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The importance of conserved residues in human liver UDPglucose pyrophosphorylase

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Comparison of the amino acid sequences of five eukaryotic UDPglucose pyrophosphorylases has identified a number of conserved residues that may be important for substrate binding or catalysis. Using the cloned cDNA for the human liver enzyme, we have investigated the role of several of these residues by site-directed mutagenesis. Changing the single conserved cysteine (residue 123) to serine resulted in an active enzyme, as did mutating the single conserved histidine (residue 266) to arginine. The two conserved tryptophans were each altered to serine; W218S is active while W333S is not. In the latter case, the enzyme does not appear to fold correctly, and a similar result was obtained by mutation to lysine at one (residue 391) of the four conserved arginines. The other three arginines are not essential, as judged by the observation that R389H, R422Q and R445H are all active. The kinetic properties of each active mutant were investigated and in most cases were found to be similar to those of wild-type. The most dramatic change is a sevenfold increase in the K_m for magnesium pyrophosphate with C123S. Overall, none of these conserved residues appears to be essential for activity, although such a role cannot be ruled out for W333 and R391 where mutation resulted in defective folding.

Keywords: conserved residues; enzyme kinetics; mutagenesis; UDPglucose pyrophosphorylase.

UDPglucose pyrophosphorylase (UDP-Glc*PP*ase) catalyses the transfer of a glucose moiety from Glc1*P* to MgUTP, forming UDP-Glc and $MgPP_i$. This reaction is necessary in several tissues. In liver and muscle, UDP-Glc is the direct precursor of glycogen, while in lactating mammary gland it is converted to UDPgalactose and thence lactose. Liver also requires UDP-Glc for the formation of UDPglucuronate, which then acts as a source for the formation of soluble glucuronides of xenobiotic and endobiotic metabolites destined for excretion.

The amino acid sequence of the enzyme from several sources has been adduced from that of the genomic or cDNA. These studies have revealed that the enzyme in eukaryotes is distinctive from the prokaryotic form, as there is no significant sequence similarity between the two types (Konishi et al., 1993). In the absence of a three-dimensional structure of UDP-Glc*PP*ase, there are two principal ways in which functional residues might be identified: chemical modification and sequence analysis. As discussed below, the former technique has been applied to the enzyme in several studies. In the latter method, there are two approaches involving either sequence motifs or conserved residues. Comparison of the sequence of human liver UDP-Glc*PP*ase with the PROSITE database has not revealed any consensus sequences such as a nucleotide-binding motif. There are, however, a number of conserved residues. An alignment of the sequences from five eukaryotic species is shown in Fig. 1, from which it can be seen that there are many conserved residues (indicated by *, # or □) that may be important for substrate binding, catalysis or both.

There are six conserved lysines at positions 107, 112, 127, 177, 319 and 396 (numbering according to the human liver enzyme sequence); these are indicated by # in Fig. 1. Chemical modification studies of the potato tuber enzyme with the affinity labels UDP-pyridoxal, UTP-pyridoxal, and pyridoxal-P_2-Glc (Kazuta et al., 1991 a, b) have shown that five lysines are reactive but only one of these (K396) corresponds to a conserved lysine in Fig. 1. Two of the others that are reactive (K291 and K357), as well as K396, are also reactive to UTP-pyridoxal in the bovine liver enzyme (Konishi et al., 1993). Mutagenesis studies on the potato tuber enzyme (Katsube et al., 1991) have revealed that alteration of these same three lysines results in major changes in the kinetic properties. While these studies provide consistent evidence for the importance of K396, the role of K291 and K357 is less clear because they are replaced by threonine and proline, respectively, in the *Dictyostelium discoideum* enzyme.

Rabbit muscle UDP-Glc*PP*ase has been shown to be inactivated by thiol reagents (Bergamini and Signorini, 1991), suggesting that one or more of the cysteine residues are important. This view is reinforced by the observation that there is protection against inactivation by either of the nucleotide substrates. Although the identity of the reactive cysteine(s) has not been established, there is only one cysteine that is conserved in all

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Abbreviations. UDP-Glc*PP*ase, UDPglucose pyrophosphorylase; Glc1*P*, glucose 1-phosphate; Glc6*P*, glucose 6-phosphate; Glc1,6P_2, glucose 1,6-bisphosphate.

