FISH MUSCLE MICROSOMES CATALYZE THE CONVERSION OF TRIMETHYLAMINE OXIDE TO DIMETHYLAMINE AND FORMALDEHYDE

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1. Introduction

The *N*-oxides of aliphatic amines are widely distributed in biological systems, but little is known about their function, synthesis, or degradation. In mammalian systems, the microsomal fraction of liver cells is the site of an oxidative dealkylation of tertiary amines requiring molecular oxygen and NADPH. Liver homogenates oxidize tertiary amines to amine oxides and cleave the resultant *N*-oxides to secondary amines and aldehydes [1]. Marine teleosts and elasmobranchs contain high concentrations of trimethylamine oxide (TMAO) in their tissues which is thought to play an osmoregulatory role in these organisms [2] although this has been questioned [3]. The biosynthetic and biodegradative pathways of TMAO have been the subject of much controversy. The mechanism of TMAO breakdown in marine fish muscle has practical consequences since the process is accelerated during frozen storage [4], and the formaldehyde produced is thought to crosslink the fish muscle protein leading to a rapid toughening of the texture of some species, particularly gadoids [5]. Both enzymic and non-enzymic processes have been suggested as important in this process [6,7]. Enzymes catalyzing the breakdown of TMAO to dimethylamine and formaldehyde have been reported from fish organs such as the pyloric caeca, kidney and liver [6,8,9]. We report the isolation of a microsomal fraction from the skeletal muscle of red hake (*Urophycis chuss*) which causes a rapid breakdown of TMAO to dimethylamine and formaldehyde in vitro.

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2. Materials and methods

We procured red hake, which had been iced after catching, from Gloucester fishermen a few hours after they had been caught. They were transported to the laboratory on ice and were immediately filleted. The fillets were frozen and stored at −80°C until use. Reagent grade chemicals were used in all cases. The microsomal membrane fraction was prepared from red hake as in [10] except that homogenization by Waring blender was replaced by mincing with a Rival electric meat grinder.

The breakdown of TMAO by the microsomal fraction was followed by measurement of dimethylamine (DMA). The components of the reaction media are noted below. The reaction was initiated by the concurrent addition of trimethylamine oxide (TMAO) and microsomes. The reaction was stopped with 1 ml 25% trichloroacetic acid added to 4 ml assay medium which was then centrifuged at 2000 × *g* for 10 min to yield a clear supernatant fraction. DMA production was linear for ⩾30 min up to ~10 mg protein/ml at 20°C. Activities are reported as mmol DMA produced . mg protein^{-1} . h^{-1}. Since there was some variation in the activities from different preparations, results are reported as percentages. Dimethylamine was analyzed as in [11] on an appropriate aliquot of the clear trichloroacetic acid extract with the following modifications. The aqueous layer was partitioned with 12 ml 5% carbon disulfide in benzene and shaking was obtained with a Cole-Palmer Roto-Torque model 7637 set at maximal speed. Formaldehyde was determined on the cleared trichloroacetic acid extract as in [12]. We were not able to get completely reliable formaldehyde analyses, however, because of interference in the presence of either ferrous or ferric ion even when

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the chromophore was extracted in *n*-butanol as suggested. It was clear, nevertheless that when appreciable amounts of DMA were formed, production of formaldehyde was also observed. Protein was determined by a modified Lowry procedure [13] with bovine serum albumin as standard.

3. Results and discussion

The factors necessary for the microsomes to convert TMAO to DMA and formaldehyde are illustrated in table. 1. Either cysteine or ascorbate can serve as substrate, and there is a requirement for iron. It made no difference whether the iron was in the +2 or +3 valence state when added to the reaction medium. In the absence of the microsomes or in the presence of heated microsomes, no DMA was produced. Although we were unable to get quantitative results, it was clear that whenever DMA was produced in significant amounts, formaldehyde was also.

The production of DMA from TMAO as a function of ascorbate and cysteine concentrations is shown in fig.1. Complex kinetics are displayed by both compounds in this reaction. The ascorbate system activates TMAO breakdown at very low concentrations, i.e., <1 mM, whereas the response of cysteine is more sigmoidal, with little effect at low concentrations but approximating that of ascorbate at ~8–10 mM. At low concentrations of these substrates, the activity with ascorbate was as much as an order of magnitude higher than that of cysteine.

