The Soluble and Membrane-bound Transhydrogenases UdhA and PntAB Have Divergent Functions in NADPH Metabolism of Escherichia coli

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Pentose phosphate pathway and isocitrate dehydrogenase are generally considered to be the major sources of the anabolic reductant NADPH. As one of very few microbes, Escherichia coli contains two transhydrogenase isoforms with unknown physiological function that could potentially transfer electrons directly from NADH to NADP⁺ and vice versa. Using defined mutants and metabolic flux analysis, we identified the proton-translocating transhydrogenase PntAB as a major source of NADPH in E. coli. During standard aerobic batch growth on glucose, 35–45% of the NADPH that is required for biosynthesis was produced via PntAB, whereas pentose phosphate pathway and isocitrate dehydrogenase contributed 20–25%, respectively. The energy-independent transhydrogenase UdhA, in contrast, was essential for growth under metabolic conditions with excess NADPH formation, i.e. growth on acetate or in a phosphoglucoisomerase mutant that catalyzed glucose through the pentose phosphate pathway. Thus, both isoforms have divergent physiological functions: energy-dependent reduction of NADP⁺ with NADH by PntAB and reoxidation of NADPH by UdhA. Expression appeared to be modulated by the redox state of cellular metabolism, because genetic and environmental manipulations that increased or decreased NADPH formation down-regulated pntA or udhA transcription, respectively. The two transhydrogenase isoforms provide E. coli primary metabolism with an extraordinary flexibility to cope with varying catabolic and anabolic demands, which raises two general questions: why do only a few bacteria contain both isoforms, and how do other organisms manage NADPH metabolism?

About 1,000 anabolic reactions synthesize the macromolecular components that make up functional cells (1, 2), but only 11 intermediates of central carbon metabolism and the cofactors ATP, NADH, and NADPH constitute the core of this intricate biochemical network (3, 4). These intermediates and cofactors must be supplied through the catabolism of different substrates at appropriate rates and stoichiometries for balanced growth; hence, anabolism and catabolism are delicately balanced and regulated to enable growth under fluctuating environmental conditions. Although chemically very similar, the redox cofactors NADH and NADPH serve distinct biochemical functions and participate in more than 100 enzymatic reactions (5). The electrons of the main respiratory cofactor NADH are transferred primarily to oxygen, thereby driving oxidative phosphorylation of ADP to ATP (3, 4, 6). NADPH, in contrast, exclusively drives anabolic reduction reactions. Despite the important role in linking the fundamental processes of catabolism and anabolism, however, even a qualitative understanding of NADPH metabolism is still missing for most organisms.

The primary NADPH-generating reactions are considered to be the oxidative pentose phosphate (PP) pathway and the NADPH-dependent isocitrate dehydrogenase in the TCA cycle (Fig. 1; Refs. 4, 7, 8). Additionally, nicotinamide nucleotide transhydrogenases may be involved, but ever since their discovery, their physiological role has been a source of speculation and often a matter of controversy (9–11). The transhydrogenase reaction [NADPH] + [NAD⁺] + [H⁺] or ↔ [NADP⁺] + [NADH] + [H⁺] may be catalyzed by either a membrane-bound, proton-translocating or a soluble, energy-independent isoform (9, 12). Microbes often contain one isoform or none, with the Enterobacteriaceae as the only known exception that contain both isoforms encoded by the pntA (13) and udhA (14) genes, respectively. In eukaryotes, the proton-translocating transhydrogenase seems to have a flexible function as a buffer against dissipation of either the mitochondrial redox potential or energy supply (9). In prokaryotes, however, the physiological role of the two isoforms remains an exciting matter of debate, and several potential functions were put forward (8, 9, 15–17).

