Physical and Genetic Interactions of Cytosolic Malate Dehydrogenase with Other Gluconeogenic Enzymes*

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Natalie Gibson and Lee McAlister-Henn‡

From the Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas 78229-3900

A truncated form (∆nMDH2) of yeast cytosolic malate dehydrogenase (MDH2) lacking 12 residues on the amino terminus was found to be inadequate for gluconeogenic function in vivo because the mutant enzyme fails to restore growth of a ∆mdh2 strain on minimal medium with ethanol or acetate as the carbon source. The ∆nMDH2 enzyme was also previously found to be refractory to the rapid glucose-induced inactivation and degradation observed for authentic MDH2. In contrast, kinetic properties measured for purified forms of MDH2 and ∆nMDH2 enzymes are very similar. Yeast two-hybrid assays indicate weak interactions between MDH2 and yeast phosphoenolpyruvate carboxykinase (PCK1) and between MDH2 and fructose-1,6-bisphosphatase (FBP1). These interactions are not observed for ∆nMDH2, suggesting that differences in cellular function between authentic and truncated forms of MDH2 may be related to their ability to interact with other gluconeogenic enzymes. Additional evidence was obtained for interaction of MDH2 with PCK1 using Hummel-Dreyer gel filtration chromatography, and for interactions of MDH2 with PCK1 and with FBP1 using surface plasmon resonance. Experiments with the latter technique demonstrated a much lower affinity for interaction of ∆nMDH2 with PCK1 and no interaction between ∆nMDH2 and FBP1. These results suggest that the interactions of MDH2 with other gluconeogenic enzymes are dependent on the amino terminus of the enzyme, and that these interactions are important for gluconeogenic function in vivo.

Three differentially compartmentalized isozymes of malate dehydrogenase (MDH)1 in Saccharomyces cerevisiae catalyze the NAD(H)-specific interconversion of malate and oxaloacetate. Mitochondrial MDH1 catalyzes a reaction of the tricarboxylic acid cycle, and disruption of the corresponding gene results in an inability to grow with acetate as a carbon source (1, 2), a phenotype shared with yeast mutants containing disruptions in genes encoding other tricarboxylic acid cycle enzymes (3–5). Cytosolic MDH2 is a gluconeogenic enzyme and is required for growth on minimal medium with ethanol or acetate as the carbon source (6), as are the other yeast gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PCK1) (7) and fructose-1,6-bisphosphatase (FBP1) (8). Peroxosomal MDH3 is proposed to catalyze a step in the glyoxylate pathway, which permits formation of C4 metabolites from C2 precursors (9, 10), and has also been shown to be necessary for growth with oleate as the carbon source (11), suggesting an additional role in providing NADH for peroxisomal β-oxidation.

The yeast MDH isozymes are all homodimers with similar subunit $M_r$ values ($33,500$ for MDH1; $40,700$ for MDH2; and $37,200$ for MDH3), and they share primary sequence identities of 43 to 50%. Distinct differences among the enzymes include a 17-residue mitochondrial targeting sequence for MDH1 that is removed upon import (2), and a carboxyl-terminal tripeptide targeting sequence (Ser-Lys-Leu) required for peroxisomal import of MDH3 (11). In addition, MDH2 has a 12-residue extension on the amino terminus that is not present on mature MDH1 or on MDH3 (Fig. 1). We have previously shown that removal of this 12-residue extension, generating a truncated enzyme designated ∆nMDH2, appears to have no effect on cellular levels of activity of the enzyme (12). However, the ∆nMDH2 enzyme is resistant to catabolite inactivation, a regulatory phenomenon in yeast involving glucose-induced inactivation and degradation of gluconeogenic and glyoxylate cycle enzymes (13, 14). In addition, when glucose is added to a culture of a strain expressing ∆nMDH2, adaptation to utilization of glucose as a carbon source is slowed (12), suggesting that catabolite inactivation may provide a physiological advantage by rapidly removing enzymes that interfere with optimal rates of glycolysis. In this report, we also provide evidence that the catalytically active ∆nMDH2 enzyme may not function efficiently in gluconeogenesis in vivo.

Because of the unfavorable equilibrium for formation of oxaloacetate from malate ($\Delta G^\circ \approx +7 \text{ kcal/mol}$), it has been proposed that physical interactions between malate dehydrogenase and the next enzyme in the same metabolic pathway may be necessary to ensure direct transfer of oxaloacetate (15), a metabolite present in very low cellular concentrations. In support of this proposal, substantial experimental evidence has been presented for interactions between the mitochondrial tri-carboxylic acid cycle enzymes, malate dehydrogenase and citrate synthase, in yeast and other organisms (16–19). In the current study, we present evidence for physical interactions of MDH2 with PCK1 and FBP1. These results suggest an association of these cytosolic enzymes in vivo that may facilitate metabolic flux through gluconeogenesis.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—Gene disruption mutants were constructed using the parental yeast strain S17-3B (MATa, leu2-3,112, his3-1, ura3-1, trp1-901, his3-1101) (20). Disruption of the MDH2 gene (MDH2::HIS3) was previously described (2). PCK1 and FBP1 genes were independently disrupted by transformation with PCR products containing ~45 nucleotides from the 5’ and 3’ ends of each coding region fused with the kanMX4 coding region (21). Gene disruptions...
Clontech. The time required for colony color development was monitored (26). Transformants were restreaked for colony filter assays of plasmids (25) containing PCK1 plasmid carrying PCK1 as illustrated in Fig. 4. Control strain was obtained by transformation with plasmid pCL1, a growth on YNB plates lacking tryptophan and/or leucine. A positive pAS/pACT combinations into Y190, and transformants were selected by ligation into plasmid generate the 5′ ends of plasmids were verified by DNA sequence analysis.

