On the Mechanism of the Acridine Orange Sensitized Photodynamic Inactivation of Lysozyme II. Kinetics in Presence of N-Acetylglucosamine

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Dedicated to Prof. Dr. J. Stauff *on the Occasion of His 65th Birthday*

Photodynamic Effect, Singlet Oxygen, Lysozyme, Acridine Orange, Kinetics

The photodynamic deactivation of lysozyme in presence of acridine orange is caused by a reaction between singlet oxygen formed via the dye triplet state and the protein. In order to identify the region where the singlet oxygen reacts with the protein we have investigated the kinetics of the deactivation in presence of the inhibitor of the enzymatic reaction N-acetylglucosamine (GlcNAc). The overall experimental rate constant becomes slower with increasing saccharide concentrations. As we can exclude experimentally that this kinetical effect is caused in presence of the saccharide by a physical quenching of singlet oxygen or of the dye triplet state it has to be assumed that GlcNAc protects the surrounding of its binding place at subsite C of the enzymatic center sterically against an attack of singlet oxygen. In this region three tryptophan residues are located, which could be sensitive against singlet oxygen. Surprisingly, however, it has been found that only those species are protected, in which a second saccharide molecule is bound to the protein, probably at subsite E at the enzymatic center, where no sensitive amino acid side chains are located.

Introduction

In a previous paper we have investigated the kinetics of the photodynamic deactivation of lysozyme with acridine orange as sensitizer [1, 2]. We have shown that singlet oxygen is formed during the excitation via the triplet state of the dye. It subsequently reacts chemically and physically with the enzyme. However, the chemical reaction responsible for the deactivation is not completely understood until now [3]. Therefore the present publication is devoted to this process.

In order to identify the region where singlet oxygen reacts with the protein we have investigated the kinetics of the photodynamic deactivation in presence of N-acetylglucosamine (GlcNAc), an inhibitor of the enzymatic reaction [4]. The inhibitor is bound at the enzymatic center of the protein. There are three tryptophan residues located, which could be sensitive against singlet oxygen. If the photodynamic reaction occurs in absence of the inhibitor at its binding region then it is expected that the reaction becomes noticeably slower in presence of the saccharide because the sugar protects the surrounding of its binding place sterically against an attack of singlet oxygen. However, this kind of experiments can only lead to unequivocal conclusions concerning the reaction place of singlet oxygen if simple physical quenching processes of singlet oxygen or of the dye triplet state caused by GlcNAc can be ruled out. These experiments are described in the present paper.

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Methods

Most of the experimental details have been described previously [2]. The inhibitor of the enzymatic reaction N-acetylglucosamine (Fluka, puriss. $[\alpha]_D = +41°$) has been used without further purification.

The kinetics of the photodynamic reaction in presence of the inhibitor were investigated as follows. In every experiment the apparent 100% activity is the enzymatic activity of the lysozyme/GlcNAc solution before the irradiation. The actual concentration of native lysozyme during the photodynamic reaction has been measured using unilluminated diluted protein solutions containing identical amounts of the saccharide as standard of the activity. Thus it is not necessary to know the exact mechanism of the enzymatic reaction in presence of the inhibitor. Because of the large excess of GlcNAc (0.1 – 0.3 M) with respect to lysozyme (1×10^{-4} M) the results of the kinetical evaluation do not depend on whether or not the inhibitor is still bound to the photooxidation products of the enzyme.

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The evaluation of the kinetics of the photodynamic deactivation of the enzyme is not influenced by an addition of GlcNAc after the illumination of the lysozyme/acridine orange solution.

Results and Discussion

The enzymatic activity of lysozyme decreases by an overall pseudo-first order rate process during the illumination of the acridine orange containing solution of the protein with visible light. The mechanism can be described by the scheme [2] reproduced in Table I. F_0, ^1F, ^3F denote respectively ground, excited singlet and triplet state of the dye, $^1\Delta$ excited singlet oxygen, P native lysozyme (initial concentration P_0), PO_2 photooxidation products of the enzyme and φ_T the quantum yield of step (1). Using quasi-stationary conditions for the triplet state of the dye and the singlet state of the oxygen the experimental pseudo-first order rate "constant" is given by equation (a)

$$k^0_{exp} = I_{abs} \cdot \varphi_T \frac{k_4[O_2]}{k_2 + (k_3 + k_4)[O_2] + k_8[P_0]} \cdot \frac{k_6}{k_5 + k_6[P] + k_7[P_0]} \quad (a)$$

In presence of he competitive inhibitor of the enzymatic reaction N-acetylglucosamine (GlcNAc $\equiv$ S) the kinetics of the photodynamic deactivation becomes noticeably slower as shown in Fig. 1 *. Three processes could be responsible for this effect.

