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Review

Enzymatically Produced Trimethylamine N-Oxide: Conserving It or Eliminating It

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Abstract: Trimethylamine N-Oxide (TMAO) is the product of the monooxygenation reaction catalyzed by a drug-metabolizing enzyme, human flavin-containing monooxygenase 3 (hFMO3), and its animal orthologues. For several years, researchers have looked at TMAO and hFMO3 as two distinct molecules playing specific but separate roles, the former to defend saltwater animals from osmotic or hydrostatic stress and the latter to process xenobiotics in men. The presence of high levels of plasmatic TMAO in elasmobranchs and other animals was demonstrated a long time ago, whereas the actual physiological role of hFMO3 is still unknown because the enzyme has been mainly characterized for its ability to oxidize drugs. Recently TMAO was found to be related to several human health conditions such as atherosclerosis, cardiovascular, and renal diseases. This correlation poses a striking question of how other vertebrates (and invertebrates) can survive in the presence of very high TMAO concentrations (micromolar in humans, millimolar in marine mammals and several hundred millimolar in elasmobranchs). Therefore, it is important to address how TMAO, its precursors, and FMO catalytic activity are interconnected.

Keywords: flavoprotein; FMO; TMAO; protein folding; cardiovascular; osmolyte; monooxygenase; microbiome; enzyme catalysis; drug metabolism

1. Introduction

One of the key environmental elements affecting the health of human body is the food intake. Food enters our body and its transformation is essential for our health and sustainment. Only recently has increased knowledge of the symbionts living in our body, the gut microbiota, supported their fundamental role in the metabolic processing of food [1]. The microbial community of the gut is very diverse, highly susceptible to dietary exposure, and its composition can deeply affect metabolic pathways yielding to various disorders like obesity [1], atherosclerosis [2], or cardio-renal dysfunction [3]. A precise metabolic role was attributed to the gut microbiota in the transformation of carnitine and choline (abundant molecules in animal derived food) to trimethylamine (TMA). TMA is oxidized (Scheme 1) to trimethylamine N-Oxide (TMAO) by flavin-containing monooxygenase 3 (hFMO3) in the liver.

Scheme 1. Conversion of trimethylamine to trimethylamine N-Oxide catalyzed by hFMO3.

TMAO can be introduced directly through diet. Historically TMAO has been first described as a major component of plasma in elasmobranchs, and subsequently explained as a component together

with urea of the high osmolarity [4] required by these fish that are not osmoregulators like osteichthyes. It is therefore found in high concentrations in seafood products [5], or it can be synthetized from its precursors that are very abundant in western diets, based on animal-derived product rich in saturated fats, refined carbohydrates and low in fibers. Several studies underline the possible direct correlation between high plasma levels of TMAO and acute or chronic pathological conditions. In this scenario, FMO3 enzymes and TMAO seem to be directly correlated to obesity and other metabolic dysfunctions like insulin resistance, diabetes, cardiovascular diseases (CVD), and chronic kidney disease (CKD) that are common conditions in metabolic syndrome. Recent studies have demonstrated a possible association of TMAO in the human body to atherosclerosis and cardiovascular disease [6–15]. In this work, we focus mainly on hFMO3 as a catalyst providing a new outlook for the enzyme. The hFMO3 enzyme is presented not only as a drug-metabolizing enzyme, but also as a possible protein folding hub of the endoplasmic reticulum.

Special attention is dedicated to the main substrate and product of the enzyme, TMA and TMAO, which are directly involved in protein stability and pathogenesis. The overall view aims at providing new perspectives for the role of FMO3 and its metabolite TMAO in cells and organisms.

2. Flavin-Containing Monooxygenases

2.1. Classification

Flavoprotein monooxygenases are able to participate to many different chemical reactions [16]. For this reason, it becomes crucial to classify each new protein and understand how it compares to a database of known enzymes. The classification of flavoprotein monooxygenases can be carried out on the basis of several parameters: structural information, substrate specificity, reaction catalyzed [17]. Six distinct flavoprotein classes (A–F) were previously identified by comparing both structural and functional elements [17]. Class A monooxygenases are flavin adenine dinucleotide (FAD) dependent and utilize either NADPH or NADH as electron donor. The oxidized electron donor is released after flavin reduction followed by C4-hydroperoxyflavin electrophilic attack on substrate. Class B monooxygenases differ from Class A because they are exclusively dependent on NADPH as electron donor and $NADP^+$ is released only after the monooxygenation is operated. Microbial N-hydroxylating monooxygenases (NMOs), and Baeyer-Villiger monooxygenases (Type I BVMO) belong also to Class B monooxygenases [16,18,19]. Class C monooxygenases are encoded by more than one gene that code for a reductase and one or two monooxygenases. Class C enzymes are flavin mononucleotide (FMN) dependent and can use NADPH or NADH as electron donors. Classes D–F differ from Class C because they are encoded by only two genes: a reductase and monooxygenase. Flavin-containing monooxygenases (EC 1.14.13.8) are oxidoreductases belonging to Class B flavoprotein monooxygenases. These enzymes are found in a variety of organisms such as vertebrates, invertebrates, plants, fungi and bacteria. Eukaryotic enzymes are bound to the membrane of the smooth endoplasmic reticulum where they catalyze the oxidation of drugs, xenobiotics and diet derived compounds [20].

Substrates of mammalian FMOs are typically nitrogen or sulfur containing molecules, including drugs that are detoxified through the monooxygenation reaction and excreted. Five functional FMO genes are present in humans [20–22]. FMO1 is expressed in kidney and small intestine [20,23–25]. FMO2 is expressed in lung and kidney [20,26–28]. FMO3 is present in liver [20,29], the most important detoxification site of human body. FMO4 is expressed in many tissues, but at low level [20,22,26,27]. FMO5 is highly expressed in the liver [20,26,27]. Historically, pig liver FMO1 (pFMO1) was the first isoform characterized biochemically. The data generated by the groups of Ziegler and Ballou in the 1970s and 1980s [30,31] described the catalytic cycle of FMO and mechanism of reaction. For many years, FMOs were thought to exert catalysis like pig liver FMO1. Nevertheless, while in general pig and human FMOs orthologues share a high level of sequence identity, pFMO isoform 1 shares only 56.10% sequence with human isoform 3 (Table 1).

