Homology Modeling Studies on Human Galactose-1-phosphate Uridylyltransferase and on Its Galactosemia-Related Mutant Q188R Provide an Explanation of Molecular Effects of the Mutation on Homo- and Heterodimers

Anna Marabotti* and Angelo M. Facchiano

Laboratory of Bioinformatics, Institute of Food Science, Italian National Research Council, Via Roma 52 A/C, 83100 Avellino, Italy, and Interdepartmental Research Center for Computational and Biotechnological Sciences (CRISCEB), Second University of Naples, 80138 Naples, Italy

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We have created theoretical models of the three-dimensional dimeric structure of human galactose-1-phosphate uridylyltransferase as well as of homo- and heterodimers carrying the Q188R mutation by using comparative modeling procedures. These mutants are associated to the most frequent form of the genetic disease galactosemia. We have analyzed the impact of this mutation both on enzyme−substrate interactions as well as on interchain interactions in the heterodimers and in the homodimer. We suggest a molecular explanation for the altered function, caused by different enzyme−substrate interactions, and for the partial dominant negative effect of the mutant allele that is present in heterozygotes for this gene, related to a substantial loss of interchain hydrogen bonds. These results can be considered a starting point for a more extensive characterization at the molecular level of the other mutations linked to this genetic disease.

Introduction

The enzyme galactose-1-phosphate uridylyltransferase (E.C. 2.7.7.12; GALT) catalyzes the conversion of galactose-1-phosphate and uridine-5′-diphosphate (UDP)-glucose into glucose-1-phosphate and UDP-galactose.[1,2] This enzyme is a member of the histidine triad superfamily, characterized by the sequence motif HhHhHhh (where H represents histidine and h represents hydrophobic amino acid). GALT belongs to branch III of this superfamily, characterized by a specific nucleoside monophosphate transferase activity rather than a hydrolase activity that is typical of the other branches.[3] The catalytic mechanism of this enzyme has been extensively studied[4,5] and is best described by a ping-pong kinetic mechanism (Scheme 1). In the first step, the histidine of the active site attacks the α-phosphorus of UDP-glucose, displaces glucose-1-phosphate, and forms a covalent intermediate that reacts with galactose-1-phosphate in the second step to produce UDP-galactose.

The genetic disorder called “classical galactosemia” or “galactosemia I” (OMIM: 230400) is linked to the impairment of this enzyme. This disease can be potentially lethal if not detected early and is revealed through symptoms such as gastrointestinal complaints, hepatomegaly, cataracts, mental retardation, and ovarian failure in females. These last two dysfunctions can persist even with a life-long dietary restriction.[2,6] Classical galactosemia is characterized by a high allelic heterogeneity, and to date more than 150 different base changes were recorded in several populations and ethnic groups.[1] However, extensive investigations on the structural effects of galactosemia-causing mutations at a molecular level have been impaired up until now due to the fact that the three-dimensional (3D) structure of the human GALT (hGALT) enzyme has still not been experimentally solved. However, the 3D structures of wild-type GALT from *E. coli* as well as of the inactive H166G site-directed mutant, complexed with different ligands, have been solved through X-ray crystallography.[7−9] All of these structures are currently available from the Protein Data Bank (PDB).[10] Considering that hGALT shares 46% sequence identity with the bacterial enzyme and that the two sequences are unequivocally superimposable starting from the 20th amino acid of the human sequence,[7] the homology modeling strategy can be successfully applied to create a suitable model of the 3D structure of hGALT enzyme. In fact, as confirmed in the comparative assessments of protein structure prediction techniques, CASP5, this strategy can give very good results, especially when applied under conditions of sequence identity higher than 25%.[11,12] However, no reports on hGALT modeling studies have been published to date. To investigate the molecular effects of the mutation Q188R on hGALT (Q188R-hGALT), a simulation was performed on the 3D structure of *E. coli* GALT, and results were extrapolated for the human enzyme.[5] The interest for the Q188R variant is due to it being the most diffuse galactosemia-linked mutation, especially in the Caucasian population.[1,6] This mutant has been extensively studied from a biochemical point of view, and it is characterized by an almost complete loss of GALT activity in in vitro assays and for individuals homozygous in vivo.[13−19] Moreover, people heterozygous for Q188R/wild-type protein show an activity of about 15−20% of the wild-type activity, indicating a partial dominant negative effect of this mutation on heterodimer function.[14,15]

* Author to whom correspondence should be addressed. Phone: +39-0825-299651. Fax: +39-0825-299813. E-mail: amarabotti@isa.cnr.it.

