Heterodimer formation and activity in the human enzyme galactose-1-phosphate uridylyltransferase

(yeast/galactosemia/mutations)

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ABSTRACT One of the fundamental questions concerning expression and function of dimeric enzymes involves the impact of naturally occurring mutations on subunit assembly and heterodimer activity. This question is of particular interest for the human enzyme galactose-1-phosphate uridylyltransferase (GALT), impairment of which results in the inherited metabolic disorder galactosemia, because many if not most patients studied to date are compound heterozygotes rather than true molecular homozygotes. Furthermore, the broad range of phenotypic severity observed in these patients raises the possibility that allelic combination, not just allelic constitution, may play some role in determining outcome. In the work described herein, we have selected two distinct naturally occurring null mutations of GALT, Q188R and R333W, and asked the questions (i) what are the impacts of these mutations on subunit assembly, and (ii) if heterodimers do form, are they active? To answer these questions, we have established a yeast system for the coexpression of epitope-tagged alleles of human GALT and investigated both the extent of specific GALT subunit interactions and the activity of defined heterodimer pools. We have found that both homodimers and heterodimers do form involving each of the mutant subunits tested and that both heterodimer pools retain substantial enzymatic activity. These results are significant not only in terms of their implications for furthering our understanding of galactosemia and GALT holoenzyme structure–function relationships but also because the system described may serve as a model for similar studies of other complexes composed of multiple subunits.

One of the fundamental questions raised by studies of the human enzyme galactose-1-phosphate uridylyltransferase (EC 2.7.7.12; GALT) concerns the role of subunit interaction on holoenzyme function and the impact of naturally occurring mutations on those interactions. Human GALT, impairment of which results in the potentially lethal disorder galactosemia, has been demonstrated to function as a dimer (1–7), ostensibly composed of identical subunits. However, the marked heterogeneity of naturally occurring point mutations identified in the GALT loci of galactosemia patients (e.g., ref. 8) indicates that many, if not most, of these patients are compound heterozygotes rather than true molecular homozygotes. The GALT enzymes expressed in these individuals, therefore, would be expected to consist of mixtures of homodimers and compound heterodimers, present in proportions reflective of the relative abundance, stability, and affinities of the individual subunits involved. This added level of heterogeneity may contribute to the broad range of phenotypic severity (for review, see ref. 9) observed for patients with galactosemia.

That normal human GALT functions as a dimer has been determined by gel filtration column chromatography, SDS/

PAGE after chemical cross-linking, and sucrose density gradient ultracentrifugation of purified or enriched preparations derived from a variety of tissues (1–7). Holoenzyme molecular weight estimates from these studies have ranged from 67,000 to 88,000, with respective monomer sizes, as determined by SDS/PAGE, at between 31,000 and 46,000. The unmodified 379-amino acid polypeptide predicted from the sequence of the cloned human GALT cDNA (10, 11) has a predicted molecular weight of 43,179.

GALT enzymes from *Escherichia coli* and *Saccharomyces cerevisiae* also have been studied, with the conclusion that both function as dimers with holoenzyme and subunit molecular weights of 80,200 and 41,000 (12) and of 86,100 and 38,000 (13), respectively. Indeed, Wedekind *et al.* (14) recently crystallized the *E. coli* enzyme and determined its structure at 1.8-Å resolution as that of a dimer composed of identical subunits.

To determine whether each GALT subunit contains its own active site or whether the two subunits work in concert to create a single active site, Frey *et al.* (15) performed measurements of the first half-reaction for the purified *E. coli* enzyme. Their results, that 2 mol of glucose 1-phosphate were released per mol of holoenzyme, strongly suggested that each subunit contains its own active site. These data did not, however, address the question of possible synergistic or regulatory interactions between subunits and their respective active sites.

