On the Mechanism of the Acridine Orange Sensitized Photodynamic Inactivation of Lysozyme

I. Basic Kinetics

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The kinetics of the photodynamic desactivation of lysozyme in presence of acridine orange as the sensitizer have been investigated in detail varying oxygen, protein, dye concentration, ionic strength and pH value. The kinetics can be approximately described as an over all pseudo-first-order rate process. Changing the solvent from water to D_2O or by quenching experiments in presence of azide ions it could be shown that the desactivation of lysozyme is caused exclusively by singlet oxygen. The excited oxygen occurs via the triplet state of the dye with a rate constant considerably lower than that to be expected for a diffusionally controlled reaction. Singlet oxygen reacts chemically (desactivation, $k = 2.9 \times 10^7\ \mathrm{M^{-1}\,sec^{-1}}$) and physically (quenching process, $k = 4.1 \times 10^8\ \mathrm{M^{-1}\,sec^{-1}}$) with the enzyme. The kinetical analysis shows that additional chemical reactions between singlet oxygen and lysozyme would have only little influence on the kinetics of the desactivation as long as their products would be enzymatically active and their kinetical constants would be less than about $1 \times 10^8\ \mathrm{M^{-1}\,sec^{-1}}$.

Introduction

The participation of singlet oxygen in sensitized photoreactions can be proved kinetically by changing the solvent from water to D_2O. This results in a considerable increase of the rate constant of the photooxidation [1]. The reaction can be quenched completely by azide ions. By these methods it could be derived that singlet oxygen is the major oxidizing species in a series of photodynamic systems [2–7].

In a brief communication [2] we showed that such reactions take place in the photodynamic inactivation of lysozyme in presence of acridine orange. Similar results have been obtained with eosin as sensitizer by Kepka and Grossweiner [3]. Protein binding of this dye can be observed by absorption spectroscopy in contrast to acridine orange. Radical reactions occur simultaneously in the presence of eosin.

Because of fluorescence measurements we suggested that in the system acridine orange/lysozyme singlet oxygen reacts with a tryptophan side chain near the active center of the enzyme [2]. Different conclusions have been drawn from similar facts by Hopkins and Spikes [9] using other sensitizers.

In this paper we want to present a more detailed investigation of the kinetics of the photodynamic inactivation of lysozyme in presence of acridine orange (part I). Subsequently (part II) it shall be demonstrated that singlet oxygen actually reacts with an aromatic amino acid side chain at the active center.

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Methods

Hen egg-white lysozyme (EC 3.2.1.17) was employed as purchased from Boehringer (Mannheim) without further purification. The actual concentration in solution has been determined by absorption spectroscopy at 280 nm in phosphate buffer pH 5.9 using a value of $E_{280\,\mathrm{nm}}^{1\%,\,1\mathrm{cm}} = 26.9$ [10] and a molecular weight of 14 500 [11]. The enzymatic activity has been measured with Micrococcus luteus (lyophilyzed, Boehringer Mannheim) through the change of the turbidity of the suspension in dependence of the time [12] at 600 nm in phosphate buffer pH 7.0 ($I = 0.07$) at 20 ± 0.2 °C.

All buffer solutions were prepared as described in the literature [13, 14] using triple-distilled water.

Acridine orange (Merck, Darmstadt or Eastman Kodak) has been purified twice by precipitation of the base/cation and subsequently by chromatographic methods. The concentration in solution was also determined by absorption spectroscopy [15]. Its absorption spectrum in the visible range is not influenced by lysozyme.

The protein-dye solutions were illuminated using a high pressure xenon lamp (Osram XBO 900) in connection with a stabilized power supply. The light

current was controlled by a photodiode. The fairly parallel light beam was passed through interference line filters (Schott & Gen., PIL 492 nm/13 nm bandwidth or PIL 468 nm/12 nm bandwidth). In some cases the samples were illuminated with polychromatic light using cut-off filters (Schott & Gen.). To prevent heating of the sample an aqueous solution of $CuSO_4$ (2%) or a filter with pure water and an additional IR reflecting filter (Schott & Gen., 115 Tempax 3 mm) were employed.

