Covalent Heterogeneity of the Human Enzyme Galactose-1-phosphate Uridylyltransferase*

Received for publication, June 16, 2000, and in revised form, July 5, 2000
Published, JBC Papers in Press, July 6, 2000, DOI 10.1074/jbc.M005259200

Jenny M. Henderson‡§, Lance Wells‡§, and Judith L. Fridovich-Keil**

From the ‡Graduate Program in Nutrition and Health Sciences, the §Graduate Program in Biochemistry and Molecular Biology, and the ¶Department of Genetics, Emory University School of Medicine, Atlanta, Georgia 30322

Galactose-1-phosphate uridylyltransferase (GALT) acts by a double displacement mechanism, catalyzing the second step in the Leloir pathway of galactose metabolism. Impairment of this enzyme results in the potentially lethal disorder, galactosemia. Although the microheterogeneity of native human GALT has long been recognized, the biochemical basis for this heterogeneity has remained obscure. We have explored the possibility of covalent GALT heterogeneity using denaturing two-dimensional gel electrophoresis and Western blot analysis to fractionate and visualize hemolysate hGALT, as well as the human enzyme expressed in yeast. In both contexts, two predominant GALT species were observed. To define the contribution of uridylylated enzyme intermediate to the two-spot pattern, we exploited the null allele, H186G-hGALT. The Escherichia coli counterpart of this mutant protein (H166G-eGALT) has previously been demonstrated to fold properly, although it cannot form covalent intermediate. Analysis of the H186G-hGALT protein demonstrated a single predominant species, implicating covalent intermediate as the basis for the second spot in the wild-type pattern. In contrast, three naturally occurring mutations, N314D, Q188R, and S135L-hGALT, all demonstrated the two-spot pattern. Together, these data suggest that uridylylated hGALT comprises a significant fraction of the total GALT enzyme pool in normal human cells and that three of the most common patient mutations do not disrupt this distribution.

Galactose-1-phosphate uridylyltransferase (GALT) catalyzes the second step of the Leloir pathway of galactose metabolism, converting UDP-glucose (UDP-Glc) and galactose-1-phosphate (Gal-1-P) into glucose-1-phosphate (Glc-1-P) and UDP-galactose (UDP-Gal) (Fig. 1). Impairment of human GALT results in the potentially lethal disorder galactosemia. Both the bacterial and human GALT enzymes function as homodimers (1, 2) and display ping-pong kinetics with catalysis that proceeds via a covalent intermediate (3, 4). In the first-half reaction, a histidine residue at the active site (His-166 in Escherichia coli GALT and His-186 in the human enzyme) serves as a nucleophile, attacking the α-phosphorus of UDP-Glc. Glc-1-P is released, leaving behind a uridylylated enzyme intermediate (5, 6). In the second-half reaction, Gal-1-P enters the active site cleft and attacks the uridylylated nitrogen on the imidazole ring of the active site histidine, forming UDP-Gal. This second product then dissociates, regenerating free enzyme (2, 7).

It has been known for more than 30 years that human GALT derived from red blood cells or other tissues is heterogeneous when fractionated under native conditions and visualized with an activity overlay stain (8). Studies using starch, agarose, or acrylamide isoelectric focusing gels have revealed a complex pattern of up to six bands (9–12). Additionally, specific variants of human GALT, such as the Duarte and Los Angeles alleles, have been characterized in part by their shifted banding patterns (8). Despite its common observance and diagnostic utility, the underlying basis of hGALT heterogeneity has remained obscure.

One obvious possibility involves covalent modification of the protein. Indeed, Banroques and colleagues (13) hypothesized that GALT might be post-translationally modified in a tissue-specific manner, based on discrepancies in the native banding patterns they observed for enzymes derived from different tissue types. The fact that human GALT is covalently heterogeneous is the focus of this report.

Previously, we have explored native human GALT heterogeneity using a null-background yeast expression system for the human enzyme. We have observed that wild-type human GALT expressed in yeast displays a native banding pattern indistinguishable from that seen in normal human hemolysates, and that the N314D-substituted human enzyme expressed in yeast demonstrates the characteristic “Duarte” shift reported for patient samples (14).

In the studies reported here, we have explored the possibility of covalent heterogeneity of human GALT. Using denaturing two-dimensional gel electrophoresis followed by Western blot analysis of normal human hemolysate-derived GALT, we have observed a reproducible pattern of two intense spots, with some fainter spots also visible on long exposures. What is more, we have demonstrated in parallel experiments that wild-type human GALT expressed in yeast presents a pattern indistinguishable from that seen in hemolysates, again confirming the utility of this model system.