Based on the isoform distribution in different organisms and the generally more reduced state of the NAD(P)H pool compared with the NADH pool (10), it has been hypothesized (15) that the physiological function of membrane-bound and soluble transhydrogenases in microbes might be generation and reoxidation of NADPH, respectively. Indeed, organisms lacking transhydrogenases, such as the yeast Saccharomyces cerevisiae, cannot tolerate imbalances between catabolic NADPH production and anabolic NADPH consumption (18, 19), unless a soluble isoform is expressed (19, 20). Further means of NADPH reoxidation must exist, however, because isotopic tracer-based carbon flux analysis revealed that at least some bacteria without transhydrogenase homologues exhibit a similar uncoupling (21–25). In Escherichia coli, overexpression of the soluble transhydrogenase UdhA was shown to partially restore the growth phenotype of a phosphoglucoisomerase mutant with impaired NADPH metabolism (26), but its phys-

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1 The abbreviations used are: PP pathway, pentose phosphate pathway; TCA cycle, tricarboxylic acid cycle; GC-MS, gas chromatography-mass spectrometry; ED pathway, Entner-Doudoroff pathway; PEP, phosphoenolpyruvate; Pgi, phosphoglucose isomerase; UdhA, energy-independent, soluble transhydrogenase; PntAB, energy-dependent, membrane-bound transhydrogenase; Zwf, glucose-6P dehydrogenase.

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### TABLE I

**Table: Constructed E. coli mutants and their maximum specific growth rate during exponential growth on glucose**

| Strain           | Genetic markers | Growth rate (h⁻¹) |
|------------------|-----------------|-----------------|
| MG1655           | F⁻ λ⁻ rph-1 (wild-type) | 0.67 ± 0.01 |
| UdhA             | MG1655 ΔudhA (soluble transhydrogenase deficient) | 0.67 ± 0.02 |
| PntAB            | MG1655 ΔpntAB (membrane-bound transhydrogenase deficient) | 0.45 ± 0.03 |
| UdhA-PntAB       | MG1655 ΔudhA ΔpntAB | 0.52 ± 0.04 |
| EDP              | MG1655 (sol eda) (Entner-Doudoroff pathway deficient) | 0.67 ± 0.03 |
| Zwf-EDP          | MG1655 Δzwf edd eda (Zwf = glucose-6P dehydrogenase deficient) | 0.52 ± 0.04 |
| Zwf-EDP-UdhA     | MG1655 Δzwf edd eda ΔudhA | 0.56 ± 0.01 |
| Zwf-EDP-PntAB    | MG1655 Δzwf edd eda ΔpntAB | 0.05 ± 0.03 |
| Pgi              | MG1655 Δpgi (phosphoglucone isomerase deficient) | 0.18 ± 0.02 |
| Pgi-EDP          | MG1655 Δpgi Δ(edd eda) | 0.20 ± 0.03 |
| Pgi-Zwf-EDP      | MG1655 Δpgi Δzwf edd eda | 0 |
| Pgi-UdhA         | MG1655 Δpgi ΔudhA | 0 |

*Correction deletion was verified by PCR with genomic DNA and by in vitro enzyme assays. Generally, the mutants grew well in LB medium and did not exhibit any distinct morphological phenotype under the light microscope or on plates.*

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*Standard deviation was from at least three experiments.*

**EXPERIMENTAL PROCEDURES**

**Strains and Growth Conditions—**Wild-type *E. coli* MG1655 and mutants derived thereof were used in all physiological experiments (Table 1). All physiological experiments were conducted in M9 minimal medium (33), supplemented with 5.0 g/liter of either glucose, acetate, glycerol, or glucyrate as the sole carbon source. Unless indicated otherwise, batch cultures were grown in 500-ml baffled shake flasks with a 50 ml inoculum at 37 °C on a gyratory shaker at 200 rpm. Where necessary, 50 mg/liter ampicillin, 50 mg/liter kanamycin, or 25 mg/liter chloramphenicol were added.