Nadler *et al.* (1) addressed the issue of functional interaction of subunits when they demonstrated interallelic complementation of naturally occurring mutant GALT enzymes in hybrid cells derived by pairwise fusion of skin fibroblasts from seven galactosemic patients. Of 28 hybrids, 3 exhibited detectable GALT activity, ostensibly reflecting the presence of active compound heterodimers composed of subunits derived from each of the two fused lines. The GALT activity produced by these hybrid cells was similar to that of the normal enzyme in terms of K_m values, pH optimum, and electrophoretic mobility on starch gels but differed in values for specific activity and V_{max} , as well as properties of thermal stability (1). While these data are compelling, they are nonetheless extremely complex to interpret due to the lack of molecular characterization of the patients involved and the lack of information regarding extent of subunit interaction in the hybrids. Furthermore, unless all of the patients were true molecular homozygotes, which seems unlikely, each pairwise fusion could have involved GALT subunits derived from as many as four different alleles.

In an effort to test more directly both the extent of physical association of subunits encoded by known alleles of GALT and the impact of those interactions on enzymatic activity, we have modified our previously reported yeast expression system for human GALT (16, 17) to enable the coexpression of epitope-tagged alleles within single cells. Here we report the results of a series of experiments designed to investigate subunit inter-

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Abbreviation: GALT, galactose-1-phosphate uridylyltransferase.

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action with proteins derived from wild-type human GALT sequences as well as GALT sequences carrying each of two previously identified naturally occurring null mutations, Q188R and R333W. These data were obtained from experiments involving coprecipitation of epitope-tagged subunits coupled with activity assays and Western blot analyses, gel filtration column chromatography, and a modified form of the yeast two-hybrid system. Our results demonstrate not only that both homodimers and heterodimers do form involving each of these two mutants subunits but also that both heterodimer pools retain substantial enzymatic activity.

MATERIALS AND METHODS

Yeast Strains, Plasmids, and Expression Studies. All yeast strains (yJFK1 and yJFK18) and yeast expression plasmids (pPEy1, pLMY2, pLMY5, and pPEy3) employed in this study have been described (16–18). All procedures including yeast transformations, culture manipulations, extract preparations, and GALT enzymatic assays were performed as described (16–18). The Q188R mutation was introduced into the human GALT cDNA as described (16). The R333W mutation was introduced into the human GALT cDNA by site-directed mutagenesis as described elsewhere (19). Sequence encoding the 12CA5 epitope tag after a start codon (MYPYDVPDYA) was introduced in-frame onto the 5' end of the human GALT coding sequence by site-directed mutagenesis (19). The sequence encoding the HIS6 epitope tag after a start codon (MHHHHHH) was introduced onto the 5' end of the human GALT coding sequence by PCR. All plasmid manipulations were performed using standard techniques (20).

Yeast Two-Hybrid System Studies. A modified form of the yeast two-hybrid system/interaction trap (21, 22) was used to probe human GALT subunit interaction, as described (18).

β -Galactosidase Activity Assays. All β -galactosidase assays were performed in quadruplicate, representing extracts prepared from two separate colonies, each assayed at two different concentrations within the linear range of the assay defined for that sample, as described (18). Activity values were derived by comparing raw data from each spectrophotometric assay with a standard curve generated using purified enzyme. Finally, all data were normalized according to the average activity detected in extracts representing wild-type human GALT homodimeric interactions.

Western Blot Analysis. Western blot analysis was performed essentially as described (18), except that after transfer the nitrocellulose membrane was blocked in IM2 buffer (50 mM Tris-HCl/150 mM NaCl/5 mM EDTA/0.25% gelatin/1% Tween 20) for 12 h. 12CA5 epitope-tagged GALT proteins were detected by Western blot analysis using as primary and secondary antibodies, respectively, an anti-12CA5 monoclonal antibody (Boehringer Mannheim) at 1 μ g/ml and a sheep anti-mouse Ig horseradish peroxidase coupled antibody (Amersham) at 1:5000 dilution. For quantitation, autoradiograms were analyzed using a Molecular Dynamics Personal Densitometer SI scanning laser densitometer.