Next, we probed the biochemical basis of hGALT covalent heterogeneity, testing the hypothesis that uridylylated enzyme intermediate might account for one or more of the observed spots. Toward this end, we created a substitution of glycine for histidine (H186G) at the active site, thereby precluding formation of the covalent intermediate. Previous crystallographic studies of the corresponding E. coli substituted enzyme (H166G) have demonstrated an otherwise normal structure for...
this protein (15). Upon expression in the yeast system followed by two-dimensional PAGE analysis, H186G-hGALT displayed a single predominant spot, implicating the covalent intermediate as the basis for the second spot in the wild-type pattern.

Finally, we have applied the yeast expression system to test the ability of each of three common patient-alleles, Q188R, S135L, and N314D, to form and accumulate covalent intermediates. Comparing each to the wild-type protein, we found similar, if shifted, patterns of spots for all three proteins. The intensities of the spots relative to one another also were unchanged. Collectively, these data demonstrate that normal human hemolysate GALT, as well as both the normal and substituted human proteins expressed in yeast, accumulate marked levels of uridylylated intermediate. These data not only offer insight into the normal mechanism of the wild-type enzyme, but they also provide a straightforward test for the ability of altered human GALT proteins to form and accumulate the product from the first half of the transferase reaction.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmid Manipulation—All recombinant human GALT proteins used in this study were expressed in yJK1, a haploid strain of Saccharomyces cerevisiae that lacks endogenous GALT and has been described previously (16). Mutations encoding the substitutions Q188R, N314D, S135L, and H186G were each introduced into the human GALT cDNA according to the method of Kunkel (17). All GALT alleles were expressed from the yeast glyceraldehyde-3-phosphate dehydrogenase (G3PD) promoter on a low copy number (centromeric) plasmid, MM22, derived from the yeast-bacterial shuttle vector YEpPlac 22 (18). Plasmid manipulations and transformations into yeast were performed according to standard protocols (19) as described previously (14). Yeast were cultured at 30 °C in synthetic dextrose medium deficient in tryptophan to mid-log density and harvested by centrifugation.

Enrichment for Human GALT from Red Blood Cells—Whole blood was collected into sodium-heparin tubes from normal volunteers previously defined by enzyme assay as wild-type for GALT. hGALT was then enriched from these samples using a DEAE-Sephascl column, essentially as described previously (11).

Yeast Lysates and Western Blot Analyses—Soluble yeast extracts were made essentially as described previously (20) using as lysis buffer 20 mm HEPES-KOH, pH = 7.5, 1 mm dithiothreitol, 0.3 mg/ml bovine serum albumin supplemented with the protease inhibitors pepstatin (0.63 μg/ml), leupeptin (0.50 μg/ml), phosphoramidon (0.44 μg/ml), chymostatin (0.2 μg/ml), phenylmethylsulfonyl fluoride (1 mm), and trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane (2.88 μg/ml). Western blot analyses were performed as described previously (21) using as primary antibody a rabbit polyclonal antiserum (EU70) directed against human GALT. The secondary antibody used was horseradish peroxidase-coupled sheep anti-rabbit IgG antiserum (Roche Molecular Biochemicals), and signals were visualized using the enhanced chemiluminesent (ECL) system from Amersham Pharmacia Biotech. Where indicated, following Western blot analysis, filters were stripped at 50 °C for 30 min in stripping buffer (2% sodium dodecyl sulfate, 0.625 M Tris, pH 6.8, 7.1 μl/ml β-mercaptoethanol) and then stained with 0.1% India ink in TNT (25 mm Tris, pH 7.5, 0.16 μl NaCl, 0.05% Tween 20) to reveal a constellation of more than 150 abundant endogenous yeast proteins. Selected subsets of these endogenous proteins were used where indicated to provide markers against which to measure the migration of the hGALT signals.

Two-dimensional Gel Electrophoresis—Separation of yeast extracts by denaturing two-dimensional gel electrophoresis was performed essentially as described previously (22), with the following modifications. First-dimension tube gel isoelectric focusing gradients were established using a mixture of 3-10 and 5-6 amphoteries (Amersham Pharmacia Biotech) combined in a 2.7:1 ratio (4 and 1.5% of total volume, respectively). Gels were focused for 1 h at 500 volts followed by 5 h at 2000 volts. The second dimension involved separation on SDS-polyacrylamide (10% acrylamide) slab gels using a mini-V gel system (Life Technologies, Inc.). Gels were electroblotted onto nitrocellulose and visualized as described above.

