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# Quantitative Relationship between Trimethylamine Oxide Aldolase Activity and Formaldehyde Accumulation in White Muscle from Gadiform Fish during Frozen Storage

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The accumulation of formaldehyde and the resulting deterioration of seafood products during frozen storage are primarily caused by the enzymatic activity of trimethylamine oxide aldolase (TMAOase). A screening of muscle samples from 24 species showed TMAOase activity in only the nine gadiform species that were analyzed. Enzyme activities in the major white muscle of gadiform fish showed large variations between species as well as between individuals. A frozen storage experiment showed a similarly large variation in the rate of formaldehyde accumulation, which could be accounted for by the endogenous white muscle in situ TMAOase activity. This TMAOase activity also correlated with the rate of insolubilization of otherwise high ionic strength soluble protein. A simple model describing the accumulation of free formaldehyde during frozen storage of gadiform fish is proposed. The model is based on a storage time-dependent decay of substrate-saturated white muscle TMAOase activity.

**KEYWORDS: TMAO; trimethylamine *N*-oxide; DMA; TMAO demethylase; TMAOase; Gadidae; enzyme; seafood**

## INTRODUCTION

Formaldehyde and dimethylamine (DMA) are breakdown products from trimethylamine *N*-oxide (TMAO) and can be formed in some species post-mortem. Formation of these products may cause severe quality changes or even spoilage during prolonged frozen storage at insufficiently low temperatures. Formaldehyde-deteriorated fish meat is characterized as tough, hard, fibrous, and dry (for reviews, see refs *1*−*5*). The formaldehyde content in seafood products is generally considered to be nontoxic, but acceptable levels of formaldehyde are in some cases exceeded, causing rejection of products and consequently economical losses.

Formaldehyde can react with a number of chemical groups, including a number of amino acid residues, terminal amino groups, and various low molecular weight compounds leading to denaturation and possibly cross-linking of proteins. This again results in a reduced solubility of especially myofibrillar proteins (*2*, *4*, *5*). However, the mechanism causing deterioration of seafood by formaldehyde is not yet completely understood. The spoilage potential of DMA is considered to be less than that of formaldehyde, although DMA as a secondary amine is expected to be a reactive compound.

TMAO is an endemic substance in marine animals, but it also occurs in some freshwater species (*6*−*8*). Its decomposition can be catalyzed nonenzymatically by iron and various reductants (*9*−*14*) or enzymatically by trimethylamine oxide aldolase (TMAOase, EC 4.1.2.32) (*15*, *16*). The nonenzymatic reaction has been reported to be of significance during heating (*10*−*12*, *14*) as well as in frozen model systems (*13*). However, most studies on the accumulation of formaldehyde and DMA during frozen storage concern species in which TMAOase activity has been found. In addition, the ability to produce formaldehyde and DMA has been induced by the addition of TMAOase-rich tissues to fish species that are otherwise without the ability to produce DMA and formaldehyde (*17*−*20*).

TMAOase has been found in a limited number of species, many of which have high economical importance and belong to the order of gadiform fish [lately reviewed by Sotelo and Rehbein (*21*)]. In gadiform fish, the highest activities are found in the inner organs: kidney, spleen, intestine, and, for some species, also the gall bladder. Increasingly lower enzyme activities have been found in blood and in red and white muscle. Due to insufficient assay sensitivity, TMAOase activity in white muscle has mostly been demonstrated indirectly via measurements of DMA and formaldehyde accumulation. Only a few direct TMAOase activity measurements on muscle (*14*, *22*−*25*) and particularly on white muscle (*19*, *20*, *26*, *27*) have been

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reported. The reported TMAOase activities of white muscle were orders of magnitude lower than the TMAOase activity in some visceral organs (*19*, *26*, *27*) and, on the basis of the rate of DMA formation, also lower than in red muscle (*28*).

The low and partly undocumented enzyme activity of white muscle has led to speculations if other factors than in situ TMAOase activity can account for the accumulation of formaldehyde and DMA in white muscle. Alternative explanations could be diffusion of TMAOase or its reaction products from other organs such as kidney, red muscle, and blood, or it could result from nonenzymatic reactions. Rey-Mansilla et al. (*27*) found increased TMAOase activity in muscle samples that were anatomically located near the kidney of blue whiting. The TMAOase activity in kidney tissue correlated with the accumulation of DMA in white muscle of blue whiting during cold storage (*29*, *30*). These results can be interpreted as supportive for an extramuscular origin of either DMA or TMAOase, but the results could also be indirect, due to a correlation between TMAOase activity in muscle and in kidney. Rehbein et al. (*20*) compared prestorage TMAOase activities in minced muscle of gadoid fish with the accumulation of formaldehyde during frozen storage and did not find a significant correlation. In favor of TMAOase from the muscle being responsible for the formation of DMA and formaldehyde, Parkin and Hultin (*22*) demonstrated that the in situ TMAOase activity was sufficient to account for the formation of DMA in red hake.

In the present study, quantitative measurements of TMAOase activity in seafood products have been correlated with chemical changes during frozen storage of seafood products. A simple model for the accumulation of formaldehyde during frozen storage is suggested, and the ability to predict the accumulated formaldehyde from in vitro enzyme activity measurements is demonstrated.