Gel-Filtration Chromatography. Two hundred microliters of yeast whole cell lysate at 6.8 μ g/ μ l containing 12CA5 epitope-tagged human GALT protein expressed from the high copy number plasmid pJFy3 [identical to pJFy1 (16) except for the elimination of an unwanted *SalI* site and conversion of the *XhoI* site to a *SalI* site] was applied to a Pharmacia Sephacryl S-200 Superfine gel-filtration column (90 cm $\times$ 1 cm) equilibrated with running buffer (20 mM Hepes, pH 7.5/200 mM NaCl) and calibrated using protein standards from the Combithek Proteins II range calibration kit (Boehringer Mannheim). All elutions were performed at 4°C with 500- μ l fractions collected at a flow rate of 178 μ l/min. Fractions were monitored by Western blot analysis, as described above, as well as by spectrophotometric-coupled GALT activity assays, per-

formed essentially as described (4). In particular, 15 μ l of an appropriate dilution of GALT extract was added to 270 μ l of assay buffer (114 mM glycylglycine, pH 8.7/5.7 mM dithiothreitol/5.7 mM magnesium chloride/909 μ M NADP/5.7 μ M glucose 1,6-diphosphate/455 μ M UDP glucose/909 μ M galactose 1-phosphate) and 15 μ l of secondary enzyme mixture [phosphoglucosyltransferase (3 units/ml)/glucose-6-phosphate dehydrogenase (4 units/ml)], and the reaction was followed by monitoring absorbance at 340 nm. All assays were zeroed against parallel reactions involving the appropriate amount of yeast extract from a strain deficient in GALT. All results were normalized with respect to the peak value detected for that assay.

35 S Labeling of Proteins. A 25-ml yeast culture was grown at 30°C in synthetic glycerol/ethanol medium deficient in leucine (SGE-leu) to an OD₆₀₀ value between 0.8 and 1.0. Cells were harvested by centrifugation for 5 min in an Adams Compact II centrifuge and the pellet was resuspended in 15 ml of synthetic minimal glycerol/ethanol medium including adenine, lysine, and tryptophan (SMGE+ade, lys, trp). Cells were then grown for 1 h, harvested, and resuspended in 1 ml of SMGE+ade, lys, trp to which 150 μ Ci (1 Ci = 37 GBq) of EXPRE 35 S 35 S protein labeling mixture (NEN) had been added. This 1-ml culture was grown for 30 min with agitation at 30°C, harvested, washed once with distilled water, and then resuspended in 300 μ l of lysis buffer [20 mM Hepes, pH 7.5/1 mM dithiothreitol/bovine serum albumin (300 μ g/ml)] and protein extracts were made as described (16).

Nickel-Affinity Precipitation. Yeast extract diluted to 480 μ l with precipitation buffer (20 mM Hepes, pH 7.5/25 mM imidazole/200 mM NaCl) was mixed with 20 μ l of nickel-NTA resin (Qiagen) preequilibrated with precipitation buffer. Precipitations destined for either GALT activity assays or Western blot analysis with the anti-12CA5 antibody were performed using 30 μ g of yeast extract; precipitations destined for Western blot analysis with the anti-GALT polyclonal antiserum were performed using 100 μ g of yeast extract. Samples were incubated with gentle end-over-end rotation on a GLAS-COL laboratory rotator at 4°C for 3 h. Precipitate was allowed to settle on ice for 10 min, and then supernatant was removed using a Lo-Dose 0.5-ml U-100 insulin syringe with a 28G $_{1/2}$ Micro-fine i.v. needle (Becton Dickinson). The precipitate was washed three times with 500 μ l of wash buffer (20 mM Hepes, pH 7.5/200 mM NaCl/30 mM imidazole), on the final wash the slurry was transferred to a fresh tube, and the supernatant removed as described above. For Western blot analysis, each pellet was resuspended in 15 μ l of sample buffer (1 M Tris-HCl, pH 7.5/2% SDS/10% sucrose/saturating bromophenol blue). For GALT activity assays, the pellet was resuspended in 37.5 μ l of lysis buffer and then analyzed as described (16).