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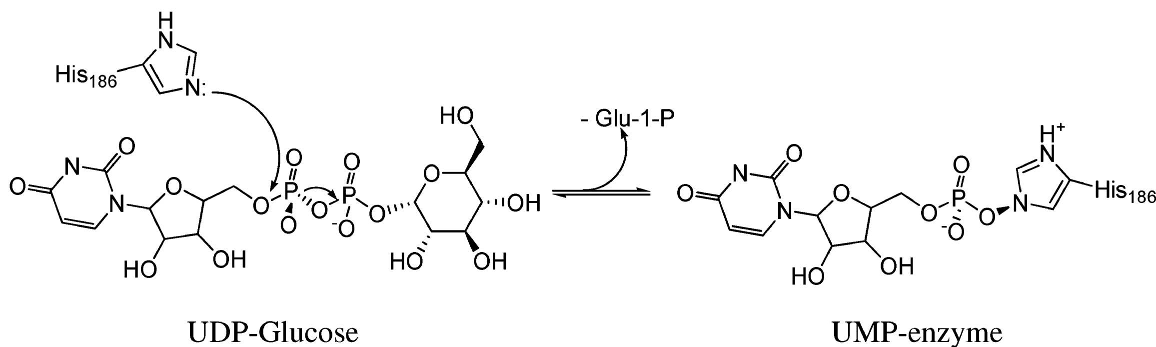
Scheme 1. Scheme of the Two Steps of the Reaction Catalyzed by Galactose-1-phosphate Uridylyltransferase



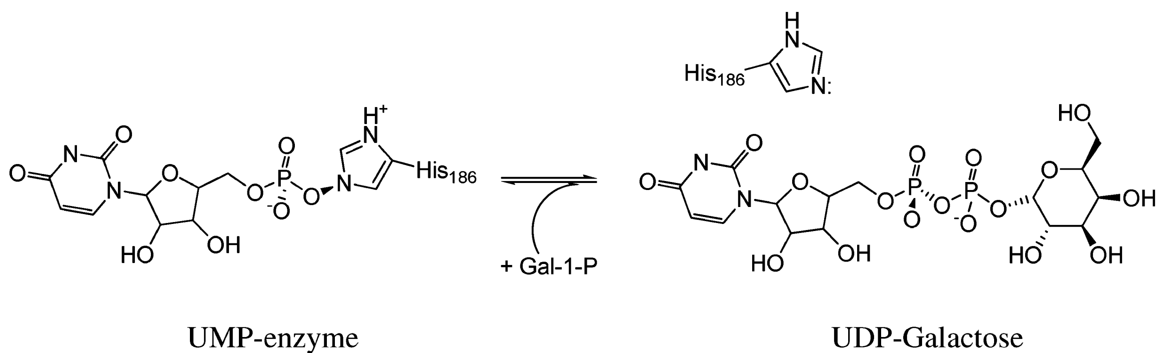


Table 1. Distribution of Residues of the Models in the Ramachandran Plot after PROCHECK Calculation and Z-Scores Evaluated with ProsaII

	Ramachandran parameters					ProsaII
models	% residues in most favored regions (A, B, L)	% residues in additional allowed regions (a, b, l, ρ)	% residues in generously allowed regions (−a, −b, −l, ρ)	% residues in disallowed regions	total	z-score
1GUP.pdb[a]						−8.39
1GUQ.pdb[a]						−8.38
hGALT (monomer)	91.1	8.9	0	0	100	
hGALT (homodimer)	86.2	13.4	0.3	0	100	−8.20
Q188R-hGALT (monomer)	92.0	7.6	0.3	0	100	
Q188R-hGALT (homodimer)	90.8	8.3	0.6	0.3	100	−7.93
AhGALT/BQ188R (heterodimer 1)	86.4	12.9	0.6	0	100	−8.09
AQ188R/BhGALT (heterodimer 2)	85.9	13.3	0.8	0	100	−7.80

[a] PROCHECK analysis was not performed on templates.