Enzymes. UDPglucose pyrophosphorylase, UTP:α-D-glucose-1-phosphate uridylyltransferase (EC 2.7.7.9); phosphoglucomutase, α-D-glucose 1,6-bisphosphate:α-D-glucose-1-phosphate phosphotransferase (EC 5.4.2.2); glucose-6-phosphate dehydrogenase, D-glucose-6-phosphate:$NADP^+$ oxidoreductase (EC 1.1.1.49).

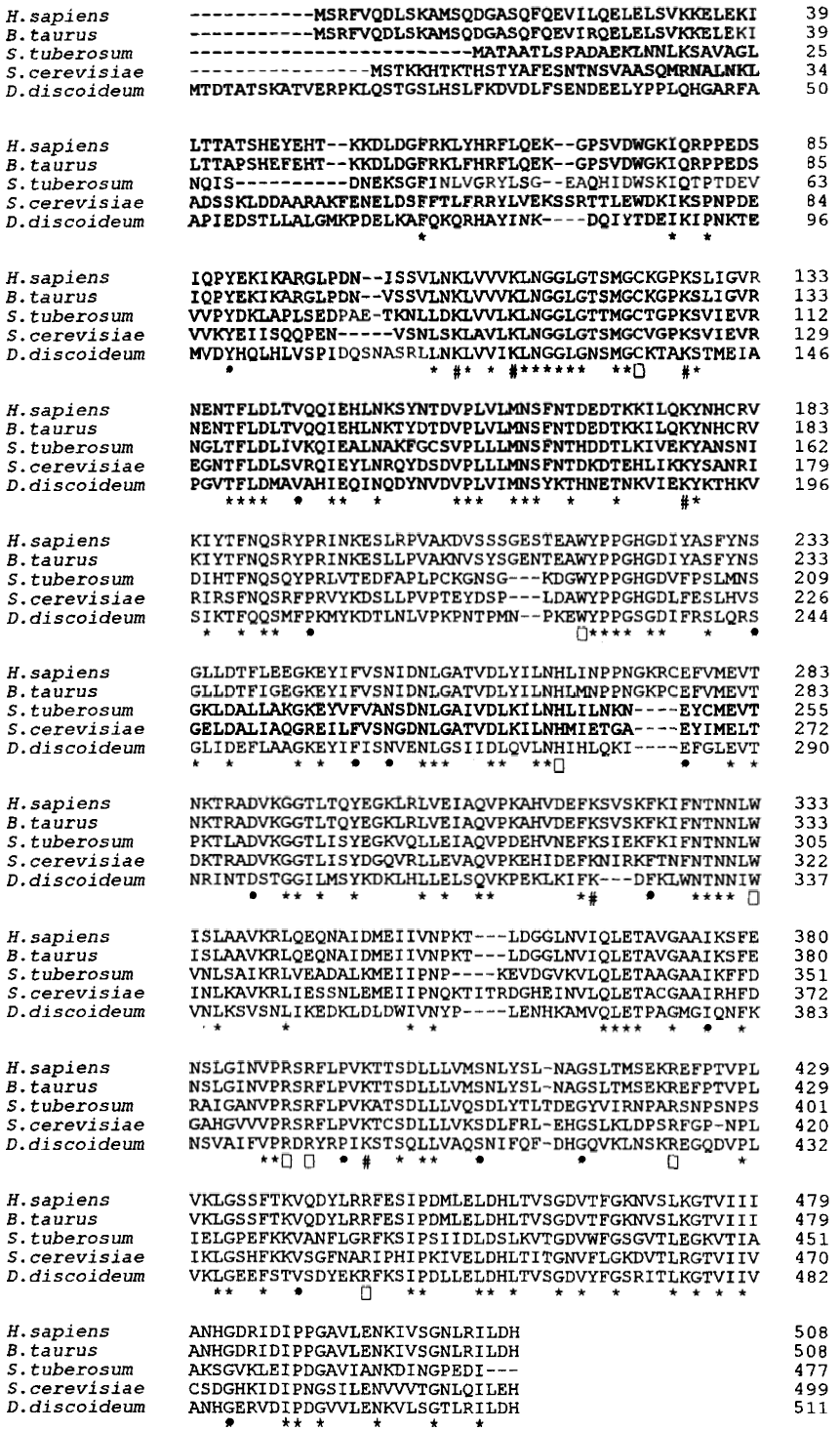

Fig. 1. Alignment of eukaryotic UDP-Glc*PP*ase protein sequences. The sequences of *Homo sapiens*, *Bos taurus*, *Solanum tuberosum*, *Saccharomyces cerevisiae* and *Dictyostelium discoideum* were obtained from GenBank and aligned using the ClustalW program (Thompson et al., 1994). Lysines conserved in all five species are shown by the symbol #, while the conserved residues that were mutated in the present study are indicated □; other conserved residues are shown by *.

eukaryotes, located at position 123 (Fig. 1). Thus, if a cysteine is essential for activity in this enzyme, it is likely to be C123. One of the objectives of the present study was to mutate C123 in order to assess its importance.