RESULTS

Epitope-Tagged Alleles of Human GALT. To facilitate the recognition and isolation of subunits encoded by individual alleles of human GALT, we have introduced sequence encoding each of two small tags, 12CA5 (23) and HIS6 (e.g., ref. 24), onto the 5' ends of both wild-type and mutant coding sequences for the human enzyme. The 12CA5 tag is recognized by a high-affinity commercially available monoclonal antibody (Boehringer Mannheim), while the HIS6 tag facilitates efficient and highly specific isolation of tagged proteins by virtue of its strong and reversible interaction with a charged nickel resin (ref. 25; Qiagen).

To ensure that neither tag impairs either the activity or expression level of human GALT in the yeast system, activity assays and Western blot analyses of extracts prepared from yeast expressing untagged (native), 12CA5-tagged, or HIS6-tagged GALT proteins were performed (Fig. 1). Expression levels of identical GALT sequences encoded by each of the two

FIG. 1. Impact of plasmid backbone and epitope tags on human GALT expression in yeast. Yeast whole cell lysates prepared from cells transformed with each of the plasmids indicated were analyzed for GALT activity (A) and on Western blots for both total GALT protein (10 μ g of extract per lane) (C) and 12CA5-tagged protein (1 μ g of extract per lane) (D). B presents the ratio for each extract of GALT activity (A) to abundance of GALT protein as determined by densitometric analysis of the autoradiogram shown in C. In both A and B, actual values graphed are indicated above each corresponding bar. The number of independent assays performed on each sample (A) are indicated in parentheses.

plasmids used in these studies, pLMY2 and pPEY1, also were investigated. As illustrated, extracts prepared from cells expressing wild-type untagged GALT from either the pLMY2 or pPEY1 plasmids demonstrated virtually indistinguishable activities and immunoreactive signals. Furthermore, neither tag impaired either the activity or abundance of GALT; indeed, both tags appear to have improved GALT abundance (and corresponding activity) to a small degree, although the reason for this observation remains unclear. Finally, both the Q188R and R333W alleles facilitated expression of significant GALT protein levels, indicating that the absence of activity associated with both of these mutations does not reflect a corresponding absence of GALT protein. Indeed, in tagged form both mutant alleles expressed levels of GALT protein virtually indistinguishable from those of the tagged wild-type enzyme (Fig. 1).

Size Determination of 12CA5-Tagged Human GALT Holoenzyme Expressed in Yeast. To determine the holoenzyme size of epitope-tagged human GALT expressed in yeast, we subjected a sample of crude yeast lysate prepared from cells overexpressing 12CA5-tagged human GALT to gel filtration column chromatography. Fractions were monitored both for GALT enzymatic activity and for anti-12CA5 immunoreactive signal as visualized on a Western blot. As illustrated in Fig. 2, a single species of GALT eluting with an estimated molecular weight of between 85,000 and 110,000, was detected by both methods. The predicted size of the tagged dimer is about 90,000. Neither GALT activity nor tagged GALT protein was detected in fractions predicted to contain monomers (molecular weight $\approx$ 45,000) or higher-order multimers.

Analysis of Human GALT Homodimer and Heterodimer Formation with Coprecipitation Experiments. Interaction of subunits in human GALT was tested directly by coprecipitation experiments involving differentially tagged subunits co-expressed in the same cells. Lysates prepared from yeast coexpressing both HIS6-tagged and 12CA5-tagged GALT

FIG. 2. Gel filtration column chromatography of whole cell lysate prepared from yeast expressing 12CA5-tagged human GALT. Elution profiles are presented for both GALT activity and immunoreactive signals. All fractions tested were normalized to the peak value for that assay. The predicted elution positions for GALT monomer and dimer are indicated.

subunits, wild type or mutant, were affinity-precipitated using a nickel resin, and the resultant pellets were probed for presence of the 12CA5-tagged subunit by Western blot analysis with an anti-12CA5 monoclonal antibody (Fig. 3).