RESULTS

Human GALT Exhibits Covalent Heterogeneity—As a first step to address the possibility of covalent heterogeneity in human GALT, normal hemolysates were fractionated by denaturing two-dimensional gel electrophoresis and then probed by Western blot analysis with a polyclonal antiserum against the human protein. As illustrated in Fig. 2, a clear pattern of two predominant spots, separated in the first dimension, was observed. The relative intensities of both spots were comparable. Extended exposures of the films sometimes revealed several additional fainter spots of unequal intensity that together accounted for less than 10% of the total signal (data not shown). The significance of these additional spots remains unclear.

To test whether human GALT expressed in yeast would demonstrate a pattern similar to that seen in hemolysates, samples of recombinant enzyme were analyzed as described above; the patterns were indistinguishable (Fig. 2, A and B). Finally, mixing experiments were performed to test the identity of the hemolysate and recombinant hGALT patterns; the spots were coincident (Fig. 2C).

Uridylylated Enzyme Intermediate Accounts for Much of the Covalent Heterogeneity Observed in Human GALT—To test the hypothesis that accumulation of the uridylylated GALT enzyme intermediate might account for the observed covalent heterogeneity, we created and utilized a substituted form of the protein H186G, which lacks the active site nucleophile and therefore cannot form covalent intermediate. X-ray crystallographic studies of the corresponding E. coli GALT (H166G) protein have confirmed its proper folding and structure (15). The H186G-substituted human GALT protein was expressed in yeast, fractionated on denaturing two-dimensional gels, and visualized by Western blot analysis as described above. Instead of the usual two-spot pattern, a single predominant spot was revealed (Fig. 3). These results strongly implicate the uridylylated enzyme intermediate as responsible for one of the two predominant spots observed for wild-type hGALT.

Three Common Naturally Occurring Substitutions in Human GALT-Q188R, N314D, and S135L All Form and Accumulate Covalent Intermediates—To test the ability of three common patient alleles, Q188R, N314D, and S135L human GALT, to form and accumulate the uridylylated intermediate, each was expressed in the null background yeast strain described above. Soluble lysates from these cultures were then fractionated by denaturing two-dimensional gel electrophoresis, and the human GALT species were visualized by Western blot analysis. As illustrated in Fig. 4, all three substituted proteins tested maintained the two-spot pattern, consistent with forma-
Human GALT Displays Covalent Heterogeneity

FIG. 3. Uridylylated intermediate accounts for most of the covalent heterogeneity found in human GALT. A, two-dimensional PAGE/Western blot analysis of wild-type human GALT expressed in yeast (15 μg of total protein); B, two-dimensional PAGE/Western blot analysis of H186G human GALT expressed in yeast (50 μg of total protein); C, two-dimensional PAGE/Western blot analysis of a mixed sample representing lysates of yeast expressing wild-type and H186G human GALT.

FIG. 4. Three common patient alleles of human GALT expressed in yeast remain competent to form and accumulate uridylylated intermediate. Soluble extracts of yeast expressing the indicated hGALT alleles were analyzed either individually (upper panels) or following mixing with wild-type samples (lower panels). In each case, 7 μg of extract were loaded on the gels. Arrows in the lower panels indicate signals corresponding to wild-type GALT.

DISCUSSION

In the experiments presented here, we have explored the possibility of covalent heterogeneity of human GALT. These studies were motivated by the well documented native heterogeneity of the human enzyme (23) and represent a first step toward addressing the basis of that heterogeneity. Our data demonstrate three main points. First, normal human hemolysate GALT exhibits covalent heterogeneity, with two predominant species of similar abundance represented. Second, one of the predominant species in this pattern likely reflects uridylylated enzyme intermediate, as demonstrated by the loss of the second spot when the active site nucleophile is genetically removed. Finally, each of three common naturally occurring substituted forms of human GALT-S135L, Q188R, and N314D, retained the two-spot pattern, although in Q188R- and N314D-hGALT both spots were shifted commensurate with amino acid charge changes predicted by the respective substitutions.

Covalent Heterogeneity of Human GALT—Banroques and colleagues (13) hypothesized over 15 years ago that human GALT might be covalently modified, but until now this possibility has remained unexplored. The results presented here demonstrate two predominant species of wild-type human GALT in red blood cells and in a yeast expression system. One spot represents ostensibly free enzyme, whereas the other represents the uridylylated intermediate. Whether this same pattern also would be observed in other human tissues or in other species remains a focus for future work.