The resulting reverse two-hybrid vectors permit in-frame cloning of coding region flanked by EcoRI restriction sites for subcloning. Similar vectors (pASFP1 and pACTFP1 containing FP1 sequences were generated using PCR with yeast genomic DNA as the template to generate the FP1 coding region with 5′ and 3′ XhoI restriction sites for ligation into plasmid SacI or XhoI sites. Coding sequences in two-hybrid plasmids were verified by DNA sequence analysis.

The two-hybrid plasmids were transformed singly and in pairwise as described in protocols provided by Clontech, respectively, to generate the PKC1 coding region flanked by EcoRI restriction sites for subcloning. Similar vectors (pASFP1 and pACTFP1 containing FP1 sequences were generated using PCR with yeast genomic DNA as the template to generate the FP1 coding region with 5′ and 3′ XhoI restriction sites for ligation into plasmid SacI or XhoI sites. Coding sequences in two-hybrid plasmids were verified by DNA sequence analysis.

Affinity Purification and Kinetic Analyses—To insert tags for affinity purification, MDH2 and ΔMDH2 genes on a YEp352 plasmid, and the PKC1 gene on a YEp352 plasmid, were used for oligonucleotide-directed mutagenesis to add codons for six histidine residues between the 5′ and the stop codon of each gene. Each plasmid contained ~500 bp of the authentic 5′ promoter sequence for expression of each gene. A 2.0-kb DNA fragment containing the FP1 coding region and adjacent promoter sequences was cloned using PCR from yeast genomic DNA and inserted into pRS426. Oligonucleotide-directed mutagenesis was subsequently used to insert nine codons for the residues in a streptavidin tag (28) into the 5′ ends of PCK1 and FP1 genes. Each plasmid was transformed into the yeast strain containing a disruption in the corresponding MDH2, PCK1, or FP1 chromosomal locus.

For affinity purification, transformants were grown in 10-ml cultures of YNB glucose medium to maintain plasmid selection, then transferred to a glucose-limited medium to support expression of gluconeogenic enzymes (6). The cells were harvested at A600nm = 4–5, and cell pellets were stored at ~20°C. For purification of histidine-tagged enzymes, the cell pellets were lysed with glass beads, and the lysate was diluted 1:1 with a buffer containing 100 mM sodium phosphate (pH 8.0), 300 mM sodium chloride, and 20 mM imidazole. Bound protein was eluted with 1.0 ml of the same buffer containing 250 mM sodium chloride. For purification of tagged enzymes, 200 μl of streptavidin was added to the cell lysates, and the lysates were loaded onto 1.0-ml streptavidin agarose columns (immunopure affinity Pak immobilized streptavidin, Pierce). The columns were washed with 5 ml of phosphate buffer (100 mM sodium phosphate, 50 mM sodium chloride, and 1 mM phenylmethylsulfonyl fluoride). We found that the streptavidin-tagged enzymes bind weakly to these columns and are eluted with a second 5-ml phosphate buffer wash. Yields for the affinity purified enzymes were ~0.5 mg of histidine-tagged enzymes and 0.1–0.2 mg of streptavidin-tagged enzymes per 1.2–1.7 g of cells harvested from 250-ml cultures. Protein concentrations were determined using absorbance at A280 nm and calculated extinction coefficients (29). Samples containing affinity purified enzymes were run on 10% polyacrylamide/sodium dodecyl sulfate gels for Coomassie Blue staining or for immunoblotting.

Enzyme activities for malate dehydrogenase (1), phosphoenolpyruvate carboxykinase (30), and fructose-1,6-bisphosphatase (31) were measured as previously described. Activities measured for affinity purified enzymes were 200–250 units/mg for MDH2His and ΔMDH2His ~8 units/mg for PKC1His and PKC1His, and ~10 units/mg for FP1His. These values are 2-3-fold lower than those reported for conventionally purified MDH2 (6), PCK1 (30), and FP1 (31).

For determination of kinetic parameters, 0.15 μg of MDH2His or ΔMDH2His were used in 1.0-ml assays containing 50 mM potassium phosphate (pH 7.5). NADH was used as the electron donor. 25 mM in the presence of 0.35 mM oxaloacetate, or oxaloacetate concentrations were varied from 0 to 1.0 mM in the presence of 0.4 mM NADH. Units are defined as micromole of NAD+ produced per minute.