All experiments were performed with 3.00 ml of the dye-enzyme solution and were illuminated in a rectangular 1 cm standard quartz cuvette (Hellma, Mühlheim) at 20 ± 0.5 °C. To remove suspended particles the solution had been centrifuged for ten minutes with 12 000 rpm before the photodynamic studies. Because the enzymatic test requires only $5-40$ µl the volume remains constant within the experimental error during the photodynamic reaction.

The number of the incident light quanta were measured using a compensated thermopile (Kipp & Zonen CA 1) in connection with a sensitive recorder (Kipp & Zonen BD 5). The thermopile was calibrated by monochromatic illumination of a ferrioxalate actinometer instead of the dye/enzyme solution. The actinometric measurements were carried out as described in the literature [16] and repeated several times (experimental error about 2%).

Because the experimental arrangement was the same in the actinometric as well as in the photodynamic experiments in the case of monochromatic illumination the EMF of the thermopile can be directly related to the number of the incident light quanta. The number of the absorbed light quanta can be determined by substracting the quanta passing the reaction mixture from those transmitted when the cuvette is filled with pure water. In most cases the acridine orange concentration was 1×10^{-4} M. Under these circumstances the number of the absorbed light quanta is practically equal to the number of the incident quanta.

During the photodynamic experiments the transmission of the solution were recorded continuously as described above. In this way photobleaching of the dye in dependence of the time could be corrected if necessary.

The enzymatic activity in every photodynamic experiment was measured in a blank test before the illumination (stock solution activity = 100%). The kinetics of the photodynamic inactivation have been followed comparing the activity of the illuminated sample with diluted probes of the nonilluminated stock solution in dependence of the time of exposure. To eleminate errors due to self-clearing of the Micrococcus suspension the reference measurements of the diluted stock solutions have been carried out parallel to the photodynamic reaction.

In all experiments the oxygen content was varied bubbling a definite oxygen-nitrogen gas mixture through the reaction solution with a constant number of gas bubbles per minute. Simultaneously the gas was passed over the surface of the solution. The exact oxygen concentration of the gas mixture was determined by an oxygen sensor (Beckman Oxygen Analyzer Model 777). The solution was stirred in the cuvette by a small glas stirrer dipping into a region of the solution not illuminated.

The absorption and fluorescence spectra were recorded with a Cary 15 absorption spectrometer and a modified Farrand Spectrofluorimeter (Mark I) respectively. The ESR measurements were carried out using a Varian E 12 X-band spectrometer with standard equipments [17].

When the kinetics of the photochemical reactions could not be described by simple pseudo-first-order rate processes the corresponding differential equations have been solved quantitatively by analog computation (hybrid analog computer EAI 680).

Results and Discussion

1. Kinetics of the photodynamic reactions

The photodynamic desactivation of lysozyme in presence of acridine orange can be approximately evaluated as a simple pseudo-first-order rate process with respect to the protein concentration.

As will be shown below our experimental data are consistent with the kinetical scheme presented in Table I, where F_0, ^1F, ^3F denote respectively ground, excited singlet and triplet state of the dye, $^1\Delta$ excited oxygen of the electronic configuration $^1\Delta_g$, P native lysozyme (initial concentration P_0), PO_2 photooxidation products of the enzyme and φ_T the quantum yield of step (1). The following differential equation describes the photodynamic inactivation of lysozyme

$$\frac{d[P]}{dt} = A([P_0]) \cdot \frac{k_6}{k_5 + k_6[P(t)] + k_7[P]} \cdot [P(t)] \equiv k_{exp}(t) \cdot [P(t)] \quad (1)$$

with

$$A([P_0]) = I_{abs} \cdot \varphi_T \frac{k_4[O_2]}{k_2 + (k_3 + k_4)[O_2] + k_8[P_0]} .$$

We assumed quasi-stationary conditions for the triplet state of the dye and for the singlet state of the oxygen. But we have to take into consideration that

Table I. Reaction scheme and rate constants for the acridine orange sensitized photodynamic desactivation of lysozyme.