It is important to recognize that our isoelectric focusing and GALT detection conditions were by definition limited, so that additional minor yet distinct populations of wild-type human GALT, beyond the two we have reported, may also exist. Indeed, loading higher amounts of protein, or longer exposures of the film following Western blot analysis, sometimes revealed one or more faint spots flanking the two predominant species (Figs. 2–4, and data not shown). Similarly, minor spots flanking the predominant product were sometimes evident in analyses of the H186G-hGALT protein (Fig. 4). The significance of these additional minor species remains unclear. Finally, although our studies have implicated uridylylated intermediate as the underlying basis for the second predominant hGALT spot observed, these data in no way rule out the possibility of additional post-translational modifications of human GALT. Ongoing collaborative studies to generate a high resolution crystal structure for human GALT should provide significant insight into this issue.

Frey, Rayment, and colleagues (3, 24–26) were the first to observe and characterize the uridylylated E. coli GALT intermediate, providing extensive biochemical and crystallographic characterization of this moiety. It is clear from their work that formation of the covalent intermediate is an essential step in the transferase reaction, with important implications for the kinetic properties of the enzyme. What has remained unclear, however, is the steady state abundance of the covalent intermediate in living cells, be they bacterial or human. Intermediates, covalent or otherwise, are often considered transient moieties at best, forming and resolving quickly through the catalytic cycle of the enzyme. The fact that uridylylated GALT enzyme accounts for approximately half of the soluble enzyme detected in normal human hemolysates is striking, and forces the question, why? It may be relevant to note that in normal hemolysates, the first GALT substrate, UDPG, although present at very low levels, is more abundant than is the second substrate, Gal-1-P, which is generally undetectable (27). The accumulation of covalent intermediate may reflect differential availability of the two substrates. Alternatively, there may be some other, as yet unknown, biological rationale for the extraordinary accumulation observed.

Impact of Common Galactosemia Patient Mutations on Covalent hGALT Heterogeneity—The mechanism of impairment for most patient-derived alleles of human GALT remains largely unknown. Considering that the transferase reaction involves two discrete steps, individual patient mutations could interfere with one, with the other, or with both steps. Clearly, additional classes of mutations that impair dimerization, stability, or other properties of the protein might also exist.

The data reported here offer a rapid screen for the ability of given mutant enzymes to form and accumulate the covalent intermediate. Although the examples given represent the two ends of a spectrum, namely wild-type distribution of GALT signal between the two spots or complete absence of one spot, quantitative differences in ratios of the two spots also may be possible. Further studies of additional mutations will be required to explore this possibility.
Human GALT Displays Covalent Heterogeneity

Of the three common patient substitutions analyzed here, two (N314D and S135L) have previously been associated with significant levels of GALT enzymatic activity (14, 28–32), consistent with the appearance of both “free” and “uridylylated” spots in these samples. In contrast, Q188R-hGALT has undetectable catalytic activity both in patients and in yeast (16), leaving open the question of whether or not this moiety is competent to form and accumulate covalent intermediate. Recently, two groups, Geeganage and Frey (33) and Lai and colleagues (34), have both explored the basis of this impairment. Although these authors have reported disparate conclusions regarding several aspects of residual activity for the mutated protein, both groups agree that the Q188R-hGALT (or Q168R-eGALT) protein remains competent to form an uridylylated intermediate, again consistent with the results reported here.

Native Heterogeneity—Finally, these studies were initiated as a first step toward understanding the basis of native heterogeneity in human GALT. By demonstrating that substantial levels of uridylylated hGALT accumulate in cells, if we assume a random assortment of uridylylated and free subunits in the dimer pool, at least three predominant species of native dimer should result: free:free, free:uridylylated, and uridylylated:uridylylated. Further studies will be required to confirm the validity of this assumption and therefore the actual contribution of uridylylated GALT intermediate to the complex native IEF pattern observed. Future work also will be required to define the basis of the additional bands typically seen in native preparations of human GALT, although one or more of the minor GALT species reported here may be involved.

Acknowledgments—We thank Dorothy Pettay for excellent technical assistance in collecting blood, David Pallas, for invaluable advice and encouragement regarding two-dimensional gels, Kristen Riehman for developing GALT constructs, Hazel Holden for critical reading of the manuscript, and Perry Frey for helpful discussion.