Table 1. Sequence identity among human and pig FMO sequences. Alignments were constructed with the BLAST algorithm at https://blast.ncbi.nlm.nih.gov/Blast.cgi.

	pFMO1	pFMO2	pFMO3	pFMO4	pFMO5
hFMO1	87.97%	56.42%	53.00%	53.57%	52.45%
hFMO2	57.47%	87.83%	58.93%	54.70%	54.61%
hFMO3	56.10%	57.12%	82.83%	52.72%	56.77%
hFMO4	51.88%	57.34%	50.09%	84.19%	52.27%
hFMO5	51.04%	57.22%	53.98%	52.13%	87.24%

Moreover, pFMO3 and hFMO3 share the lowest sequence identity when compared to the other isoforms (Table 1). Since each isoform can be expressed in a specific tissue in high or low amount depending on the animal that is subject of analysis, each FMO isoform should be studied as a unique protein not only for its specific substrates, but also for the catalytic cycle and reaction mechanism. Therefore, in this review we focus mainly on hFMO3 because among all the five isoforms it is the most important one in terms of contribution to catabolic function, having a primary role in the metabolism of drugs and xenobiotics. Indeed, together with cytochromes P450, FMO3, contributes to Phase I drug metabolism producing S- or N-Oxides that are often not toxic and can be readily excreted by the organism [4]. Moreover, FMO3 is the only human enzyme able to catalyze the oxidation of trimethylamine to trimethylamine N-Oxide [32].

2.2. Single Nucleotide Polymorphisms of hFMO3

Drug metabolism is strictly connected to genetic polymorphism. Significant differences in drug clearances were previously reported for drug-metabolizing enzymes [33–36]. Human FMO3 is highly polymorphic since more than 20 single nucleotide polymorphisms (SNPs) were reported for this enzyme in the SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP). ASP132HIS, GLY180VAL, GLU158LYS, VAL257MET, VAL277ALA, GLU308GLY and GLU362GLN are the most common hFMO3 variants [33,37–43], (Figure 1). The crystal structure of hFMO3 is not available, but molecular modelling can give significant hints on the location of the amino acids on the structure of the enzyme. Common polymorphisms are usually on the surface of the protein structure and they can alter the oxidation activity of drugs, but they do not affect the transformation of TMA into TMAO. Active site mutations of hFMO3 were also reported. These mutants have a dramatic loss of activity against TMA [39].

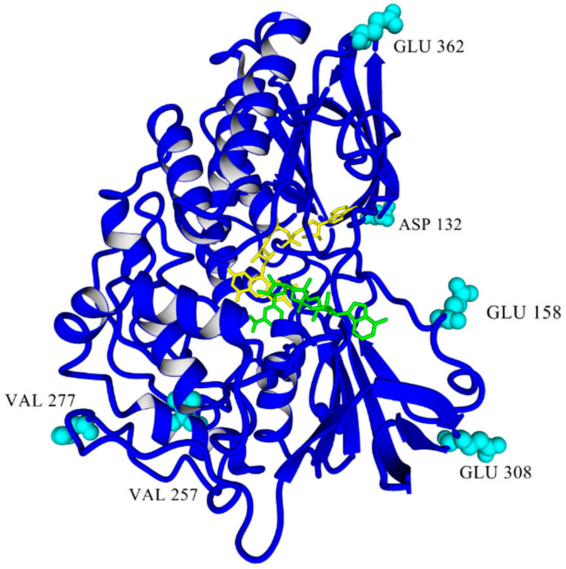


Figure 1. Location of common hFMO3 single nucleotide polymorphic variants on the homology model of the enzyme, developed in [44]. FAD cofactor is shown in yellow and NADPH in green.

2.3. Catalytic Cycle

Human FMO3 catalytic cycle consists of a reductive half reaction and an oxidative half reaction [31]. During the reductive half reaction, the enzyme binds NADPH, receives two electrons on the FAD cofactor, and binds molecular oxygen [31] (Figure 2).

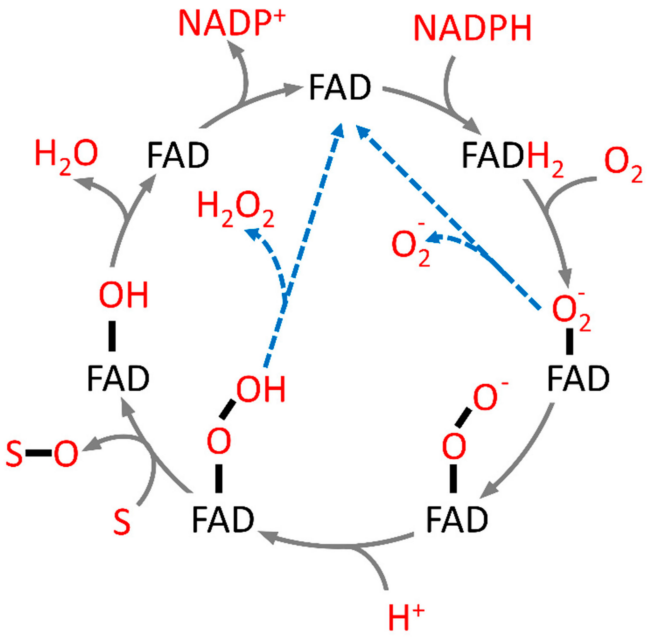


Figure 2. Catalytic cycle of hFMO3.

The binding of O_2 yields the C4a-hydroperoxyflavin intermediate that is the intermediate performing the monooxygenation reaction [31]. In the oxidative half reaction, any suitable S- or N-containing substrate that gains access to the active site of the enzyme receives an atom of oxygen. After monooxygenation $NADP^+$ leaves the active site together with a water molecule regenerating the oxidized FAD cofactor [31]. In this process, the stability of the C4a-hydroperoxyflavin intermediate-$NADP^+$ complex is crucial because a premature departure of the electron donor can lead to uncoupling reactions, diminished product formation, and the production of reactive oxygen species.