For ¹³C-labeling experiments, cultures were grown in 30 ml of M9 medium, supplemented with 3 g/liter [¹³C]glucose or with a mixture of 0.6 g/liter [¹³C]glucose and 2.4 g/liter unlabeled glucose. After inoculation with 7 ml (100 %) of an M9 preculture and strictly exponential growth at the maximum rate up to an optical density at 600 nm (A₆₀₀) of 1.0-1.5, culture aliquots were harvested, centrifuged at 2,500 x g and 4 °C for 8 min, washed with 10 ml of 5 M NaCl, and stored at -20 °C.

**Genetic Methods—**All recombinant DNA techniques followed standard protocols (34). For colony PCR, part of a colony from a LB plate was dissolved into 50 μl of ddH₂O and incubated at 99 °C for 10 min. 0.5-2.0 μl of the lysate were then used as the template for PCR. Knockout mutants were generated by marker-free deletion of genes from start to stop codon (35). Briefly, PCR-fragments of the Flp recognition target-flanked kanamycin gene from plasmid pKD13 were amplified with primers that contained sequence extension that match the flanking regions of target genes or operons. Correct plasmid integration and excision were verified in all mutants by colony PCR.

Total RNA for semi-quantitative RT-PCR was purified from 1-ml aliquots of bacterial cultures with an A₆₀₀ of about unity using the RNeasy Mini Kit (Qiagen). The RNA solution was frozen in liquid nitrogen and stored at -70 °C for further use. RNA quantification and purity were controlled in agarose gels with formamide (34). For RT-PCR, we used the OneStep RT-PCR kit (Qiagen) with 100 ng of total RNA and 0.6 μl of gene-specific primers. Reverse transcription was allowed to proceed for 30 min at 50 °C, and the cDNA was then amplified by cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min/kb at 72 °C, using the following primers: for *udhA* 5’-AAATGTGGGCGCGGTTGC-3’ and 5’-CATC-GTCGGTAGTTAAGGGTTGT-3’; for *pntA*, 5’-CCAAAGAGCG- GTTAACAACTGAAA-3’ and 5’-ATAGACCCGGGATAAATCTAAGG- AA-3’; for the *rpoD* gene that encodes the RNA polymerase subunit α (37), 5’-GATCCACGCAATGGATTGAGTGTG-3’ and 5’-CTTTCCCC-GATGTGCTGTTGCACATA-3’. For the *rpoD* gene that encodes the RNA polymerase subunit α (37), 5’-GATCCACGCAATGGATTGAGTGTG-3’ and 5’-CTTTCCCC-GATGTGCTGTTGCACATA-3’. For the *rpoD* gene that encodes the RNA polymerase subunit α (37), 5’-GATCCACGCAATGGATTGAGTGTG-3’ and 5’-CTTTCCCC-GATGTGCTGTTGCACATA-3’. For the *rpoD* gene that encodes the RNA polymerase subunit α (37), 5’-GATCCACGCAATGGATTGAGTGTG-3’ and 5’-CTTTCCCC-GATGTGCTGTTGCACATA-3’. For the *rpoD* gene that encodes the RNA polymerase subunit α (37), 5’-GATCCACGCAATGGATTGAGTGTG-3’ and 5’-CTTTCCCC-GATGTGCTGTTGCACATA-3’.

**Metabolic Flux Ratio Analysis by GC-MS—**Direct analytical interpretation of GC-MS-derived ¹³C-labeling pattern in proteinogenic amino acids was used to quantify independent flux partitioning ratios (36, 37). Biomass pellets harvested from ¹³C-labeling experiments were resuspended and hydrolyzed in 1.5 ml of 6 M HCl at 105 °C for 24 h in sealed 2.2-ml Eppendorf tubes. After drying in a vacuum centrifuge at room temperature, hydrolysates were derivatized at 85 °C in 50 μl of dimethylformamide (Fluka) and 50 μl of N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide (Fluka) for 60 min. The mass isotope distribution in the derivatized amino acids was then analyzed by gas chromatography-mass spectrometry (GC-MS), and the GC-MS-derived mass distributions of proteinogenic amino acids were corrected for naturally occurring isotopes (36). The corrected mass distributions were related to *in vivo* metabolic activities using algebraic equations and statistical data treatment which quantified 14 ratios of fluxes through converging reactions and pathways to the synthesis of eight intracellular metabolites from [¹²C]glucose and [¹³C]glucose experiments (36).

