a general modification of the membrane properties like a change in lipid composition (for review see Ref. 18). Moreover, another membrane enzyme, such as succinate dehydrogenase, does not display the same change in kinetic parameters after removal of Gut2p and Nde1p/Nde2p (data not shown). The result seems to indicate that the close physical contact between the two dehydrogenases previously reported (13) influences the apparent kinetic properties of these membranal enzymes. Thus the increased affinity may be explained by an improved accessibility of the enzyme for its substrate in the absence of the alternative dehydrogenase. A similar observation, *i.e.* an increased affinity for NADH of the external NADH dehydrogenase correlated to a decrease in the amount of Gut2p, was obtained in a chemostat study at different dilution rates and with nitrogen or glucose as the limiting substrate (11).

The most important result from this study is that NADH addition inhibited mitochondrial glycerol 3-phosphate consumption. However, there was no direct effect of NADH on Gut2p activity (Fig. 2), but the presence of both the external NADH dehydrogenase and its substrate were needed to observe this effect (Figs. 1 and 2). Hence an active external NADH dehydrogenase is a prerequisite for inhibition of the Gut2p in isolated mitochondria. These observations are in contrast to the results obtained with dehydrogenases facing the matrix, *e.g.* when using succinate in combination with ethanol, an additive effect is instead seen on respiratory activity (19). However, the fact that a (unphysiologically) high NADH concentration completely blocks Gut2p activity does not rule out the possibility that both systems are in use simultaneously *in vivo*. The intracellular NADH concentration is most probably very low. During anaerobic conditions, which are known to provoke an elevated NADH level (20), an NADH concentration of ~0.2 mM (assuming an intracellular volume of 2 ml/g, dry weight) has been reported (21). The $K_{0.5}$ values of the NADH respiration ranging from 0.02 to 0.07 mM in this study also suggests that *in vivo* NADH concentrations are very low. Consequently, at such low NADH concentrations it is not obvious that the external NADH activity completely blocks the Gut2p. Indeed, when keeping NADH levels low by using the NADH-generating system it was possible to obtain simultaneous activity of both the external NADH dehydrogenase and the Gut2p although at saturating concentrations of glycerol 3-phosphate (Fig. 3). Furthermore, simultaneous activity of the Gut2p and external NADH dehydrogenase has been indicated during growth on ethanol and especially at low dilution rates (9, 22).

Gut2p inhibition by the external NADH dehydrogenase activity strongly supports the idea that the dehydrogenases are in physical contact with each other. As a consequence the regulation depends on the relative proportion of the two enzymes. Indeed it was shown that the amount of Gut2p decreased more than the Nde1p/Nde2p when increasing the specific growth rate in glucose-limited chemostats (11). In other words, this result also proposes that the role of Gut2p is most significant at low growth rates.

In conclusion, this study clearly established that the kinetic properties of one membrane enzyme were dependent on the presence of another membrane enzyme with a similar physiological function. Furthermore, the activity of the external NADH dehydrogenase had an inhibitory effect on the external glycerol-3-phosphate dehydrogenase. Consequently, NADH is an indirect effector of also the alternative cytosolic NADH oxidizing system, *i.e.* the glycerol 3-phosphate shuttle.

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