Arginine residues are often important for binding anionic groups of substrates to enzymes as, for example, in lactate dehydrogenase (Holbrook et al., 1975), aldolase (Sygusch et al., 1987) and aconitase (Lauble et al., 1992). Both of the substrates of UDP-Glc*PP*ase (in either direction of reaction) contain one or more phosphate groups and there are four conserved arginine residues shown in Fig. 1, at positions 389, 391, 422 and 445. A second objective of the present study was to determine whether any of these plays any essential role in the enzyme.

A number of enzymes utilise the imidazole side-chain of histidine for acid-base catalysis; although there is no evidence for such an involvement in UDP-Glc*PP*ase, there is a single histi-

dine (H266) conserved amongst the eukaryotic enzymes. Hence, if a histidine is involved in catalysis, it must be H266; the third aim of this study was to test the effect of replacement of this histidine.

The reaction catalysed by UDP-Glc*PP*ase involves the interconversion of two nucleotides, UTP and UDP-Glc. Binding of these aromatic compounds may well involve interactions with the side-chains of aromatic amino acids in the protein. There are 24 positions where all five of the eukaryotic sequences contain one of the aromatic amino acids and it would be an undertaking of some magnitude to investigate all of these by mutagenesis. As a first step, in the fourth aim of this study, we have mutated the two tryptophan residues (W218 and W333) that are conserved absolutely.

MATERIALS AND METHODS

Recombinant DNA techniques, mutagenesis and DNA sequencing. All restriction enzymes were obtained from Boehringer-Mannheim. The cDNA was subcloned into M13 vectors and the sequence determined with the Sequenase kit (Amersham) with either universal M13 primer or synthetic oligonucleotides. The sequence was confirmed in both strands and the question of compressions was resolved by sequencing with dITP. Nucleotide sequences were analysed with the DNASTAR program using a Macintosh LCII computer.

The plasmid pHC309, containing cDNA encoding the entire sequence of human liver UDP-Glc*PP*ase, is one of several clones that were obtained by screening of a human liver cDNA library as described previously (Peng and Chang, 1993). *Escherichia coli* JM109 harbouring this plasmid was induced with isopropyl β-D-thiogalactopyranoside and used as the enzyme source.

Site-directed mutagenesis was performed using the Altered Sites II kit (Promega). A *Bam*HI−*Xho*I fragment of human liver UDP-Glc*PP*ase cDNA (pHC308) was subcloned into the pALTER-1 vector and the single-stranded DNA of the resulting plasmid was prepared following the instructions of the manufacturer. The template DNA was annealed to a phosphorylated mutagenic oligonucleotide and an ampicillin-repair primer and the mutant strand synthesised using the T4 DNA polymerase and four dNTPs. The reaction mixture was transformed sequentially to the *E. coli mutS* strain ES1301 and then to JM109. Plasmid DNA was made from the ampicillin-resistant clones and the mutant cDNAs were identified by DNA sequencing. The mutation was then moved into the expression vector by shuffling an *Acc*I−*Acc*I fragment from pALTER/UDP-Glc*PP*ase to pHC309. Oligonucleotide primers used to make mutations were shown below with the mutated base in the lower case.

C123S	5′ AGT ATG GGC aGC AAA GGC C 3′
W218S	5′ CA GAA GCT TcG TAC CCC CC 3′
H266R	5′ TT CTT AAT CgT CTA ATC AA 3′
W333S	5′ AC AAC CTA TcG ATC TCT CT 3′
R389H	5′ AT GTT CCA AaG AGT CGT TT 3′
R391K	5′ CA AGG AGT CaT TTT CTG CC 3′
R422Q	5′ GT GAA AAG CaG GAG TTT CC 3′
R445H	5′ AC CTA AGG AaG TTT GAA AG 3′

Enzyme preparation and assays. Tetrasodium pyrophosphate was purchased from Nacalai Tesque Inc. (Kyoto, Japan), triethanolamine was from Ajax Chemicals (Auburn, Australia). Other substrates, coenzymes, coupling enzymes and buffers were obtained from Sigma. The enzyme was partially purified from *E. coli* clones containing plasmid-encoded UDP-Glc*PP*ase as described elsewhere (Duggleby et al., 1996).