Table 1
Production of DMA by microsomes from red hake muscle

Components	DMA (%)	Formaldehyde
Microsomes, TMAO, Fe^{2+}, ascorbate, cys	100	+
Microsomes, TMAO, Fe^{2+}, ascorbate	56	+
Microsomes, TMAO, Fe^{2+}, cys	4.4	+
Microsomes, TMAO, Fe^{2+},	<1	tr
Microsomes, TMAO ascorbate, cys	<1	tr
Microsomes, Fe^{2+}, ascorbate, cys	0	
TMAO, Fe, ascorbate, cys	<1	tr
Heated microsomes, TMAO, Fe^{2+}, ascorbate, cys	<1	tr

The concentrations of the components when used in the reaction media were 10 mM TMAO, 0.1 mM $FeCl_2$, 1 mM ascorbate, and 1 mM cysteine. All samples contained 0.12 M KCl, 50 mM histidine at pH 6.9 and 2.0 mg protein/ml

The absolute values corresponding to 100% DMA production ranged from 86–500 nmol . mg protein^{-1} . h^{-1} in 5 expt

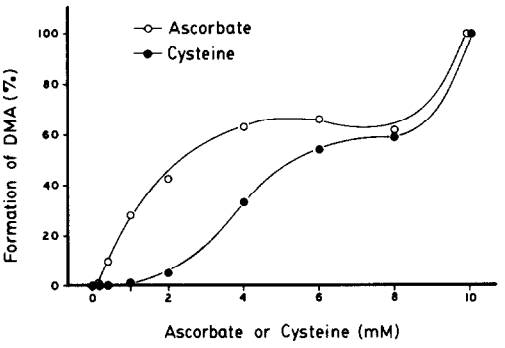

Fig.1. Formation of DMA as a function of ascorbate and cysteine concentration. Fish muscle microsomes were prepared as in [10]. The reaction media contained 10 mM TMAO, 0.1 mM $FeCl_2$, 0.12 M KCl, 5 mM histidine at pH 7.0, 1 mg protein/ml and ascorbate or cysteine as indicated. Results are av. 4 expt.

On the other hand, the kinetics of the reaction with TMAO appeared to be Michaelis-Menten in a reaction medium containing 0.2 mM $FeCl_2$, 2.0 mM ascorbate and 2.0 mM cysteine at pH 6.9. The apparent Michaelis constant of the reaction for TMAO under these conditions was 4 mM. Optimal activity was observed at pH ~ 6.6.

The activity of the microsomal system as a function of $[P_i]$ is shown in fig.2. The response is very complex >70% of the microsomal activity is inhibited at ~5 mM. An increase in activity between 0.1–1 mM

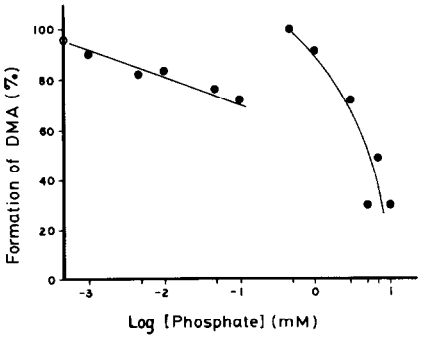

Fig.2. Inhibition of DMA formation by P_i. The reaction media contained 10 mM TMAO, 0.4 mM $FeCl_2$, 2 mM cysteine, 2 mM ascorbate, 0.12 M KCl, 5 mM histidine at pH 7.0, 1 mg protein/ml and P_i as indicated. Results are av. 4 expt.

was consistently observed. The steepest slope of this phosphate inhibition curve occurred over a $[P_i]$ range which is roughly that reported in muscle in situ [14]. A change in activity of from 70 ~ 30% of the maximal rate occurred over ~3–8 mM phosphate. Thus, in situ the content of free phosphate may be an important modifying factor in muscle tissue and the rate of hydrolysis of phosphate compounds in fish muscle after death may play an important role in the rate at which trimethylamine oxide is degraded.

