COPE elimination reaction observed in the biodegradation of quaternary ammonium surfactants

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The biodegradation of dialkyldimethylammonium compounds in aqueous solutions indicates that part of the degradation pathway includes the Cope elimination reaction, which does not occur under mild conditions in the absence of microorganisms.

This report details the first report of the Cope reaction mediated by microorganisms and starting with long-chained quaternary ammonium compounds. This reaction appears to be a key step in the biodegradation of quaternary ammonium compounds ('quats'). The quats which are the subject of this study are either dialkylethoxymethylammonium, dialkylamidoammonium or dialkylmethylimidazolinium quats.

Long-chained quaternary ammonium compounds have found use in a variety of products and sometimes end their life-cycle by disposal into wastewater or surface waters. Thus the biodegradation of these compounds has been the subject of many studies. It has been found that a wide variety of microorganisms can utilise quats as energy sources. It is also known that many microorganisms can utilise alkylamines as a substrate and use an inducible monooxygenase (EC 1.14.13.8) which is capable of oxidising the substrate to the corresponding *N*-oxide.[1] We have discovered that a key step in the biodegradation of these quats is the formation of an *N*-oxide which is formed once the quats have first degraded to an alkyldimethylamine. During our investigations of the biodegradation of dialkydimethylammonium salts (DADMA X; X = F, Cl, Br, I, OH), and subsequent determination of the metabolic pathway by fermentation of these compounds, we isolated the corresponding *N*-oxides and cleavage products of these *N*-oxides, *e.g.* the C_{18} alkene and the corresponding hydroxylamine. The *N*-oxide is converted to the corresponding trialkylamine and formaldehyde (which are mineralised to CO_2 and H_2O) by an amine *N*-oxide aldolase (EC 4.1.2-) functioning as a demethylase.[2] The degradation of the quats was done using acclimated bacterial strains, fungi and yeasts for fermentation, typical of those known to be present in the environment.

The Cope reaction is the cleavage of dialkylamine *N*-oxides to produce alkenes and hydroxylamines.[3] Mild conditions are used to reduce side reactions, and the generated olefins do not normally rearrange. In this report we show the transformation of dialkyldimethylammonium salts to *N*-oxides, followed by cleavage of the *N*-oxides to the corresponding alkenes in a Cope-like process using a crude enzymatic preparation from microorganisms of environmental origin. We also show an enhancement of the kinetics of olefin formation due to the presence of the DADMA X micelles (primarily for X = OH).

Fermentation was performed with 12 strains of microorganism§ known to be present in soil and aquatic systems, including waste treatment (*Xanthomonas compestis*, salt regulative #33951, *Bacillus subtilis vulgatus* #6984, *Torulopsis* spp. #34356, together with aerobic and faculative anaerobic bacterial genera *e.g. Actinobacter, Agrobacterium, Alcaligenes, Nocardia, Arthrobacter, Cellulomonas, Mycobacterium* and *Candida albicans*). The cells were harvested, centrifuged, washed with 0.1 M KH_2PO_4–K_2HPO_4, pH 7.5 and stored at −10 °C. The intracellular contents were purified by $(NH_4)_2SO_4$ fractionation (25–65% of a saturated solution at 5 °C). The portions containing the monooxygenase activity were found in the fractions collected in 48–60% saturated $(NH_4)_2SO_4$ solutions. These fractions were used to prepare the S-100 supernatant at 5 °C. The supernatant was used as a crude enzyme preparation and stored in 10% (v/v) glycerol in the presence of 0.01 M KH_2PO_4–K_2HPO_4, 5 mM $MgCl_2$ and 1 mM β-mercaptoethanol (pH 7.8, 20 °C). The S-100 fraction containing 10–15 mg ml^{-1} protein was used in the enzymatic experiments in the presence of glycerol¶ and the distearyldimethylammonium salt (DSDMA OH; 5 mM) at pH 7.8 (37–40 °C) for studying enzyme mediated olefin formation, eqn. (1), where R = C_nH_{2n+1} and n = 8, 10, 12, 14, 16

$$(R)_2N^+CH_3{\rightarrow}O \longrightarrow (R)CH_3NOH + CH_2{=}CH(CH_2)_{15}CH_3 \quad (1)$$