## MODEL DESCRIPTION

The in situ formation of formaldehyde ($\Delta P$) in a time interval from zero to $t_R$ is

$$\Delta P = \bar{v} t_R \tag{1}$$

where $\bar{v}$ is the average in situ enzyme activity defined by the average reaction rate during the time $t_R$.

Neither $\bar{v}$ nor $\Delta P$ can be directly and accurately measured. Instead, the in situ enzyme kinetics can be analyzed on the basis of in vitro enzyme activity measurements and the determination of accumulated formaldehyde in muscle samples. If the ratio between $\bar{v}$ and the average in vitro reaction rate at optimal reaction conditions (the in vitro activity, $\bar{a}$) is named $Q$, eq 1 can be reformulated as

$$\Delta P = Q \bar{a} t_R \tag{2}$$

$Q$ is constant only if the effect of changing reaction conditions on enzyme activity is insignificant.

Formaldehyde is both reactive and volatile. The accumulation of free (acid-extractable) formaldehyde will therefore not necessarily reflect its rate of formation (see also the Discussion). However, under conditions likely to be valid in muscle tissue, that is, high concentration of reactive sites and a small equilibrium constant between "bound" and free formaldehyde, the accumulation of free formaldehyde ($\Delta P_F$) is proportional to the (total) formation of formaldehyde:

$$\Delta P_F = \beta \Delta P \tag{3}$$

Substituting $\Delta P_F$ for $\Delta P$ in eq 2 gives

$$\Delta P_F = \beta Q \bar{a} t_R \tag{4}$$

The enzyme is gradually inactivated during storage, causing a decrease in $a$ with time. The inactivation can often be described by a simple first-order process:

$$\text{active enzyme} \xrightarrow{k} \text{inactive enzyme} \tag{5}$$

This gives the following equations for $a$ and $\bar{a}$:

$$a = a_0 \, e^{-kt} \tag{6}$$

$$\bar{a} = \frac{1}{t_R}\int_0^{t_R} a \, dt = \frac{1}{t_R}\int_0^{t_R} a_0 \, e^{-kt} \, dt = a_0 \frac{(1 - e^{-kt_R})}{kt_R} \tag{7}$$

In the present investigation, $k$ was determined from paired values of $a$ and $t$ using eq 6. $\bar{a}$ was subsequently calculated from eq 7. In addition to the direct measurement of the initial (prestorage) in vitro activity ($a_0$) for each experimental fish, $a_0$ was also calculated for individual samples using values for $a$ and $t$ in eq 6. In this way, $\Delta P_F$ and estimates of $\bar{a}$ from identical samples could be related, thereby avoiding errors due to sample variation. As seen from eq 4, plotting $\Delta P_F$ against $\bar{a}t_R$ gives a straight line through the origin and with a slope of $\beta Q$. From this slope and an independent determination of $\beta$, the ratio, $Q$, between the in situ and in vitro activities was calculated. Assuming that the falling TMAO concentration was only due to the formation of formaldehyde ($\Delta P$), $\beta$ was estimated from the negative slope of $\Delta P_F$ versus $\Delta$TMAO in accordance with eq 3.

Inserting the expression for $\bar{a}$ (eq 7) in eq 4 gives a complete model for the accumulation of free formaldehyde during the storage time ($t_R$):

$$\Delta P_F = \beta Q \bar{a} t_R = \beta Q a_0 \frac{(1 - e^{-kt_R})}{k} \tag{8}$$

According to eq 8, the accumulation of free formaldehyde during storage for the time $t_R$ can be predicted from measurements of the initial assay activity, $a_0$. When using $a_0$ instead of $\bar{a}$, $\Delta P_F$ is no longer strictly proportional to $t_R$, but to $(1 - e^{-kt_R})/k$, which also has the dimension "time".

## MATERIALS AND METHODS

**Sample Preparations and Measurements.** Raw fish and seafood products were primarily caught in seas in the North Atlantic region. Exceptions to this were burbot (*Lota lota*) from freshwaters in Denmark and giant tiger prawn (*Penaeus monodon*) from the Indian Ocean. All specimens were bled and cleaned immediately after capture. Thereafter they were either frozen at sea (−30 °C or in dry ice) or cleaned and kept fresh in melting ice until use within 3 days. All experimental animals and the applied postharvest treatments are listed in **Table 1**.

Frozen samples for the storage experiment were transported to the laboratory in dry ice packages and used for experiments within 8 days from capture. Large fish were split transversally in up to five chops of 200−500 g. Contamination of the white muscle segments with other tissues, for example, kidney, was avoided. Each chop was gently cleaned in water, vacuum-packed in polyethylene bags, and frozen at −10 ± 0.3 °C for up to 217 days. Small specimens (fish and invertebrates) were frozen in similarly sized lots of single or multiple individuals. Squid mantles were deskinned and frozen whole. Prawns and lobsters were decapitated and frozen without peeling.