Immunoblotting and Co-immunoprecipitation—Immunoblot analysis of MDH2 or ΔMDH2 was conducted using a polyclonal antisera prepared against ΔMDH2 (6). The antisem was preadsorbed with extracts from a Δndh2 strain and used at dilutions of 1:250. A polyclonal antisem against FPB1 was obtained from Dr. K. Eschrich (32) and was used at dilutions of 1:500. Histidine-tagged enzymes were also detected using a monoclonal penta-histidine antisem obtained from Qiagen. Immunocomplexes were detected with the enhanced chemiluminescence method (Amersham Biosciences).

For co-immunoprecipitation experiments, cells were harvested from 1-10 ml of cultures, and cell pellets were lysed with glass beads, then resuspended and boiled in 50 mM of SDS electrophoresis sample buffer. After centrifugation to remove the beads, the supernatants were used for gel electrophoresis and immunoblot analysis.
**Hummel-Dreyer Analyses**—Gel filtration chromatography was conducted on a Superdex 200 HR 10/30 column (24 ml bed volume) attached to an Applied Biosystems HPLC model 120A. The chromatography buffer contained 50 mM sodium phosphate (pH 7.4) and 50 mM sodium chloride. Samples and standard proteins (at concentrations of 4 μM for calibration and otherwise as indicated in the text) were injected with a 50-μl loop and chromatographed at a flow rate of 0.25 ml/min. For some experiments, 4.0 μM MDH2 was included in the chromatography buffer.

**Surface Plasmon Resonance Analyses**—Experiments were performed on a Biacore 2000 surface plasmon resonance (SPR) instrument using steptavidin and carboxymethyl dextran (CM5) research grade chips (Biacore, Piscataway, NJ). Runs were conducted at 25 °C with HBS buffer (10 mM HEPES, pH 7.0, 150 mM sodium chloride, 3 mM EDTA, and 0.005% surfactant P20) at a flow rate of 5 μl/min. Proteins were immobilized on steptavidin chips by injection in 50 mM HEPES (pH 8.0) containing 300 mM sodium chloride. CM5 chips were activated by a 6-min injection of 50% N-hydroxysuccinimide and 50% Ν-ethyl-N’-(dimethylaminopropyl)carbodiimide. Proteins (ligands) were immobilized on activated CM5 chips at 1000–3500 response units (RU; 1 RU = a change of ~1 ng/mm² in surface protein concentration) at pH 7.5. After immobilization, the surfaces were blocked by a 6-min injection of 1.0 mM ethanolamine (pH 8.5). Soluble analyte proteins or bovine serum albumin as the control were passed over the surfaces at the indicated concentrations for 3 min. The surfaces were regenerated between binding runs by a 3-min injection of 1 mM sodium chloride or 4 mM guanidine HCl.

**RESULTS**

**Differential Function of MDH2 and ΔMDH2 in Vivo**—Removal of the amino-terminal 12-residue extension of yeast MDH2 was previously shown to have little effect on activity of the enzyme, because levels of malate dehydrogenase activity and immunodetectable MDH2 were similar in extracts from cells expressing the authentic or truncated enzyme (ΔMDH2) (12). However, unlike authentic MDH2, ΔMDH2 was found to be resistant to glucose-induced degradation, as were the activities of fructose-1,6-bisphosphatase and isocitrate lyase in cells expressing ΔMDH2. The latter phenotype suggested a dominant effect on enzymes normally subject to catabolite inactivation (13, 14). To further investigate potential growth phenotypes that might be associated with a general effect on gluconeogenesis, we constructed yeast mutants with disruptions in MDH2, FBP1, or PCK1 genes encoding the major gluconeogenic enzymes. As previously reported (6–8), these mutant strains were found to grow well on plates containing rich medium with C₆ carbon sources (ethanol or acetate), but the strains fail to grow on plates of minimal medium with the same carbon sources. This phenotype was attributed to the requirement for gluconeogenesis for growth in minimal medium that lacks carbon compounds sufficiently abundant in rich medium to allow for growth of these strains. (Note, no growth phenotypes are observed with glucose media because gluconeogenic enzymes are not expressed or functional under this growth condition.)

We additionally found that transformation of the Δmdh2 strain with a centromere-based plasmid containing the MDH2 gene restores the ability to grow on minimal medium plates with ethanol or acetate as carbon sources. In contrast, transformation of the same strain with a similar plasmid bearing the ΔMDH2 gene fails to restore growth under these conditions. These results were extended by measuring culture doubling times in minimal medium. When shifted from minimal glucose to minimal ethanol medium, the parental strain and the Δmdh2 strain expressing MDH2 exhibit a lag of ~24 h before attaining doubling times of 5.9 and 6.9 h, respectively (Table I). In contrast, all three gluconeogenic gene disruption strains and the Δmdh2 strain expressing ΔnMDH2 fail to grow under these conditions. Similar results were obtained when strains and transformants were shifted to minimal medium with acetate as the carbon source (Table I), although doubling times for the parental strain and for the Δmdh2 strain expressing MDH2 were significantly longer on this medium. These results suggest that ΔMDH2 does not compensate for the normal gluconeogenic function of MDH2.