The kinetic properties were assessed for each of the substrates using assays based on those described by Tsuboi et al. (1969). Assays were conducted at 25 °C in 0.1 M triethanolamine/HCl pH 8.0 in the presence of 1 mM dithiothreitol. In what is conventionally designated the forward direction (using Glc1*P* and MgUTP as substrates), the reaction was monitored at 340 nm by addition of NAD^+ (2 mM) and UDP-Glc dehydrogenase (0.25 U/ml). In the reverse reaction, (UDP-Glc and $MgPP_i$ as substrates) the reaction was monitored at 340 nm by addition of phosphoglucomutase (5 U/ml) and its cofactor Glc(1,6)P_2 (2 μM), Glc6*P* dehydrogenase (5 U/ml) and $NADP^+$ (1 mM). For assays in both directions, $MgCl_2$ was added to a total concentration calculated so as to give a free magnesium ion concentration of 1 mM, using the stability constants cited by Aksamit and Ebner (1972) for the Mg complexes of the reactants.

Preparation of antibody and dot-blot analysis. Antiserum against UDP-Glc*PP*ase was prepared by subcutaneously injection of a New Zealand rabbit with 25 μg homogeneous bovine liver enzyme in Freund's complete adjuvant (both were purchased from Sigma). The injection was repeated three times at 10-day intervals and blood was collected 2 weeks after the last injection.

Serial dilutions of the protein were transferred to nitrocellulose membranes (Hybond-C; Amersham) in a dot-blot apparatus (Life Technologies, Bethesda MD). The membrane was blocked in phosphate-buffered saline containing 2.5% bovine serum albumin, incubated with 1/500-diluted antiserum and developed with an anti-(rabbit IgG) serum coupled to alkaline phosphatase (Organon Teknika, Turnhout, Belgium).

Data analysis. Kinetic data were analysed using the nonlinear regression computer program DNRPEASY that is an adaptation of the DNRP53 program described elsewhere (Duggleby, 1984). The following equations were fitted to the data.
Michaelis-Menten kinetics:

$$v = V_m[S]/(K_m + [S]). \quad (1)$$

Competitive inhibition:

$$v = V_m[S]/\{K_m(1 + [I]/K_i) + [S]\}. \quad (2)$$

Ligand titration:

$$y = y_\infty + (1 - y_\infty)/(1 + [L]/K_l). \quad (3)$$

RESULTS

Wild-type. We have shown previously (Duggleby et al., 1996) that wild-type human liver UDP-Glc*PP*ase, expressed in *E. coli*, shows complex kinetic properties. The substrate saturation curves with respect to each of the substrates in the reverse direction, and with respect to Glc1*P* in the forward direction, are non-hyperbolic. However, replacement of the first nine residues with a 42-residue peptide derived from the pBluescript LacZ gene simplifies the kinetics greatly. We have used this fusion protein as the reference enzyme for investigating the properties of the mutants described below.

The kinetics of the wild-type fusion enzyme were assessed with respect to several properties. In both reaction directions, the enzyme displays simple Michaelis-Menten kinetics (Fig. 2) from which an apparent K_m for each substrate (Glc1*P* and MgUTP in the forward direction, and UDP-Glc and $MgPP_i$ in the reverse direction) were determined. Replicate determinations of each of these values were performed. In each of these experiments, the non-varied substrate was present at a fixed concentration that may not be fully saturating. For example, the K_m for Glc1*P* was determined with MgUTP at 1.0 mM, compared to the K_m for MgUTP of 0.563 mM. In those cases where the

