PATHS TO PYRIMIDINES

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Editor's Corner

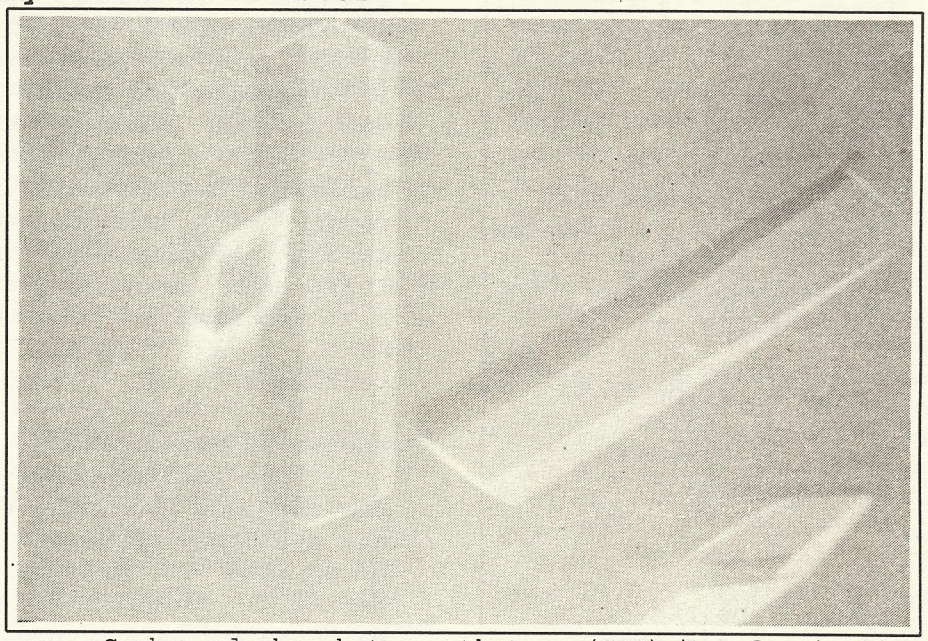
Happy New Year!

Last year we only got out two issues of the newsletter. The goal for this year is three issues scheduled for January, May and September. Please feel free to submit articles, research up-dates, and announcements. For the May 1995, September 1995 and January 1996 issues, I need to receive your material by April 1st, August 1st and December 1st, respectively. If you have figures that are computer generated, please send the file on disk. Scanned figures are often of poor quality.

Inside

Structural and Mechanistic Investigations of Carbamoyl Phosphate Synthetase from E. coli

by Frank M. Raushel



Carbamoyl phosphate synthetase (CPS) is a fascinating enzyme that catalyzes the following reaction:[1]

$2ATP + HCO_3^- + Glutamine + H_2O$ -----> $2ADP + P_i + Glutamate + Carbamoyl\text{-}P$

Monovalent and divalent cations are required for enzymatic activity[2] and ammonia may replace glutamine as the nitrogen source.[3] The carbamoyl phosphate produced in this reaction is subsequently utilized in two separate biosynthetic pathways. The molecule may react with either aspartate, in a reaction catalyzed by aspartate transcarbamoylase, for the eventual synthesis of pyrimidine nucleotides. Or, alternatively, the carbamoyl phosphate reacts with ornithine for the subsequent synthesis of arginine and/or urea. In lower organisms a single enzyme catalyzes the synthesis of carbamoyl phosphate for both pathways whereas in higher organisms there is a separate enzyme for each pathway.