Reaction		Rate constant	Reference
	$F_0 \rightarrow {}^1F$	I_{abs}	15
	${}^1F \rightarrow F_0$	$\tau = 4.5$ nsec (alcohol)	28, 29
		2.4 nsec (water)	28
(1)	${}^1F \rightarrow {}^3F$	$\varphi_T = 0.10$	30
(2)	${}^3F \rightarrow F_0$	$k_{2a} = 4 \times 10^3\ sec^{-1}$	21
		$k_{2b} = 300\ sec^{-1}$	22
(3)*	${}^3F + O_2 \rightarrow F_0 + O_2$ or X	$k_{3a} = 1.4 \times 10^8\ M^{-1}\ sec^{-1}$	
		$k_{3b} = 1.0 \times 10^7\ M^{-1}\ sec^{-1}$	
(4)*	${}^3F + O_2 \rightarrow F_0 + {}^1\Delta$	$k_{4a} = 2.2 \times 10^8\ M^{-1}\ sec^{-1}$	
		$k_{4b} = 1.7 \times 10^7\ M^{-1}\ sec^{-1}$	
(5)	${}^1\Delta \rightarrow O_2$	$k_5(H_2O) = 5 \times 10^5\ sec^{-1}$	
		$k_5(D_2O) = 5 \times 10^4\ sec^{-1}$	
(6)	$P + {}^1\Delta \rightarrow PO_2$	$k_6(H_2O) = 2.9 \times 10^7\ M^{-1}\ sec^{-1}$	
		$k_6(D_2O) = 4.7 \times 10^7\ M^{-1}\ sec^{-1}$	
(7)	$P + {}^1\Delta \rightarrow P + O_2$	$k_7(H_2O) = 4.1 \times 10^8\ M^{-1}\ sec^{-1}$	
		$k_7(D_2O) = 5.9 \times 10^8\ M^{-1}\ sec^{-1}$	
(8)	${}^3F + P_0 \rightarrow F_0 + P_0$	$k_{8a} = 4.7 \times 10^7\ M^{-1}\ sec^{-1}$	
		$k_{8b} = 3.5 \times 10^6\ M^{-1}\ sec^{-1}$	

* $k_3 + k_4$ has been measured in H_2O while the ratio $k_4/(k_3+k_4)$ results from our experiments in methanol (compare section 5). It has been assumed that the value of this ratio is the same in H_2O as well as in methanol. Under this assumption k_3 and k_4 were calculated for water as the solvent.

$[{}^1\Delta]$ is a slight function of time because of the time dependence of [P].

Thus it follows that corresponding to the above equation the evaluated experimental k_{exp} will actually be a "constant" only under special conditions. In the general case, however, the "pseudo-first-order rate constant" could depend more or less pronounced on the time.

2. *Dependence of the kinetics on the light intensity*

The evaluation of the experimental data can be simplified if the quantum yield of the photodynamic reaction is independent of the intensity of the exciting light. Such a behavior can be expected only at relatively small intensities when second-order processes with respect to the excited molecules (*e. g.* triplet-triplet annihilations) can be neglected. Actually we found that as usual [18] there is a linear relation between the experimental pseudo-first-order rate constant k_{exp} and the number of the absorbed quanta per second (dose rate). (These samples were illuminated with polychromatic light $\lambda > 420$ nm in order to employ light intensities as high as possible.) This means that the quantum yield

$$q = \frac{1}{I_{abs}} \cdot k_{exp} \cdot [P_0]$$

of the photodynamic inactivation is independent of light intensity.

Because of these results it is not essential to keep the dose rate constant during the different photodynamic experiments in order to compare the kinetics of the various reactions. Thus we shall discuss our measurements on the basis of the experimental pseudo-first-order rate constants extrapolated to a constant number of absorbed light quanta per second of 5.6×10^{-5} Einstein $\cdot l^{-1}\ sec^{-1}$. This value is about the actual dose rate.