Initial studies using the homologue pig liver enzyme pointed towards a high stability of the C4a-hydroperoxyflavin intermediate that was found to last hours before decaying into the oxidized FAD cofactor [39]. Recently several other studies performed using the recombinant human FMOs have highlighted the importance of the uncoupling reaction in the catalytic cycle [28,45,46].

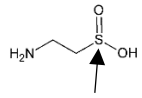
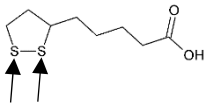
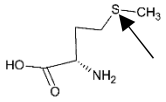
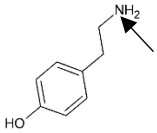
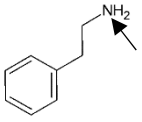
The uncoupling reaction is the wastage of electrons and oxygen that does not result in product formation. The diminished amount of product is accompanied by the production of reactive oxygen species (ROS) like superoxide or hydrogen peroxide. In a study involving different isoforms human of FMO, FMO 1, 2 and 3 were found to waste 30–50% of O_2 as H_2O_2 after receiving 2 electrons from NADPH [47]. Other works have also reported the production of superoxide [45,48] causing an even earlier shunt from the catalytic cycle, counting for up to 18% of electrons donated by NADPH [41], with no production of a C4a-hydroperoxyflavin intermediate.

2.4. Endogenous Substrates

FMOs have shown the ability to perform the monooxygenation of several endogenous molecules, including methionine, lipoic acid, trimethylamine, tyramine and phenethylamine, (Table 2). In a recent work by Shephard's group it was demonstrated that hFMO1 is also able to convert hypotaurine (a cysteine-derived molecule) to taurine [49]. Taurine is an organic osmolyte [50] that can exert different roles in the cells: regulation of cell volume and intracellular calcium concentration, formation of bile

salts, protein structure stabilization, and defense against reactive oxygen species [51–53]. For many years, the synthesis of taurine remained the subject of scientific debate. On one other hand earlier studies postulated that the enzyme hypotaurine dehydrogenase was thought to catalyze the reaction [54,55], whereas other researchers more recently have hypothesized a non-enzymatic conversion driven by the presence of strong oxidants such as hydrogen peroxide or superoxide [56,57]. NADPH oxidation in the presence of hFMO1 and hypotaurine as substrate resulted in the determination of a K_M of ~4.1 mM and kcat of ~55 min^{-1} [49]. Further, hFMO1 is much more specific than hFMO3 or hFMO5 in performing the monooxygenation of hypotaurine to taurine [49]. Interestingly the reaction seems to be carried out by either NADPH or NADH as electron donor [49]. These results constitute the first evidence of a cofactor promiscuity by hFMO1 and open a new path in the characterization of the structure-function activity. [58]. Most of the literature on FMO focusses on its role in the metabolism of drugs and xenobiotics and this has probably delayed the discovery of a physiological role for this enzyme especially in humans. A detailed understanding of the effects of increased or decreased levels of endogenous metabolites in connection to hFMO3 activity will help to shed light on the actual physiological function of the enzyme.

Table 2. Catalytic parameters for the reaction of flavin-containing monooxygenase (FMO) on endogenous substrates. Arrows indicate the site of oxygenation.

Substrate	Reaction	K_m	Enzymatic Rate of Reaction	Catalyst	Ref.
Hypotaurine		4000 µM	55 min^{-1}	hFMO1	[49]
Lipoic acid		120 µM	n/a	pFMO1	[59]
Methionine		20,000 µM	20 nmol/min/nmol prot.	hFMO3	[60]
Tyramine		231 µM	110 min^{-1}	hFMO3	[61]
Phenethylamine		90 µM	28.2 nmol/min/mg prot.	hFMO3	[62]
Trimethylamine		28 µM	36.3 nmol/min/nmol prot.	hFMO3	[32]

2.5. Current Limitations to Understanding the Physiological Role of hFMO3

Detailed information about the function of the enzyme can be obtained by expressing and purifying the enzyme. The pure protein can be used to investigate functional and structural problems. Functionally hFMO3 has been looked at as a drug-metabolizing enzyme that is resident in the ER of the human liver ready to exert catalysis on any suitable substrate that gains proximity to the enzyme [61]. The broad substrate specificity and lack of a proper ligand binding mechanism complicate the identification of the key residues of the active site responsible for catalysis. Indeed, unlike cytochromes P450, hFMO3 does not have a binding pocket and it exposes the flavin to the solvent without recognizing a substrate before performing catalysis [63]. Efforts have been made to circumnavigate this problem by implementing colorimetric or calorimetric assays that, exploiting

substrate competition for the active site, can also give some indications on possible inhibitors of the enzyme [63,64]. In fact, proper enzyme inhibition could help the characterization of the enzyme function also in vivo, as it could selectively block its activity without influencing its scaffold structure needed for potential signal transduction within the cell. On the other hand, cell biology information about hFMO3 physiological partners in the cells also remains to be determined. Protein–protein interactions studies are strongly needed to map the interaction network of hFMO3 in the cell and understand more about the involvement of this enzyme in other pathways that are not related to drug metabolism. For structural characterization, the most important information missing for hFMO3 is the three-dimensional crystal structure. Homology modelling can somehow provide guidance in the absence of an experimentally determined structure. Unfortunately, hFMO3 displays poor sequence identity to the closest homologue structure [44]. We can still rely on the homology models of this enzyme to design experiments [65] and have an idea about the overall folding, but we cannot use this information to perform structure-guided inhibitor design. The lack of the crystal structure is mainly due to the difficulties in producing regular protein crystals from membrane proteins. Protein engineering was applied to hFMO3 to produce soluble enzymes that have better chances to crystallize [44], but to date crystallization attempts have failed.