Regulation

Since the product of the reaction catalyzed by CPS is at the branch point for two separate biosynthetic pathways, the catalytic activity of this enzyme is highly regulated.[3] The enzyme from *E. coli* is activated by ornithine (the first substrate in the arginine/urea pathway) and inhibited by UMP (the ultimate product in the pyrimidine pathway).[3]

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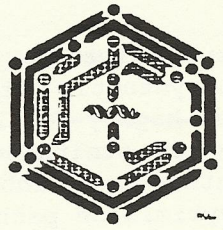


CPS is regulated by a different array of metabolites depending on the specific organism. For example, the trifunctional CAD protein from mammals is regulated by UTP and PRPP whereas the mammalian CPS utilized for arginine biosynthesis requires N-acetylglutamate for catalytic activity.[4-5] Preliminary experiments indicate that these allosteric regulators modify the activity of the enzyme primarily by alteration of the K_m for ATP. In *E. coli*, ornithine and IMP decrease the K_m of ATP whereas UMP increases the K_m of ATP.[6] The physical basis for the mechanism of regulation is not understood at the molecular level but it appears not to require oligomerization of the protein to dimers or tetramers.[7]

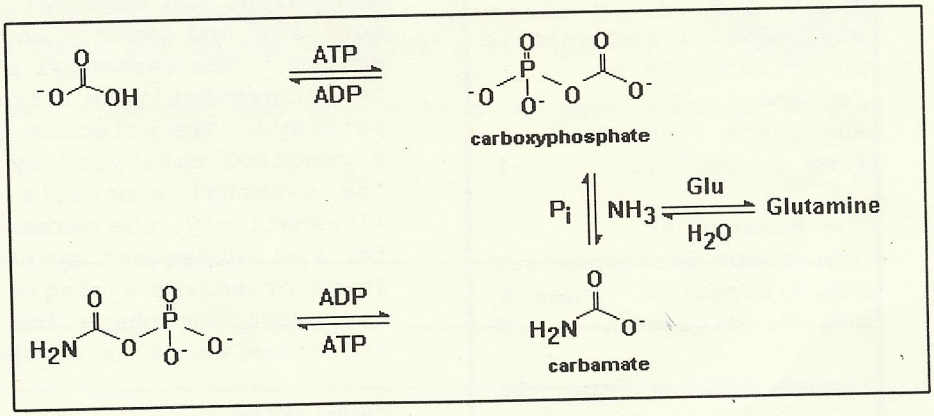
Mechanism of Action.

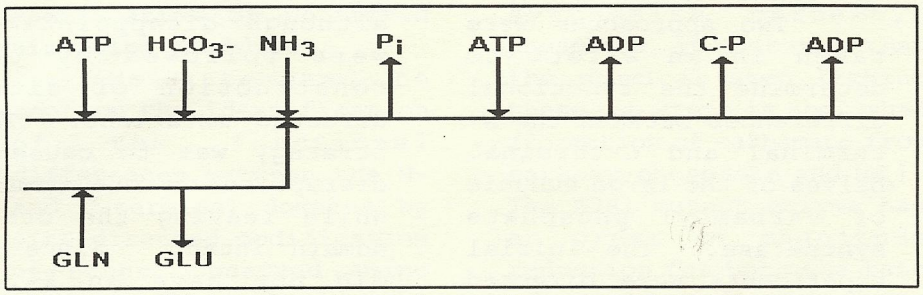
The working model of the chemical mechanism for the synthesis of carbamoyl phosphate is presented below.[1] Experimental evidence for this mechanism has been obtained from the observation of three partial reactions catalyzed by this enzyme[3] and subsequent positional isotope exchange (PIX) experiments.[8-10] The three partial reactions catalyzed by this enzyme include a bicarbonate-dependent hydrolysis of ATP in the absence of a nitrogen source, synthesis of ATP from ADP and carbamoyl-P, and the hydrolysis of glutamine.[11] There are two postulated chemical intermediates in this mechanism, *carboxyphosphate* and *carbamate*. The formation of the carboxyphosphate intermediate is consistent with the partial ATPase reaction noted above and the transfer of oxygen-18 from the bicarbonate to the product, P_i.