3. *Standard reactions and experimental error*

For repeated checks of the experimental conditions we measured a standard photodynamic reaction eight times. The standard conditions were: lysozyme concentration 1×10^{-4} M, acridine orange concentration 1×10^{-4} M in phosphate buffer pH 5.9 ($I = 0.07$) at 20 °C, air-saturated solution (2.9×10^{-4} M O_2), excitation wavelength 492 nm (bandwidth of the interference filter 13 nm). The experimental pseudo-first-order rate constant referred to a dose rate of 5.6×10^{-5} Einstein $\cdot l^{-1}\ sec^{-1}$ was

$$k_{exp} = (1.75 \pm 0.03) \times 10^{-4}\ sec^{-1}.$$

Thus considering the uncertainty of the actinometric measurements of about 2% the experimental error of the absolute value of the rate constant under standard conditions is less than $\pm 4\%$. For typical photodynamic reactions the relative experimental error has been estimated to be less than $\pm 5\%$. It becomes $\leqq 10\%$ at very high or very low concentrations of the reactants.

It has been proved that the enzymatic activity does not change in the dark after the illumination, although the solution became turbid under special circumstances after some time. This effect occured

especially when the solution had not been centrifuged carefully before the experiments. Probably the reason is an aggregation of the denatured protein.

4. Oxygen dependence

The oxygen content of the aqueous solution is related to the composition of the gas mixture bubbling through the solution (see experimental section) due to Henry's law by [19]

$$c_{O_2}[M] = 1.38 \times 10^{-3} \frac{\text{Vol-\% } O_2}{100}$$

at 20 °C and 1 atm total pressure.

As usual in photodynamic reactions [20] in absence of oxygen exposure to light does not influence the enzymatic activity of lysozyme. A linear relation exists between the reciprocal experimental rate constant and the reciprocal oxygen concentration (Fig. 1) in agreement with the proposed singlet oxygen mechanism (Table I)

$$\frac{1}{k_{exp}} = \frac{1}{I_{abs} \cdot \varphi_T}\left(\frac{k_2}{k_4[O_2]} + \frac{k_8}{k_4[O_2]} \cdot [P_0] + \frac{k_3 + k_4}{k_4}\right)\left(\frac{k_6[P] + k_7[P]}{k_6} + \frac{k_5}{k_6}\right). \quad (2)$$

Since in an air saturated solution the part of the acridine orange fluorescence quenched by oxygen is less than 1% it has to be assumed that the excited species responsible for the formation of singlet oxygen is the triplet state T_1 of the dye.

The apparent rate constant $(k_3 + k_4)$, which corresponds to the quenching of the dye triplet state by ground state oxygen, can be calculated at sufficiently low protein concentrations from the ratio of the slope m and the intercept b of the straight line (Fig. 1)

$$\lim_{[P] \to 0} \frac{m}{b} = \frac{k_2}{k_3 + k_4}$$

if k_2 is given.

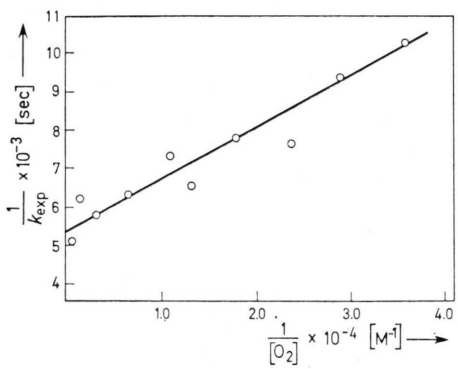

Fig. 1. Oxygen dependence of the photodynamic inactivation of lysozyme (1.0×10^{-4} M). Acridine orange 1.0×10^{-4} M, phosphate buffer (I=0.07) pH 5.9, illumination 492 nm, temperature 20 °C.

As to be expected from Eqn. (2), m/b depends on the protein concentration. From Fig. 2, the quenching constant of the dye triplet state by lysozyme k_8 can then be calculated.

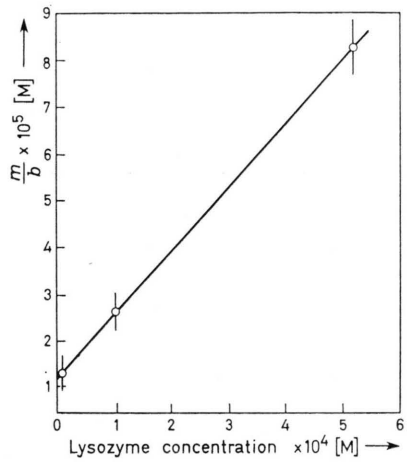

Fig. 2. Protein dependence of m/b (see text). Slope of the straight line 0.13, intercept 1.1×10^{-5} M.