**Table 1.** Specimens Included in the Screening and Storage Experiments[a]

| common name | Latin name | tissue type | TMAOase activity[b] | screening expt | | storage expt | |
|---|---|---|---|---|---|---|---|
| | | | | *N*[c] | treatment | *N*[d] | treatment |
| cod, North Sea | *Gadus morhua* | fillet, spleen | + | 9 | frozen | | |
| cod, Baltic Sea | *Gadus morhua* | fillet, spleen | + | 8 | frozen | | |
| cod, Oeresound | *Gadus morhua* | fillet | + | 7 | fresh < 1 day | 10 | fresh < 1 day |
| ling | *Molva molva* | fillet, spleen | + | 3 | frozen | 4 | frozen |
| saithe | *Pollachius virens* | fillet | + | 1 | frozen | 4 | fresh < 3 days |
| blue ling | *Molva dypterygia* | fillet | + | | frozen | 4 | frozen |
| cusk | *Brosme brosme* | fillet, spleen | + | 1 | frozen | 1 | frozen |
| whiting | *Merlangius merlangus* | fillet, spleen | + | 8 | frozen | | |
| burbot | *Lota lota* | fillet[e] | + | 3 | frozen | | |
| haddock | *Melanogrammus aeglefinus* | fillet, spleen | + | 7 | frozen | | |
| green pollack | *Pollachius pollachius* | fillet, spleen | + | 4 | frozen | | |
| angler | *Lophius piscatorius* | fillet, jar | – | 4 | fresh < 3 days | 3 | fresh < 3 days |
| redfish | *Sebastes marinus* | fillet | – | | | 3 | frozen |
| dogfish | *Squalus acanthias* | fillet | – | | | 2 | fresh < 3 days |
| halibut | *Hippoglossus hippoglossus* | fillet | – | | | 2 | frozen |
| plaice | *Pleuronectes platessa* | fillet | – | | | 2 | fresh < 3 days |
| catfish | *Anarhichas lupus* | fillet | – | | | 2 | fresh < 3 days |
| northern squid | *Loligo forbesi* | mantle | – | | | 2 | frozen |
| Norway lobster | *Nephrops norvegicus* | tail | – | | | 1 lot | fresh < 3 days |
| scallop | *Pecten maximus* | adductor | – | | | 1 lot | frozen |
| shrimp | *Pandalus borealis* | tail | – | | | 1 lot | frozen |
| tiger prawn | *Penaeus monodon* | tail | – | | | 1 lot | frozen |
| rainbow trout | *Oncorhynchus mykiss* | fillet | – | 2 | fresh < 3 days | | |
| mackerel | *Scomber scombrus* | fillet | – | 2 | fresh < 3 days | | |
| dab | *Limanda limanda* | fillet | – | 2 | fresh < 3 days | | |
| flounder | *Platichthys flesus* | fillet | – | 2 | fresh < 3 days | | |

[a] The table also shows the qualitative result of screening for TMAOase activity in the principal muscle (in fish, the white muscle part of the fillet). [b] + indicates TMAOase activity was above the detection limit of ~0.05 nkat/g. [c] Number of individuals for screening of white muscle. The numbers of individuals for screening of spleen were frequently lower, see **Table 2**. [d] Number of individuals or (when specified) number of lots prepared from multiple individuals. [e] Contamination with red muscle cannot be ruled out.

Shrimps were frozen whole. Samples were thawed in random order. Samples for "prestorage" analysis originated from samples that were thawed after 16 h at −40 °C.

Bellyflaps and exposed muscle surfaces within a depth of at least 5 mm and all blood-stained tissue were discharged, and 65−75 g of white muscle was isolated. Muscle samples were blended for 10 s in a kitchen blender, and the mince was used for all analyses.

A number of seafood products were screened for TMAOase activity in the muscle but were not included in the storage experiment. In species with distinct red and white muscle sections only white muscle was analyzed. For the invertebrates the principal muscle was used. Muscle activity was thus measured in 9 fish species (74 individuals) of the Gadidae family (order of gadiforms), 10 fish species representing 5 other orders, and 5 invertebrate species including 2 mollusks (scallop and squid) and 3 crustaceans (shrimp, prawns, and Norway lobster); see **Table 1**. In addition, TMAOase activities of spleen and muscle were compared in 39 of the gadiform fish representing 6 species. The isolated spleens were frozen at −80 °C until analysis. **Table 1** shows a complete list of the investigated species and their replicate numbers. The weight of all fish was recorded.

All frozen samples were thawed in plastic bags in circulating water at 10 °C. All analyses were performed in duplicate at 0−5 °C unless otherwise stated. All chemicals used were of analytical grade.

In vitro TMAOase activity was measured according to "procedure A" in Nielsen et al. (*31*). Samples were homogenized (UltraTurax, 20000 rpm) in 5 volumes of 50 mM acetate, 1 M NaCl, pH 4.5. The homogenate was mixed with Triton X-100 until a final concentration of 2% (w/v). The crude homogenate was directly used in the in vitro TMAOase assay. The reaction conditions were as follows: 0.73 M NaCl, 62.5 mM acetate, 50 mM TMAO, 1 mM ascorbic acid, 1 mM l-cysteine, 100 $\mu$M $FeCl_2$, 52 $\mu$M methylene blue, 1.46% (w/v) Triton X-100, pH 4.5, 25 °C.

Low ionic strength protein solubility was measured by centrifugation of 1 g of mince for 15 min at 49500*g*. The supernatant was collected, and the pellet was rehomogenized (UltraTurax, 20000 rpm) in 20 volumes of water and centrifuged again. The second pellet was rehomogenized in a Dounce homogenizer (large clearance) in 9 mL of 10 mM $Na_2CO_3$, 40 mM $NaHCO_3$, and 600 mM NaCl and stirred for 30 min before the third centrifugation. The protein in the first and second supernatants was referred to as low ionic strength soluble protein. The protein in the last supernatant was referred to as high ionic strength soluble protein.