In addition to these intermediates the mechanism for the hydrolysis of glutamine has been proposed to involve the formation of a thioester intermediate with an active-site cysteine residue.[11] The primary evidence for this postulate has come from the inactivation of the glutamine-dependent activity upon reaction with a chloroketone derivative of glutamine[12] and site-directed mutagenesis of cysteine-269.[13]

Kinetic Studies

The steady state kinetic mechanism has been determined for the enzyme from *E. coli*.[2] These studies have demonstrated that the order of addition and release of products is sequential. ATP binds first, followed by the





addition of bicarbonate and ammonia, phosphate is released, the second ATP binds and then ADP, carbamoyl-P, and the last ADP are released in that order. These results are summarized above using the Cleland notation. This kinetic mechanism is fully consistent with all of the partial reactions catalyzed by this enzyme and it furthermore demonstrates that the two molecules of ATP used in the overall reaction must occupy separate binding sites. This last conclusion has been supported by active site labelling studies with FSBA (a chemically reactive ATP analog) which showed two moles of label per mole of inactivated protein.[14]

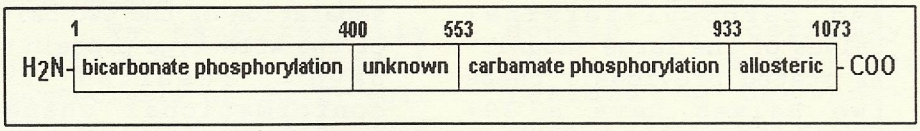
The steady state turnover of the *E. coli* enzyme is rather slow (~4/second) and it is unknown what steps actually limit the overall reaction. A rapid-quench analysis of the ATPase partial reaction indicated that formation of the carboxy-phosphate intermediate was fast relative to the net turnover of ATP.[15] No detailed kinetic studies have been conducted on the hydrolysis of glutamine. Experiments by Meister's laboratory have demonstrated that a complex of enzyme and glutamine can be precipitated with acid.[11] These studies have been confirmed by Lusty and may indicate that the thioester intermediate can be trapped[16].

Structural Studies

The carbamoyl-phosphate synthetase from *E. coli* is a heterodimer.[17] The large and small subunits of this enzyme have molecular weights of 118,000 and 42,000, respectively. The enzymes from other organisms have somewhat different molecular structures. For example, in the rat liver enzyme the two domains are fused into a single polypeptide chain.[18] The mammalian enzyme, utilized for the biosynthesis of pyrimidine, is similar but it is also fused to aspartate transcarbamoylase and dihydroorotase.[19] Physical separation of the two subunits comprising the *E. coli* enzyme has demonstrated that the small subunit catalyzes the hydrolysis of glutamine while the large subunit catalyzes everything else and, in addition, contains the binding sites for all of the allosteric activators.[20] Separation of the two subunits can be accomplished either by partial denaturation of the protein followed by size exclusion chromatography and renaturation or, alternatively, by expression of the genes on separate plasmids. It is unknown how catalytic turnover at the active sites on these two subunits are coordinated but it is known that the ammonia produced on the small subunit does not dissociate into solution before reaction with the phosphorylated intermediate.[21]

Genetic Studies

Carol Lusty has isolated, cloned, and sequenced the genes encoding for carbamoyl phosphate synthetase from *E. coli*.[22] The *car*A and *car*B genes code for the small and large subunits, respectively. The sequences have now been determined for hamster, rat, slime mold, yeast, fruitfly, and *B. subtillus*.[23-24] The sequences have proven to be quite interesting. The small subunit has been found to have a high degree of sequence identity with the *trp*G-type amidotransferases.[25] This is a class of enzymes that utilizes glutamine to deliver ammonia to an activated reaction center. Enzymes in this class include anthranilate synthetase and CTP synthetase. The large subunit has been found to contain a large tandem repeat. The amino acid sequence that spans residues 1-400 is 39% identical to the region which encompasses residues 553-933. This observation has suggested that the present day enzyme arose via a duplication and fusion of an ancestral gene. The 150 amino acid linker regions are not homologous to each other. Recent experiments in our own laboratory have indicated that the two homologous domains each contain a binding site for one of the two ATP molecules used in the overall biosynthetic