Unfortunately the value of k_2 in solution published in the literature varies between

$k_{2a} = 4 \times 10^3 \text{ sec}^{-1}$ [21] and $k_{2b} = 300 \text{ sec}^{-1}$ [22].

It has been evaluated by flash spectroscopy. Probably the reason for this uncertainty is the high light intensity used in flash experiments so that second-order reactions of the excited species dominate over the first-order decay of the dye triplet state.

Thus we calculated

$(k_3 + k_4)_a = (3.6 \pm 1.2) \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$

and $k_{8a} = (4.7 \pm 2.6) \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$

or

$(k_3 + k_4)_b = (2.7 \pm 1.0) \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$

and $k_{8b} = (3.5 \pm 1.9) \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$

with k_{2a} and k_{2b} respectively.

In the case of thionine and eosin the corresponding values of k_8 are $3.8 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}$ and 7.2×10^8 [23] $- 1 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}$ [3] respectively.

It is well known that acridine orange forms dimers and higher aggregates in dependence on the concentration [15, 17]. The absorption spectra of these species differ strongly. In order to prove whether the reaction between the dye triplet state and oxygen depends on the different dye species we measured the oxygen dependence of the photodynamic reaction under various conditions of illumination. The ratio m/b did not depend on whether the exposure had been performed with monochromatic light at 492 nm or with polychromatic light $\lambda > 375$ nm. With a protein and a dye concentration of 1×10^{-4} M each the corresponding values are

$$\frac{m}{b_{492}} = (2.3 \pm 0.4) \times 10^{-5}\ \text{M}$$

and

$$\frac{m}{b_{\text{poly}}} = (1.9 \pm 0.4) \times 10^{-5}\ \text{M}\,.$$

Both values are well within the experimental error. Thus, since in both cases monomers and aggregates participate differently in the absorption process it can be concluded that oxygen quenches both species equally efficient supposing k_2 remains constant. The last assumption seems to be quite reasonable because of ESR measurements at low temperatures. It has been shown that the lifetime of the triplet state of the monomers and the dimers respectively of acridine orange [17] as well as other acridines [24] are practically identical.

As pointed out above the participation of the dye triplet state in the formation of singlet oxygen has been merely deduced from negative fluorescence quenching experiments. If, nevertheless, the excited singlet state of acridine orange instead of its triplet state was involved in this process the evaluation of Figs 1 and 2 would had to be modified in the following sense

$$\frac{m}{b} = \frac{k_s}{k_3' + k_4'}\,.$$

$k_s = 4.2 \times 10^8\ \text{sec}^{-1}$ [28] is the decay constant of the excited singlet state of acridine orange. Olmsted [29] determined the fluorescence quenching constant by oxygen (in ethanol) $k_q = (k_3' + k_4') = 7.7 \times 10^9\ \text{M}^{-1}\,\text{sec}^{-1}$. These values would lead to $\lim_{[P]\to 0}(m/b) = 5.5 \times 10^{-2}$ M far different from the experimentally obtained 1.1×10^{-5} M.

5. Evaluation of k_3 and k_4

As pointed out in the previous section only the sum of the rate constants $(k_3 + k_4)$ can be obtained by investigating the oxygen dependence of the photodynamic reaction. Solely k_4 is responsible for the formation of singlet oxygen and therefore significant for the photodynamic effect.

Supposing the ratio $k_4/(k_3 + k_4)$ does not depend on the solvent this constant can be obtained by measuring the dependence of the acridine orange sensitized photooxidation of 1,3-diphenylisobenzofuran (DPBF) upon its concentration in methanol. The reaction between DPBF and singlet oxygen in methanolic solution has been investigated by several authors [25, 26, 34]. The rate constant of the first-order decay of singlet oxygen in methanol k_5 (Table I with P ≙ DPBF) published in the literature varies between $8.8 \times 10^4\ \text{sec}^{-1}$ and $1.6 \times 10^5\ \text{sec}^{-1}$ [25–27, 32–34]. We will use an average of $k_5 = 1.1 \times 10^5\ \text{sec}^{-1}$. k_8 and also k_7 with respect to k_6 (Table I) can be neglected.

