LIGHT-INDUCED CATALYTIC INHIBITION OF ACETYLCHOLINE ESTERASE

Maurice P. GOELDNER and Christian G. HIRTH

Laboratoire associé au CNRS, Institut de Chimie, Université Louis Pasteur, 1 rue Blaise Pascal, Strasbourg, France

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1. Introduction

Catalytic enzyme inhibitors are defined as chemically-inert substrate analogs. Inhibition of the enzyme is due to exclusive active-site alkylation by a species generated after the enzymatic reaction. These inhibitors, first discovered by Bloch and his group [1] have the potential advantage of being more specific than the classical affinity-labelling inhibitors. Most of these inhibitors have been developed for enzymes involving proton abstraction in the first catalytic steps [2]. More recently glucotriazene [3] and *N*-nitrosolactam [4] have been used as catalytic active-site inhibitors of β-galactosidase and chymotrypsin, respectively, by in-situ generation of a carbonium ion from an alkyl diazonium. As well, acylated chymotripsin carrying a photolabile group in the active site was described some years ago [5]. In this paper we describe a new type of inhibitor which combines the reaction mechanisms of enzyme catalysed transformation and product photolability. We relate here the irreversible, light-promoted, catalytic inhibition of acetylcholine esterase (EC 3.1.1.7) by methyl (acetoxymethyl) nitrosamine (DMN-OAc) 1 (fig.1).

2. Materials and methods

DMN-OAc was synthesised according to Keefer et al. [6]. All other chemicals were of the best available grade. Electric eel acetylcholine was obtained from Sigma Chemical Co. (grade V, 1400 units/mg) and used without purification. Enzyme assays were performed in 50 mM phosphate buffer, pH 7.2 at room temperature in the presence of acetylthiocholine (5×10^{-4} M) and 5,5′dithio-bis(2-nitrobenzoic acid). The liberation of thiocholine was followed spectrophotometrically by recording the absorption of the thionitrobenzoate anion at 405 nm.

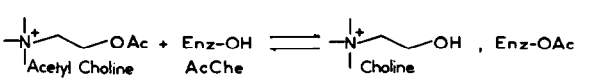
Irradiation was carried out in pyrex glassware in 50 mM phosphate buffer, pH 7.2, at room temperature with a Phillips lamp HPK 125W type 572033/00

Hydrolysis of DMN-OAc 1 was followed either by proton titration or by ultraviolet spectroscopy to follow the disappearance of the *N*-nitroso chromophore at 235 nm.

4,5-Dicarbomethoxy pyrazole was synthesised adding dimethyl acetylenedicarboxylate to an excess of diazomethane in ether at 0°C. The formed pyrazole crystallised out and was recrystallised twice in ether: m.p. 141. 5–142. 5°C; ultraviolet (H_2O) 225 nm, $\epsilon = 6380$.

3. Results and discussion

We found that DMN-OAc 1 was slowly hydrolysed by acetylcholine esterase (K_m 10^{-2} M; V_{max} 4% V_{max} acetylthiocholine) giving compound 2 which is a precursor of alkylating species [6] (fig.1), but no inhi-



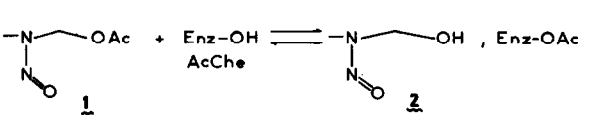


Fig.1.