In this work, we present the results of the comparative modeling of the homodimeric hGALT by using as a template the structure of the enzyme from *E. coli*. The structural features of this protein are discussed, focusing on differences and similarities between the bacterial and the human enzyme. We also investigated the structural properties of the Q188R-hGALT mutant in both homodimeric and heterodimeric forms, to highlight the effect of this mutation on catalysis and subunit interactions. We also propose an interpretation of the role of this residue in the destabilization of the dimer association.

Results and Discussion

3D Model of Native and Mutant GALT Structures. The models of the monomers of hGALT and Q188R-hGALT were obtained with MODELLER (Experimental Section). We selected, through a PROCHECK analysis, the best in terms of average stereochemical properties, which show more than 90% of residues in most favored regions of the Ramachandran plot (Table 1). This result is comparable with high-quality crystallographic structures determined at a resolution of more than 2 Å.[20] The PROCHECK analysis revealed a high stereochemical quality also of the assemblies of the homo- and heterodimeric structures of GALT, obtained as described in the Experimental Section (Table 1). Moreover, the analysis with ProsaII program performed on the templates and models showed that their z-scores are comparable (Table 1), which means that the model structures are consistent with their fold.[21] With the same program we calculated the pseudoenergy profiles based on knowledge-based mean fields for the templates and models, finding that they are strictly similar (data not shown). All of these data confirmed the high quality of our models as well as their reliability.

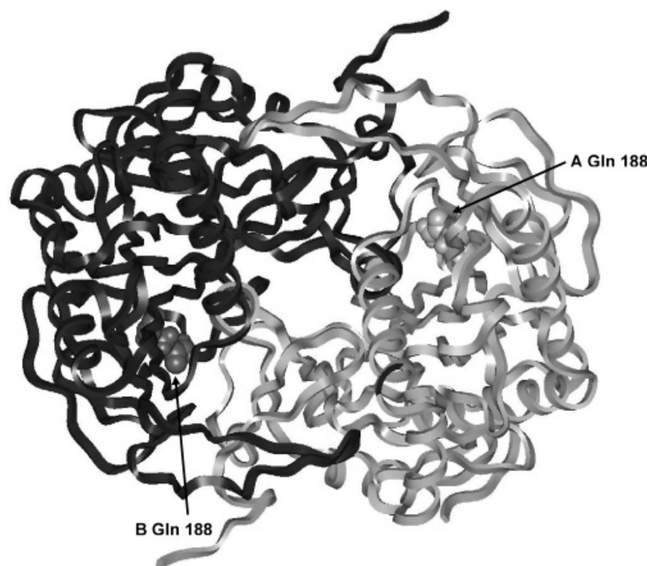


Figure 1. Schematic view of the three-dimensional structure of the final model of hGALT. Chain A is represented in light gray, chain B in dark gray. Gln 188 of both chains is highlighted in CPK mode.

The dimeric structure of the hGALT model was submitted to the PDB, and having passed the ADIT validation, it was accepted with the PDB ID code 1R3A. Its structure is presented in Figure 1.

The dimeric structure of hGALT was superimposed to the reference structure of the 1GUP file. The average root-mean-square deviation (rmsd) between the Cα of the two structures is 0.60 Å, indicating that no major differences are present in their tertiary and quaternary structure. The most relevant differences lie in two segments: (i) 100−120 of hGALT (corresponding to residues 80 to 100 in GALT from *E. coli*), in which the rmsd difference between the Cα of the two structures is about 1.0 Å, with a maximum of 2.94 Å for Leu 116; (ii) segment 363−371 of hGALT, which is six amino acids longer in human than in bacterial protein.