Similar levels of malate dehydrogenase activity (7–8 units/mg of protein) were measurable with cellular extracts from the Δmdh2 strains expressing MDH2 or ΔMDH2. However, because of expression of other MDH isoforms in those strains, immunoblot analysis was conducted to specifically examine levels of expression of MDH2 and ΔMDH2. As illustrated in Fig. 2, this analysis indicates no significant difference between levels of MDH2 (lane 2) and levels of ΔMDH2 (lane 3) in extracts from respective transformants. Levels of the plasmid-expressed enzymes exceed that of chromosomally expressed MDH2 in extracts from the parental strain (lane 1) by 2–3-fold, and MDH2 is clearly absent in the Δmdh2 strain (lane 4). Fig. 2 also illustrates the differences in electrophoretic mobilities of authentic MDH2 (lanes 1 and 2) relative to ΔMDH2 (lane 3) and relative to purified MDH2 carrying a histidine tag (lane 5), as described below.

To examine activity and kinetic properties of the MDH2 and ΔMDH2 enzymes in more detail, we constructed plasmids for production of carboxyl-terminal histidine-tagged versions of both enzymes. The enzymes were affinity purified with similar yields using Ni²⁺-NTA chromatography. Basic kinetic properties of the purified enzymes were compared using standard assay conditions. In assays with increasing concentrations (0–300 μM) of oxaloacetate, both enzymes exhibit hyperbolic kinetic curves, as illustrated in Fig. 3A. Higher concentrations of oxaloacetate produce an inhibition of activity (inset in Fig. 3A), resulting in velocities lower than the apparent Vmax as also reported by others (33). Using data from the hyperbolic regions of the curves, similar apparent Vmax and Km values for oxaloacetate were obtained for MDH2 and ΔMDH2 (Table II). With NADH as the varied assay component, the wild-type and mutant enzymes exhibit similar kinetic curves (Fig. 3B), and both enzymes are inhibited at high concentrations of NADH (inset in Fig. 3B). On average, the ΔMDH2 enzyme exhibits slightly higher apparent Km and Vmax values with NADH than the MDH2 enzyme (Table II). The kinetic parameters obtained for the affinity purified wild-type enzyme are similar to those previously reported for the conventionally purified enzyme (9), indicating that the histidine tag has little effect on catalytic properties of the enzyme.

We also examined kinetic parameters for the reverse reaction as would be required for function in gluconeogenesis. With NAD⁺ as the variable assay component (0–1.0 mM) and a malate concentration of 50 mM, MDH2 and ΔMDH2 enzymes exhibit similar apparent Vmax values (460 and 490 units/mg, respectively) and Km values for NAD⁺ (83 and 120 μM, respectively). For malate (concentrations varied from 0 to 200 μM with an NAD⁺ concentration of 1.0 mM), similar apparent Km values were obtained (59 μM for MDH2 and 55 μM for

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### Table I

| Relevant genotype | Carbon source | Ethanol | Acetate |
|------------------|---------------|---------|---------|
| Parental         |               | 5.9     | 19.0    |
| ΔMDH2            | NG³          | NG      | NG      |
| ΔPKC1            | NG           | NG      | NG      |
| ΔFBP1            | NG           | NG      | NG      |
| ΔMDH2 + pRS314MDH2 | 6.9      | 26.4    |         |
| ΔMDH2 + pRS314ΔnMDH2 | NG   | NG      |         |

*²% ethanol or 2% acetate were used as carbon sources with minimal YNB medium.

*³ NG, no growth over a 60-h period.

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inherent catalytic differences or to differences in steady state levels of expression in ethanol-grown cultures.

Two-hybrid Analyses of MDH2 and ΔnMDH2—Among possible explanations for differential function of the authentic and truncated forms of MDH2 in vivo despite their kinetic similarities are differences in interactions with other cellular components. Because of the dominant effect of MDH2 truncation on apparent glucose-induced turnover of other gluconeogenic enzymes (12), and the inability of the ΔnMDH2 enzyme to support gluconeogenesis as described above, we hypothesized that co-localization and/or interactions among gluconeogenic enzymes in the cytosol might facilitate pathway function and/or regulated turnover, and that the amino-terminal extension of MDH2 might be essential for these processes. To investigate these possibilities, we initially tested possible interactions among gluconeogenic enzymes using the yeast two-hybrid system because this provides a sensitive in vivo assay.