3. Trimethylamine and Trimethylamine N-Oxide

3.1. Sources of TMA

It has been suggested that the synthesis of TMA, a tertiary amine, derives mainly from the choline moiety (a quaternary amine) in itself derived from any of several common biomolecules such as phosphatidylcholine, glycine-betaine, and carnitine among others (Figure 3).

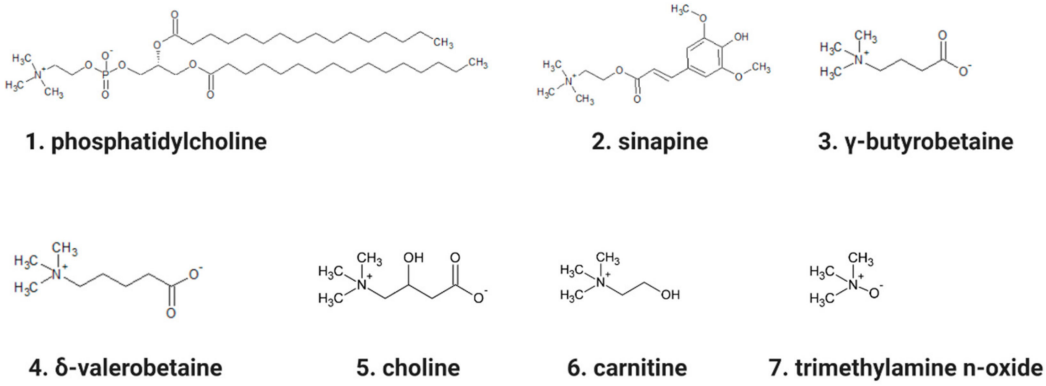


Figure 3. Molecular sources of trimethylamine that are commonly found in food.

A recent hypothesis of one of the alternative uses of the phosphatidylcholine pathway is its role in obtaining acylglycerol ethers that will be further processed [66] toward lipid storage.

3.2. TMA and Its Toxicity

Long before understanding the role of TMAO on osmotic balance and cell function it was noted how the spoilage of fish and other foods is manifest in a strong and repulsive smell, and this smell is due to amine compounds and mainly trimethylamine (TMA). A recent paper has characterized a very remarkable source of TMA and TMAO and its bacterial processing. It has been known for a long time that the meat of the Greenland shark *Somniosus microcephalus* is poisonous to humans and domestic animals [67], nevertheless it is regularly processed and consumed. The complexity of the microbial consortia involved in the detoxification through fermentation was recently analyzed [68].

Remarkably, TMAO instead has a very faint odor, and contrary to the reduced molecule it is not volatile. A recent discovery has been the identification of specific olfactory receptors (TAAR) for trace amines such as TMA [69], implying a possible behavioral importance for these molecules and their

regulation. The potency of small amounts of TMA to evoke olfactory awareness may be an avoidance behavior toward spoiled foods, but it has also been postulated as part of a pheromonal pathway. TAAR-5 related receptor family is specifically expanded in mammals, but related receptors are present in most vertebrates [70]. The connection between TMA and TMAO has been slow to develop as the oxidation of TMA is not easily obtained by simple chemical reactions, at least in the presence of other electron donors [71]. Only the discovery of a microsomal fraction derived from liver has shown that this oxidation is readily obtained in animal tissues. Moreover, accumulation of TMA in the human body is known as trimethylaminuria (TMAU) [72]. Patients affected by TMAU present an unpleasant body smell due to high levels of TMA in sweat, urine and breath. Biochemical characterization studies have demonstrated that the loss of activity against TMA can be explained by a diminished ability to bind $NADP^+$ and stabilize the C4a-hydroperoxy intermediate in the catalytic cycle of hFMO3 [45].

3.3. TMAO: The Discovery of Organic Compatible Osmolytes

Bacteria, plants, and animals have different extracellular solute compositions respect to the environment and therefore have to cope with diverse osmotic and other water-related stresses. Independently from evolutionary distance, the organic osmotic solute composition shares many properties and key molecules. Polyalcohols, amino acids, and a combination of urea and methylamines are the most commonly observed organic osmolytes [73,74] (Figure 4). Only halobacteria relate mainly on inorganic osmolytes.

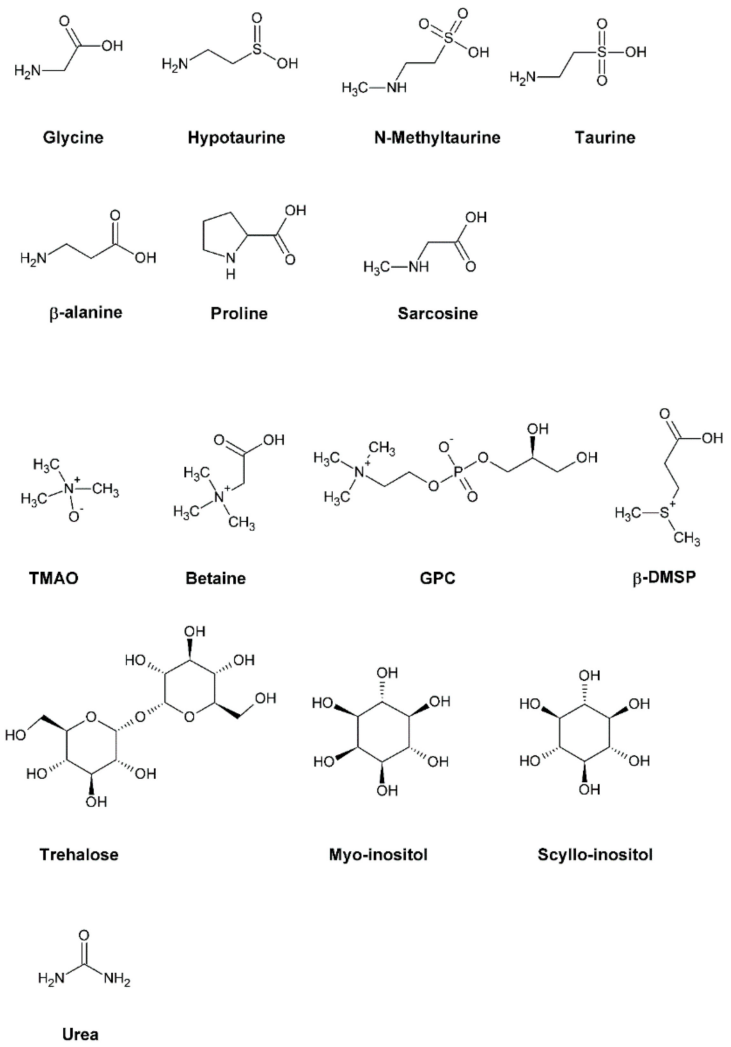


Figure 4. Organic osmolytes used by human and marine organisms.

