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PROTEIN REPAIR AND INBORN ERRORS OF METABOLISM

The study of the mechanism of formation of an intriguing phosphate ester, fructose 3-phosphate, led us to discover fructosamine 3-kinase, an enzyme serving to remove sugar adducts from proteins. Part of our effort is now devoted to understanding the function of 'fructosamine-3-kinase-related protein', an enzyme that apparently serves to repair ribulosamines. Besides this, our group aims also at elucidating the cause of inborn errors of metabolism. In this respect, a lot of efforts have been made to identify new enzymes. Furthermore, the elucidation of the cause of a metabolic disease, L-2-hydroxyglutaric aciduria, brought us to study another form of repair, 'metabolite repair'.

PROTEIN DEGLYCATION

M. Veiga-da-Cunha, Y. Achouri, E. Van Schaftingen

Free sugars spontaneously react with amino groups of proteins to form Schiff bases, which rearrange to become Amadori products. The Amadori product that is formed when glucose reacts resembles fructose and is therefore named fructosamine. A few years ago, we uncovered an enzyme, fructosamine 3-kinase (FN3K), that phosphorylates the third carbon

of fructosamines, making them unstable and causing them to detach from proteins. FN3K is therefore a protein repair enzyme, a conclusion that was supported by the finding that protein-bound fructosamines are significantly more abundant in FN3K^{-/-} mice than in control mice.

We have also identified several other enzymes that are potentially involved in protein deglycation. A first one is fructosamine-3-kinase-related protein (FN3K-RP; ref 2). This enzyme shares about 65 % sequence identity with FN3K and is encoded by a gene that is present next to the FN3K gene on human

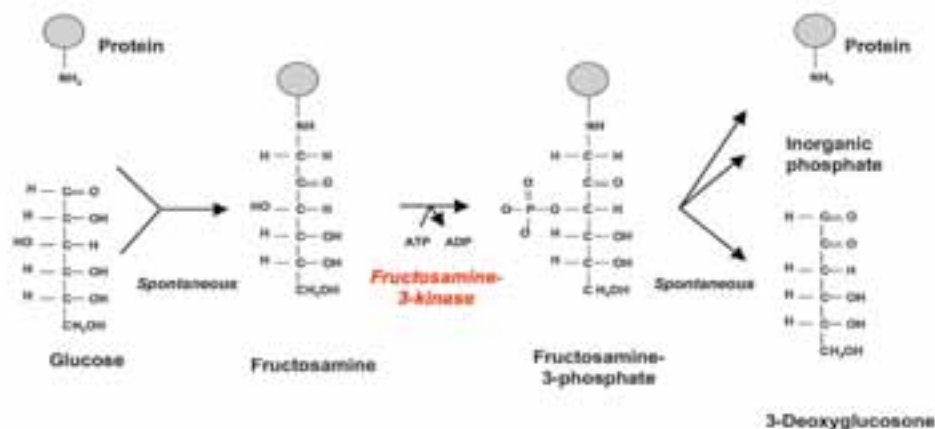


Figure 1. Formation and breakdown of fructosamines

chromosome 17q25. A similar gene arrangement is found in other mammals and in chicken, although not in fishes, indicating that a gene duplication event occurred during or after the fish radiation. FN3K-RP is also a ketoamine 3-kinase, acting best on ribulosamines and erythrosamines, but not at all on fructosamines. An enzyme with a similar substrate specificity is found in many fishes, in plants and in a significant proportion ($\approx 25\%$) of bacteria. All ketoamine 3-phosphates are unstable and their spontaneous decomposition regenerates the free amino group, indicating that FN3K-RP is also a protein repair enzyme (7).

It is unlikely that the physiological substrates of FN3K-RP are formed through a reaction of amines with free ribose or erythrose, because these sugars are present at very low concentrations ($< 10\ \mu\text{M}$) in tissues. They are most likely formed through a reaction of proteins with ribose 5-phosphate or erythrose 4-phosphate, two extremely potent glycation agents that react ≈ 80 and 500-fold more rapidly than glucose. The ribulosamine 5-phosphates (Fig. 2) and erythrosamine 4-phosphates that are formed from phosphorylated intermediates need to be dephosphorylated before being phosphorylated on their third carbon by FN3K-RP, and thereby destabilized and removed from proteins. The phosphatase catalyzing

this reaction has recently been identified as LMW-PTP (low-molecular-weight protein-tyrosine-phosphatase). One of our goals is to understand the physiological significance of FN3K-RP-mediated deglycation.

NEUROMETABOLIC DISORDERS

Y. Achouri, G. Noël, R. Rzem, M. Veiga da Cunha, E. Van Schaftingen

D- and L-2-hydroxyglutaric acidurias are distinct neurometabolic diseases characterized by the accumulation of abnormal amounts of either D- or L-2-hydroxyglutarate in cerebrospinal fluid, blood and urine. Work in our lab has led to the elucidation of the metabolism of these compounds (Fig. 2). Both of them are converted to α -ketoglutarate by distinct FAD-linked dehydrogenases. The dehydrogenase acting on L-2-hydroxyglutarate is bound to mitochondrial membranes and mutations in its gene are found in virtually all cases of L-2-hydroxyglutaric aciduria (7). The dehydrogenase acting on D-2-hydroxyglutarate is in the mitochondrial matrix and most likely transfers its electrons to the respiratory chain via electron-transfer-flavoprotein (1). It is mutated in about half of the patients with D-2-hydroxyglutaric aciduria.