reaction. The N-terminal half uses ATP for the phosphorylation of bicarbonate while the C-terminal half uses ATP to phosphorylate carbamate.[26] Rubio has provided evidence to indicate that the terminal 150 amino acids contain the binding site for the allosteric activator UMP.[27] The precise location of the active site(s) within these regions is unknown. In the folded molecule the active sites may all be contiguous or alternatively the intermediates may have to diffuse through channels as has been observed in the three-dimensional structure of tryptophan synthetase.[28]

Dissection of Functional Domains

Our own efforts with carbamoylphosphate synthetase have recently focussed on mapping of the active sites and identification of specific amino acids critical for the catalytic and regulatory properties of this enzyme. This has been accomplished by advanced kinetic analyses of the wild-type and mutant proteins. The initial objective was to identify the functional differences between the amino-terminal and carboxyl-terminal halves of the large subunit. As stated previously these two halves have a 39% sequence identity and thus the folded tertiary structures of these two domains are expected to be very similar. Our initial hypotheses were that either each half of the protein has an ATP binding site (one for each ATP used in the overall reaction) or, alternatively, that one half contained the catalytic components while the other half was involved primarily in regulation.

Two approaches were taken in an effort to determine the functional differences between the N-terminal and C-terminal halves of the large subunit of carbamoyl phosphate synthetase. The initial experiments were directed at the construction of chimeric or truncated proteins containing only defined portions of the full length protein. A truncated protein containing amino acids 1-556 (N-protein) was made by deletion of an *Acc*I restriction fragment (followed by religation) from the plasmid containing the gene for the *E. coli* carbamoyl phosphate synthetase. This procedure created a new stop codon immediately following the codon for amino acid 556. The truncated protein was over-expressed, purified, and found to catalyze *none* of the partial or full reactions exhibited by the wild-type protein. The truncated protein, containing only those amino acids for the C-terminal half, was constructed by a similar procedure but again no catalytic activities were observed. Since the N- and C-terminal fragments were inactive it was felt that separation of the two halves of the wild-type protein may have resulted in conformational changes which destroyed any potential catalytic activity. Alternatively, the catalytic and regulatory components may have shared residues from both domains. Nevertheless, we constructed a tandem repeat of the N-terminal half in an effort to overcome the first possibility. The N-N tandem repeat protein was found to possess no catalytic activity.[29]

These experiments, although disappointing, were followed by the construction of site-directed mutations. The strategy was to cause a disruption on one domain while leaving the other domain intact. Since we did not have an X-ray crystal structure to guide our mutagenesis targets we relied on previous observations which have indicated that there is a glycine rich loop in the nucleotide binding site of many proteins which use ATP as a substrate.[30-31] Two such glycine-rich regions were identified within the large subunit of CPS. The conserved glycine residues at positions 176, 180, and 722 were individually mutated by site-specific mutagenesis to isoleucine residues. The mutants were overexpressed, purified to homogeneity and kinetically characterized. For all three mutations the overall rate of carbamoyl phosphate synthesis was reduced by greater than 10-fold.[26] The two mutant proteins on the N-terminal half (G180I and G176I) were also defective in the bicarbonate dependent ATPase reaction while the ATP synthesis reaction was not disrupted. Conversely, the mutant protein on the C-terminal half (G722I) was defective in the ATP synthetase activity while the ATPase activity occurred at wild-type rates. These results were interpreted to suggest that the N-terminal domain is primarily responsible for the phosphorylation of bicarbonate in the formation of carboxyphosphate while the C-terminal domain is primarily responsible for the phosphorylation of carbamate for the formation of carbamoyl phosphate.