Analysis of Contacts with Substrates. Looking at the active site of GALT in human and bacterial enzyme, it is possible to note that the backbones of the amino acids for all the models and templates are nearly superimposed, but some differences are present in the orientations of their side chains. To gain insight on the interactions of our models with substrates, we simulated the complexes of UDP-galactose and UDP-glucose into the binding site of both hGALT and Q188R-hGALT, evaluating the presence of hydrogen bonds in the complexes formed by the bacterial and the human enzymes with the program HBPLUS. Results on chains B of all the complexes are representative of the global analysis and are available in Table A in the Supporting Information. From these results, it is possible to note that several hydrogen bonds are well conserved in all complexes (e.g., those with Ala 81, Asn 97, Asp 98, Asn 173, and Ser 181 (Val 61, Asn 77, Asp 78, Asn 153, and Ser 161 in GALT from *E. coli*)). Interestingly, the first three residues are involved in missense mutations causing galactosemia.[1,6] On the contrary, other hydrogen bonds appear to be more variable in the different complexes. For example, hydrogen bonds with Arg 48 and Arg 51 are present alternatively in the complexes of hGALT; when the first is present, generally the other is not and vice versa. Hydrogen bonds between Lys 334, Tyr 339 (Lys 311, Tyr 316 in the bacterial enzyme), and glucose are not present in chain B of hGALT, but they are present in chain A of the same complex, and they are also present in the contacts with galactose. The contacts made by His 166 are not present in the table for the bacterial enzyme because the structures analyzed are those of the mutant H166G.

Our analysis was then focused on the contacts of residue 188. In the bacterial enzyme, the side chain of Gln 168 forms two hydrogen bonds with the oxygens of the α- and β-phosphate (Figure 2a). From biochemical and structural studies,[4,5,7−9] the role of this residue appears to be the stabilization of the enzyme intermediate as well as probably increasing the susceptivity of phosphate for the double displacement reaction (Scheme 1). The two hydrogen bonds between Gln 168 and the two negatively charged phosphate oxygens disperse the electron density, favoring the creation of an electronic gap on the phosphorus atom. Thus, the nucleophilic attack of galactose-1-phosphate is favored. The analysis on our model of the human protein is in agreement with these experimental data. The hydrogen bonds between Gln 188, O2A, and O1B in hGALT (Figure 2b) are consistent with the X-ray crystallography on the bacterial enzyme.

The hydrogen bond pattern of Arg 188 in the Q188R-hGALT model appears to be altered. This residue is unable to interact with the phosphoric moiety of ligands in the same way as Gln 188. Moreover, it is also involved in other hydrogen bonds (e.g., with the oxygen named O5′ that links the uridylyl moiety to the phosphate chain (Figure 2c)). Obviously, the validity of our analysis is strongly dependent on the correct position of residue 188 in our models. We verified this by comparing the position of Gln 188 and Arg 188 in all the models produced by MODELLER. The side chain of Gln 188 is orientated with the geometry shown here in almost all models; thus we considered this as the most probable geometry. A larger variability among the models is present for the position of the Arg 188 side chain. However, when we used other monomers to simulate the enzyme/substrate interactions and we compared the results, we always observed an alteration of the hydrogen bond pattern analogous to that described here, thus confirming that our deductions are correct and reproducible.

A molecular interpretation of the effect of the Q188R human mutation was previously attempted by simulating the simple replacement of Gln 168 with an arginine residue in the structure of the active site of *E. coli* GALT covalently linked to uridine-5′-monophosphate (UMP) (PDB code 1HXQ).[5] In that work, the search for hydrogen bonds performed with SYBYL (Tripos Associates) showed that the amidic group of Gln 168 could interact with two phosphoryl oxygens of the uridyl moiety, whereas Arg 168 could form only one hydrogen bond. The authors deduced that the cause of the lack of stability of the intermediate and, as a consequence, of the functional impairment of the Q188R human mutant was simply the lower number of hydrogen bonds created by the Arg 188 residue. We analyzed the file 1HXQ using HBPLUS but surprisingly found a different