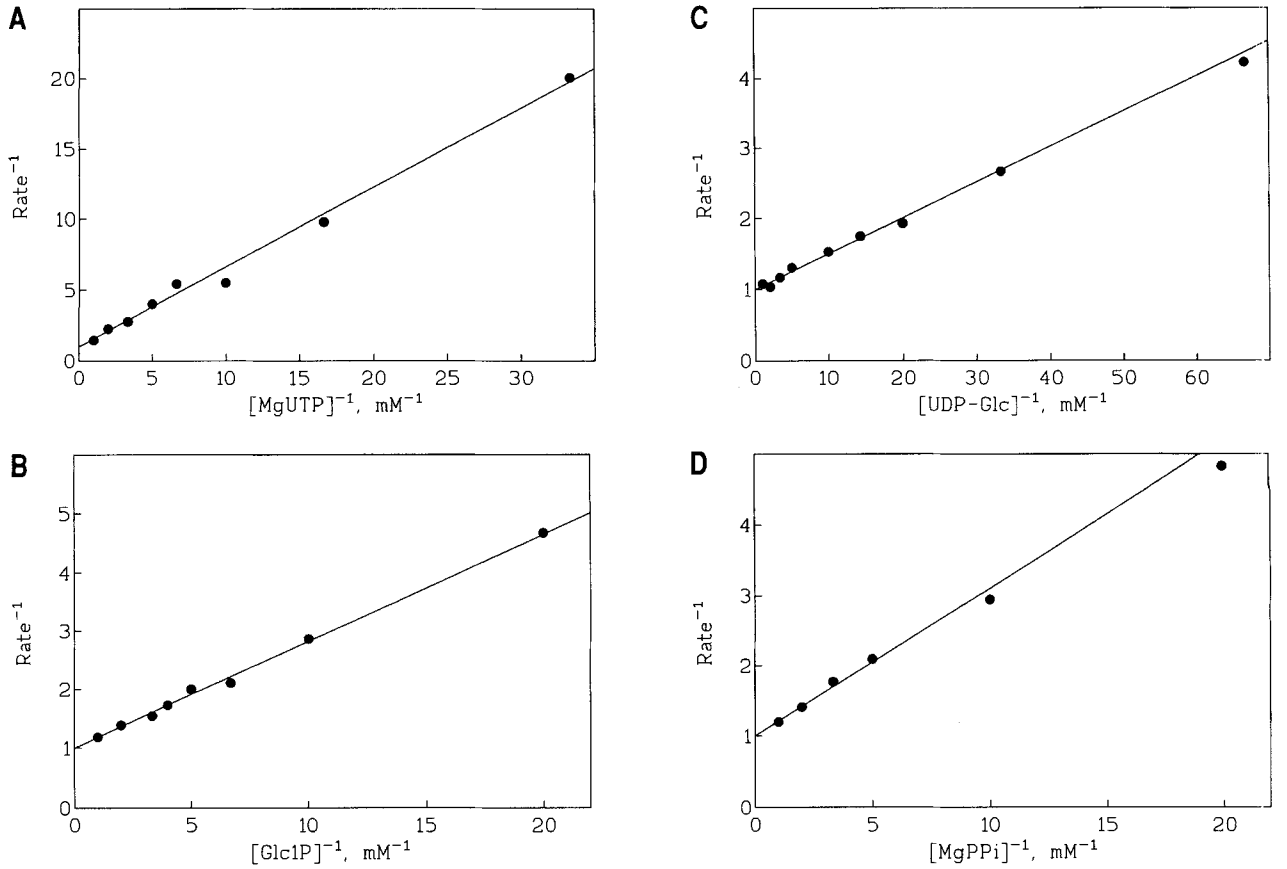

Fig. 2. Kinetics of wild-type human liver UDP-Glc*PP*ase with respect to its substrates. The rate of the forward reaction was measured as a function of (A) the MgUTP concentration, at a constant Glc1*P* concentration of 1 mM; or (B) the Glc1*P* concentration, at a constant MgUTP concentration of 1 mM. The rate of the reverse reaction was measured as a function of (C) the UDP-Glc concentration, at a constant $MgPP_i$ concentration of 1 mM; or (D) the $MgPP_i$ concentration, at a constant UDP-Glc concentration of 1 mM. In each case, results are shown as a double-reciprocal plot, with the rate expressed as a fraction of the maximum velocity and the lines representing the best fit of Eqn (1) to the data.

apparent K_m was likely to be affected by the concentration of the non-varied substrate, the true K_m was calculated by extrapolation.

When studied in the reverse direction, MgUTP is a product inhibitor that shows competitive inhibition with respect to UDP-Glc (Fig. 3); the K_i for this inhibition was determined. These measurements also yielded another estimate of the K_m for UDP-Glc.