Table I. Reaction scheme and rate constants for the acridine orange sensitized photodynamic deactivation of lysozyme [2].

Reaction		Rate constant	Reference
	$F_0 \rightarrow {}^1F$	I_{abs}	8
	$^1F \rightarrow F_0$	$\tau = 4.5$ nsec (alcohol) 2.4 nsec (water)	9
(1)	$^1F \rightarrow {}^3F$	$\varphi_T = 0.10$	10
(2)	$^3F \rightarrow F_0$	$k_{2a} = 4\times10^3$ sec^{-1}	11
		$k_{2b} = 300$ sec^{-1}	12
(3)	$^3F + O_2 \rightarrow F_0 + O_2$ or X	$k_{3a} = 1.4\times10^8$ M^{-1} sec^{-1}	
		$k_{3b} = 1.0\times10^7$ M^{-1} sec^{-1}	
(4)	$^3F + O_2 \rightarrow F_0 + {}^1\Delta$	$k_{4a} = 2.2\times10^8$ M^{-1} sec^{-1}	
		$k_{4b} = 1.7\times10^7$ M^{-1} sec^{-1}	
(5)	$^1\Delta \rightarrow O_2$	$k_5(H_2O) = 5\times10^5$ sec^{-1}	13
		$k_5(D_2O) = 5\times10^4$ sec^{-1}	
(6)	$P + {}^1\Delta \rightarrow PO_2$	$k_6(H_2O) = 2.9\times10^7$ M^{-1} sec^{-1}	
		$k_6(D_2O) = 4.7\times10^7$ M^{-1} sec^{-1}	
(7)	$P_0 + {}^1\Delta \rightarrow P_0 + O_2$	$k_7(H_2O) = 4.1\times10^8$ M^{-1} sec^{-1}	
		$k_7(D_2O) = 5.9\times10^8$ M^{-1} sec^{-1}	
(8)	$^3F + P_0 \rightarrow F_0 + P_0$	$k_{8a} = 4.7\times10^7$ M^{-1} sec^{-1}	
		$k_{8b} = 3.5\times10^6$ M^{-1} sec^{-1}	

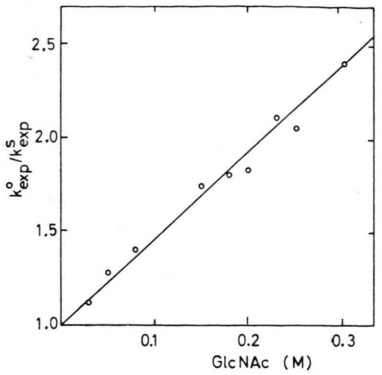

Fig. 1. k^0_{exp}/k^s_{exp} in dependence on the saccharide concentration. Lysozyme concentration 1×10^{-4} M, dye concentration 1×10^{-4} M in phosphate buffer/H_2O, air saturated.

(i) GlcNAc could react as quencher of the triplet state of the dye

$$^3F + S \xrightarrow{k_q} F_0 + S. \quad (I)$$

(ii) It could quench singlet oxygen in solution physically

$$^1\Delta + S \xrightarrow{k_s} O_2 + S. \quad (II)$$

Both reaction (I) and (II) would lower the "stationary" singlet oxygen concentration. Therefore in presence of the inhibitor a decrease of the experimental rate constant k^s_{exp} would result compared with k^0_{exp} in formula (a).

(iii) GlcNAc is bound at the enzymatic center of the protein [4]

$$P_f = S \rightleftharpoons PS \qquad K = \frac{[PS]}{[P_f]\cdot[S]}. \quad (III)$$

If the saccharide shields the sensitive amino acid side chains at the enzymatic center sterically, a chemical reaction between singlet oxygen and PS becomes impossible **.

$$P_f + {}^1\Delta \rightarrow PO_2, \qquad PS + {}^1\Delta \not\rightarrow PSO_2. \quad (IV)$$

* Monosaccharides not acting as inhibitors (glucose, sorbite, erythrite) do not lower the experimental rate constant at concentrations comparable with that used for GlcNAc.