Protein was determined according to the method of Lowry et al. (*32*), and free formaldehyde was determined according to the method of Nash (*33*). pH was measured at 25 °C with a glass electrode in a suspension of mince in 3 volumes of water. TMAO was determined as trimethylamine (TMA) essentially by the procedure of Conway and Byrne (*34*) after reduction of TMAO to TMA by titanium trichloride. The TMAO content in burbot was verified by capillary electrophoresis as described by Timm and Jørgensen (*35*).

**Data Analysis.** All values reported are mean ± standard deviation (SD) unless otherwise stated. Where the standard error of the mean (SEM) is used, it is calculated under the assumption of a normal distribution of average values.

Significant differences between series of values from different species were tested with the nonparametric Kruskal−Wallis one-way analysis of variance (*36*). Where significance at the $p < 0.05$ level was found, the series were compared in pairs by Dunn's multiple-comparison test (*37*).

Two types of linear regression were used: regression using mean-centered abscissa and ordinate values (averages taken over values from single individuals or values from single species) or regression of a proportionality model, that is, the regression line is forced through (0,0). Whether slopes of the regression lines were significantly different from zero or from each other was judged from the confidence intervals ($p > 0.95$). Where slopes were not found to be significantly different between species, a common regression line was calculated. For the data set with abscissa values that could not be considered to be virtually error-free, the regression result was compared to that of an inverse regression. In all cases, the resulting parameter was found to be within the confidence limits of the parameter originating from the original regression.

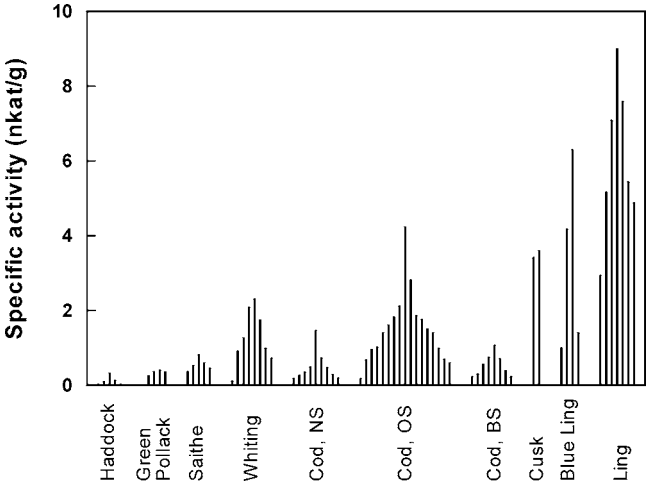


**Figure 1.** TMAOase activity in white muscle from various species of gadoid fish. Each bar represents a single individual. The values are grouped according to species. The values for cod are also grouped according to place of catch: NS, North Sea; OS, Oeresund; BS, Baltic Sea.

## RESULTS

TMAOase activity was documented in white muscle from all of the investigated gadiform species. TMAOase activity was not found in the principal muscle of any of the nongadiform species. The qualitative results are listed in **Table 1**.

Within species from the family Gadidae, TMAOase activities in white muscle ranged from below the detection limit of ~0.05 to 9 nkat/g. Large variations in TMAOase activity of the white muscle were found between species as well as between individuals from the same species. Within single species the TMAOase activity exceeded 10-fold differences. The variation between species partly followed the classification of species in subfamilies of Gadidae (*38*). The highest average TMAOase activities were found in ling, blue ling, and cusk, which all represent the subfamily Lotinae. Significantly lower average TMAOase activities were found in haddock, green pollack, and saithem representing the subfamily Gadinae. Samples from cod and whiting also representing Gadinae exhibited intermediate TMAOase activities. The TMAOase activities in frozen samples of cod from the North Sea and the Baltic Sea were lower than those in samples of cod from the Oeresound (located between the North Sea and the Baltic Sea), which had been held in ice for a maximum of 24 h. The average TMAOase activities in cod from the North Sea and from the Baltic Sea did not differ. The variation between samples from the same individual showed no correlation to its longitudinal anatomical location or to any other recorded characteristics of the sample, supporting the validity of the procedure. The quantitative data are presented in **Figure 1**.

A surprisingly high TMAOase activity was found in muscle from three individuals of the freshwater fish burbot, belonging to the family Gadidae and the subfamily Lotinae (data not shown); however, it cannot be ruled out that these muscle samples had been contaminated by minor amounts of red muscle. Burbot muscle also contained $27 \pm 3$ $\mu$mol of TMAO/g (Conway method), which was later confirmed by capillary electrophoresis to be 32 $\mu$mol of TMAO/g (single replicate).

As shown in **Table 2**, the ratio between TMAOase activity in spleen and muscle from the same individuals was between $447 \pm 72$ (mean $\pm$ SEM) for three ling and $5644 \pm 979$ for eight cod from the Baltic Sea. The mass of the spleen was ~0.1% of that of the white muscle but contained more TMAOase activity than the white muscle in total. Nonparametric analysis (*36*) showed a significant ($p < 0.0005$) influence of species on the ratio (**Table 2**).