From these experiments, several estimates of the extrapolated maximum velocity in each direction were also obtained. However, the individual values are not useful for comparing different mutants, as each will depend on the enzyme concentration which will vary for different preparations. In contrast, the ratio of maximum velocities should be independent of the enzyme concentration so this ratio was also determined. The kinetic properties of the wild-type fusion enzyme, as well as those of the mutants described below, are summarised in Table 1.

C123S. In view of the suggested role of a cysteine in the activity of UDP-Glc*PP*ase, we were surprised to find that mutating C123 to a serine resulted in an active enzyme. Moreover, there is no significant effect on the K_m for either substrate in the forward direction or that for UDP-Glc in the reverse direction. The K_i for MgUTP was approximately double that of wild type but the most notable difference was a sevenfold increase in the K_m for $MgPP_i$.

If C123 corresponds to the cysteine identified by Bergamini and Signorini (1991) as that responsible for conferring sensitivity of rabbit muscle UDP-Glc*PP*ase to sulfhydryl reagents, then the C123S mutant should be resistant to such compounds. This was confirmed; for example, the wild-type enzyme lost 56% activity upon incubation for 30 min with 0.1 mM iodoacetam-

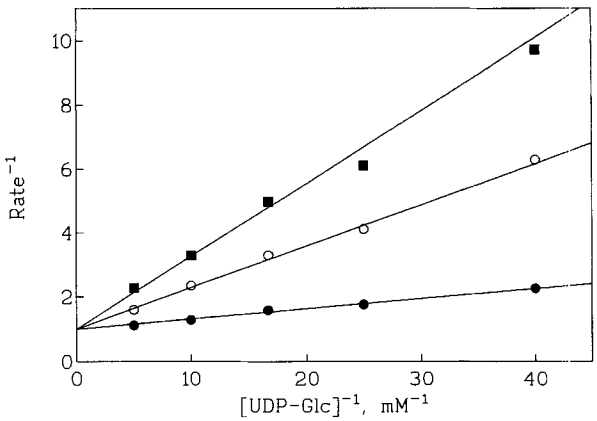

Fig. 3. Product inhibition of wild-type human liver UDP-Glc*PP*ase by MgUTP. The rate of the reverse reaction was measured as a function of the UDP-Glc concentration, at a constant $MgPP_i$ concentration of 1 mM and MgUTP concentrations of 0 (●), 1.5 (○) or 3.0 (■) mM. The results are shown as a double-reciprocal plot, with the rate expressed as a fraction of the maximum velocity and the lines representing the best fit of Eqn (2) to the data.

Table 1. Kinetic properties of wild-type and mutants of human liver UDP-Glc*PP*ase.

Parameter	Value for UDP-Glc*PP*ase						
	wild-type	C123S	H266R	W218S	R389H	R422Q	R445H
K_m (UDP-Glc)	51 ± 4	36 ± 2	47 ± 2	116 ± 6	55 ± 2	31 ± 0.2	43 ± 2
μM	55 ± 9	64 ± 9	39 ± 3	136 ± 14	44 ± 4	54 ± 5	52 ± 5
	31 ± 4			129 ± 9			41 ± 3
K_m (MgPPi)	210 ± 17	1337 ± 279	79 ± 3	121 ± 8	102 ± 7	65 ± 3	200 ± 17
μM	172 ± 25			166 ± 16			223 ± 18
K_m (Glc1*P*)	172 ± 11	224 ± 31	195 ± 25	136 ± 8	449 ± 64	74 ± 4	163 ± 13
μM	174 ± 30						
K_m (MgUTP)	563 ± 115	617 ± 38	365 ± 9	2083 ± 679	458 ± 32	138 ± 10	490 ± 97
μM	692 ± 243						
K_i (MgUTP)	477 ± 41	901 ± 65	381 ± 23	1774 ± 96	239 ± 17	411 ± 27	598 ± 30
μM							
V_{fwd}/V_{rev}	0.2602	0.1452	0.2388	0.1505	0.2878	0.3396	0.1764

ide, while C123S lost little or no activity under the same conditions.

H266R. Mutation of the single conserved histidine to arginine resulted in an active enzyme with little or no change in the kinetics towards the sugar-containing substrates, UDP-Glc and Glc1*P*. There is a small decrease in the K_m and K_i for MgUTP, and a somewhat larger decrease in the K_m for $MgPP_i$. Overall, these changes were relatively minor suggesting that H266 has no major role in enzymic activity.