As illustrated in Fig. 4A, strong signals were detected in lanes representing wild-type GALT homodimers (lane 5), wild-type/Q188R-GALT heterodimers (lane 6), Q188R-GALT homodimers (lane 7), wild-type GALT/R333W-GALT heterodimers (lane 8), and R333W-GALT homodimers (lane 9), indicating interallelic subunit interaction. As a control for specificity of the nickel-HIS6 interaction, extracts containing 12CA5-GALT, but no HIS6-GALT, also were analyzed, revealing no detectable signal (lane 3). Similarly, as a control for potentially spurious interaction of preformed GALT dimers during the affinity-precipitation procedure, separate extracts containing 12CA5-tagged GALT and HIS6-tagged GALT were mixed just prior to start of the precipitation procedure. Again, no 12CA5 signal was detected (lane 4). To confirm the presence of 12CA5-tagged GALT in each of these control lysates, both also were subjected to Western blot analysis with an anti-12CA5 antibody prior to affinity precipitation (lanes 1 and 2). As expected, although both demonstrated strong signals, the band apparent in lane 1, representing two copies

FIG. 3. Schematic of the GALT coprecipitation procedure. Open squares represent 12CA5-tagged subunits of human GALT; solid circles represent HIS6-tagged subunits of human GALT.

FIG. 4. Nickel affinity precipitation of defined GALT homodimers and heterodimers. (A) All samples were subjected to Western blot analysis with the antibodies indicated to the left. As marked, samples loaded in lanes 1 and 2 (1 μ g and 2 μ g, respectively) were not subjected to nickel affinity precipitation; samples loaded in lanes 3–9 represent the pellets remaining after nickel affinity precipitation of 30 μ g (anti-12CA5 panel) or 100 μ g (anti-GALT panel) of crude extract. Lanes represent extracts from yeast expressing the following human GALT proteins: 1, 12CA5hGALT + 12CA5hGALT; 2, 12CA5hGALT mixed at $t = 0$ with HIS6hGALT; 3, 12CA5hGALT + 12CA5hGALT; 4, 12CA5hGALT mixed at $t = 0$ with HIS6hGALT; 5, HIS6hGALT + 12CA5hGALT; 6, HIS6hGALT + 12CA5hGALTQ188R; 7, HIS6hGALTQ188R + 12CA5hGALTQ188R; 8, HIS6hGALT + 12CA5hGALTR333W; 9, HIS6hGALTR333W + 12CA5hGALTR333W. (B) Nickel affinity precipitation of 35 S-labeled yeast extract prepared from cells expressing HIS6-tagged human GALT. The first lane represents 11.6 μ g of crude extract; the second lane represents the pellet after affinity precipitation of GALT from 100 μ g of crude extract.

of 12CA5-tagged GALT, is noticeably more intense than is that in lane 2, representing one 12CA5-tagged copy of GALT. Finally, as a control for efficiency of the nickel affinity precipitations, parallel samples representing all lanes also were subjected to Western blot analysis with an anti-GALT polyclonal antiserum that detects both 12CA5- and HIS6-tagged forms of the protein. As expected, strong signals were detected in all lanes except lane 3 (Fig. 4A).

As a final control to rule out nonspecific protein–protein interactions with HIS6-tagged GALT, an affinity precipitation experiment was performed using a metabolically radiolabeled yeast lysate containing HIS6-GALT. As illustrated (Fig. 4B), although the crude lysate contained innumerable bands, the affinity-purified sample demonstrated only a single band migrating to the correct position on the gel for HIS6-GALT.

Modified Yeast Two-Hybrid Experiments. As an independent test of subunit interaction in human GALT, we have applied a modified form of the yeast two-hybrid system, as described (18, 21, 22). In brief, cells were cotransformed with two plasmids, each of which encoded a defined subunit of human GALT fused in-frame with one-half of a transcriptional activator—either the DNA binding domain of bacterial LexA or the acidic activating domain of yeast *GAL4*. In each cotransformed cell, if the two encoded subunits of GALT interact, their fusion partners should be brought into close approximation and a functional transcription factor reconstituted capable of activating expression of a β -galactosidase reporter gene. If the two GALT subunits do not interact, the two halves of the transcription factor should remain indepen-

dent and the reporter gene should remain silent. As illustrated in Fig. 5, extracts representing all of the appropriate controls were negative, and extracts representing interactions corresponding to wild-type GALT homodimer, wild-type GALT/Q188R-GALT heterodimer, Q188R-GALT homodimer, wild-type GALT/R333W-GALT heterodimer, and R333W-GALT homodimer were positive, again demonstrating interaction between all of the subunits tested.