**Metabolic Net Flux Analysis—**Intracellular net carbon fluxes were estimated from the physiological data with a stoichiometric matrix containing 24 intracellular fluxes, 3 extracellular fluxes, and 22 metabolite balances (partly shown in Fig. 1). Fluxes into biomass were calculated from the known metabolite requirements for macromolecular compounds (3) and the growth rate-dependent RNA and protein content of *E. coli* (27, 38). The system of linear equations was solved uniquely with constraints obtained from six of the above calculated flux ratios (i.e. serine through glycolysis, pyruvate through Entner-Doudoroff pathway (ED pathway), oxaloacetate from phosphoenolpyruvate (PEP), PEP from oxaloacetate, pyruvate from malate, and PEP through PP pathway) that were formulated as linearly independent equations as described previously (23, 39, 40). Briefly, the sum of the weighed square HDN Metabolism of *E. coli*...
maximal growth rate \( (\mu_{\text{max}}) \) as the regression coefficient. To obtain specific production rates, \( A_{\text{bio}} \) values were converted to cellular dry weight using a predetermined correlation factor of 0.51 g/liter cellular dry weight per \( A_{\text{bio}} \) unit. The specific substrate uptake and product formation rates were calculated by log-linear regression of substrate/product versus biomass concentration, dividing the regression coefficient by the maximal growth rate. Errors are calculated as standard deviations or as average error of the mean, in which case the error was calculated as for a general function \( f(x,y) \).

In Vitro Enzyme Activities—Crude cell extracts were prepared from M9 batch cultures that were harvested at \( A_{\text{bio}} \) of around unity. Cell pellets were washed once with 9 g/liter NaCl and 10 mM MgSO\(_4\) and disrupted by sonication at 100 W on ice for 2 min. The specific enzyme activities of phosphoglucone isomerase and ED pathway activities were determined in supernatants of crude cell extracts after centrifugation at 10,000 \( g \) for 30 min as described previously (41). Because one isofrom is membrane-bound, transhydrogenase activity was determined in cell extracts without centrifugation (42).

**RESULTS**

Physiological and Biochemical Characterization of Transhydrogenase Mutants—To elucidate the function of the soluble and the membrane-bound transhydrogenase, marker-free deletion mutants of the \( \text{udhA} \) and \( \text{pntAB} \) genes were constructed in \( E. coli \) wild-type MG1655. The mutants grew without any apparent phenotype on LB medium, and successful deletion was verified by the absence of the corresponding mRNA in the mutants with RT-PCR. As expected, in vitro reduction of \( \text{NAD}^+ \) from \( \text{NADH} \) was abolished in cell extracts obtained from the UdhA-PntAB double mutant during growth on glucose, but some activity was retained in the UdhA and PntAB mutants (Fig. 2). Thus, both isofroms appear to be present during growth on glucose, with the membrane-bound transhydrogenase PntAB as the major one. The isoenzyme proportion of PntAB may be even higher because activities of soluble proteins are typically better determined with in vitro assays than the activities of membrane-bound enzymes.

The maximum growth rate of the UdhA mutant on glucose was indistinguishable from that of the parent, but was significantly reduced for the PntAB and UdhA-PntAB mutants (Table I). To elucidate the intracellular carbon flux response, we used metabolic flux ratio analysis by GC-MS (36). By comparing \( ^{13}\text{C} \)-labeling pattern in proteinogenic amino acids, this strictly local analysis quantifies ratios of converging fluxes at the junction of two or more pathways or reactions (43, 44).