or, as in the example given here, 18. The formation of the $(R)_2N(CH_3){\rightarrow}O$ compounds have also been studied at pH 6.0 in 0.01 M borate buffer at a protein concentration of 1 mg ml^{-1}, in the presence of 5 mM $NADH_2^+$ (or 3.5 mM NAD^+), and a $NADH_2^+$ (NAD^+) regenerating system at 37 °C.‖ Formation of the olefins has not been observed in the absence of the crude, enzymatic mixture at 37 °C under the same conditions. However, by refluxing the *N*-oxides in xylene in the presence of 1 mM NaOH for two hours the corresponding olefin can be obtained in an almost quantitative yield. Since the formation of *N*-oxides operates only at pH 6.0–6.5 (37 °C) and the olefin formation is seen mainly in the presence of 10% (v/v) glycerol and pH 7.5–8.0, both pathways can be studied separately. The *N*-oxides studied have also been prepared chemically by reacting the appropriate amine with 30% hydrogen peroxide at 0 °C for 10 min and characterised using standard methods. The *N*-oxides obtained from the enzymatic reactions were characterised in the same fashion.

Fig. 1 shows the yields of the olefin $CH_2{=}CHC_{16}H_{33}$ generated from distearylmethylamine *N*-oxide (10 mg ml^{-1}) using the crude enzymatic fraction in the presence of 5 mM DSDMA X (X = OH, F, Cl, Br, I) (pH 7.8, 37 °C) and 10% (v/v) glycerol. The most effective yield is obtained in the presence of 5 mM DSDMA OH [10% (v/v) glycerol], whereas at pH 6.5 the yield is very low as it was for the Br. Furthermore, addition of 1–10 mM NaCl to the reaction medium reveals inhibition of olefin generation (C_i = 6 mM NaCl, 50% inhibition). The influence of NaCl can be related to: (i) the salt induced change of the colloidal state of DSDMA OH from vesicle to micelle,[4,5] and (ii) the shifting of the equilibrium of DSDMA OH + Cl^- ⟷ DSDMA Cl + OH^-, toward the insoluble DSDMA Cl and colloidal state of both the hydroxide and chloride.

The biotransformation of DADMA X is strongly dependent on the critical micelle concentration (CMC) pH, temperature and ionic strength.[6] The initial rate constants for the anion exchange step where DSDMA X ⟶ DSDMA OH (X = Cl,

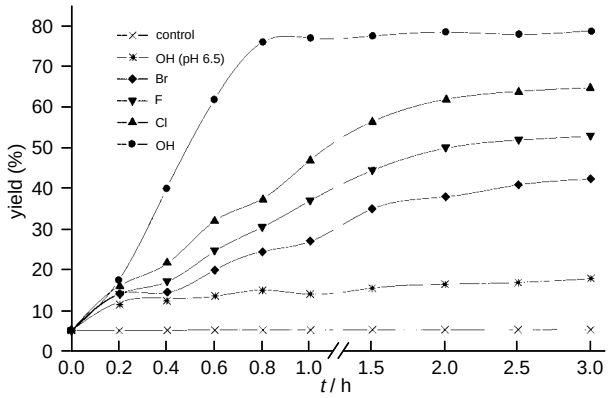

Fig. 1 Olefin generation (CH_2=$CHC_{16}H_{33}$) from distearylmethylamine *N*-oxide in the presence of 5 mM DSDMA X (X = OH, F, Cl, Br) at pH 7.5, 37 °C in 0.01 M K_2HPO_4, 5 mM $MgCl_2$, containing 10 mg ml^{-1} protein. Aliquots from the reaction vessel (10 ml) were taken after 15 min, analysed for remaining *N*-oxide and CH_2=$CHC_{16}H_{33}$ by GC–MS techniques.

Br) are strongly pH dependent, *e.g.* for DSDMA Cl at pH 5.0: k^*_{app} = 20.1 × 10^3 M^{-2} s^{-1} and at pH 8.0: k^*_{app} = 0.105 M^{-2} s^{-1}. Measurements below the CMC revealed a rate constant of k = 6.79 × 10^9 l mol^{-1} s^{-1} for Cl^- (OH^-) and k = 6.25 × 10^9 l mol^{-1} s^{-1} for Br^-. These values are typical for a diffusion limited rate process between ions. Furthermore, a plot of log k *vs.* $I^{1/2}$ reveals the influence of DSDMA Cl (Br) + OH^- on the ionic strength, and is linear with a slope of 1.03 at pH 7.5–8.0 below the CMC, and 0.35 above the CMC. Furthermore, the fraction of exchange of Cl^- (α) by OH^- at pH 6.0 (25 °C) at equilibrium is 1.56%, and at pH 7.5 it is 30.6%. So the second rate constant for exchange is $k_q\alpha$, which is the rate for forming the ternary complex (DSDMA Cl + OH^- ⟷ DSDMA OH + Cl^-) times the fraction of that complex leading to exchange, *e.g.* at pH 6.0 (25 °C) to 318.6 M^{-2} s^{-1} and at pH 8.0 to 0.379 M^{-2} s^{-1}, respectively.