Modification of Conserved Histidine Residues

We attempted to confirm the identification of the functional differences between the N- and C-terminal domains by site-directed modification of other conserved amino acids. The next logical choice was histidine because of the 15 histidine residues in the large subunit of the *E. coli* CPS only three of these residues are fully conserved. Histidine residues can participate directly in the catalytic process via acid/base catalysis, complexation of metal ions and hydrogen bonding to substrates. Histidine residues were also deemed potentially important since diethylpyrocarbonate (a histidine specific chemical modification reagent) was shown by us to inactivate all of the partial activities catalyzed by the *E. coli* CPS. Two of the conserved histidine residues (H781 and H788) are located on the C-terminal domain while the other conserved histidine (H243) is located within the N-terminal domain (H243 and H788 are homologous residues in the sequence alignment). We mutated these three conserved histidine residues to asparagine. [32] The H243N mutant protein was expressed, purified to homogeneity and kinetically characterized. This protein is unable to synthesize carbamoyl phosphate at a significant rate but the activities of the two partial reactions involving ATP (bicarbonate dependent ATPase and ATP synthesis reactions) are catalyzed at wild type rates. Since both steps involving ATP are fully functional, but the enzyme is unable to synthesize carbamoyl phosphate, it must mean that the chemical step linking these two steps is abolished (formation of carbamate from carboxy-phosphate and NH_3). The H781 mutant enzyme has an order of magnitude reduction for both the rate of carbamoyl phosphate formation and ATP synthesis which is consistent with the proposal that the C-terminal half of the large subunit is primarily involved in the phosphorylation of the carbamate intermediate. The modification of the third histidine residue (H788) results in a mutant with somewhat different properties. This mutant (H788N) catalyzes the synthesis of carbamoyl-phosphate essentially at wild-type rates but the K_m for the nucleotide involved in the phosphorylation of carbamate has been increased by about an order of magnitude. These selective mutations of conserved glycine and histidine residues have specifically impaired each of the three chemical reactions catalyzed by the large subunit of CPS.

Partial Proteolysis of Carbamoyl Phosphate Synthetase

We have recently utilized partial proteolysis of the *E. coli* CPS to probe for fragments that may be catalytically active. Incubation of CPS with trypsin for short periods of time provides the following results. A 105 kDa polypeptide is observed that is formed by cleavage of a 13 kDa fragment from the C-terminal end of the holoenzyme. After formation of the 105 kDa polypeptide, an 80 kDa fragment is formed via cleavage of a 25 kDa peptide from the C-terminal end. The 80 kDa polypeptide is then cleaved at residue 373 to form two fragments of molecular weight 45 kDa and 35 kDa. The 35 kDa fragment then undergoes further cleavages at the C-terminal end to form fragments of molecular weights 32,000 and 31,000. The small subunit is, however, quite resistant to proteolysis. After the large subunit is completely degraded the small subunit is cleaved at residue 93 to form a 32 kDa fragment that is still catalytically active (Shadreck Mareya unpublished experiments). Proteolysis experiments have also been initiated with endoproteinase asp-N. The initial cleavage sites are at the C-terminal end of the protein but these large truncated fragments are catalytically active. These studies demonstrate that the extreme C-terminal end of CPS is not required for catalytic activity and are consistent with other proposals that indicate that this region of the large subunit contains the binding sites for the allosteric regulators[27].

Active Site Mutagenesis of Small Subunit

The amidotransferase domain of carbamoyl phosphate synthetase resides on the small subunit. Previous evidence has been provided to support the proposal that the formation of NH_3 from glutamine involves a thioester intermediate with Cys-269.[11] Thus, the amide bond would be hydrolyzed using a mechanism very similar to papain. We have attempted to identify the active site residues that participate in proton transfers and binding by mutating all four of the