The effect of several other compounds which were shown to be activators or inhibitors of DMA production by the microsomal system of red hake muscle are given in table 2. Phenazine methosulfate and methylene blue were the most potent activators of the system. These are compounds which can participate in electron transfer reactions and methylene blue is frequently used in the assay for this enzyme to increased activity. Trimethylamine and choline have similar structures to the substrate trimethylamine oxide and could be functioning as competitive inhibitors. EDTA and citrate could possibly inhibit by chelating the Fe^{2+} in the reaction medium, and the inhibitory effect of Cu^{2+} might indicate that sulfhydryl groups were involved in the enzyme.

Conversion of trimethylaime oxide to DMA by the red hake muscle microsomal fraction was examined in the frozen and unfrozen state (table 3). DMA production in samples at temperatures above freezing were assayed after 1 h whereas samples in the frozen state were assayed after 3 h. These values, however, were all calculated on the basis of DMA yield/h reaction. The last column in the table is an estimate of the amount of DMA that could be produced in 100 g fish flesh over 24 h. The calculation is based on the determined rate of DMA formation and the yield of microsomal protein obtained from this quantity of fish muscle. It represent an underestimation of the 'potential' TMAO converting power of this membrane fraction in the fish flesh since 100% yields would be most unlikely. This 'potential' activity is sufficient to more than account for the rates of TMAO breakdown to DMA that are observed in the storage of fish muscle, i.e., the reaction is not limited by the amount of enzymic protein.

Table 2
Activation and inhibition of DMA production by microsomes

Compound	No. Expt	mM	% of control[a]
Phenazine methosulfate	2	0.1	614
Methylene blue	1	0.02	274
Trimethylamine	3	10	58
Choline	4	10	24
EDTA	3	1	32
Citrate	2	1	29
Cu^{2+}	3	0.4	39

[a] The control contained 10 mM TMAO, 0.1–0.4 mM Fe^{2+}, 2.0 mM ascorbate in 0.12 M KCl, 5 mM histidine buffer (pH 6.9) at 1.0 or 2.0 mg protein/ml. Due to varying activities of the membrane preparations, increasing $[Fe^{2+}]$ to 0.4 mM and [protein] to 2 mg/ml was sometimes necessary to produce measurable DMA and reliable data

Table 3
Production of DMA by microsomes from red hake muscle as a function of temperature

Temp. (°C)	μmol DMA . mg microsomal protein^{-1} . h^{-1}	mmol DMA . 100 g fish^{-1} . 24 h^{-1}
20	0.30	1.25
8	0.21	0.88
−6	0.39	1.65

The reaction medium contained 20 mM TMAO, 0.2 mM $FeCl_2$, 2.0 mM ascorbate, 2.0 mM cysteine, 0.12 M KCl, 5 mM histidine at pH 6.9 and 1.0 mg protein/ml (−6°C) or 2.0 mg protein/ml (8°C, 20°C)

The frozen muscle tissue of cusk (*Brosme brosme*) produces dimethylamine at a slower rate than that of red hake [4]; the isolated microsomal fraction from cusk had an activity of ~10% of that of red hake. The microsomal fraction of winter flounder (*Pseudopleuronectes americanus*) had minimal activity (<1% of that of the red hake), and flounder muscle does not degrade TMAO to dimethylamine and formaldehyde to any significant extent during frozen storage [15].

Many of the conditions under which the breakdown of TMAO to dimethylamine (and presumably formaldehyde) by the microsomal fraction of red hake muscle occurs are similar to those expected in post mortem muscle in situ; e.g., substrate and cofactor concentrations and optimal pH. The relatively low app. K_m for TMAO of the system would indicate that the enzyme could function at a relatively high activity until most of the trimethylamine oxide is converted. [TMAO] in red hake muscle is usually 70–100 mM. There was a faster rate of TMAO breakdown at −6°C than at either 8°C or 20°C: −6°C is almost optimal for the breakdown of TMAO in frozen fish muscle [16].

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