The aggregation of acridine orange is strongly influenced by the solvent. Therefore the behavior of the sensitizer in lysozyme solutions and in the DPBF solution can only be compared if the oxygen quenching processes of the dye triplet state are independent of the degree of aggregation of acridine orange. This supposition had been proved in the previous section.

Because of the high solubility of oxygen in methanol compared with water its quenching of the excited singlet state of acridine orange cannot be neglected furthermore. Assuming that this process merely leads to an enhancement of the triplet quantum yield [29, 38] of the dye

$$^1\text{F} + \text{O}_2 \xrightarrow{k_q} {}^3\text{F} + \text{O}_2$$

the oxygen dependent triplet yield φ'_T has to be taken into consideration instead of $\varphi_T = 0.10 \pm 0.03$ (30) *. (Other authors [31] found a value of $\varphi_T = 0.30 \pm 0.02$, but some of their experimental conditions were not specified in detail.) We get $\varphi'_T = 0.17$ with $k_q = 7.7 \times 10^9\ \text{M}^{-1}\,\text{sec}^{-1}$ [29], the decay time of the excited singlet state of acridine orange $\tau = (4.5 \pm 0.3)$ nsec [28, 29], $[\text{O}_2] = 2.6 \times 10^{-3}$ M (air saturated methanolic solution [19] and the data of Soep *et al.* [30]).

As to be expected from Eqn (1) the kinetics of the acridine orange sensitized photooxidation of DPBF at high substrate concentrations cannot be described by a simple pseudo-first-order rate process

* The reaction $^1\text{F} + \text{O}_2 \rightarrow {}^3\text{F} + {}^1\Delta$ can be neglected because the energy difference between the first excited singlet and triplet state is less than 8000 cm^{-1}, which would be necessary in order to excite oxygen to its $^1\Delta_g$ state.

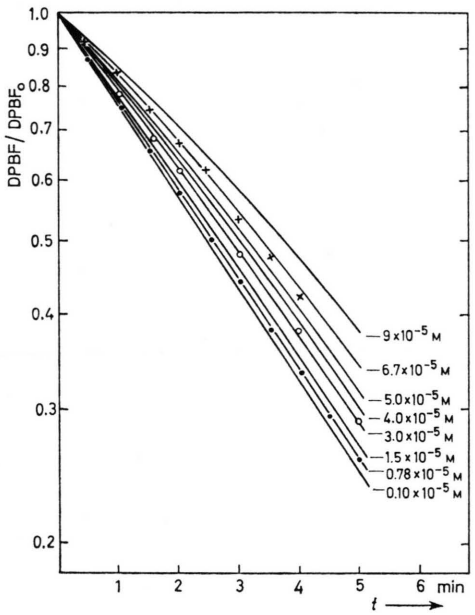

Fig. 3. Kinetics of the acridine orange sensitized photooxidation of DPBF in dependence on its concentration. Analog computation (solid lines) with $k_6 = 1.0 \times 10^9$ M^{-1} sec^{-1}, $k_{4a} = 2.2 \times 10^8$ M^{-1} sec^{-1}, $k_5 = 1.1 \times 10^5$ M^{-1} sec^{-1}. Experimental: DPBF $= 6.7 \times 10^{-5}$ M (×), 3.0×10^{-5} M (○), 7.8×10^{-6} M (●); acridine orange 1×10^{-4} M, air saturated methanolic solution, illumination: PIL 492 + GG 475 nm.

(Fig. 3). With $\varphi'_T = 0.17$, $(k_3 + k_4)$ from section 4 and $k_5 = 1.1 \times 10^5$ sec^{-1} the experimental curves agree satisfactorily with those calculated by analog computation according to Eqn (1) when $k_6 = (1.0 \pm 0.1) \times 10^9$ M^{-1} sec^{-1} and respectively $k_{4a} = (2.2 \pm 0.3) \times 10^8$ M^{-1} sec^{-1} or $k_{4b} = (1.7 \pm 0.2) \times 10^7$ M^{-1} sec^{-1} are used.