For two-hybrid assays, MDH2 and ΔnMDH2 were expressed as fusion proteins with DNA-binding and transcriptional activation domains of GAL4 using conventional two-hybrid plasmids. These constructs result in GAL4 domains fused to the amino termini of MDH2 and ΔnMDH2 enzymes. Assays used to calculate kinetic parameters were conducted with oxaloacetate concentrations from 0 to 300 μM. Assays conducted with oxaloacetate concentrations ranging from 0 to 1 mM (inset) show inhibition of activity with substrate concentrations above 300 μM. B, NADH-dependent activities of MDH2 and ΔnMDH2 enzymes. Assays used to calculate kinetic parameters were conducted with NADH concentrations from 0 to 200 μM. Assays conducted with concentrations of NADH to 300 μM (inset) illustrate inhibition of activity at higher concentrations of NADH.

ΔnMDH2), and the mutant enzyme exhibits a higher V_{max} value (130 units/mg) than the wild-type enzyme (44 units/mg). Collectively, these data suggest that the authentic and truncated MDH2 enzymes are kinetically quite similar, and that any differences in function in vivo cannot be attributed to
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moderately high, and (c) that addition of the GAL4 domains onto the amino termini may slightly retard homodimeric interactions.

To test possible heteromeric interactions of MDH2, we constructed conventional two-hybrid plasmids containing PK1 and FBP1 coding regions fused to the 3' end of GAL4 domain sequences. These plasmids were co-transformed in all possible pairwise combinations with the conventional and reverse two-hybrid plasmids containing MDH2 and ΔMDH2 gene fusions. As illustrated in Table IV, most of the assays were negative. However, positive results were observed with two pairs of constructs. Both contain the GAL4 DNA-binding domain fused to the amino terminus of MDH2, which appears to interact with the GAL4 transcription-activation domain fused to either PK1 or FBP1. With both types of assays, the levels of β-galactosidase activity in these transformants are lower than those detected in a transformant expressing full-length GAL4 or the ΔMDH2 homodimers (Table III), but they are substantially higher than background levels detected with negative controls obtained with pairs of empty two-hybrid plasmids or with pairs expressing only one fusion protein. The MDH2/FPB1 pair exhibits lower levels of β-galactosidase activity in enzyme assays than does the MDH2/PCK1 pair. We also tested and found no evidence with this method for an interaction between PCK1 and FBP1 (data not shown).

Because putative interactions for MDH2 with PCK1 or with FBP1 can be detected only when MDH2 is fused to the carboxyl terminus of the GAL4 DNA-binding domain (Table IV, section A), this suggests that the GAL4 transcription-activation domain in this position may block the interactions. With similar conventional two-hybrid constructs, no interactions are detectable for ΔMDH2 with PK1 or FBP1 (Table IV, section B), suggesting that the amino-terminal extension of authentic MDH2 is essential for such interactions. Neither MDH2 nor ΔMDH2 in reverse two-hybrid fusion proteins exhibits interactions with PK1 or FBP1 in these assays (Table IV, sections C and D), suggesting for MDH2 that relatively large GAL4 domains fused to the carboxyl terminus may block interactions observed when the GAL4 DNA-binding domain is fused to the amino terminus of MDH2. The latter suggestion seems reasonable in light of the proximity of amino and carboxyl termini in three-dimensional structures of related malate dehydrogenases (35, 36).

In summary, these data from two-hybrid analyses suggest that free amino termini enhance homodimer formation for both MDH2 and ΔMDH2. The apparent interactions between MDH2 and PK1 or FBP1 are observed only in certain pairs of possible domain combinations and orientations. These interactions appear to require the amino-terminal 12-residue extension of MDH2.

Physical Assays for Protein Interactions Involving MDH2—

To initiate physical approaches to examine possible interactions among yeast gluconeogenic enzymes, we generated constructs to permit affinity purification of PCK1 and FBP1. Plasmids were constructed for expression in yeast of PK1 carrying a carboxyl-terminal penta-histidine tag, and for expression of PK1 and FBP1 carrying nine-residue streptavidin tags on their carboxyl termini. Electrophoretic analyses of fractions...
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obtained during affinity purification of MDH2His, FBP1Strep, PCK1His, and PCK1Strep are illustrated in Fig. 5 (panels A–D, respectively). We found that the histidine-tagged enzymes are efficiently purified with good yields, whereas the steptavidin-tagged enzymes are purified with significantly lower yields. All purified enzymes are active in standard enzyme assays, confirming retention of the native states during purification.