conserved histidine residues to asparagine.[33] The conserved histidines are found at positions 272, 312, 341, and 353. The H272N and H341N enzymes were found to be identical to the wild-type enzyme and thus these residues are not essential for catalytic activity. The H353N protein was unable to catalyze the hydrolysis of glutamine and thus this histidine is probably involved in proton transfers. This histidine is also conserved in all other *trp*G-type amido-transferases. However, glutamine was still able to bind to the active site of this mutant since we were able to observe an enhancement of the bicarbonate-dependent ATPase activity upon addition of glutamine to the assay mixtures. This result demonstrated that H353 contributes very little to the binding of the substrate and that binding of ligands to the small subunit causes alterations in the reactivity of the large subunit. Modification of H312 to asparagine results in a protein with an elevated K_m for glutamine (100-fold) but no significant change in the V_{max}. In addition, the acid side of the bell-shaped pH rate profile for V/K_{gln} has disappeared. These results have suggested that H312 is involved primarily in the binding of glutamine to the small subunit.

In collaboration with Carol Lusty of the Public Health Research Institute we have made a kinetic evaluation of the mutants which she has constructed for the replacement of the active site cysteine residues with either glycine or serine. As expected, no glutaminase activity was observed with these mutants but unexpectedly the bicarbonate-dependent ATPase activity of large subunit increased by over an order-of-magnitude.[34] The origin of this dramatic increase in reactivity for the hydrolysis of ATP was probed using the positional isotope exchange (PIX) technique. We reasoned that if the observed increase in ATP turnover was due to a *stabilization* of the free energy level of the transition state for the reaction of water with carboxyphosphate then we would observe a diminution in the PIX rate relative to the chemical rate for product formation. However, if the increase in the ATPase reaction was due to the *destabilization* of the ground state for enzyme bound carboxyphosphate (or CO_2) then there would be no change in the relative PIX rate compared to the wild type enzyme since both the PIX rate and the ATPase rate would be altered by exactly the same factor. We found no difference in the ratio of the PIX rate relative to the k_{cat} for the ATPase reaction compared to the wild-type protein and thus modification of cysteine-269 on the small subunit has altered the free energy profile by destabilization of the ground state for the carboxyphosphate intermediate[35].

Allosteric Regulation

In collaboration with Professor Gregory D. Reinhart of the University of Oklahoma we have quantified the effects of the allosteric ligands UMP, IMP, and ornithine on the partial reactions catalyzed by *E. coli* carbamoyl phosphate synthetase. Both of these partial reactions, a bicarbonate-dependent ATPase reaction and a carbamoyl-P dependent ATP synthesis reaction, follow bimolecular ordered sequential kinetic mechanisms. The initial velocity kinetic studies for the ATP synthesis reaction indicate that ADP binds before carbamoyl-P in an equilibrium ordered mechanism except in the presence of ornithine. We have found that IMP has little effect on the overall reaction of either of these two partial reactions. UMP and ornithine, which have a pronounced affect on the apparent K_m for ATP in the overall reaction, both substantially change the thermodynamic dissociation constant for ADP from the binary E-ADP complex, K_{ia}, in the ATP synthesis reaction, with UMP increasing K_{ia} 15-fold and ornithine decreasing K_{ia} by 18-fold. By contrast, only UMP substantially affects the K_{ia} for ATP in the ATPase reaction, increasing it by 5-fold. These effects are independent of the concentration of the unaffected substrate carbamoyl-P, in the AT synthesis reaction and bicarbonate in the ATPase reaction, and do not reflect the influence that the allosteric ligands have on the K_m for nucleotide in each case. The UMP effect on the ATPase reaction and the ornithine effect on the AT synthesis reaction derive primarily from changes in the off-rate constant for the nucleotide.

Crystallization of Carbamoyl Phosphate Synthetase

In collaboration with Professor Ivan Rayment and Hazel Holden of the Institute for Enzym

Research, University of Wisconsin-Madison we have crystallized CPS in a form that is suitable for high resolution x-ray diffraction studies.[36] The crystals belong to the orthorhombic space group $P2_12_12_1$ with unit cell dimensions of a = 154 Å, b = 167 Å and C = 339 Å and diffract to beyond 2.8 Å resolution. This is a very exciting development. The solution of the 3-dimensional crystal structure will enable us to identify the site of interaction between the large and small subunits and the symmetry relationship between the two homologous halves of the large subunit. The molecular mechanism for the allosteric control should also become clearer. Perhaps Professor Davidson will ask us to write another review when the structure of carbamoyl phosphate synthetase is finally solved.