**Table 2.** Ratio ($a_{Sp}/a_M$) between TMAOase Activity in Spleen ($a_{Sp}$) and White Muscle ($a_M$) of Gadiform Fish and Significance Level of Correlation Coefficient between $a_{Sp}$ and $a_M$ in the Same Individual

| species | N [a] | ratio (mean ± SEM) | correlation [b] |
|---|---|---|---|
| cod, North Sea | 8 | 4566 ± 577 | $p < 0.01$ |
| cod, Baltic Sea | 8 | 5644 ± 979 | NS |
| whiting | 7 | 913 ± 621 | NS |
| haddock | 6 | 853 ± 538 | NS |
| ling | 3 | 447 ± 72 | NS |
| green pollack | 3 | 647 ± 60 | NS |
| cusk | 1 | 1218 | |

[a] Number of individuals. [b] NS, not significant ($p > 0.05$).

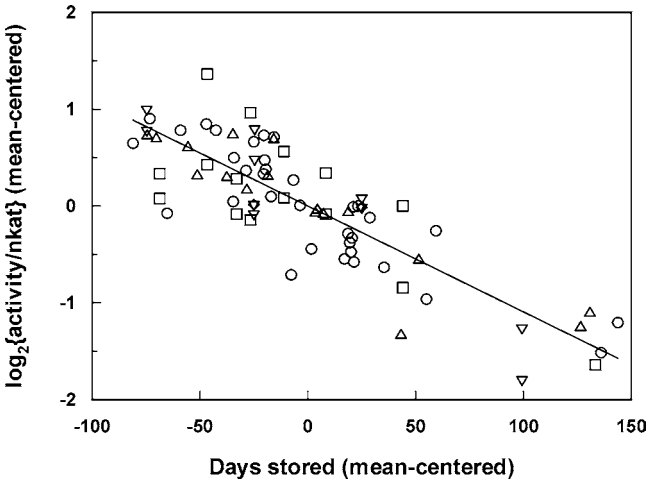


**Figure 2.** Changes in enzyme activity during frozen storage: ○, cod; □, saithe; △, ling; ▽, blue ling. TMAOase activity was measured in a group of individuals at different frozen storage times. Each point is the base-2 logarithm to the activity value of one individual at a specific time. To remove the dependence of initial activity, both the abscissa and ordinate values for each individual are mean-centered by subtracting the average value of measurements on that individual. The line shown is the linear regression line common to all of the species measured.

The TMAOase activity in spleen and white muscle showed no relationship except among the group of eight cod from the North Sea ($p < 0.01$). Neither the TMAOase content in white muscle nor that in the spleen was correlated to the size of the fish.

The inactivation of TMAOase at −10 °C appeared to follow first-order kinetics. Residual TMAOase activity was found throughout the whole storage period. Linear regression of the logarithm to the TMAOase activity versus storage time resulted in a common monoexponential inactivation constant, $k = 7.5 \pm 0.5 \times 10^{-3}$ days$^{-1}$, corresponding to a half-life of 92 days (**Figure 2**). The $k$ estimates of single species did not differ significantly. Estimated values of $k$ for single individuals of fish did not correlate with other experimental variables such as the TMAO concentration and the rate of formaldehyde accumulation.

Accumulation of formaldehyde during frozen storage was detected in white muscle of all individuals of gadiform fish but not in any other seafood products in the experiment. The free formaldehyde accumulated in gadiform species in accordance with the suggested model (eq 8, **Figure 3**). The model described small accumulations of free formaldehyde (**Figure 3B**) as well as large accumulations of free formaldehyde during >6 months of storage at −10 °C (**Figure 3A**).

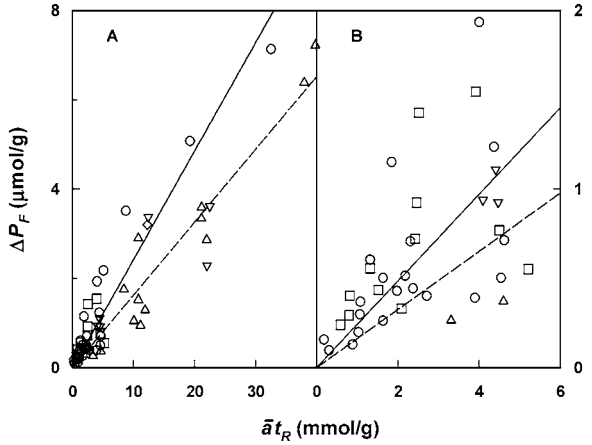


**Figure 3.** Effect of average enzyme activity ($\bar{a}$) and frozen storage time ($t_R$) on the accumulation of free formaldehyde ($\Delta P_F$): (**A**) full range of values; (**B**) values in the low range [same lines as in (A)] (○, cod; □, saithe; △, ling; ▽, blue ling; black oval, cusk). The regression lines shown are forced through (0,0). The solid line applies to cod and saithe, the dashed line to ling and blue ling.

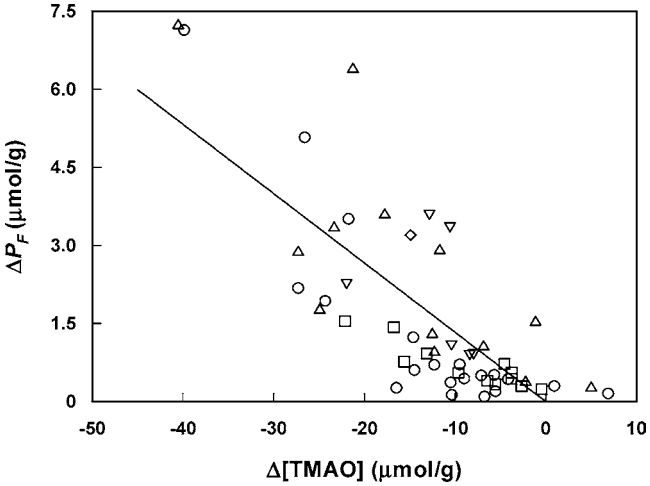


**Figure 4.** Relationship between formation of free formaldehyde ($\Delta P_F$) and degradation of TMAO: ○, cod; □, saithe; △, ling; ▽, blue ling; black oval, cusk. Each point is the measured value after storage *minus* the initial value of the same individual. The regression line shown is forced through (0,0) and common to all species included.