Our values of k_6 and $\beta = k_5/k_6 = 1.1 \times 10^{-4}$ M agree very well with those published in the literature [25, 26, 34]. At low DPBF concentrations a pseudo-first order rate constant of $\lim_{\text{DPBF} \to 0} k_{exp} = 4.7 \times 10^{-3}$ sec^{-1} ($I_{abs} = 5.0 \times 10^{-6}$ Einstein · l^{-1} sec^{-1}) results from analog computation compared with $(5.1 \pm 0.5) \times 10^{-3}$ sec^{-1} from the plot $1/k_{exp}$ *versus* DPBF concentration.

However, the actual uncertainty of k_4 (not of k_6) is considerable higher than that obtained by analog computation because of the experimental error *e. g.* of φ'_T and the experiments described in section 4. A critical consideration of these data leads to $k_{4a} = (2.2 \pm 1.0) \times 10^8$ M^{-1} sec^{-1} with $(k_3 + k_4)_a = 3.6 \times 10^8$ M^{-1} sec^{-1} or $k_{4b} = (1.7 \pm 0.7) \times 10^7$ M^{-1} sec^{-1} with $(k_3 + k_4)_b = 2.7 \times 10^7$ M^{-1} sec^{-1}. From the above values $k_{3a} = (1.4 \pm 1.0) \times 10^8$ M^{-1} sec^{-1} and $k_{3b} = (1.0 \pm 0.7) \times 10^7$ M^{-1} sec^{-1} respectively can be calculated. Furthermore due to the experimental error of $(k_3 + k_4)$ the uncertainty of k_4 will become about $\pm 60\%$.

Because of theoretical reasons it has to be expected that k_3 is some magnitudes less than k_4 [35]. Therefore it seems to be more probable that step (3) is caused by radical reactions of the sensitizer triplet state [40] than by a physical quenching process.

6. *Influence of the ionic strength. Protein binding of the dye*

The photodynamic reaction is independent of the ionic strength under standard conditions (Fig. 4).

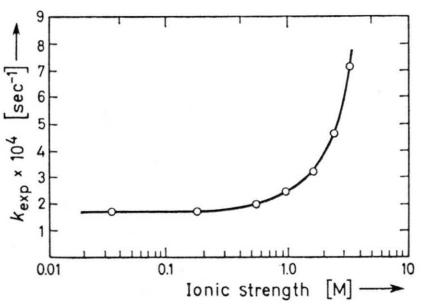

Fig. 4. Dependence of k_{exp} upon the ionic strength (lysozyme 1×10^{-4} M, acridine orange 1×10^{-4} M, phosphate buffer pH 5.9, air saturated).

This means that at least one of the reactants is uncharged in agreement with the assumption of the participation of singlet oxygen corresponding to Table I. On the other hand the increase of k_{exp} at high buffer concentrations can be explained by the linear salt effect appearing at high salt concentrations in reactions between ions and neutral molecules.

The absorption spectrum of acridine orange is not influenced by lysozyme. In spite of this fact a weak dye-protein binding could be detected unequivocally by equilibrium dialyzation when sufficiently high ionic strengths were employed (10-fold as normal). High ionic strengths are necessary in order to suppress effects arising from membrane potentials caused by the cationic dye in presence of the positively charged protein [36]. (The Donnan equilibrium pretends negative binding ratios at low ionic strengths.)

Thus because of these experimental difficulties it cannot be concluded unambiguously whether there is actually a noticeable dye binding under standard conditions.

7. *Protein dependence. Reaction in H_2O and D_2O*

As to be expected due to Eqn (2) the experimental rate constant decreases with increasing protein concentration in water as well as in D_2O (Figs 5 and 6). In all cases the kinetics can be described quite well by a pseudo-first-order rate process.