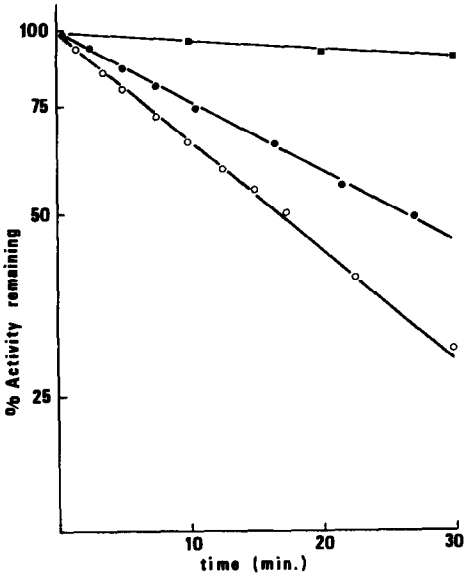


Fig.2. Inhibition of irradiated acetylcholine esterase at different concentrations of DMN-OAc 1. Log $100A/A_0 f(t)$, where A/A_0 is the percentage of remaining activity. [Enz] 2×10^{-2} mg ml^{-1}. [DMN-OAc 1]: (■) no inhibitor; (●) 1.5×10^{-3} M; (○) 5×10^{-3} M.

bition of the enzyme was noticeable: (1.2×10^{-7} M enzyme; 5×10^{-3} M DMN-OAc; reaction time 30 min). However irradiation of the enzyme solution in the presence of DMN-OAc led to a time-dependent loss of enzyme activity which followed first-order kinetics (fig.2).

We checked that under our irradiation reaction conditions

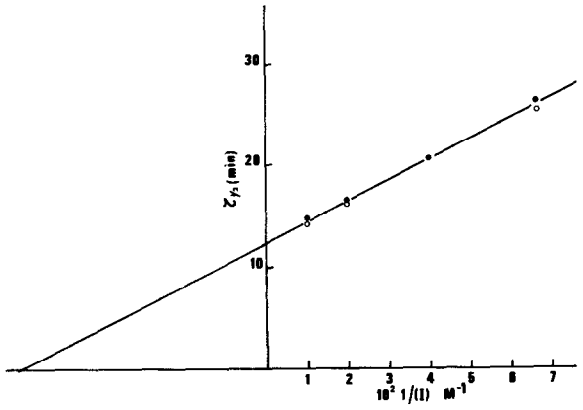


Fig.3. Relationship between the half-life of enzyme activity and the reciprocal of inhibitor concentration: (○) In the absence of BSA. (●) In presence of 5 mg. ml^{-1} of BSA.

i. The enzyme activity was only slightly affected.
ii. Bovine serum albumin (BSA) slightly protected the enzyme.
iii. Inhibitor 1 remained unchanged in the absence of acetylcholine esterase [7] and preirradiation of a solution of DMN-OAc before the addition of enzyme did not affect the rate of inactivation, this excluded the possibility of an inhibition due to some minor photosensitive impurity.
iv. Finally dialysis of the reaction medium overnight did not regenerate the activity of the enzyme.

We conclude that the loss of activity was independent of enzyme purity: addition of up to 5 mg.ml^{-1} of BSA did not significantly change the time course of inactivation (fig.3). The following reaction scheme for enzyme inactivation can be formulated:

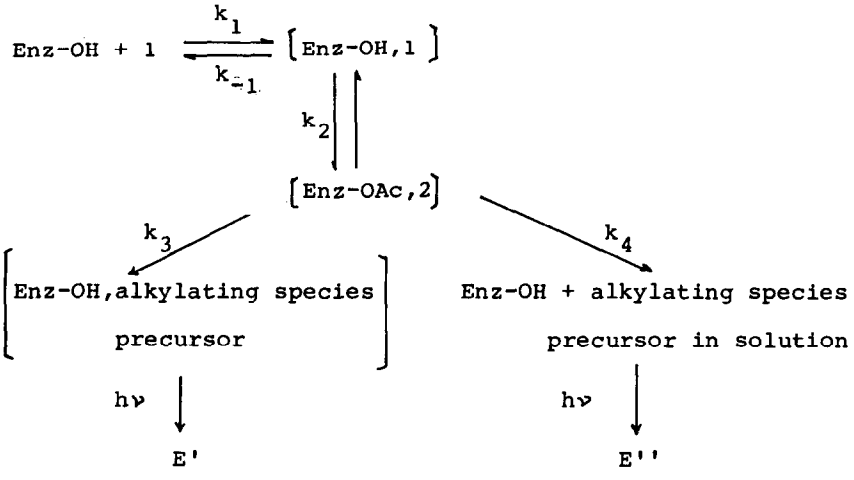


Fig.4.