The accumulation of free formaldehyde in frozen samples could be predicted with a relative root-mean-square error of prediction (RMSEP) of 0.65 when the in vitro prestorage activity ($a_0$) was measured in a random sample from the same individual. RMSEP was reduced to 0.52 when $a_0$ was estimated from a residual activity in the same sample, which was also used for the measurement of free formaldehyde.

The formation of free formaldehyde did not progress similarly in all species of fish, which could be divided into two groups according to their different values of $\beta Q$, as represented by the slope of the appropriate regression line in **Figure 3**. Cod and saithe of the subfamily Gadinae showed significantly higher $\beta Q$ values than did ling and blue ling, representing the subfamily Lotinae. The single result for cusk fell inside the range of both groups.

Comparison of individuals showed large differences in the accumulation of formaldehyde during storage at −10 °C. The differences were mostly explained by their differences in white muscle TMAOase activity. During the first ∼40 days of storage six fish with the highest TMAOase activity accumulated formaldehyde at a rate that was 7 times higher than that of the six fish with the lowest TMAOase activity. The highest concentration of free formaldehyde was 7.3 μmol of free formaldehyde/g, which was measured in a ling after storage for 201 days. In comparison, a saithe with low TMAOase activity accumulated only 0.57 μmol/g after 202 days.

Estimates of $\beta Q$ from single individuals showed no correlations with the residual concentrations of TMAO or with any other measured variables including muscle pH and longitudinal sample location. The TMAO concentration decreased as expected throughout the storage period. The lowest residual TMAO concentration found was 4.5 μmol/g in ling stored for 201 days.

The decrease in TMAO concentration during storage exceeded the accumulation of free formaldehyde. The estimates of $\beta$ did not differ significantly between species and showed no significant correlation with $\Delta P_F$ (**Figure 4**). Neither $\beta Q$ nor $\beta$ nor $Q$ showed any significant correlation with storage time. The estimates of $\beta Q$, $\beta$, $Q$, and $k$ are listed in **Table 3**.

The relative model residuals calculated from the parameters presented in **Table 3** were independent of the measured values of $\Delta P_F$, suggesting constant relative errors of prediction. An analysis of the residuals showed these distributed in two populations, each characterized by the amount of muscle homogenate that had been used for the activity measurement. This difference in sample size was a necessary consequence of the large variation in TMAOase activity between samples and had no systematic influence on the predictions of $\Delta P_F$.

**Table 3.** Model Parameter Estimates[a]

| | $\beta Q$ | ± 95% confidence interval |
|---|---|---|
| cod and saithe | $2.4 \times 10^{-4}$ | $2.3 \times 10^{-4}$ |
| ling and blue ling | $1.6 \times 10^{-4}$ | $1.8 \times 10^{-4}$ |
| | $\beta$ | ± 95% confidence interval |
| all species | 0.13 | 0.03 |
| | $Q$ | ± 95% confidence interval |
| cod and saithe | $1.8 \times 10^{-3}$ | $0.3 \times 10^{-3}$ |
| ling and blue ling | $1.2 \times 10^{-3}$ | $0.2 \times 10^{-3}$ |
| | $k$ ($day^{-1}$) | ± 95% confidence interval |
| all species | $7.5 \times 10^{-3}$ | $1 \times 10^{-3}$ |

[a] $\beta Q$ values are the slopes of the lines in **Figure 3**, and $-\beta$ is the slope of the line in **Figure 4**. $Q$ is calculated as $(\beta Q)/\beta$.

The amounts of high ionic strength soluble protein decreased more quickly and to a greater extent in species with detectable TMAOase activity than in species without TMAOase activity (**Figure 5**). Among gadiform fish the high ionic strength protein solubility decreased more quickly in the individuals with the highest TMAOase activity. The solubility decreased rapidly in the first part of the storage period, whereas only minor changes occurred after 1 month. No lag phase was observed. The low ionic strength protein solubility decreased only to a lesser extent during the storage period regardless of the presence of TMAOase (data not shown).