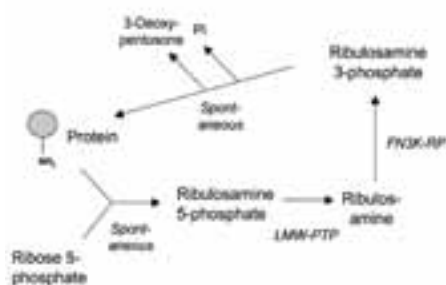


Figure 2. Formation and breakdown of ribosamines

The other half of the patients with D-2-hydroxyglutaric aciduria have a peculiar neomutation in isocitrate dehydrogenase 2 (3) (a mitochondrial enzyme). This mutation changes its catalytic activity: the mutated enzyme is no longer able to catalyse the oxidative decarboxylation of isocitrate to alpha-ketoglutarate, but will instead catalyse the NADPH-dependent reduction of alpha-ketoglutarate to L-2-hydroxyglutarate. This production exceeds the capacity of D-2-hydroxyglutarate dehydrogenase, thereby causing accumulation of D-2-hydroxyglutarate in body fluids. The neomutation had initially been found in different types of tumours. Remarkably, D-2-hydroxyglutaric aciduria, unlike L-2-hydroxyglutaric aciduria, does not seem to be associated with an increased cancer risk.

The formation of L-2-hydroxyglutarate is catalyzed by mitochondrial L-malate dehydrogenase (Fig. 3). This enzyme is not completely specific for oxaloacetate : it also reduces, at a very low rate, alpha-ketoglutarate to L-2-hydroxyglutarate. This activity is sufficient to account for the daily formation of L-2-hydroxyglutarate. Since L-2-hydroxyglutarate does not appear to have any role, but to have only toxic effects, L-2-hydroxyglutarate dehydrogenase is a ‘repair enzyme’ and L-2-hydroxyglutaric aciduria is a disorder of metabolite repair.

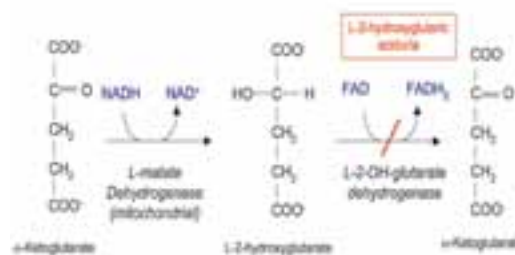


Figure 3. Formation and breakdown of L-2-hydroxyglutarate

We have recently produced a mouse model of L-2-hydroxyglutaric aciduria, which we are analysing in order to confirm the origin of L-2-hydroxyglutarate and the pathophysiological mechanisms of this disease.

IDENTIFICATION OF ENZYMES POTENTIALLY IMPLICATED IN METABOLIC DISEASES

F. Collard, C. Linster, A. Marbaix, G. Tahay, E. Wiame, G. Connerotte, K. Peel, M. Veiga-da-Cunha, E. Van Schaftingen

Synthesis of N-acetyl-aspartate

The brain-specific compound NAA (N-acetylaspartate) is the second most abundant organic molecule in brain. It occurs almost exclusively in neurons, where its concentration reaches ≈ 20 mM. Its abundance is determined in patients by MRS (magnetic resonance spectroscopy) to assess neuronal density and health. The molecular identity of the N-acetyltransferase that catalyses NAA synthesis has remained unknown, because the enzyme is membrane-bound and difficult to purify.

Using a database search approach we have identified its gene. Briefly, we have searched

the human and mouse genome for putative N-acetyltransferases that would be membrane-bound and exclusively expressed in brain. Two candidates were selected in this way (NAT8L and NAT14). They were expressed in HEK cells and NAT8L was shown to be the N-acetylaspartate-producing enzyme (10). A patient deficient in N-acetylaspartate was shown to have a homozygous 19 bp deletion in the coding sequence of the NAT8L gene, further proving that NAT8L is responsible for NAA production. In collaboration with D. Tyteca and P. Courtoy (CELL Unit), we also showed that this enzyme is associated with the endoplasmic reticulum, and not with mitochondria, as often stated previously. The molecular identification of this enzyme will lead to new perspectives in the clarification of the function of N-acetyl aspartate in neurons and for the diagnosis of hypoacetylaspartia in other patients.