The coding sequence of this mutant was cloned into the Novagen vector pET30 and expressed, resulting in the formation of a fusion protein containing an N-terminal polyhistidine tag. This protein was purified to homogeneity by metal ligand affinity chromatography. This form of the mutant protein was active although it exhibited some differences in kinetic properties from those reported in Table 1. In particular, the $MgPP_i$ saturation curve is sigmoidal; this is consistent with our previous suggestion (Duggleby et al., 1996) that the N-terminal region is involved in subunit interactions that are reflected in the kinetic properties. The other major difference noted was a fourfold decrease in the K_m and K_i for MgUTP.

The tryptophan fluorescence properties of this histidine-tagged form were investigated and it was observed that there was a 25% fluorescence decrease upon adding MgUTP. This decrease followed a simple hyperbolic curve (Fig. 4) with a half-saturating concentration of 163 μM, similar to the kinetic K_i of 95 μM.

Tryptophan mutants. Changing W218 to serine yielded an active enzyme that exhibits little or no change in the K_m for $MgPP_i$ or Glc1*P*. There are clear increases in the K_m for the nucleotide substrates (UDP-Glc and MgUTP) and a similar increase in the K_i for MgUTP. These results suggest that W218 may interact with the UDP portion of these substrates, possibly by ring-stacking with the uridine ring. This proposal would be consistent with the tryptophan fluorescence quenching by MgUTP that was mentioned above for H266R. However, considering the major change in the mutated side-chain in W218S, there must be many other interactions involved in binding of the nucleotide substrates.

If W218 is involved in substrate binding then W218S could have relaxed substrate specificity. This proved not to be the case; both wild-type and mutant enzymes had similar, and very low activity (less than 2% of that with UDP-Glc), using CDP-Glc or TDP-Glc as substrates. The results obtained for the wild-type enzyme are similar to those reported by Knop and Hansen (1970) for the native human liver enzyme.

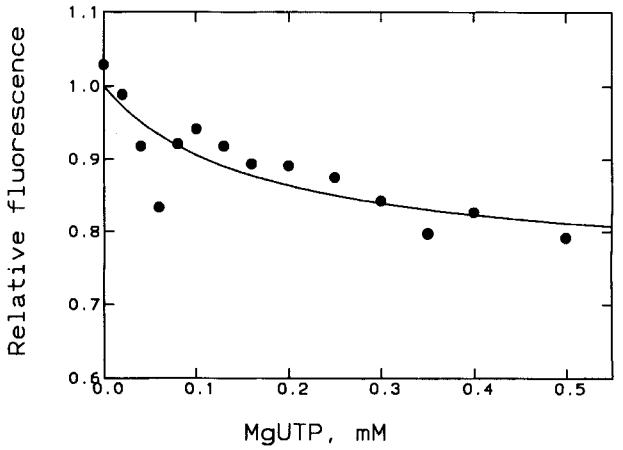

Fig. 4. Tryptophan fluorescence quenching of UDP-Glc*PP*ase by MgUTP. Fluorescence was measured at 337 nm, using an excitation wavelength of 300 nm at an enzyme concentration of 0.5 mg/ml. Measurements were made after successive additions of small volumes of 10 mM MgUTP. The line represents the best fit of Eqn (3) to the results, after normalising the fluorescence to a starting value of 1.0.

The W333S mutant was found to be totally inactive, a result that correlates with the observation that the protein was found to be in the insoluble phase of extracts of *E. coli* cells expressing this mutant form. Thus it appears that W333 is involved in folding of the protein and the radical substitution with serine disrupts this process. It would be of interest to examine the effect of conservative substitutions, such as phenylalanine or tyrosine, on the protein.

We attempted to obtain the protein in a soluble form by expressing it as a polyhistidine fusion protein but again found it to be both inactive and insoluble. Induction at lower temperatures failed to produce a soluble form. Addition of this histidine tag would not of itself be expected to impair folding, as judged by the effect of this modification on H266R mutant described above. The histidine-tagged W333S protein could be solubilised in 6 M urea and was purified to homogeneity by metal-ligand affinity chromatography. Upon slow removal of urea by dialysis, the protein precipitated and no recovery of activity was observed.

Arginine mutants. Expression of R391K gave similar results to those observed for W333S, i.e. an inactive and insoluble protein.