## DISCUSSION

The occurrence of TMAOase activity in gadiform fish suggests that enzymatic formation of formaldehyde and DMA is a problem that is primarily restricted to gadiform species. However, other studies have shown formation of DMA in a

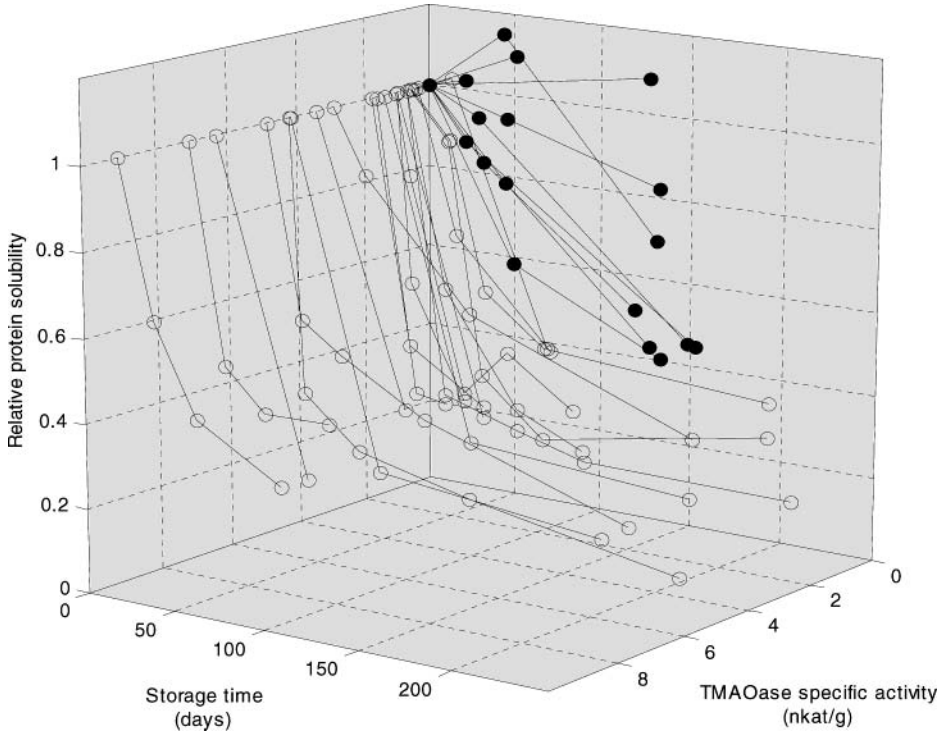


**Figure 5.** High ionic strength protein solubility as a function of frozen storage time and initial TMAOase activity. Each curve shows protein solubility versus storage time of a single individual. The solubility is given relative to the initial solubility of "pre-storage" samples (time = 0). The curves are placed according to the initial TMAOase activity of the fish muscle. Individuals from species for which no enzyme activity was detected are indicated by black symbols.

wider range of species including internal organs of shrimp (*39*) and bivalve mollusks (*40*) and mantle muscle of squid (*Loligo pealei* and *Loligo opalescens*) (*41*). These findings were not supported by measurements on the Northern squid, *Loligo forbesi* (representing the same genus), in the present study. Neither muscle TMAOase activity nor significant amounts of free formaldehyde were measured during the storage of squid at −10 °C for 126 days.

The TMAOase activities in white muscle shown in **Figure 1** are in accordance with previously reported values from blue ling, cod, and saithe (*19*). The documented TMAOase activity in white muscle of haddock and whiting elucidates previous undetected values (*19*). The reason for the large individual variation found among most species is as yet unknown but may be caused by individual periodic variations. The lack of correlation between fish size and TMAOase activity in white muscle is in agreement with a previously published result that was based on TMAOase activity in internal organs of hake (*42*). The average TMAOase activity differed significantly between species ($p < 0.0001$). The ranking of species according to their average TMAOase activities (**Figure 1**) resembles the ranking of species according to their rate of DMA formation. A similar ranking based on formation of DMA was found for haddock, cod, and cusk (but not saithe) (*28*) as well for haddock and cod (*43*).

The higher average TMAOase activity found in cod from Oeresound than in cod from the North Sea and from the Baltic Sea may reflect differences between populations or may be due to the differences in postharvest treatment described in **Table 1**. The result emphasizes the necessity of systematic sampling in investigations of the biological variation of the distribution of TMAOase in gadiform fish.

Cod from the Baltic Sea and from the North Sea had similar TMAOase activities despite lower TMAO contents in cod from the Baltic Sea (*44*, *45*). This finding suggests that TMAOase activity in white muscle has no regulatory effect on, nor is it induced by, the TMAO level in vivo.

Burbot belongs to the minority of freshwater fish that accumulates TMAO within the normal range for marine gadiforms. It also represents the first discovery of TMAOase in freshwater fish. Because freshwater fish are all hyperosmotic, the accumulation of TMAO may either be a manageable genetic leftover or fulfill physiological functions other than regulation of the osmotic pressure. Because TMAO is scarce in freshwater prey organisms (*44*), it must be synthesized by the burbot at an average rate that exceeds the in vivo activity of TMAOase.

The ratio of TMAOase contents in white muscle and spleen confirms that TMAOase is most abundantly located in the viscera (*21*). Even minor contaminations of the white muscle with such tissue may therefore raise the TMAOase activity of white muscle tissue dramatically. The correlation between TMAOase activity of muscle and spleen of cod from the North Sea was not shown in other gadiform species. Therefore, white muscle TMAOase activity can generally not be predicted from measurements of the much higher activities in the spleen.

**Figure 2** shows that TMAOase decays monoexponentially with a half-life of 92 days at −10 °C. This fairly long half-life supports the general picture of TMAOase as a chemically and physically robust enzyme (*21*). The effect of TMAO on the stability of TMAOase during freezing could be dual. TMAO is an effective cryostabilizer of fish muscle proteins (*46*), but TMAO also constitutes the substrate of TMAOase and thereby increases the potential formation of protein denaturizing formaldehyde. However, the result shows neither any effect by the initial TMAO concentration nor an effect by formaldehyde on the in situ rate of TMAOase. The experimental variation seen in **Figure 2** mostly represents sample variation.