While amino acids are easily understood in terms of their availability and of their diverse chemical properties, the role of nitrogen containing urea and methylamines might appear less obvious. Urea is an easily available and abundant solute that can be considered a common waste product of amino acid metabolism. Ammonia could be considered as the precursor to urea and is considerably more toxic. As a strategy to reduce its toxicity, many animals convert ammonia to urea that can be safely transported in the blood to the kidneys where it is eliminated in urine. While urea can be defined as not toxic, nevertheless it has an effect on macromolecules and biochemical reactions, by weakly bonding to many large organic compounds, surrounding them in an inert 'shell' and so inhibiting intermolecular hydrogen bonding. Most biochemical processes are slowed down and/or inhibited in the presence of urea. While mammals excrete most of urea, elasmobranch fishes (such as sharks and rays), and other groups of salt-water animals keep urea in the blood as a major osmolyte to a level that makes the plasma approximately isoosmotic to sea water. It has been observed that such animals have one or more other organic solutes in the plasma and the most common have a trimethylamine moiety and are trimethylglycine (TMG, also called betaine-glycine or simply betaine) and trimethylamine N-Oxide. The concentration of the latter solutes is approximately one third of the concentration of urea. It is well established that the addition of TMG or TMAO to a urea containing solution is able to recover biological processes, such as enzyme activity [74].

High concentrations of TMAO are present in the plasma of all salt-water elasmobranch [75], of *Latimeria chalumnae* (coelacanth, a lobed-finned fish [76]), and in deep sea osteichthyes with an increasing concentration as a function of depth. According to Treberg [75], total methylamine content (summed values for TMAO, betaine and sarcosine) in marine elasmobranch muscle ranges from approximately 100 to 160 μmol/g, while in fresh water elasmobranch TMAO concentration is negligible. It has been observed that in elasmobranch and *Latimeria* the role of TMAO is mainly as a urea compensating osmolyte, while in deep-sea bony fish its role is related to resistance to hydrostatic pressure. It does not come as a surprise that as freezing is a further water related stress TMAO is also a component of the freezing avoidance response of smelt (*Osmerus mordax*) [77].

Recently, a renewed interest in TMAO content in relevant marine traded fish and other aquatic animals has targeted a more detailed analysis. The main reason for this interest is linked to the spontaneous decomposition in stored frozen fish of TMAO that results in noxious levels of formaldehyde and dimethylamine that markedly reduce the quality of this important food resource. The decomposition in several marine species is increased by the enzymatic activity of TMAOase (aspolin1 [78]), a muscle enriched protein characterized by a very high aspartic acid content and active even at less than −20 °C. Chung and Chan [79] have presented data on a large number of marine fish species indicating that TMAO concentrations are generally in the range of 1% to 5% of the muscle tissue (dry weight). In the same study, TMAO was not detected in most freshwater fish species except *Oreochromis niloticus*, *Micropterus salmoides*, and *Siniperca chuatsi*. The latter two freshwater fish species were found to contain relatively high levels of TMAO in the range 400–760 mg/kg.

As animal cells cannot survive osmotic stress, intracellular osmolytes have to be present to eliminate water stress across the cell membranes. It has been posited that in several animals, intracellular organic osmolytes have a relevant role in this context, but specific analysis of their nature is less complete. It would be of great interest to investigate whether non-aquatic animals may use TMAO as a stress-relieving agent too, but the data are extremely limited.

3.4. TMAO in Animal Body Fluids

From the preceding discussion on the physiological requirement of TMAO as a stress protection osmolyte, it can be assumed that even high concentrations are not globally toxic to many animals and it has been well studied in fish. The highest concentrations found are from 200 mmol/L in elasmobranchs, up to 400 mmol/L in deep dwelling Actinopterygi such as rough grenadier (*Coryphaenoides yaquinae*, Macrourinae), found at even 7000 m of depth [80].

The TMAO concentration in body fluids of mammals has been studied only in relatively few species, and the data are relatively imprecise. It is interesting that one of the few data on a marine mammal (*Leptonychotes weddellii*, Weddell seal) shows a highly variable level of plasmatic TMAO, that is strongly dependent from the nutrition state [81]. The fasting plasmatic TMAO level of lactating seals was 45 ± 20 μmol/L, while peak levels upon feeding ranged in a specific individual from 83 to 1040 μmol/L, (mean 324 ± 110 μmol/L). A report shows that dogs fed with krill (*Euphausia superba*) have a marked increase in choline levels and in plasmatic TMAO (krill 32.4 ± 12.6 vs. control 4.4 ± 4.7 μmol/L, six-week treatment) [82].

3.5. TMAO and Human Pathologies

Prevention and innovative therapies are starting to significantly affect oncological pathologies, but cardiovascular disease (CVD), type 2 diabetes and other metabolically related conditions are still on the rise, as is their death toll on people. This issue has recently attracted the attention toward dietary role in these pathologies that show a clear differential increase related to specific lifestyles. Regarding this approach, researchers have pointed out that the role of the microbiome in the conversion of dietary precursors toward absorbed molecules may lead to possible unexpected mechanisms. Elevated plasmatic TMAO in humans was evaluated in several cohorts of patients and it is suspected to have a role in several CVD and metabolic diseases, or at least to be a relevant marker. What is not obvious instead is whether there is a clear distinction between normal and potential pathologically relevant concentrations of TMAO both in plasma and in urine, and very little is known about tissue concentrations. While the ingestion of potential precursors of TMA is certainly a dietary requirement at least for choline [83], the abundance and variety of trimethylamine moiety donors makes the study more complex. The ingestion of TMAO itself is potentially relatively high in fish eating populations, and it is strongly correlated with plasmatic and urinary TMAO levels. It has been shown using labelled TMAO on healthy volunteers that the gut without a requirement for conversion steps can readily directly absorb it. The labelled substance in the same study [84] was found to be rapidly excreted. Plasma TMAO level depends mostly on the following three factors. First, the liver produces TMAO from TMA, a gut bacteria metabolite of dietary choline and carnitine. Second, plasma TMAO increases after ingestion of dietary TMAO from fish and seafood. Finally, plasma TMAO depends on TMAO and TMA excretion by the kidneys.