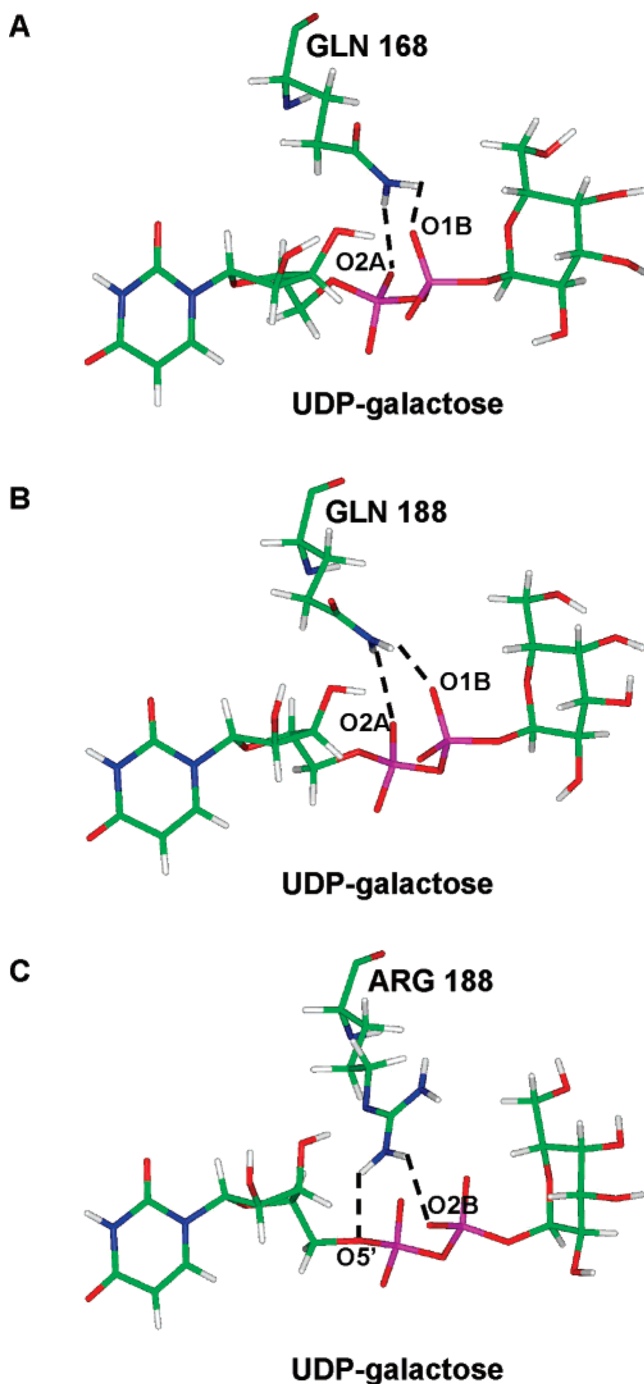


Figure 2. Closeup of the interactions between UDP-galactose and the residue 188 (168 for bacterial enzyme) in three different complexes: (A) GALT from *E. coli* (crystallographic structure); (B) hGALT; (C) Q188R-hGALT. Molecules are represented in atom code color (carbon, green; nitrogen, blue; oxygen, red; hydrogen, white; phosphorus, magenta). Hydrogen bonds calculated with HBPLUS are highlighted with broken lines and interacting atoms on the substrate are labeled.

hydrogen bond pattern between Gln 168 and UMP (Table A in Supporting Information). The explanation of this discrepancy may be that the analysis performed by SYBYL for hydrogen bond formation in the complexes took into account different parameters from those evaluated by HBPLUS, and as a consequence it produced different criteria for the hydrogen bonds definition. However, from our analysis it is evident not only that Arg 188 has a limited interaction with the phosphate moiety of ligands but also that other hydrogen bonds are formed only in the mutant enzyme. This causes an incorrect stabilization of the intermediate as well as the difficulty to proceed with the second step of the reaction. The hydrogen bond of Arg 188 with the O5′ oxygen is unable to disperse the negative charge of phosphoric moiety; therefore the phosphorus atom becomes less electrophilic. Thus, the nucleophilic attack of galactose-1-phosphate is not favored, and the reaction is impaired. These deductions agree with experimental data.[4]