Activity of Defined GALT Homodimer and Heterodimer Pools. We next tested each defined homodimer and heterodimer pool for GALT activity (Fig. 6). Nickel-affinity precipitations were performed in triplicate on extracts prepared from yeast coexpressing the indicated HIS6-tagged mutant alleles of GALT with 12CA5-tagged alleles of either wild-type GALT or mutant GALT. From each set, one pellet was subjected to Western blot analysis with the anti-12CA5 antibody, one pellet was subjected to Western blot analysis with the anti-GALT polyclonal antiserum, and one pellet was analyzed for the presence of GALT activity. As a reference point against which to measure the mutant homodimer and heterodimer activities, extracts expressing both HIS6-tagged and 12CA5-tagged forms of wild-type GALT also were investigated. Finally, all activity assays were repeated three times for quantitative analysis.

Pellets derived from each nickel-affinity precipitation procedure, termed “heterodimer pellets,” would be expected to include a mixture of two forms of GALT: (i) HIS6-tagged GALT homodimers and (ii) HIS6-tagged GALT/12CA5-tagged GALT heterodimers. As demonstrated in Fig. 4, no 12CA5-tagged GALT homodimers should be present due to the specificity of the nickel affinity precipitation. Furthermore, no 12CA5-tagged GALT homodimers should have been able to reform from subunits dissociated from heterodimers during the course of the 30-min activity assay, as demonstrated by incubating “heterodimer pellets” in reaction buffer at the appropriate temperature for 40 min, centrifuging the sample to separate any dissociated 12CA5-tagged subunits from the bound HIS6-tagged subunits, and then analyzing both fractions by Western blot with the anti-12CA5 antibody. No diminution of signal was observed in pellets analyzed after the 40-min incubation, and no 12CA5 signal was detected in lanes representing supernatants (data not shown). Finally, because

FIG. 5. Reporter gene expression assays resulting from a modified yeast two-hybrid system assessment of GALT subunit interaction. β -Galactosidase activity was measured in extracts prepared from yeast cotransformed with derivatives of the plasmids pLMY5 and pPEY3 encoding the GALT alleles indicated. All activity values were normalized to the average activity value obtained for the wild-type sample. Any normalized activity values calculated to be negative numbers were truncated at zero.

FIG. 6. Activity associated with defined homodimer and heterodimer pools of human GALT. Pellets resulting from nickel affinity precipitations of 30 μ g (anti-12CA5 panel and activity assays) or 100 μ g (anti-GALT panel) of crude extract prepared from yeast coexpressing the indicated alleles of human GALT were subjected to GALT activity assay as well as Western blot analyses using each of the antibodies indicated. For each extract, the HIS6-tagged allele is indicated above the line; the 12CA5-tagged allele is indicated below the line. All GALT activity values were normalized according to the wild-type control.

homodimers of both Q188R- and R333W-GALT are catalytically inactive (Figs. 1 and 6), any activity detected in each pellet must reflect activity of the corresponding heterodimer pool.

As illustrated in Fig. 6, strong signals were detected in all lanes with both antibodies, indicating that all precipitations worked well. Furthermore, wild-type GALT homodimer exhibited substantial activity, both Q188R and R333W mutant GALT homodimers exhibited no detectable activity, and both heterodimer pellets exhibited between 20 and 30% of the activity seen with wild-type homodimers.

DISCUSSION

The experiments presented herein explore the impact of two naturally occurring mutations, Q188R and R333W, on human GALT dimer formation and activity. The Q188R mutation was selected for study because it accounts for 60–70% of the classic galactosemia alleles identified in Caucasian patients studied to date (26, 27). Furthermore, it impacts a position only two codons from the presumed active site of the enzyme. The R333W mutation was selected for study because, in contrast to Q188R, it impacts sequence distal to the active site of the enzyme, both in linear terms and as predicted by homology to the bacterial enzyme structure (14).