**NADPH Metabolism in Transhydrogenase Mutants**—Metabolism-wide NADPH production and consumption in the mutants was then assessed by quantifying absolute intracellular carbon fluxes from the extracellular fluxes in and out of the cells, based on quantitative physiological data and the flux ratios of Fig. 3 (39, 40). In contrast to previous isotopomer-based net flux analysis in \( E. coli \) (27, 28, 45, 46), we have sufficient data from separate \( \text{[1-^{13}\text{C}]} \text{glucose} \) and \( \text{[U-^{13}\text{C}]} \text{glucose} \) experiments to resolve the fluxes through both the ED pathway and the glyoxylate shunt. For wild-type \( E. coli \), the molar flux estimates revealed that about 25% of glucose catabolism proceeds by means of the PP pathway and about 2% by means of the ED pathway (Fig. 4). Only a relatively small fraction of the consumed glucose was oxidized to \( \text{CO}_2 \) within the TCA cycle, as was described previously for batch cultures of the same strain using a comprehensive isotopomer model (37). Consistent with the flux ratios (Fig. 3), the absolute in vitro fluxes were very similar in wild-type and UdhA mutant (data not shown). The PntAB and
UdhA-PntAB mutants, however, channeled significantly more carbon flux through the oxidative PP pathway (Fig. 4). The in vivo production rate of NADPH was then estimated by summing up carbon fluxes through the NADPH-producing catabolic reactions (Fig. 5). Because the two isoforms of malic enzyme exhibit different redox cofactor specificities, the calculated flux constitutes a bound for additional NADPH that could maximally be produced in this reaction, but is probably much smaller than that suggested by the hatched bars in Fig. 5. The consumption rate of NADPH is directly accessible from the known biochemical requirement of NADPH for growth rate-dependent macromolecule biosynthesis (3, 27, 38). For wild-type and UdhA mutant growing at a specific rate of 0.67 h⁻¹, the NADPH requirements of 10.6 ± 0.2 mmol g⁻¹ h⁻¹ exceeded by far the estimated NADPH production of 6.2 ± 1.5 mmol g⁻¹ h⁻¹, clearly demonstrating that another reaction must produce NADPH during exponential growth on glucose (Fig. 5). Because NADPH production and consumption were balanced within the resolution of the method in the PntAB and PntAB-UdhA mutants, the additional NADPH-producing reaction in the wild type must be catalyzed by the membrane-bound transhydrogenase (Fig. 5). Wild-type E. coli thus produces 35–45% of the required NADPH by means of the membrane-bound transhydrogenase during exponential growth on glucose (Fig. 5). Because NADPH production and consumption were balanced within the resolution of the method in the PntAB and PntAB-UdhA mutants, the additional NADPH-producing reaction in the wild type must be catalyzed by the membrane-bound transhydrogenase (Fig. 5). Wild-type E. coli thus produces 35–45% of the required NADPH by means of the membrane-bound transhydrogenase during exponential batch growth on glucose, although the reactions of the oxidative PP pathway and isocitrate dehydrogenase contribute 35–45% and 20–25%, respectively. Upon knockout of pntAB, increased carbon fluxes through the oxidative PP pathway (Fig. 4) quantitatively compensated the lack of PntAB-based NADPH production (Fig. 5).

**Knockout Mutants with Perturbed NADPH Metabolism**—Although the membrane-bound transhydrogenase was shown to be an important NADPH-generating reaction during batch growth on glucose, the function of the soluble transhydrogenase remained obscure. Therefore, we constructed two mutant families with perturbed NADPH metabolism. In the first, the oxidative PP pathway was blocked by deleting the glucose-6P dehydrogenase and the entire ED pathway (Fig. 1). This Zwf-EDP mutant catabolized glucose exclusively by means of glyceraldehyde-3P dehydrogenase and the entire ED pathway (Fig. 1). This Zwf-EDP mutant catabolized glucose exclusively by means of glycolytic pathways. In the second, the oxidative PP pathway was blocked by deleting the glucose-6P dehydrogenase and the entire ED pathway (Fig. 1). This Zwf-EDP mutant catabolized glucose exclusively by means of glycolytic pathways.