It is surprising that this conservative change has such a drastic effect on the enzyme. Mutation of each of the remaining conserved arginine residues gave an active enzyme in all cases. Each of these mutants shows unchanged kinetics towards UDP-Glc and, for R445H, there is little or no change in the affinity for any of the substrates. For R389H, there is a halving of the K_m for $MgPP_i$ and the K_i for MgUTP, and a 2.6-fold increase in the K_m for Glc1*P*. In the case of R422Q there is a two–threefold decrease in the K_m for $MgPP_i$, Glc1*P* and MgUTP, but little effect on the K_i for MgUTP.

Maximum velocities. The relative values of the forward and reverse maximum velocities (Table 1) appear to fall into two distinctive groups. H266R, R389H and R422Q are each similar to wild type with a V_{fwd}/V_{rev} ratio of approximately 0.3. This value is similar to that reported for the human erythrocyte enzyme (Tsuboi et al., 1969). For the three remaining mutants (C123S, W218S and R445H) this ratio was approximately 0.15, a value that is half of that observed for the wild-type enzyme.

These measurements do not detect possible effect of the mutations on the catalytic capacity of UDP-Glc*PP*ase. Direct quantitation of k_{cat} could not be performed since the enzyme preparation used for these studies was partially purified only. However, there are two lines of evidence to suggest that none of the mutants (apart from the inactive W333S and R391K) had major impairments of catalytic function. First, the activity of cell extracts of *E. coli* expressing the mutants was similar to that of the wild type. Since induction conditions were the same, the amount of UDP-Glc*PP*ase protein is expected to be comparable, arguing for similar specific activities. Second, we made semi-quantitative estimates of the amount of UDP-Glc*PP*ase in the partially purified preparations by immunoassay. Using antibody raised against the pure bovine enzyme, dot blots were performed and the intensity of these blots compared by eye with a series of standards composed of the bovine enzyme. From these measurements, the wild-type specific activity of 133 U/mg was found to be similar to that of the mutants, which ranged from 40 U/mg (R445H) to 392 U/mg (R422Q). These variations are of the same magnitude as the estimated uncertainty (a factor of approximately three) in visual evaluation of blot intensity. While we cannot rule out some changes in catalytic efficiency, it is clear that none of the mutants is substantially different from wild-type in this respect.

DISCUSSION

In this study we have mutated several conserved residues of human liver UDP-Glc*PP*ase. These include the single conserved cysteine and histidine, both conserved tryptophans, and all four conserved arginine residues. Two of these mutants (W333S and R391K) are totally inactive and this appears to be due to defective folding rather than a direct effect on substrate binding or catalysis. Unless these mutants can be expressed in a soluble form, or the insoluble form refolded, it will not be possible to draw any conclusions about whether these residues are important for activity *per se*.

Each of the remaining mutants is active and none shows any drastic change in the kinetic properties. The most substantial alteration is the sevenfold increase in the K_m for $MgPP_i$ exhibited by the mutant C123S, followed by the fourfold increase in K_m and K_i for MgUTP in the W218S mutant. These results strongly suggest that none of the residues targeted for mutagenesis in this study plays any major role in the function of the enzyme.

One class of residues that we have not investigated here is that with carboxylate side-chains. These may be important in view of the observation (Signorini et al., 1989) that the rabbit muscle enzyme is inactivated upon chemical modification of a single carboxylate. There are 12 such residues conserved in the alignment shown in Fig. 1, and a further seven for which the residue is either glutamate or aspartate. Before undertaking mutagenesis studies, it would be advantageous to reduce the number of possibilities to be investigated by identifying the labelled peptide.

The most interesting result reported here, in view of the purported role of a cysteine in UDP-Glc*PP*ase activity (Bergamini and Signorini, 1991) is that C123S is active. It appears, therefore, that it is the attachment of a bulky substituent by chemical modification, rather than a specific requirement for the thiol moiety, that results in inactivation. While this may argue for C123 being located near the active site, conformational changes transmitted through the protein from a remote site cannot be ruled out. Other mutational changes at C123 are unlikely to distinguish between these possibilities and determination of the three-dimensional structure is the most definitive way to settle this question. As far as we are aware, such studies are not currently underway, despite the fact that the enzyme was first crystallised 30 years ago (Albrecht et al., 1966). Possibly the size of the subunits and the octameric organisation (Levine et al., 1969) has inhibited crystallographers from undertaking structure determination.

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