Two fundamental questions were asked, (i) what are the impacts of these two mutations on subunit assembly and (ii) if heterodimers do form, are they active? To address these questions, we have established and applied a yeast model system for the coexpression and analysis of defined epitope-tagged alleles of human GALT. Our results demonstrate that both mutant forms of GALT remain competent to form homodimers and heterodimers with the wild-type subunit, and that both mutant/wild-type heterodimers retain substantial enzymatic activity despite the fact that each includes one subunit that, at least in homodimeric form, is completely catalytically inactive.

Both of the epitope tags used in this study, HIS6 and 12CA5, were introduced onto the amino terminus of the human GALT sequence, a region that is highly divergent through evolution (10, 11, 28). As illustrated in Fig. 1, neither tag appears to have exerted any significant effect on specific activity of the GALT protein. The recently reported crystal structure of bacterial GALT (14) indicates that the amino termini of both subunits

reside on the surface of the holoenzyme, perhaps explaining the human enzyme's tolerance to modification reported herein.

As reported, both the Q188R (16, 17) and R333W (29) mutations are associated with complete loss of enzymatic activity in the homodimeric state. The significance of the slight diminution of protein abundance observed herein for both untagged mutant proteins remains unclear.

While GALT preparations from a number of different sources have been analyzed with respect to size (1–7, 12, 13), these analyses have followed activity of the enzyme rather than simple presence of the protein, leaving open the possibility that monomeric (or multimeric) pools of GALT exist but are inactive. The gel filtration column chromatography results reported herein (Fig. 2) demonstrate that tagged human GALT produced within the context of the yeast expression system migrates as a dimer. These data are consistent with those reported for native human GALT (2–4), suggesting that neither expression within the yeast system nor presence of the epitope tags perturbs the subunit association properties of the human enzyme. The data presented herein also are significant because no pools other than dimer were detected, despite the fact that we followed the migration of both GALT protein and GALT activity through the column. Clearly, since no monomer or higher-order pools of GALT were detected, the question remains open as to whether or not such species would be active.

We have presented herein two sets of experiments that demonstrate interaction of both wild-type and mutant subunits of human GALT. While the coprecipitation experiments have demonstrated direct physical interaction of subunits, the modified yeast two-hybrid system experiments have corroborated these results. Indeed, the indirect nature of the two-hybrid system renders it a qualitative rather than a quantitative assay, so that the small differences observed between samples representing different GALT alleles (Fig. 5) may or may not reflect true differences in the affinities of the subunits involved.

The data presented herein demonstrating activity of heterodimers (Fig. 6) yielded results quantitatively similar, although not identical, to those that would have been expected under idealized circumstances: with all subunit assortments random, the probability of affinity precipitation for any dimer independent of whether it includes one HIS6-tagged subunit or two and all heterodimers 50% as active as wild-type homodimers. In this idealized circumstance, one would have predicted the heterodimer pellets analyzed herein, composed of 2/3 heterodimers and 1/3 HIS6-tagged mutant homodimers, to exhibit 33% as much GALT activity as the positive control.

Finally, the observation that both of the naturally occurring mutant subunits studied remained competent to interact with wild-type GALT is significant because it furthers the possibility that allelic combination and not just allelic constitution may play some role in defining holoenzyme function, and thereby patient outcome. Of course, in attempting to extend from the results reported herein to the human system, it is important to recognize that the possibility remains that other factors or conditions present in human cells, and absent in yeast, or visa versa, may impact GALT expression and/or function in galactosemic patients or carriers. The results reported herein were derived using a yeast model system to overexpress a human protein and, therefore, must always be interpreted with caution. Nonetheless, in addition to the results reported herein, the system described herein lends itself, via sequential affinity precipitation, to isolation of defined heterodimer or compound heterodimer pools, thereby facilitating a more detailed study of these hybrid enzymes and their biochemical properties.

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