Long chain ammonium salts having two different chain lengths and two methyl groups, *e.g.* $(C_{12}H_{25})(C_{18}H_{37})N^+(CH_3)_2$ X^-, particularly for X = OH, have also been studied. For these materials *N*-oxidation by this enzymatic transformation could yield the chiral (*S*)-(−)- and (*R*)-(+)-*N*-oxides. Chiral analysis

of the products** shows that the amounts of the two kinds of enantiomers are dependent on the chain length. Keeping C_{18} and choosing the other chain length as between C_8 and C_{16}, the enantiomeric excesses (ees) were found to be of the order 60% (*R*) and 40% (*S*). When the second chain length was between C_4 to C_6, the ees changed to 40% (*R*) and 60% (*S*). In addition, when the *N*-oxides were reacted according to eqn. (1), it was found that the olefins produced were generated from the shorter chain length residue where the chiral *N*-oxide has the (*S*)-(−) conformatioin (80%), whereas the (*R*)-(+)-*N*-oxide generated only 10–15% alkene for R^1 = C_{18}, R^2 = C_{12}. Changing the chirality of the *N*-oxide from (*S*) to (*R*) for R^1 = C_{18} and R^2 = C_4–C_6, the generated olefins are of the order of 80%. Racemic R^1R^2 *N*-oxides generated in the average 40–45% of the corresponding alkenes.

Footnotes and References

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§ The fermentation broth consisted of 10 g glucose, 2.5 g KH_2PO_4, 0.05 g peptone, 0.01 g urea and 10 mM DSDMA OH in 2.5 l HPLC grade water (pH 7.5, 20 °C). The broth was aerated and the pO_2 and pCO_2 were also computer controlled (100 Hz).

¶ The monooxygenase activity was measured through an $NADPH_2$ regenerating system, comprised of 10 mM NADP, 1.5 M glucose-6-phosphate and 75 mM of glucose-6-phosphate dehydrogenase (6-GPD)–10 ml 0.01 M K_2HPO_4 buffer, pH 7.8 (37 °C). The 6-GPD (EC 1,1,1,49) was from Baker's yeast and Torula (100 units). The activities for monooxygenase of the crude enzyme preparation were found to be of 14.2 ± 1.2 nmol mg^{-1} for ethylmorphine *N*-dimethylase, 40.1 ± 4.2 nmol mg^{-1} min^{-1} for aminopyridine *N*-dimethylase and 45 nmol mg^{-1} min^{-1} for aniline hydroxylase, respectively, applying the Hantzsch reaction.

‖ Glucose dehydrogenase (GDH) from *B. cereus* was used for the cofactor regeneration (Sigma, St Louis, MO) since this enzyme can use glucose as a substrate and accepts either NAD^+ or $NADP^+$ as a cofactor. The regeneration of $NADH_2^+$ ($NADPH_2^+$) or synthesis of olefin can be maintained (37 °C) in a 10 ml solution containing glucose (0.35 M), 5 mM bromomercaptoethanol, GDH (700 units) in 0.01 M sodium borate buffer, pH 6.0, containing 0.05 M $MgCl_2$, 0.05 M NaCl and 0.05 M $CaCl_2$.

** The concentrations of (*S*)- and (*R*)-enantiomers of the *N*-oxides were measured by HPLC and NMR, using a Regis-DNB-phenylglycine Pirkle column, monitoring the change of the refractive index at 500 nm. Enantiomeric excess (ee) is defined as: ee = ([*R*]-[*S*])/([*R*] + [*S*]) × 100%. The NMR method used the derivatization of the protonated *N*-oxides with (*R*)-(+)-2-methoxy-2-trifluoromethylphenylacetic acid in pyridine.

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