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![Fig. 3. Origin of key metabolic intermediates in E. coli mutants during exponential growth on glucose obtained from metabolic flux ratio analysis by GC-MS.](image3)

3P-glycerate derived through glycolysis was observed in serine. The asterisks indicate values obtained from experiments with 100% [1-13C]glucose. All others were from experiments with 20% [U-13C]glucose and 80% unlabeled glucose. For wild-type and transhydrogenase mutants, the mean and standard deviation of three independent experiments are given. For the Zwf-EDP and Pgi mutants, a representative of two independent experiments is shown, and the experimental error was estimated from redundant mass distributions as described elsewhere (36).

![Fig. 4. In vivo carbon flux distribution in central metabolism of E. coli wild-type (top values) and the UdhA-PntAB mutant (bottom values) during maximum exponential growth on glucose.](image4)

The estimated net fluxes in the white rectangles are molar percentages of the mean specific glucose uptake rates, which were 7.5 ± 0.5 and 8.4 ± 0.2 mmol g⁻¹ h⁻¹ (dry weight), respectively. Relative to the glucose uptake rate, the confidence intervals were 20% for the TCA cycle and malic enzyme, and less than 5% for all other fluxes. Arrowheads indicate the direction of a given flux; the solid gray arrows indicate carbon fluxes required for biomass formation.
TCA cycle flux cannot be determined (n.d.) from the available data. The wild type during growth on glucose. This mutation forced the fastest and the slowest growing strains, respectively (27).

Error bars in independent experiments, each with 100% [1-13C]glucose or with 20% [U-13C]glucose and 80% unlabeled glucose. In the PntAB mutant, the TCA cycle flux cannot be determined (n.d.) from the available data.

colysis (Figs. 3 and 6) and grew slower on glucose than the wild type (Table I), as was described before (47). The specific rates of glucose consumption and acetic acid production, however, were rather similar to the wild type (Fig. 6). Although the flux through isocitrate dehydrogenase was much higher in Zwf-EDP than in the wild type, this reaction produced less NADPH than was required for biosynthesis, and the remainder was presumably generated through the membrane-bound transhydrogenase (Fig. 5).

Knockout of the transhydrogenases in this Zwf-EDP mutant resulted in two different phenotypes. Deletion of the soluble transhydrogenase in the Zwf-EDP-UdhA mutant had no further impact upon growth on glucose (Table I), and the flux pattern was almost identical to that of Zwf-EDP (data not shown). Deletion of the membrane-bound transhydrogenase in the Zwf-EDP-PntAB mutant, in contrast, was detrimental to growth and resulted in very poor growth rates (Table I), which was presumably the consequence of insufficient NADPH formation. This was a more pronounced phenotype than the previously described growth rate reduction in an undefined Zwf-PntAB mutant (48). Introducing a phosphoglucone isomerase knockout into the Zwf-EDP mutant generates, of course, a lethal phenotype on glucose (Table I), because no catabolic pathway remains for the conversion of glucose-6P.

The second mutant family was based on the phosphoglucone isomerase knockout, and successful deletion was verified by in vitro activities below 10 μmol min⁻¹ g⁻¹ (protein) in all strains with this mutation, compared with 980 ± 10 μmol min⁻¹ g⁻¹ in the wild type during growth on glucose. This mutation forced glucose catabolism primarily through the PP pathway (Figs. 3 and 6; Refs. 26, 36, 37). The resulting flux rerouting caused a metabolic condition with more than 100% excess NADPH formation (Figs. 5 and 6). Moreover, the Pgi mutant was the only strain with an exceptional low fraction of oxaloacetate originating from PEP and a significant fraction of oxaloacetate originating from glyoxylate (Fig. 3), as was described before (37). The resulting absolute flux distribution reveals significant activity of the recently identified PEP-glyoxylate cycle (37), i.e., flux through the glyoxylate shunt and net flux from oxaloacetate to PEP (Fig. 6). Growth of the Pgi mutant was slow on glucose (Table I; Ref. 37), but was abolished when combined with a knockout of the soluble transhydrogenase in the Pgi-UdhA mutant (Table I). Growth could be recovered, however, upon plasmid-based expression of UdhA (data not shown), demonstrating that the lethal Pgi-UdhA phenotype on glucose results from an imbalance of NADPH metabolism. Therefore, it seemed justified to conclude that the soluble transhydrogenase Udha catalyzes the reoxidation of NADPH.