Recent clinical studies show a positive correlation between elevated plasma TMAO and increased cardiovascular risk [85]. However, the mechanism of the increase and biological effects of TMAO in the circulatory system are obscure. To give a summary of recent observations about plasmatic TMAO and TMA and their presence in other human biological samples, we can examine data from recent papers. In a cohort of 171 males and 126 females (Germany), in healthy conditions plasmatic TMAO was on average respectively 3.96 μmol/L and 4.06 μmol/L with a slightly higher variability in males [86]. In a cohort of healthy subjects (Norway) instead was reported to be 5.8 μmol/L [87] and in a group of healthy women (Poland, n = 172) plasmatic TMAO was reported to be 14.04 ± 2.36 μmol/L [88]. Given these highly variable figures for control concentrations, it is not surprising that distinct dietary lifestyles can exacerbate differences, but it is even more interesting to analyze reported values in patients at risk for cardiovascular and renal pathologies. A review and meta-analysis that included 22 prospective studies with data on controls and patients at risk gave a range of plasmatic concentrations from 1.74 to 103.81 μmol/L [89].

3.5.1. Insulin Resistance and Diabetes

Among metabolic dysfunction Insulin Resistance (IR) and Type 2 Diabetes (T2D) are closely related to diet and lifestyle and recent research has pointed out the possible implication of TMAO in the onset of these pathological conditions. High plasma TMAO concentrations were found in diabetic mice, but Dambrova et al. found that in human high TMAO plasma concentrations are not strictly related with diabetic condition. In fact, it seems that several factors such as age, gender, body mass

index and nutrition contribute to augmented plasma levels of TMAO both in diabetic and non-diabetic patients [90]. Sun and colleagues found a positive correlation between TMAO plasma concentrations and IR only in hyperglycemic patients [91]. Other studies evidenced that TMAO is high in patients with hepatic insulin resistance probably due to the inverse relation between insulin and FMO3 expression, in fact genetically modified mice that do not express hepatic insulin receptor have high FMO3 expression levels in the liver and when fed a high fat diet, a high hepatic glucose output was detected [92]. So, for insulin resistance and diabetes also, it is not clear if TMAO is an independent risk factor or a risk marker of the pathology.

3.5.2. Cardiovascular Diseases

TMAO's pathophysiological roles have been studied in several cardiovascular pathologies, but principal indications are those derived from atherosclerosis. Diet derived trimethylamine N-Oxide in fact is probably involved in augmented circulating levels of triglyceride, total cholesterol, low density lipoprotein, resulting in an acceleration of aortic lesion formation [93]. Yu and colleagues found a direct relation between high urinary levels of TMAO and coronary heart disease, underlining an accelerated progression of the pathological condition when diet contribute to an augmented intake of TMAO or its precursors [94]. TMAO seems to be directly involved in platelet hyper-reactivity, thrombosis risk [95] and in the development of ischemic stroke. In this scenario, it seems reasonable that TMAO could be considered a crucial factor in the development of cardiovascular diseases. Recent studies at the cellular level have objected to these observations, showing that even very high concentrations of TMAO do not have acute harmful effects on rodent cardiac myocytes [96]. It has also been noted that TMA instead of TMAO could be the culprit as they are strictly linked [97].

3.5.3. Chronic Kidney Diseases

Among pathological conditions, renal dysfunctions are related to elevated plasma TMAO, but the role of the metabolite towards this condition is still controversial. Studies with animals or cohort studies in humans seem to underline that a previous renal failure is the cause of TMAO rising in plasma and not the contrary [98,99]. Therefore, mortality or other pathological conditions in the presence of high TMAO concentrations and renal dysfunction are probably due to this last condition rather than an all-cause damaging effect of TMAO.

3.5.4. Other Pathologies

High TMAO concentrations were also found in cerebrospinal fluids of patients with Alzheimer's clinical syndrome and TMAO is associated with biomarkers of Alzheimer's disease [100]. Further studies are needed to evaluate the role of TMAO in the development of the pathology.

4. Protein Folding: A Biochemical Process Shared by FMO and TMAO

4.1. FMO and Protein Folding

Human flavin-containing monooxygenases are membrane proteins localized in the endoplasmic reticulum (ER) [101]. ER is known to be the central protein folding hub of the cell [102]. Most of the proteins that are present in this organelle are actually dedicated to the process of protein folding. When a new protein is synthetized and targeted to the ER either molecular chaperones or folding enzymes will assist the correct folding of the target [102]. Chaperones are proteins that help other proteins gaining access to their native conformation without taking part to the final protein structure [103]. They work primarily by preventing aggregation of target proteins therefore favoring a correctly folded environment in the cell [102]. Folding enzymes act by influencing the energy landscape of the protein folding process without affecting the equilibrium [102]. The two main classes of folding enzymes are prolyl peptidyl cis–trans isomerases and oxidoreductases [102]. Yeast flavin-containing monooxygenase (yFMO) was previously found to be involved in the generation of oxidizing equivalents that drive

protein folding in the ER [101,104,105]. Yeast FMO catalyzes the oxidation of thiols like glutathione (GSH) to glutathione disulfide (GSSG) [104]. The GSH/GSSG balance is an extremely important redox buffering system that can lead to proper disulfide bond formation and protein folding [106] (Figure 5).