** It is supposed that the equilibrium (III) is established fast with respect to the photodynamic reaction.

The physical quenching process (7) and reaction (8), however, should not be influenced by reaction (III).

$$P_f + PS + PO_2 = P_0,$$
$$P_0 + {}^1\Delta \rightarrow P_0 + O_2,$$
$$P_0 + {}^3F \rightarrow P_0 + F_0.$$

First we want to show that reaction (I) and (II) cannot be responsible for the observed decrease of the experimental rate constant k^s_{exp} in presence of GlcNAc. Thus it follows that the saccharide protects the enzymatic center sterically.

Case (i). Supposing GlcNAc acts as quencher of the dye triplet state the following expression results by using similar assumptions as stated above for the evaluation of equation (a)

$$\frac{k^s_{exp}}{k^0_{exp} - k^s_{exp}} = \frac{k_2 + k_8[P_0]}{k_q[S]} + \frac{k_3 + k_4}{k_q[S]}[O_2]. \quad (b)$$

Using the rate constants reproduced in Table I and the results of Fig. 1 concerning k_q the plot $k^s_{exp}/(k^0_{exp} - k^s_{exp})$ *versus* oxygen concentration should give the straight line shown in Fig. 2 (dotted). However, experimentally the ratio is independent of the oxygen concentration (cp. Fig. 2). Hence, a

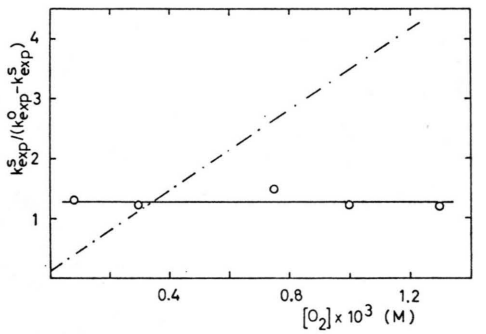

Fig. 2. $k^s_{exp}/(k^0_{exp} - k^s_{exp})$ in dependence on the oxygen concentration in solution. Lysozyme concentration 1×10^{-4} M, dye concentration 1×10^{-4} M, phosphate buffer/H_2O, GlcNAc 0.2 M. —·—·— Calculated from (b), ○—○ experimental.

quenching process of the dye triplet state cannot be the reason for the observed effect.

Case (ii) and (iii). If reaction (II) is responsible for the decrease of k^s_{exp} in presence of the inhibitor the following equation would describe the influence of the saccharide on the photodynamic kinetics.

$$\frac{k^0_{exp}}{k^s_{exp}} = 1 + \frac{k_s}{k_5 + k_6[P] + k_7[P_0]}[S]. \quad (c)$$

From Fig. 1 $k_s = 2.5 \times 10^6\ M^{-1}\,sec^{-1}$ is obtained, a reasonable value for a singlet oxygen quenching reaction [5].

However, a similar expression would result also if GlcNAc exclusively acts as a steric protector of the enzymatic center. Supposing that only the free protein P_f (not in contact with GlcNAc)

$$[P_f] = \frac{[P]}{1 + K[S]}$$

($[P] = [P_f] + [PS]$) can be deactivated photodynamically, equation (d) is responsible for the action of GlcNAc,

$$\frac{k^0_{exp}}{k^s_{exp}} = 1 + \frac{(k_5 + k_7[P_0])K}{k_5 + k_6[P] + k_7[P_0]}[S]. \quad (d)$$

From the slope of the straight line in Fig. 1 the equilibrium constant (III) for the complex formation of lysozyme and GlcNAc is calculated to be $K = 4.6\ M^{-1}$. The same value has been found with D_2O as solvent instead of H_2O.

Thus the straight line shown in Fig. 1 can be described equally well by equation (c) and (d) using appropriate constants for k_s and K respectively.

A decision between case (ii) and (iii) is possible by measuring the kinetics in H_2O and D_2O. In case (ii) the ratio of the experimental pseudo-first order rate constants in water and D_2O is given by

$$\frac{k^{s,D_2O}_{exp}}{k^{s,H_2O}_{exp}} = \frac{k_6^{D_2O}}{k_6^{H_2O}} \cdot \frac{k_5^{H_2O} + k_6^{H_2O}[P] + k_7^{H_2O}[P_0] + k_s[S]}{k_5^{D_2O} + k_6^{D_2O}[P] + k_7^{D_2O}[P_0] + k_s[S]}. \quad (e)$$