Dimeric Association. It is well-known that classical galactosemia is characterized by compound heterozygosis.[1,17] Probably the largest amount of studies on this phenomenon were carried out on enzymes carrying the Q188R mutation, and the two most important features found were that (i) GALT activity is practically absent in all patients homozygous for the Q188R mutation and (ii) the heterodimeric Q188R/wild-type enzyme shows variable activity but always lower than 50% of the wild-type activity, indicating that this mutation exerts a partial dominant negative effect on the dimer.[13−19]

Several papers point out that this was an unexpected effect because the Q188R is a mutant at the active site and, therefore, far from the intersubunit interface.[15,18,19] On the contrary, the fact that residues in the active site are in strict contact with the dimer interface was already implicit in the work of Thoden and colleagues.[9] They showed that each active site of *E. coli* GALT is made up of residues belonging to both subunits. As a consequence, a mutation in the active site could perturb not only the catalytic properties of the enzyme but also its stability and intersubunit association.

Looking at our hGALT and Q188R-hGALT models, we were able to confirm that amino acids included in a distance of 5 Å from residue 188 belong to both chains of the dimer. In particular, the nearest residue of the other chain to which residue 188 belongs is Arg 48. In Figure 3, these two residues are represented with their van der Waals radii, and as clearly shown, when residue 188 is mutated to arginine, it is in strict contact with Arg 48. Moreover, within the radius of 5 Å from residue 188, there are three positively charged residues (Arg 48, Arg 51, and Lys 127), whereas only one residue (Glu 172) is negatively charged. Thus, it is evident that both the steric hindrance due to the high proximity of two bulky residues and the repulsion due to the unfavorable electrostatic interaction may be the causes of unfavorable dimerization.

We also analyzed the hydrogen bonds present at the dimer interface in homo- and heterodimers of hGALT with HBPLUS. The result highlights that the number of hydrogen bonds decreases from the hGALT wild-type homodimer (22 hydrogen bonds) to the wild-type/Q188R heterodimers (17 and 18 hydrogen bonds) to the Q188R-hGALT homodimer (12 hydrogen bonds) (for a complete listing, see Table B in the Supporting Information). This observation further supports the hypothesis that the mutation is able to negatively perturb the dimeric interface. Moreover, the loss of hydrogen bonds at the interface is in agreement with the experimental observation that Q188R/wild-type heterodimers are less thermostable than the hGALT homodimer.[15] Interestingly, only 10 hydrogen bonds (Table 2) are common to