Acknowledgements

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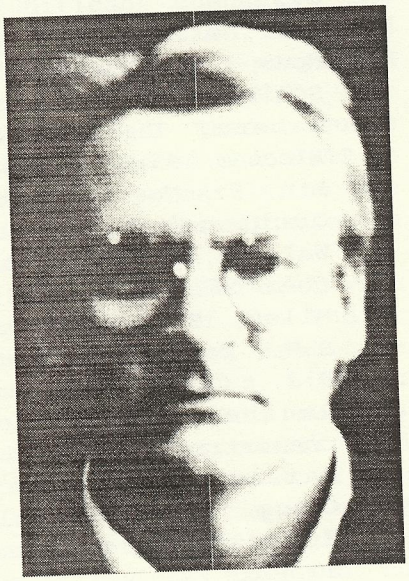
Frank M. Raushel received his undergraduate training in chemistry at the College of St. Thomas located in St. Paul, Minnesota. After graduation in 1972 he joined the laboratory of W. W. Cleland at the University of Wisconsin. His graduate studies were directed at the characterization of fructo-kinase from bovine liver. In 1976 he moved to the Chemistry Department of Penn State University for a postdoctoral position in the laboratory of Joseph J. Villafranca and started to investigate the structure and mechanism of carbamoyl phosphate synthetase. He accepted a junior faculty position in 1980 at Texas A&M University where he is now Professor of Chemistry. (Dept. of Chemistry, Texas A&M University, College Station, TX 77843). His laboratory is investigating the mechanism of phosphotriesterase and the folding pathways of ribonuclease T1 in addition to the work with CPS.



The Human UDP-glucuronosyltransferases: Detoxification, Genes and Genetic Disease.

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Summary

The UDP-glucuronosyltransferases (UGTs) are a family of microsomal enzymes responsible for the detoxification of a wide range of endogenous substrates (such as bilirubin and steroids) and foreign or xenobiotic compounds (drugs and pesticides). Detoxification is achieved by conjugation to a hydrophilic sugar group (glucuronic acid) from the nucleotide sugar donor, UDP glucuronic acid (UDPGA). The glucuronide formed (a β-D-glucopyranosiduronic acid) is generally more water soluble than the aglycone, and can be excreted from the body.

Multiple genes encoding UGTs have been isolated from several species and at least 20 different isoenzymes are encoded in the human. In man 12 of these isoforms appear to be expressed by alternative splicing from a single gene which is over 200 kilobases long. Each UGT enzyme is capable of glucuronidating a specific range of substrates, hence the UGTs have the potential to detoxify thousands of potentially harmful compounds in the body. The most critical detoxification role played by the UGTs in man is the elimination of bilirubin. Crigler-Najjar Syndrome is the result of a genetic-defect in bilirubin UGT causing a severe and lethal hyperbilirubinaemia.

The level of expression of each UGT is under complex control which is determined by developmental stage, tissue type and exposure to environmental inducing agents such as cigarette smoke.

Introduction to detoxification

Since life began, organisms have exploited their environment whilst defending themselves from chemical attack. As organisms evolved from single cells to a multicellular state, the number of endogenous compounds synthesized has dramatically increased. Many endobiotics have potentially toxic breakdown products or are toxic when ingested by other organisms. As a means of chemical defence, organisms have evolved complex two-step detoxification systems. In phase I detoxification the substrate is 'activated' by oxidation, hydration, reduction or isomerization. The active intermediate is further metabolized in a phase II reaction, in which conjugation to a polar hydrophilic group forms a less toxic and more water