Synthesis of N-acetylaspartylglutamate and beta-citrylglutamate

N-acetylaspartylglutamate (NAAG) is the most abundant dipeptide present in vertebrate central nervous system (CNS). β -citrylglutamate, a structural analogue of NAAG is present in testis and immature brain. The role of both compounds is still mysterious. Previous evidence suggests that NAAG is synthesized by an ATP-dependent ligase. As attempts to detect this ligase in brain extracts failed, we searched the mammalian genomes for putative enzymes that could catalyze this type of reaction. We found two putative ligases homologous to *Escherichia coli* RIMK, which ligates glutamates to the C terminus of ribosomal protein S6. One of them, named RIMKLA, is almost exclusively expressed in the CNS, while RIMKLB, which shares 65% sequence identity with RIMKLA, is expressed in the CNS and testis. Both proteins were expressed in bacteria or HEK293T cells and purified. RIMKLA catalyzed the ATP-dependent synthesis of N-acetylaspartylglutamate from N-acetylaspartate and L-glutamate. RIMKLB catalyzed this reac-

tion as well as the synthesis of β -citrylglutamate. The nature of the reaction products was confirmed by mass spectrometry and NMR. RIMKLA was shown to produce stoichiometric amounts of NAAG and ADP, in agreement with its belonging to the ATP-grasp family of ligases. The molecular identification of these two enzymes will facilitate progress in the understanding of the function of NAAG and β -citrylglutamate (1).

Formation of mercapturic acids

NAT8 shares about 30 % identity with NAT8L (aspartate N-acetyltransferase) is predicted to be membrane-bound and to be expressed exclusively in kidney and liver. Based on these features we hypothesized that it would correspond to the acetyltransferase that makes mercapturic acid (N-acetylcysteinyl-S-conjugates), catalysing thereby the last step in one of the major pathways of xenobiotic metabolism (9). This hypothesis was confirmed by expressing this protein in HEK cells and testing its enzymatic activity. In collaboration with D. Tyteca and P. Courtoy, we found that like NAT8L, NAT8 is associated with the endoplasmic reticulum thanks to a non-classical targeting signal that we are now characterizing. The NAT8 gene has recently been shown to be associated with chronic kidney diseases (Chambers et al. Nature Genetics, 2010). Because of the toxicity of non acetylated cysteinyl-S-conjugates, our identification provides a potential explanation for this association.

In relation with the metabolism of xenobiotics, we also carried out for the first time the molecular identification of omega amidase, the enzyme that hydrolyzes alpha-ketoglutarate, a product made by transaminases using glutamine as an alpha-amino group donor.

Carnosine synthase

Carnosine (beta-alanyl-L-histidine) is a most abundant (concentration ≈ 10 mM) dipeptide in muscle whereas homocarnosine (gamma-aminobutyryl-L-histidine) is the second most abundant dipeptide in brain of most vertebrates and some invertebrates. Their function is still not well established and the enzyme (carnosine synthase) that synthesizes them both was not well characterized and its molecular identity was unknown. To determine this identity, we have purified carnosine synthase from chicken pectoral muscle (2). We found that this enzyme hydrolyses ATP to ADP and inorganic phosphate and not to AMP and pyrophosphate, as previously assumed. Furthermore, by combining a database mining approach with a mass spectrometry analysis of the purified protein, we could show that carnosine synthase corresponds to a protein of unknown function named ATPGD1 in the databases. This was confirmed by expression and purification of human and mouse ATPGD1, which we found to catalyze the synthesis of both carnosine and homocarnosine (2). The identification of the gene encoding carnosine synthase will help getting a better understanding of the biological functions of carnosine and related dipeptides. Furthermore, it opens the perspective of testing if the low homocarnosine level found in the CSF of some patients is due to primary carnosine/homocarnosine synthase deficiency.

A specific pseudouridine 5'-phosphate phosphatase

We previously identified enzymes that metabolize pseudouridine, the fifth-most abundant nucleoside in RNA, and showed that these enzymes are not present in mammals (4). Pseudouridine is indeed not metabolized in mammals, but excreted intact in urine. We have recently identified a specific pseudouridine 5'-phosphatase, which presumably plays an important role in the formation of the free

nucleoside from RNA breakdown products. We found indeed that human erythrocyte extracts contain a pseudouridine-5'-phosphatase displaying a $K_m \leq 1 \mu\text{M}$ for its substrate. The activity of the partially purified enzyme was dependent on Mg^{2+} , and was inhibited by Ca^{2+} and vanadate, suggesting that it belonged to the 'haloacid dehalogenase' family of phosphatases. Its low molecular mass (26 kDa) suggested that this phosphatase could correspond to the protein encoded by the HDHD1 gene, present next to the STS (steroid sulfatase) gene on human chromosome Xp22. Purified human recombinant HDHD1 dephosphorylated pseudouridine 5'-phosphate with a catalytic efficiency that was at least 1000-fold higher than that with which it acted on other phosphate esters. The molecular identity of pseudouridine-5'-phosphatase was confirmed by the finding that its activity was negligible (<10% of controls) in extracts of B-cell lymphoblasts or erythrocytes from X-linked ichthyosis patients harbouring a combined deletion of the STS gene (the X-linked ichthyosis gene) and the HDHD1 gene. Furthermore, pseudouridine-5'-phosphatase activity was 1.5-fold higher in erythrocytes from women compared with men, in agreement with the HDHD1 gene undergoing only partial inactivation in females. In conclusion, HDHD1 is a phosphatase specifically involved in dephosphorylation of a modified nucleotide present in RNA (5).

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