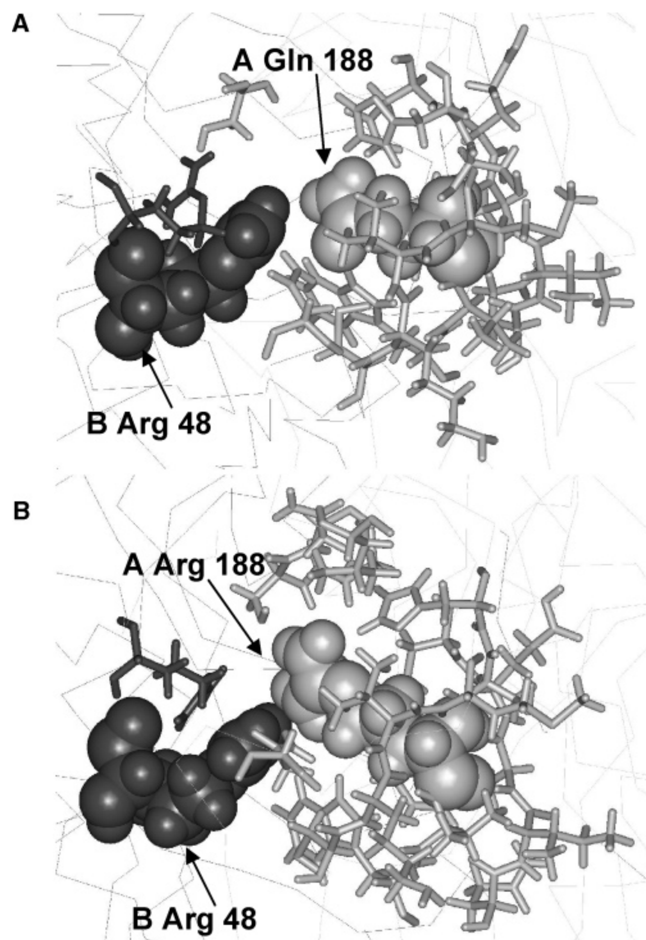


Figure 3. (A) Closeup of the contact between Gln 188 (light gray) in chain A and Arg 48 (dark gray) in chain B of hGALT. (B) Closeup of the contact between Arg 188 (light gray) in chain A and Arg 48 (dark gray) in chain B of Q188R-hGALT. Amino acids within a distance of 5 Å from residue 188 are represented in stick mode.

all hGALT dimers, and all of them involve at least one residue whose mutation has been found in galactosemia-linked enzymes.[6] This observation may suggest a crucial role of these hydrogen bond interactions in the dimer stabilization. Most of these hydrogen bonds involve backbone atoms, and the side chain substitutions can alter their geometry only if the mutation determines a different backbone conformation. This opens the way to the interpretation at the molecular level of the pathological effects of these mutations, because these amino acids are probably crucial for the correct backbone folding and dimerization of the enzyme, although a role for the creation of the active site of the protein cannot be excluded.

Therefore, it is clear from our results that the introduction of an arginine in position 188 directly perturbs the dimeric interface with the sum of negative electrostatic and steric interactions. The partial dominant negative effect exerted by this mutation in the heterodimers may be explained by the fact that not only the mutation interferes with the correct enzymatic activity in the active site in which it is inserted but it could also negatively perturb the active site of the other subunit, via perturbation of the interface geometry. The sum of this double effect can justify the activity that does not reach the expected 50% if there is neither positive nor negative interaction between the mutant and the wild-type subunits in the heterodimeric complex.

It is important to highlight that this analysis could not be possible by merely changing Gln 168 for Arg in the active site of the *E. coli* enzyme. In fact, more than 43% of the residues at the dimer interface of the two proteins are not conserved, and therefore, such a substitution would have not allowed the correct prediction of the interface effects. On the contrary, the use of a complete 3D human protein model allowed us to deepen the impact of this point mutation at a global structural level, thus providing a useful starting point to study mutations that have a limited impact on the structural features of the protein, to simulate unexpected effects of these mutations in positions far from the mutation itself, and to model mutations that could affect the entire architecture of the enzyme.

In conclusion, with the creation of a reliable model of the 3D structure of hGALT by homology modeling methods and the comparison of its structural features with those of *E. coli* enzyme, we found that although the interactions with the substrate are similar in both enzymes they are not perfectly superimposable. This suggests that the structural data inferred from the simple substitution of an amino acid into a nonhuman enzymatic environment should be treated with care. In addition, the mutant form Q188R-hGALT model has allowed us to investigate the molecular effects of this perturbation not only in substrate interaction and catalysis but also in intersubunit relationships. In fact, we were able to give a molecular explanation for the partial dominant negative effect exerted by the Q188R mutation on heterodimer activity.

Table 2. HBPLUS Analysis on the Interactions at the Dimeric Interface of hGALT Dimers[a]

donor (D)			acceptor (A)			hydrogen bond parameters		
chain	residue	atom type	chain	residue	atom type	D/A distance	H/A distance	DHA angle
A	ILE 32*	N	B	LYS 120	O	2.96	1.96	162.8
A	TYR 34	N	B	GLN 118*	O	3.31	2.42	143.5
A	LYS 120	N	B	ILE 32*	O	3.13	2.16	157.3
A	ASP 197	N	B	GLN 344*	OE1	3.17	2.30	141.8
A	ILE 198*	N	B	ALA 343	O	3.43	2.42	168.2
B	ILE 32*	N	A	LYS 120	O	3.03	2.03	163.1
B	TYR 34	N	A	GLN 118*	O	3.36	2.48	143.6
B	LYS 120	N	A	ILE 32*	O	3.02	2.01	168.2
B	ASP 197	N	A	GLN 344*	OE1	3.12	2.25	141.3
B	ILE 198*	N	A	ALA 343	O	3.40	2.39	166.9

[a] Hydrogen bonds common to all dimers are shown here, and residues whose mutation leads to a galactosemic form of the enzyme are marked with an asterisk.