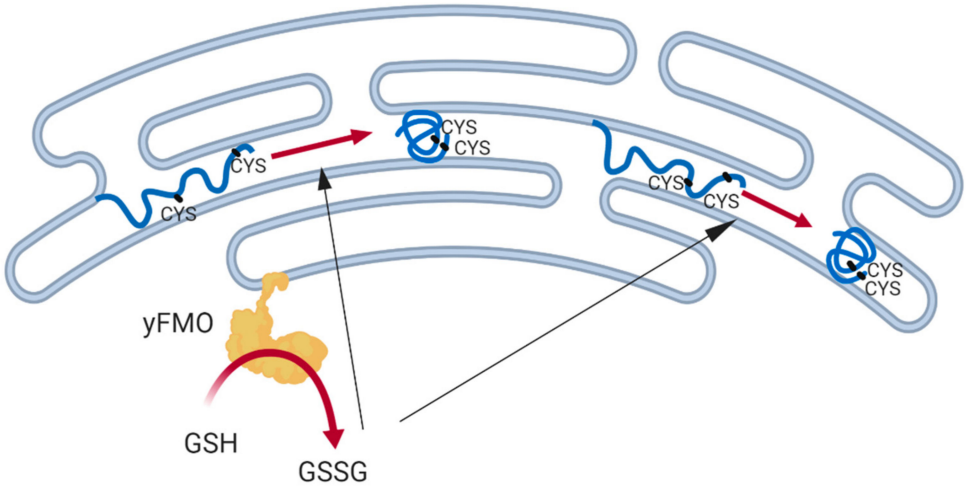


Figure 5. Role of yFMO in protein folding on the surface of the endoplasmic reticulum.

It was also found that yFMO undergoes redox regulation by interacting directly with accumulated GSSG [105]. The interaction between GSSG and yFMO is mediated by two cysteines (CYS 339 and CYS 353) [105]. The accumulation of GSSG due to yFMO activity causes the formation of a mixed disulfide with CYS 353. The disulfide can then exchange with C339 leading to an intramolecular disulfide that blocks the access of the substrate to the active site [47]. Interesting results were also obtained using pig liver FMO [107]. This enzyme was also tested for its ability to oxidize thiols [107]. A clear correlation between refolding of functional ribonuclease and pig liver FMO oxidizing activity on cysteamine as a thiols source was found [107]. Therefore, human flavin-containing monooxygenase could also have a similar role to yFMO and pFMO in ER. The TMAO producing enzyme primary sequence contains some indications that favors this hypothesis. Indeed, hFMO3 sequence contains 11 cysteines out of 532 amino acids. The presence of a large number of this functional amino acid might indicate a physiological role. A possible explanation could be the action of one or more of these amino acids as redox switches similarly to the mechanism reported for yFMO. Further investigations are needed to understand the redox status of the cysteines of the human enzyme and its activity against physiological thiols. Recently hFMO3 was found to be involved in a key reaction pathway that links the enzyme to the unfolded protein response of the cell [59]. Indeed, in this work, the product of its catalysis, TMAO was found to bind and activate PERK, a main component of the unfolded protein response. The unfolded protein response is a signaling mechanism of the cell that is activated by a series of conditions such as calcium depletion, lipid overload or unfolded proteins accumulation that lead to endoplasmic reticulum stress [58]. The UPR can be seen as an intrinsic biosensor of conditions protein homeostasis occurring in the ER that is able to monitor protein folding and transduce the signal to gene expression machinery. Three different branches are part of the UPR and work as stress sensors: ATF6, PERK, and IRE1 [108]. While ATF6 modulates transcriptional response, resulting in increased protein folding capacity, IRE1 and PERK decrease protein-folding load through translational control. In their study, Chen et al. showed that TMAO produced by hFMO3 at a concentration of 50 μM can bind and activate PERK therefore activating directly a branch of the unfolded protein response.

4.2. TMAO as a Small Chaperone

Globular proteins contain all the information to guide the correct protein folding in their primary amino acid sequence [109]. A protein in its folded form will tend to adopt the most favorable three-dimensional structure that corresponds to the lowest energy state for the reference system.

Nevertheless, the amino acid sequence can only control and determine the native state of the protein, but the solution in which the protein is immersed can influence the equilibrium between native and denatured protein ensembles. Therefore, the interaction between the solvent and the protein can yield several populations of native and denatured proteins. Small molecules, such as ligands, can also influence the proteostasis by binding protein populations and resulting in further stabilization of the protein target and lower free energy. TMAO is a small naturally occurring osmolyte that prevents harmful effects of the solvent on the protein structure. Two different models of interactions between TMAO and proteins are available. In the first model, TMAO does not interact as well as water with the protein backbone [110], so at high concentrations proteins tend to adopt a compact structure lowering unfavorable interactions [50,110]. As a result, the presence of TMAO pushes the equilibrium towards the native ensemble conformations of the protein structure [110]. A second model for the interaction between protein structure and TMAO has also been reported. In this model TMAO binds more favorably to the protein structure via hydrogen bonding [111].

In a third, more recent model, TMAO was found to stabilize folded conformations acting as a surfactant for the heterogeneous surface that emerges on protein folding [112]. The mechanism of action of TMAO still needs to be clarified and future work should shed light on how TMAO actually interacts with the protein structure. Nevertheless, the pH dependent nature of TMAO is often neglected when theoretical or experimental studies are carried out [113]. The pKa of TMAO is 4.7 ± 0.1 [113] and at acidic pH it is in the protonated form. This form is not able to stabilize the native protein state [113]. In the deprotonated form, TMAO possesses a large dipole moment and can interact very well with water, forming three strong hydrogen bonds leading to an increase in the magnitude of the solvent-excluded volume effect [113]. Indeed, Vigorita et al. have argued that when protonated, TMAO could not possibly form three bonds with water and lose its stabilizing effect caused by water attraction [113].

Physiologically TMAO is very often found in the presence of urea. In this context, TMAO is still a strong protein folding agent and it prevents unfolding by structuring the solvent around the protein structure [114]. The protective action of TMAO is thought to be exerted by discouraging urea–protein interactions [114].