Our first test for physical associations among the gluconeogenic enzymes was to assess the possibility of co-purification. As shown in Fig. 5, denaturing electrophoretic analysis of each of the affinity purified enzymes produced no evidence for co-purification of significant amounts of other gluconeogenic enzymes. For more sensitive assays, we assayed wash and elution fractions from each affinity column for activity of the other enzymes. The gel filtration column was then equilibrated with buffer containing 4.0 mM MDH2His and loaded with a sample containing 1.0 μM PCK1His plus MDH2His at concentrations ranging from 0 to 14 μM. The size of the PCK1 peak eluting in each column run increased with increasing concentrations of MDH2 in the sample. The increases in the size of the PCK1 peak were attributed to binding of MDH2 by PCK1 and were used to calculate the concentration of bound MDH2 (Fig. 6). The slope of the plot

**Fig. 5.** Affinity purification of yeast gluconeogenic enzymes. Fractions collected during affinity purification of MDH2His (A), FBP1Strep (B), PCK1His (C), and PCK1Strep (D) were used for electrophoresis and Coomassie Blue staining. Lanes 1 were loaded with protein molecular weight standards with M, values as shown. Lanes 2 in panels B–D were loaded with 15–20 μl of whole cell extracts prepared from transformants. Lane 2 in panel A and lanes 3 in panels B–D were loaded with 15–20 μl column flow-through fractions. Lanes 3–5 in panel A, lanes 4–6 in panel B, lanes 3–5 in panel C, and lanes 4–5 in panel D were loaded with 15–20 μl of sequential wash fractions. Lanes 6 and 7 in panels A and C were loaded with 10 and 20 μg, respectively, of purified MDH2His and PCK1His eluted from the Ni²⁺-NTA columns. Wash fractions containing purified FBP1Strep and PCK1Strep were precipitated using 10% trichloroacetic acid and aliquots used for electrophoresis in lanes 7 and 8 of panel B and lane 6 of panel D, respectively. Arrows indicate fractions used in subsequent analyses.

With histidine-tagged forms of MDH2 and PCK1, it was possible to purify sufficient quantities of both enzymes to apply the classical Hummel-Dreyer chromatography method for binding analyses (37–39). For this, we developed a gel filtration system for resolution of MDH2 and PCK1 and determined their elution patterns relative to a set of standard proteins (inset, Fig. 6). MDH2 eluted with a calculated Mr of 71,000 and PCK1 eluted with a calculated Mr of 212,000, values, respectively, consistent with the native dimeric and tetrameric forms of these enzymes. The gel filtration column was then equilibrated with buffer containing 4.0 μM MDH2His and loaded with a sample containing 1.0 μM PCK1His. The elution profile showed, in addition to the peak for PCK1His, a trough in the basal absorbance (data not shown), indicating binding of MDH2His in the buffer to PCK1His (39). Similar chromatography runs were conducted with the same buffer and samples containing 1.0 μM PCK1His plus MDH2His at concentrations ranging from 0 to 14 μM. The size of the PCK1 peak eluting in each column run increased with increasing concentrations of MDH2 in the sample. The increases in the size of the PCK1 peak were attributed to binding of MDH2 by PCK1 and were used to calculate the concentration of bound MDH2 (Fig. 6). The slope of the plot

Co-immunoprecipitation techniques were also tested as described under “Experimental Procedures” using polyclonal antisera against MDH2 and FBP1. We found some evidence for co-immunoprecipitation of MDH2 with the anti-FBP1 antiserum from extracts of the parental strain, and MDH2 was not precipitated with the same antiserum when extracts were prepared from the ΔFBP1 strain (data not shown). However, these results were difficult to routinely reproduce, and the converse co-immunoprecipitation of FBP1 from parental cell extracts with anti-MDH2 serum was not detected. We therefore focused on using the affinity purified enzymes for other physical assays for protein interactions.
Fig. 6. Hummel-Dreyer analysis of MDH2 binding to PCK1. Gel filtration chromatography was conducted as described under “Experimental Procedures,” and elution patterns were determined for MDH2His and PCK1His relative to those for indicated protein standards (inset). The size of the PCK1His peak was used to calculate the concentration of MDH2His bound following chromatography of samples containing 1.0 μM PCK1His and increasing concentrations of MDH2His on a column equilibrated with a buffer containing 4.0 mM HEPES, 0.76 mM NaCl, and 7.69 mM KCl. The size of the PCK1His peak was used to calculate the concentration of MDH2 bound following chromatography of samples containing 1.0 μM PCK1His and increasing concentrations of MDH2His on a column equilibrated with a buffer containing 4.0 mM HEPES, 0.76 mM NaCl, and 7.69 mM KCl. The size of the PCK1His peak was used to calculate the concentration of MDH2 bound following chromatography of samples containing 1.0 μM PCK1His and increasing concentrations of MDH2His on a column equilibrated with a buffer containing 4.0 mM HEPES, 0.76 mM NaCl, and 7.69 mM KCl. The size of the PCK1His peak was used to calculate the concentration of MDH2 bound following chromatography of samples containing 1.0 μM PCK1His and increasing concentrations of MDH2His on a column equilibrated with a buffer containing 4.0 mM HEPES, 0.76 mM NaCl, and 7.69 mM KCl. The size of the PCK1His peak was used to calculate the concentration of MDH2 bound following chromatography of samples containing 1.0 μM PCK1His and increasing concentrations of MDH2His on a column equilibrated with a buffer containing 4.0 mM HEPES, 0.76 mM NaCl, and 7.69 mM KCl.

indicates a binding to PCK1 of −0.14 μmol of MDH2/μmol of total MDH2 within these concentration ranges. Because of limitations with absorbance measurements, it was not possible to achieve saturation concentrations. However, an interaction between these two gluconeogenic enzymes is clearly measurable using this technique.