The future perspective of our work will be the characterization of all galactosemia-linked mutations at a molecular level. We hope that our model will be a useful starting point also for other people involved in studies of this disease.

Experimental Section

Four different structures of GALT from *E. coli* complexed with different ligands are published in the PDB with the codes 1HXP,[7] 1HXQ,[8] 1GUP, and 1GUQ.[9] The coordinates of the first two structures lack a portion of a surface loop that forms part of the active site, whereas in the two last structures, obtained by cocrystallizing an active site mutant enzyme, H166G, with UDP-glucose and UDP-galactose, no gaps are present in the chains. As a consequence, we chose these two last structures as templates.

The sequences of the human and bacterial enzymes were aligned with two independent programs, CLUSTAL W[22] and BLAST,[23] in order to compare the alignments and to choose the best one. The outputs of the two programs were almost identical, with the exception of one more gap in the middle of the CLUSTAL W alignment. Because this did not significantly improve the alignment score and to avoid unnecessary insertions that could give erroneous results in the following modeling step, we decided to use the BLAST result as starting point.

The program MODELLER 4[24] implemented in the Quanta molecular simulation package (Version 00.1110, Accelrys, Inc.) was used for the creation of the models. This program is able to model only single chains, so we proceeded in two steps. First of all, we used the single chain A of the templates to model 10 different monomers of both hGALT and Q188R-hGALT. The sequence of bacterial GALT is 20 residues shorter than that of the human protein, so the first 20 residues of the human sequence could not be modeled and were discarded. We set 4.0 Å as the rmsd between the crystal structure of the templates and the fully optimized models, and the program operates with multiple cycles of refinement with conjugate gradient minimization and molecular dynamics with simulated annealing (for a complete list of settings see Andrej Sali's web page http://salilab.org/modeler/manual/manual.html). The best models for monomeric hGALT and Q188R-hGALT among those obtained were chosen by evaluating their stereochemical quality with the program PROCHECK.[20]

The second step was the creation of dimeric wild-type as well as mutant homo- and heterodimeric protein models. Two monomeric chains were superimposed onto the structure of the bacterial GALT dimer, to keep the same relative orientation of the two subunits, with the aid of InsightII (Version 2000.1, Accelrys, Inc.) tools. To reduce steric clashes, an optimization of the dimeric structures was achieved by using 1000 minimization steps with the steepest descent method followed by the conjugate gradient method when the energy gradient reached the value of 0.01 kcal/mol. All atoms were allowed to relax with no constraints. The control of the final quality of all models was performed again with PROCHECK, and in addition, an analysis with ProsaII program[25] was also performed on the templates and models. Finally, the ADIT validation tool available online at the PDB (http://deposit.pdb.org/validate/) was used to test the final quality of the dimeric hGALT model before PDB submission.

To create the complexes between the human enzyme and either UDP-galactose or UDP-glucose, we performed the docking of these molecules in the binding cavity. The substrates were modeled in the binding site of hGALT and Q188R-hGALT, and the CVFF force field developed for InsightII was adopted to assign potentials and charges both to the protein and to the ligands after addition of hydrogens to the molecules. Then, to relax the reciprocal geometry of side chains as well as of the ligands with the scope of avoiding sterical clashes, a mild minimization was applied to the complexes. Steepest descent minimization, 500 steps, was run with a gradient limit of 0.1 kcal/mol. This procedure represents the best compromise between the need for relieving sterical clashes and the maintenance of a correct geometry. Again, all atoms were allowed to relax with no constraints. Identification of hydrogen bonds in all enzyme−substrate complexes analyzed and between dimer interfaces of homo- and heterodimers was carried out with HBPLUS.[26]

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Supporting Information Available: Complete HBPLUS analyses made on hGALT and Q188R-hGALT models. This material is available free of charge via the Internet at http://pubs.acs.org.

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