Redox Metabolism and Transhydrogenase Expression on Alternative Carbon Substrates—Although the soluble transhydrogenase was essential for growth of the NADPH-overproducing phosphoglucone isomerase mutants on glucose, it was clearly not necessary in wild-type strains during batch growth on glucose (Table I). To identify the physiological function of the soluble transhydrogenase, we grew transhydrogenase mutants on the alternative carbon sources (acetate and glycerol).
that are expected to affect redox metabolism. On acetate, wild-type and PntAB mutant grew at a specific rate of 0.2 h⁻¹, but the Udha mutant did not grow at all, presumably because extensive catabolism of this substrate through the TCA cycle generated more NADPH than was required for biosynthesis. This view is consistent with virtually absent oxidative PP pathway fluxes during growth of E. coli on acetate (46). On glycerol, all strains grew at about the same maximum specific rate of 0.4 h⁻¹, but the PntAB mutant assumed growth only after an unusually long lag phase of about 12 h that was not seen with the other two strains. Because this lag phase occurred also upon subcultivation in glycerol-containing minimal medium, it seems that a metabolic adaptation complements the lack of PntAB-catalyzed NADPH synthesis. Thus, the picture emerges that transhydrogenases ensure flexibility of E. coli redox metabolism and that the physiological function of the soluble transhydrogenase-encoding udha gene as an internal standard, we determined mRNA levels of udha and pntA by semi-quantitative RT-PCR. Relative to the transcription level in wild-type E. coli during growth on glucose. Gray, white, and black bars indicate batch cultures grown on glucose, acetate, or glycerol, respectively, as the sole carbon source.

Because both transhydrogenases have apparently divergent physiological functions in redox metabolism, we wondered whether their expression was affected by the redox state of metabolism. Using the house-keeping rpoD gene as an internal standard, we determined mRNA levels of udha and pntA by semi-quantitative RT-PCR. Relative to the transcription level during growth on glucose, significantly reduced transcription was seen for udha on glycerol (Fig. 7A) and for pntA on acetate (Fig. 7B). In the Pgi mutant, udha transcription was unaffected, but pntA transcription was significantly reduced (Fig. 7). The opposite pattern was apparent in the Zwf-EDP mutant, with significantly reduced transcription of udha and moderately increased transcription of pntA (Fig. 7). This increase in pntA transcription was consistent with the reported doubling of in vitro transhydrogenase activity in a Zwf mutant (16).

Overall, these expression patterns support the notion that expression of the soluble transhydrogenase-encoding udha gene is down-regulated in metabolic situations that require a higher formation of NADPH than wild-type growth on glucose, i.e. growth on glycerol or growth of the Zwf-EDP mutant on glucose. Analogously, transcription of pntA was down-regulated in situations with increased NADPH availability, i.e. growth on acetate or growth of the Pgi mutant on glucose.

**DISCUSSION**

In contrast to the common belief that the PP pathway and isocitrate dehydrogenase are the major sources of NADPH (4, 7), we show here that transhydrogenases play important roles in NADPH metabolism. Moreover, the results presented here demonstrate divergent physiological functions of the two transhydrogenase isoforms in E. coli that effectively decouple catabolic NADPH production from anabolic NADPH consumption. While the membrane-bound transhydrogenase PntAB catalyzes the energy-dependent transfer of reducing power from NADH to NADP⁺, the soluble transhydrogenase Udha catalyzes the reverse, energy-independent reaction. In standard aerobic batch culture, glucose catabolism generated less NADPH than was required for biosynthesis. Under these conditions, the membrane-bound transhydrogenase was a major pathway of NADPH formation that contributed about 35–45% of the total anabolic demand for NADPH, which goes well beyond the previously suggested specific role of the membrane-bound isocitrate in ammonia assimilation of E. coli (17). This PntAB-catalyzed NADPH formation was apparently not essential for batch growth on glucose, because PntAB mutants grew, albeit slightly slower, on this substrate. The loss of PntAB-catalyzed NADPH formation was compensated for primarily through increased PP pathway fluxes.