We also tested a second sensitive method for detection of protein-protein interactions, SPR, which requires relatively small amounts of purified proteins. The affinity tags of PCK1Strep and FBP1Strep were used initially to couple the proteins (ligands) to the surfaces of strepavidin chips. MDH2His as the soluble analyte was found to bind to both chips and not to a control chip carrying biotinylated bovine serum albumin. However, the kinetics of binding were slow, precluding accurate measurements of saturation. Therefore, PCK1Strep and FBP1Strep were covalently immobilized on carboxymethyl dextran (CM5) chips, which significantly increased the kinetic response to MDH2 binding.

The binding of MDH2His and ΔnMDH2His to CM5 chips carrying PCK1Strep was measured at equilibrium using a range of concentrations of each analyte protein. The binding of MDH2 by PCK1 was found to be dose-dependent and saturable (● in Fig. 7A), whereas no binding of bovine serum albumin by PCK1 was observed in parallel experiments (data not shown). A calculated maximum of 10,590 RU of MDH2 could be bound to a chip loaded with 1,560 RU of PCK1. Saturation was achieved with ≥8 μM MDH2His as ligand. Binding to PCK1 was also observed using ΔnMDH2His as the analyte (○ in Fig. 7A). Whereas the calculated maximum binding (10,640 RU of ΔnMDH2His by a chip loaded with 996 RU of PCK1) was similar to that observed with the authentic enzyme, the saturation curve suggests a significantly lower affinity. These binding data were used for Scatchard plot analysis (Fig. 7B) to calculate $K_r$ values of 0.63 μM for the MDH2/PCK1 interaction and of 7.69 μM for the ΔnMDH2/PCK1 interaction. Thus, affinity of PCK1 for MDH2 is dramatically reduced by amino-terminal truncation. The n value obtained from the Scatchard plot, corrected for relative $M_e$ values for the holoenzymes (81,400 for dimeric MDH2 and 243,920 for tetrameric PCK1), gives a maximum of 20 mol of authentic MDH2 bound per mol of PCK1.

Similarly, binding of MDH2His and ΔnMDH2His to CM5 chips carrying covalently bound FBP1Strep was measured at equilibrium for a range of concentrations of each analyte protein. The binding of MDH2 by FBP1 was also found to be dose-dependent and saturable (Fig. 8A), whereas no binding of bovine serum albumin by FBP1 was observed in parallel experiments (data not shown). A maximum of 10,410 RU of MDH2 could be bound to a chip loaded with 8,710 RU of FBP1. Saturation was achieved with ~8 μM MDH2. In contrast, we found that concentrations as high as 20 μM ΔnMDH2His produced no detectable binding to chips loaded with equivalent RU of FBP1. Thus, truncation of MDH2 appears to eliminate measurable interaction with FBP1. Scatchard analysis of the binding data for authentic MDH2 to immobilized FBP1 (Fig. 8B) was used to calculate a $K_r$ value of 0.58 μM and an n value of 0.56 mol of dimeric MDH2 bound per mol of monomeric FBP1 ($M_e$ = 38,260). The latter value thus implies an equimolar association of MDH2 and FBP1 polypeptides.

Collectively, these SPR results provide further evidence for physical interactions among yeast gluconeogenic enzymes. PCK1 and FBP1 exhibit similar affinities but different holoenzyme stoichiometries for binding MDH2. The affinity of PCK1 for ΔnMDH2 appears to be substantially lower than that for...
MDH2, and no interaction between FBP1 and ΔnMDH2 was detectable. Thus, these results from SPR experiments are generally consistent with those obtained with yeast two-hybrid assays using the authentic and truncated forms of MDH2.

**DISCUSSION**

Cytosolic malate dehydrogenase (MDH2) performs an essential function in gluconeogenesis in yeast, because this enzyme is required for assimilation of C₆ carbon sources such as ethanol or acetate. We have found that a truncated form of this enzyme (ΔnMDH2) lacking the first 12 amino acids fails to provide this function, despite retention of kinetic properties that are essentially indistinguishable from those of the authentic enzyme. Previous studies also indicated that the ΔnMDH2 enzyme is refractory to catabolite inactivation (12), which normally results in rapid elimination of MDH2 and other gluconeogenic enzymes when glucose is added to a yeast culture growing on non-fermentable carbon sources (13, 14). In addition, expression of ΔnMDH2 appears to have a dominant inhibitory effect on catabolite inactivation of other cellular enzymes (12). These observations led us to hypothesize that some attribute of MDH2, in addition to catalytic activity, is important for normal cellular function, and that this attribute is dependent on the amino-terminal region of the protein. We therefore investigated the possibility that MDH2 may normally interact with the other gluconeogenic enzymes, and that these interactions might be dependent on the presence of the normal amino terminus of the protein. In support of this hypothesis, we have detected interactions of MDH2 with PCK1 and FBP1 using three distinct experimental techniques. Our data also suggest that these interactions are substantially reduced by truncation of the amino terminus of MDH2.