5. Conclusions

Human flavin-containing monooxygenase 3 is a potent catalyst that can work on many different molecules due to its promiscuous active site and the lack of a proper binding site for the substrate in the enzyme structure. Catalysis is influenced by individual genetic variability and unwanted delivery of oxygen and electrons from O_2 and NADPH to superoxide and hydrogen peroxide. FMO3 is deeply involved in the metabolism of exogenous compounds such as drugs and xenobiotics, but it can also perform monooxygenation of endogenous compounds like the organic osmolytes trimethylamine and hypotaurine. Trimethylamine is a compound of dietary origin that is converted by FMO3 to trimethylamine N-Oxide (TMAO). TMAO is known to be a small molecular chaperon that assists protein folding. The molecular mechanism used by TMAO to stabilize protein structure is not known, but its requirement and beneficial effects are known in different organisms and are often coupled to a water stress condition: high solute including urea concentration, hydrostatic pressure, freezing, and potentially dehydration. Nevertheless, in the recent literature, several studies have put forward the hypothesis that elevated TMAO could be linked to several human pathologies. The issue is very relevant, as TMAO is acquired in a complex pattern not only directly but also through the processing in the gut of several nutrients. Therefore, TMAO abundance and variation in time can follow very different patterns depending on TMA derived from nutrients through gut microbiome and on FMO3 oxidation of TMA (Figure 6).

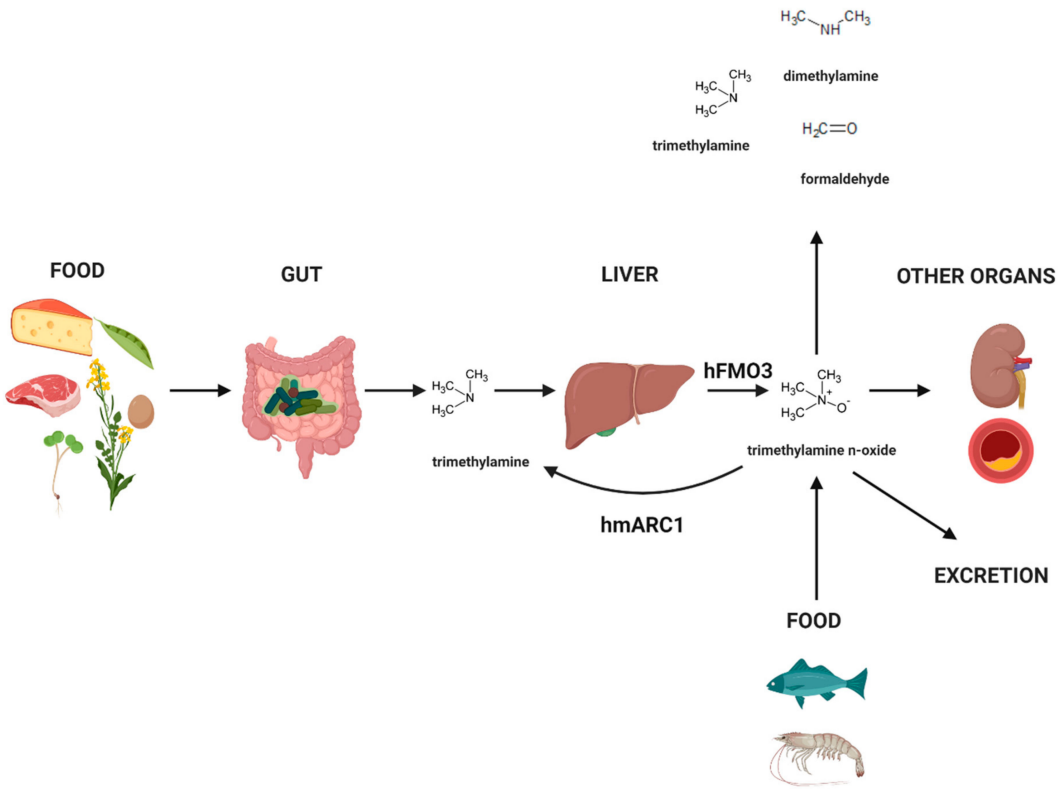


Figure 6. The journey of trimethylamine and trimethylamine N-Oxide in the human body. Trimethylamine is extracted from food by bacteria in the gut. In the liver hFMO3 converts it to TMAO. TMAO can a) be converted ubiquitously to formaldehyde, dimethylamine or trimethylamine b) be moved through the blood to other peripheral organs c) be excreted in urines by the organism d) be converted back to TMA by the mitochondrial amidoxime reducing component 1 (hmARC1) [115]. Trimethylamine N-Oxide can also enter directly the body mainly through the introduction of fish.

Further complications are created by possible impairment of the unusually rapid renal excretion of TMAO in normal conditions. It is therefore expected that not only different diets, but also individual or lifestyle variations could influence both rapid TMAO fluctuations (for instance upon fish consumption), but also long-term levels. It is of further interest that TMAO uptake in cells is not fully characterized, and back conversion of TMAO to TMA or other products is not clearly known. On the contrary, TMA is a well-known toxicant, and this might partially explain the presence of specific olfactory receptors and the strong aversive odor. Recent work hypothesized that since cardiovascular diseases are associated to higher osmotic and hydrostatic stresses TMAO could act as a compensatory response mechanism to protect cells [116].

The physiological role of the FMO3 as a TMAO forming enzyme is still unclear, but increasing data are pointing towards an active regulatory role of the enzyme within the cell that goes beyond detoxification from exogenous substrates. Among all cellular processes, protein folding homeostasis and metabolic response to stress are two possible routes that should be explored in the future in order to precisely map the complex axis involving diet, FMO3, TMAO, and pathological status. In this context, the determination of the crystal structure of the enzyme and the identification of the protein partners in the cell will be two lines of research that will provide essential information to further advance the knowledge of hFMO3 and how the modulation of its activity can be exploited for the improvement of human health.

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