Although transcribed and present during growth on glucose, the soluble transhydrogenase had no apparent function during batch growth on this substrate, but the present data do not exclude simultaneous operation of both isoforms in a proton gradient-dissipating futile cycle. Under metabolic conditions that led to excess NADPH formation, however, the soluble transhydrogenase Udha was essential for growth. Such conditions were growth on acetate or in phosphoglucose isomerase mutants that catabolized glucose almost exclusively through the PP pathway, thereby producing a vast excess of NADPH, as was also described for S. cerevisiae (20). Another metabolic condition with excess NADPH formation in E. coli is slow growth in glucose-limited chemostat cultures, because fully respiratory growth with high TCA cycle fluxes of about 80% of the specific glucose uptake rate in these cultures produced much NADPH in the isocitrate dehydrogenase reaction (27, 45). Batch cultures with extensive acetate overflow metabolism, in contrast, exhibit severalfold lower relative TCA cycle fluxes and generate much less NADPH in the isocitrate dehydrogenase reaction.

In such slow growing, glucose-limited chemostat cultures, the soluble transhydrogenase may also function as a valve to reoxidize excess NADPH, but extensive NADPH-formation can alternatively be avoided by a flux rerouting from the NADPH-producing TCA cycle to the recently described PEP-glyoxylate cycle in E. coli that does not produce NADPH (37). Although the PEP-glyoxylate cycle was also active in glucose batch cultures of the Pgi mutant (Fig. 6; Ref. 37), the lower NADPH formation does not suffice to counterbalance the extensive NADPH formation, because Udha was essential in this mutant. Although the redox-balancing function of the soluble transhydrogenase and the PEP-glyoxylate cycle seem to be partially overlapping, the Pgi mutant data indicate that the cycle is more relevant for fine-tuning of NADPH metabolism but that conditions with extensive overproduction of NADPH require Udha.
Generally, the observed expression pattern of the udhA and pntA genes indicates transcriptional regulation that responds to the redox state of cellular metabolism. Concomitant with the dramatic excess NADPH formation in the Pgi mutant on glucose and probably in wild type on acetate, we observed reduced pntA transcription. Analogously, down-regulation of udhA transcription occurred when NADPH formation was reduced in the wild type on glycerol. The fact that pntA and udhA expression responds conversely to the availability of NADPH upon genetic or environmental changes suggests an NADP-dependent repression mechanism. It has not escaped our notice that udhA transcription and in vitro activity of the soluble transhydrogenase in glucose-grown E. coli requires an additional level of regulation to prevent equilibration of the more reduced NAD(P)H pool with the more oxidized NADH pool (10). This metabolic regulation of the soluble transhydrogenase may be the strong allosteric activation of this enzyme by NADPH and inactivation by NADP+ that was described for the Pseudomonas aeruginosa homologue (49).

Because NADPH metabolism is a general network property (defined as a biological property that emerges from the interaction of network components; Ref. 50) that connects the fundamental metabolic processes of catabolism and anabolism, the results presented here are also important for faithful modeling of E. coli metabolism (1, 51–53), in particular for the generation of a whole-cell model (54). More generally, the apparent flexibility of E. coli NADPH metabolism provokes a question: why are the Enterobacteriaceae the only known group that contains both transhydrogenase isoforms? Because many species are seemingly capable of reoxidizing excess NADPH in the absence of UdhA homologues (21–25), alternative mechanisms for management of NADPH metabolism must exist.

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