Positive results from yeast two-hybrid assays are potentially the most convincing evidence for interactions of MDH2 (but not of ΔnMDH2) with PCK1 and with FBP1, because these assays are conducted in vitro. Based on colony filter and enzyme assays for reporter gene expression, these interactions are weak relative to interactions between identical polypeptides to form homodimers of MDH2 (and of ΔnMDH2). The weakness of heteromeric interactions among gluconeogenic enzymes may in part be because of localization in the nucleus in these assays. The heteromeric gluconeogenic enzyme interactions were also found to be highly dependent on orientation and the type of GAL4 domain fused to MDH2, because positive results are obtained only when the GAL4 DNA-binding domain is fused to the amino terminus of MDH2. This orientation dependence is contrary to our initial prediction that interactions might be stronger with a free amino terminus of MDH2. However, because of the proximity of amino- and carboxyl-terminal ends of malate dehydrogenase polypeptides in crystallographic analyses (35, 36), we assume that fusion of GAL4 domains onto the carboxyl terminus of MDH2 blocks heteromeric two-hybrid interactions with PCK1 and FBP1. This is not the case for homodimeric interactions, which appear to be enhanced by the free amino termini on MDH2 (and ΔnMDH2) subunits.

We used several methods to study interactions of gluconeogenic enzymes in vitro. Whereas MDH2 and FBP1 could be co-immunoprecipitated, this technique was not sufficiently reliable for further analyses. A physical interaction between MDH2 and PCK1 was demonstrated with Hummel-Dreyer chromatography. Physical interactions between MDH2 and PCK1 and between MDH2 and FBP1 were also observed using SPR. In contrast, SPR results indicated a significant reduction in affinity for binding of ΔnMDH2 by PCK1 and no binding of ΔnMDH2 by FBP1. Consistent with two-hybrid results, SPR data indicate that interactions of PCK1 and FBP1 with MDH2 are relatively weak, with Kᵦ values in the low micromolar range. In fact, these Kᵦ values are near the limit for detection with two-hybrid assays (38, 40). This limit would explain the inability of two-hybrid assays to detect even weaker ΔnMDH2/PCK1 interactions. The stoichiometries obtained for MDH2/PCK1 and MDH2/FBP1 interactions by SPR are very different and imply differences in individual subunit interactions (10:1 for MDH2 and PCK1 subunits and 1:1 for MDH2 and FBP1 subunits). The stoichiometry for the MDH2/FBP1 interaction is clearly closer to that expected for a physiologically relevant interaction. However, it will be of interest to determine how these values correlate with relative cellular concentrations of the enzymes, because one prediction for association of enzymes in a multi-enzyme complex is the maintenance of specific stoichiometries within the complex.

Our evidence for physical associations, particularly between MDH2 and PCK1, is consistent with a prediction by Srere (15) that close proximity with the next enzyme within a metabolic pathway may be important to ensure direct delivery of oxaloacetate generated in a reaction catalyzed by malate dehydrogenase. An interaction between sequential enzymes in a given metabolic pathway is frequently cited as evidence for the possibility of "channeling" of intermediates within the pathway.
(41). To our knowledge, ours are the first data demonstrating such interactions involving the cytosolic gluconeogenic isozyme of malate dehydrogenase. However, there is abundant experimental evidence for interactions between the mitochondrial tricarboxylic acid cycle isozyme of malate dehydrogenase and citrate synthase. This evidence has been obtained using a variety of methods including gel filtration chromatography and polyethylene glycol precipitation (17). In addition, kinetic models, based on yeast or mammalian fusion proteins containing citrate synthase fused to the amino terminus of malate dehydrogenase, have been presented to support the potential for channeling of oxaloacetate between the soluble enzymes (17, 18, 42). Velot and Srere (19) also used a peptide “puritubagen,” an in vivo competitor, to identify a region (residues 353–366) of yeast citrate synthase (CIT1) that appears to be important in functional interactions with MDH1 in vitro. In preliminary experiments, we have been unable to detect interactions between MDH1 and CIT1 using yeast two-hybrid assays, but results cited above obtained for the fused CIT/MDH enzymes suggest that such interactions may be highly dependent on orientation and on linker spacing between MDH1 or CIT1 and GAL4 domains.

At present, we have no evidence for a kinetic advantage of physical associations among the gluconeogenic enzymes, but their co-localization would theoretically reduce diffusion of intermediates (e.g. oxaloacetate) away from the pathway. In the current study, we have focused upon the enzymes unique to gluconeogenesis. Clearly, however, this pathway also requires several enzymes that also function in glycolysis. Therefore, it will obviously be of interest in the future to examine the importance of the amino-terminal proline residue of MDH2 in interactions with PCK1 and FBP1 using methods described in this report.

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Natalie Gibson and Lee McAlister-Henn

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