It has been suggested that DMA is a measure of the total amount of formaldehyde formed. However, as a secondary amine, DMA is expected to be somewhat reactive. Thus, the formation during storage of both formaldehyde and DMA must be expected to be higher than measured values of free formaldehyde. Instead, the formation of formaldehyde and DMA should equal the decrease in the amount of TMAO. This last assumption is the basis for calculation of $\beta$ from the apparently linear relationship depicted in **Figure 4**. The data, however, did not show any systematic influence of free formaldehyde on the ratio between concentrations of free and total formaldehyde. The calculated ratio of 13% free formaldehyde in **Table 3** is slightly smaller than was determined from recovery experiments by Rehbein (*47*), who reported that successive additions of formaldehyde to fish mince lead to increasing recoveries of free formaldehyde. The estimated constant value of $\beta$ indicates an excess of binding sites for formaldehyde in fish muscle and a small equilibrium constant between bound and free formaldehyde.

The ability of the suggested model to describe the accumulation of formaldehyde, on the basis of in vitro TMAOase activities and storage time, is a strong indication that white muscle in situ TMAOase activity is the primary cause for formation of formaldehyde and DMA in gadiform fish. If isoenzymes exist as suggested by several authors (*48*−*50*), these must therefore either exhibit a common value of $Q$ or their activities must be in constant proportions. The influence of variations in the in situ TMAOase reaction conditions also seems to be secondary to the enzyme concentration.

Different values of RMSEP for the free formaldehyde accumulation, $\Delta P_F$, were obtained depending on how the prestorage activity, $a_0$, was estimated. RMSEP was highest when $\Delta P_F$ values of thawed samples were predicted on the basis of a measured value of $a_0$ from a different sample from the same individual. A lower value of RMSEP was obtained if $a_0$ was calculated from the residual activity in a thawed sample that was also used for measurement of $\Delta P_F$. The difference between the two RMSEP values is a consequence of the random variation of the TMAOase activity between samples, which is only taken into account by the latter calculation.

During the storage period, the TMAO concentration was reduced to values near or even below the $K_M$ value of TMAOase in vitro (17 mM at 25 °C). If the in situ value at −10 °C was close to this, $Q$ and therefore $\beta Q$ would be storage-time dependent due to the depletion of TMAO. This was, however, not found, so the effect of substrate concentration, for example, in the form of the integrated Michaelis−Menten equation, was not included in the model. The result shows that TMAOase is nearly saturated with substrate in situ, suggesting a $K_M$ significantly lower than the in vitro estimate. In support of this, Phillippy and Hultin (*25*) have reported a $K_M$ value of 3 mM TMAO by using reaction conditions more similar to the expected in situ conditions. A $K_M$ value in this range would cause the enzyme to be nearly saturated with substrate during the full storage period.

In the case of a fully saturated enzyme, $Q$ represents the ratio of in situ $V_{Max}$(−10 °C)/in vitro $V_{Max}$(25 °C). Because the estimated values of $Q$ in **Table 3** are much lower than what can be explained by temperature effects alone, the in situ reaction rate must also be rate limited by other factors. This conclusion is in agreement with results from studies based on reconstituted mince products and muscle extracts (*25*, *51*, *52*). In particular, redox agents are known to be important cofactors, which are therefore likely to influence the value of $Q$. However, the in situ regulation of TMAOase activity is not as yet completely understood. The apparently constant value of $Q$ in the present study shows that the total effect of the TMAOase activity modulators seems to be conserved throughout the prolonged freezing period.

Comparing model residuals from single individuals of fish shows that these distribute around a characteristic average value for each individual. This suggests the $\beta Q$ parameters to be individual or that more factors, not already being fully accounted for in the model, exist. In the present study, the variation of $\beta Q$ and $Q$ between species as well as between individuals was minor in comparison to the effect of the TMAOase concentration.

The negative effect of formaldehyde production on protein solubility in fish is well documented and expected to be closely related to the characteristic quality changes (*2*, *4*, *5*). In the present study, the effects of prestorage TMAOase activity and storage time on the insolubilization of high ionic strength soluble protein were documented as shown by **Figure 5**. The kinetics of the insolubilization was not modeled due to a low number of measurements during the initial phase and the level of experimental variation between measurements.

Because the rate of formaldehyde formation during frozen storage of gadiform fish can be predicted on the basis of TMAOase activity levels in the white muscle, reliable statistical knowledge about the large biological variations of this parameter in populations of gadiform fish would be valuable for the fishery industry. In particular, aquaculture production of gadiform fish opens up a possibility to manipulate the level of white muscle TMAOase activity in livestocks. In this case, TMAOase activity should be an object for optimization in both breeding and growing.

## ABBREVIATIONS USED

$a$, in vitro reaction rate (activity, time dependent); $a_0$, initial in vitro reaction rate (activity); $\bar{a}$, average in vitro reaction rate (activity); $\Delta P$, formation of formaldehyde (mol/g); $\Delta P_F$, the accumulation of free formaldehyde (mol/g); $\bar{v}$, average in situ reaction rate; $t$, time; $t_R$, storage time; $Q$, ratio of $\bar{v}$ over $\bar{a}$; $\beta$, ratio of $\Delta P_F$ over $\Delta P$; $k$, inactivation rate constant.

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