REFERENCES

1. Dale, G., and Popjak, G. (1976) J. Biol. Chem. 251, 1057–1063
2. Wedekind, J. E., Frey, P. A., and Rayment, I. (1995) Biochemistry 34, 11049–11061
3. Wong, L.-J., Sheu, K.-F. R., Lee, S.-L., and Frey, P. A. (1977) Biochemistry 16, 1010–1016
4. Crews, C., Wilkinson, K. D., Wells, L., Perkins, C., and Fridovich-Keil, J. L. (2000) J. Biol. Chem. 275, 22847–22853
5. Field, T. L., Reznikoff, W. S., and Frey, P. A. (1989) Biochemistry 28, 2094–2099
6. Kim, J., Ruzicka, F., and Frey, P. (1990) Biochemistry 29, 10590–10593
7. Arahshahi, A., Brody, R. S., Smallwood, A., Tsai, T. C., and Frey, P. A. (1986) Biochemistry 25, 5583–5589
8. Segal, S., and Berry, G. (1995) in The Metabolic and Molecular Bases of Inherited Disease (Scrivner, C., Beaudet, A., Sly, W., and Valle, D., eds) pp. 967–1000, McGraw-Hill, Inc., New York
9. Schapira, F., Gregori, C., and Banroques, J. (1978) Biochem. Biophys. Res. Commun. 80, 281–297
10. Banroques, J., Gregori, C., and Schapira, P. (1981) Biochim. Biophys. Acta 657, 374–382
11. Kelley, R. I., Harris, H., and Mellman, W. J. (1983) Hum. Genet. 63, 274–279
12. Shin, Y. S., Niedermeier, H. P., Endres, W., Schaub, J., and Weidinger, S. (1987) Clin. Chim. Acta 166, 27–35
13. Banroques, J., Gregori, C., and Dreyfus, J. C. (1983) Biochimie (Paris) 65, 7–13
14. Fridovich-Keil, J. L., Quimby, B. B., Wells, L., Mazur, L. A., and Elsevier, J. P. (1995) Biochem. Mol. Med. 56, 121–130
15. Thoden, J., Ruzicka, F., Frey, P., Rayment, O., and Holden, H. (1997) Biochemistry 36, 1212–1222
16. Fridovich-Keil, J. L., and Jinks-Robertson, S. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 298–302
17. Kunkel, T., Roberts, J., and Zakour, R. (1987) Methods Enzymol. 154, 367–382
18. Gietz, R. D., and Sugino, A. (1988) Gene 74, 527–534
19. Guthrie, C., and Fink, G. (1991) in Methods in Enzymology, Academic Press, Inc., San Diego
20. Quimby, B. B., Wells, L., Wilkinson, K. D., and Fridovich-Keil, J. L. (1996) J. Biol. Chem. 271, 26835–26842
21. Elsevier, J. P., Wells, L., Quimby, B. B., and Fridovich-Keil, J. L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7166–7171
22. O’Farrell, P. (1975) J. Biol. Chem. 250, 4007–4021
23. Holton, J. B., Walter, J. H., and Tyfield, L. A. (2000) in Metabolic and Molecular Bases of Inherited Disease (Scrivner, C. R., Beaudet, A. L., Sly, S. W. and Valle, D., eds) McGraw Hill, New York, in press
24. Wong, L.-J., and Frey, P. A. (1974) J. Biol. Chem. 249, 2322–2324
25. Frey, P. A., Wong, L.-J., Sheu, K.-F. P., and Yang, S. L. (1982) Methods Enzymol. 87, 29–36
26. Wedekind, J. E., Frey, P. A., and Rayment, I. (1996) Biochemistry 35, 11560–11569
27. Shih, Y. S. (1995) Eur. J. Pediatr. 154, (Suppl. 2), S75–S76
28. Reichardt, J. K. V., and Woo, S. L. C. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2633–2637
29. Reichardt, J. K. V., Levy, H. L., and Woo, S. L. (1992) Biochemistry 31, 5430–5433
30. Fridovich-Keil, J. L., Langley, S. D., Mazur, L. A., Lennon, J. C., Dembure, P. P., and Elsas, L. J. (1995) Am. J. Hum. Genet. 56, 640–646
31. Lai, K., Langley, S. D., Singh, R. H., Dembure, P. P., Hjelm, L. N., and Elsas, L. J. (1996) J. Pediatr. 128, 89–95
32. Wells, L., and Fridovich-Keil, J. L. (1997) J. Inherited Metab. Dis. 20, 633–642
33. Geeganage, S., and Frey, P. A. (1998) Biochemistry 37, 14500–14507
34. Lai, K., Willis, A., and Elsas, L. (1999) J. Biol. Chem. 274, 6559–6566