For case (iii) a similar equation results,

$$\frac{k^{s,D_2O}_{exp}}{k^{s,H_2O}_{exp}} = \frac{k_6^{D_2O}}{k_6^{H_2O}} \cdot \frac{(k_5^{H_2O} + k_7^{H_2O}[P_0])(1 + K[S]) + k_6^{H_2O}[P]}{(k_5^{D_2O} + k_7^{D_2O}[P_0])(1 + K[S]) + k_6^{D_2O}[P]}. \quad (f)$$

With $K = 4.6\ M^{-1}$, the kinetical constants of Table I and $P_0 = 1 \times 10^{-4}$ M the ratios calculated from (e) and (f) are given in Table II. Comparing the calculated results with those obtained experimentally it has to be concluded that reaction (III) is responsible for the influence of GlcNAc on the kinetics of the photodynamic deactivation of lysozyme. This means that the decrease in k_{exp} in presence of GlcNAc is caused by the shielding of sensitive amino acid side chains against an attack of singlet oxygen.

It is generally accepted that GlcNAc is mainly bound to subsite C at the enzymatic center, the two

Table II. Comparison of the ratio $k_{exp}^{s,D_2O}/k_{exp}^{s,H_2O}$ experimentally observed with that calculated supposing that GlcNAc acts as protector of the enzymatic center and as singlet oxygen quencher respectively. Lysozyme concentration 1×10^{-4} M, dye concentration 1×10^{-4} M, air saturated.

GlcNAc [M]	Observed	$k_{exp}^{s,D_2O}/k_{exp}^{s,H_2O}$ Mechanism (calc.) Protection	Quenching
0	8.0	7.8	7.8
0.1	7.6	7.8	3.5
0.2	8.4	7.9	2.8
0.3	8.6	7.9	2.4

anomers slightly different[4, *]. The α-anomer has contact with trp 108. The β-anomer is hydrogen bonded to trp 62 and 63. These amino acid side chains could react with singlet oxygen. It has been shown recently[7] that independently a smaller part of the saccharide is bound also at subsite E. The corresponding protein-saccharide complex, however, should not be protected directly against singlet oxygen, since in the surrounding of this subsite no sensitive amino acid residues are located.

The value of the equilibrium constant for the protein-saccharide complex, $K=4.6\ \mathrm{M}^{-1}$, calculated from equation (d) does not agree with that observed by standard methods for the complex formation at subsite C. It is smaller by a factor of about 5 compared with published values[4, 7].

Our experimental figure can be explained quantitatively only if we assume that a simultaneous occupation of both binding places (subsite C and E) by the inhibitor (PS_CS_E) is necessary in order to protect the enzyme against singlet oxygen. The free protein and the other species, in which the saccharide is either bound only to subsite C (PS_C) or subsite E (PS_E) are not protected.

With these suppositions P_f and PS in equation (IV) have to be substituted

$$P_f \rightarrow P_f + PS_C + PS_E\,,$$
$$PS \rightarrow PS_CS_E + PS_ES_C\,.$$

Using the binding scheme and the corresponding single binding constants published by Ikeda and Hamaguchi[7]

$$\begin{array}{ccc} PS_E & \overset{K_{PSE}}{\rightleftharpoons} & P_f \\ K_{PSESC}\ \Updownarrow & & \Updownarrow K_{PSC} \\ PS_ES_C = PS_CS_E & \underset{K_{PSCSE}}{\rightleftharpoons} & PS_C \end{array}$$

($K_{PSC}=22.5\ \mathrm{M}^{-1}$, $K_{PSE}=4\ \mathrm{M}^{-1}$, $K_{PSCSE}=5\ \mathrm{M}^{-1}$, $K_{PSESC}=33\ \mathrm{M}^{-1}$) and apparent binding "constant"

$$K_{app}=\frac{([PS_CS_E]+[PS_ES_C])}{([P_f]+[PS_C]+[PS_E])\cdot[S]}\,,$$

$$(P_f\,, PS_C\,, PS_E) + {}^1\Delta \rightarrow PO_2\,,$$
$$(PS_CS_E\,, PS_ES_C) + {}^1\Delta \nrightarrow PO_2\,,$$

can be defined, which has to be used for calculating the part of lysozyme sensitive to singlet oxygen. In this way $K_{app}\approx 7\ \mathrm{M}^{-1}$ is obtained. This value agress satisfactorily with that of $K=4.6\ \mathrm{M}^{-1}$ measured by our kinetical experiments.

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* We used fully mutarotated solutions of GlcNAc ($[\alpha]_D=+41°$). The ratio $\alpha:\beta$ is about 60:40 at equilibrium[6].

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