This scheme implies that enzymatic hydrolysis of DMN-OAc 1 preceeds the inhibition of the enzyme (E′ + E″). The use of a 250-fold excess of BSA/AcchE is of particular interest because it would quench any reactive species which might leave the active site and give rise to non-specific inactivation (enzyme E″). The similarity in the rates of inactivation of the enzyme with or without added BSA suggests that alkylation of the enzyme occurred exclusively at the active site by a reagent that remained there (enzyme E′). Furthermore, the following experiments agree with the definition of suicide enzyme inactivators [2]:

i. The inactivation rate became independent of concentration at high inhibitor concentrations (fig.3). From these results an apparent affinity constant of inhibitor 1 can be determined: K_1 1.6×10^{-3} M; k_{cat} 10^{-3} s^{-1}. For kinetic treatment of fig.4 see ref. [8].

ii. In the presence of choline the rate of inhibition of the enzyme reaction decreased when the choline concentration was raised while the inhibitor concentration was kept constant (table 1).

4. Conclusions

Nature of the alkylating species

Ester hydrolysis of compound 1 gave rise to compound 2 which was shown under similar reaction conditions [6] to be a very short-lived species forming formaldehyde and methyl diazotate 3 a precursor of diazomethane [10] (fig.5). Diazomethane is known to be stable in water in contrast to other diazoalkanes and needs acid catalysis for its decomposition [11]. To ascertain the acetylcholine esterase catalysed formation of diazomethane, we hydrolysed a 0.5 M solution of DMN-OAc 1 in water with acetylcholine esterase

Table 1
Effect of choline on the half-life of enzyme activity

	Choline (M)		
	0	10^{-3}	5×10^{-3}
$T_{1/2}$ (min)	21	24	34

[DMN-OAc 1 2.5×10^{-3} M.
K_i choline 5×10^{-4} M [9]

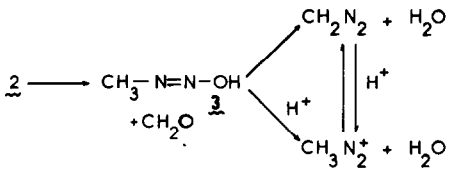


Fig.5.

(reaction followed by ultraviolet spectrophotometry). Upon addition of dimethyl acetylenedicarboxylate an absorption at 225 nm appeared immediately in agreement with the λ_{max} of the expected pyrazole formed by 1,3-dipolar addition of diazomethane to the acetylenic group [12]. We excluded the possibility of enzyme inactivation by alkylation due to affinity of diazomethane for the active site with the following experiment: irradiation of a solution of enzyme (10^{-7} M in phosphate buffer 0.1 M, pH 7.2) and diazomethane (3×10^{-4} M) led to a loss of enzyme activity of only 10%. No protection against this inactivation was observed in the presence of choline (5×10^{-3} M).

It is tempting to suggest that diazomethane is generated from compound 2 at a rate which is greater than the rate of diffusion from the active site (i.e. $k_3 \rangle k_4$) and that methylene carbene photochemically generated from it would react readily at the active site. These experiments were carried out by irradiation with polychromatic light source at $\lambda \rangle 300$ nm. However when irradiation was performed at monochromatically fixed wavelength no inactivation took place at λ 405 nm (λ_{max} for diazomethane). Irradiation at 370 nm restored the loss of enzyme activity. This result suggests that a precursor of diazomethane possibly methyl diazotate 3 [13], is photolabile and inhibits the enzyme.

This is the first example of enzyme inactivation by photoactivation of one of the reaction products. Work is in progress to determine the nature of this product as well as the nature of the alkylating species generated by irradiation.

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