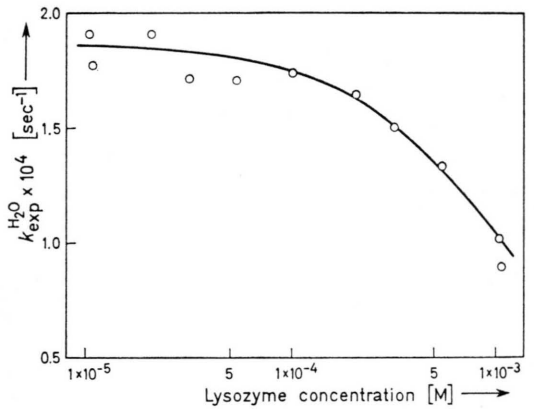

Fig. 5. Protein dependence of k_{exp} in H_2O (acridine orange 1×10^{-4} M, phosphate buffer pH 5.9 ($I = 0.07$), PIL 492 nm).

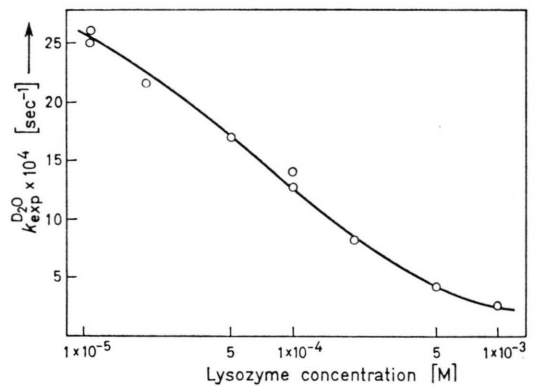

Fig. 6. Protein dependence of k_{exp} in D_2O (acridine orange 1×10^{-4} M, phosphate buffer pD 5.9 ($I = 0.07$), PIL 492 nm).

Merkel, Nilsson and Kearns [1] demonstrated that the lifetime $\tau = 1/k_5$ of singlet oxygen is 2 μsec in water compared with 20 μsec in D_2O. At low lysozyme concentrations the first-order decay of singlet oxygen dominates over second-order reactions. Therefore in this case the ratio of the two experimental rate constants in H_2O and D_2O

$$Q = \frac{k_{exp}^{D_2O}}{k_{exp}^{H_2O}} = \frac{(k_6 + k_7)\,[P] + k_5^{H_2O}}{(k_6 + k_7)\,[P] + k_5^{D_2O}}$$

depends only on the decay time of singlet oxygen in water and D_2O respectively. At high protein concentrations Q should become 1 supposing the rate constants k_6 and k_7 are independent of the solvent [37]. As shown in Fig. 7 the plot Q *versus* protein concentration shows the expected trend. It completely rules out that an uncommon high inverse kinetical isotopic effect could be responsible for the observed solvent dependence of the photodynamic reactions.

At low protein concentrations the pseudo-first-order rate constant is given by

$$\lim_{P \to 0} k_{exp} \equiv k_{exp}^0 = \frac{k_6}{k_5} \cdot A \quad [\text{see Eqn (1)}].$$

Our measurements in water (Fig. 5) led to $k_{exp}^0 = 1.9 \times 10^{-4}\ \text{sec}^{-1}$ ($\pm 8\%$). With $k_5 = 5 \times 10^5\ \text{sec}^{-1}$ [1] and the data of the previous sections a value of $k_6 = 2.9 \times 10^7\ \text{M}^{-1}\,\text{sec}^{-1}$ can be calculated. Using $k_5 = 5 \times 10^4\ \text{sec}^{-1}$ [1] a similar value is obtained with D_2O as the solvent ($k_{exp}^0 = 3.1 \times 10^{-3}\ \text{sec}^{-1}$, $k_6 = 4.7 \times 10^7\ \text{M}^{-1}\,\text{sec}^{-1}$). Taking into consideration the uncertainties of A and k_5 as described in section 5 the error of k_6 could become $\pm 60\%$ *. (Kepka and Grossweiner [3] found for the eosin sensitized photodesactivation of lysozyme $k_6 = 1.3 \times 10^8\ \text{M}^{-1}\,\text{sec}^{-1}$ and $2.5 \times 10^8\ \text{M}^{-1}\,\text{sec}^{-1}$ in H_2O and D_2O respectively.)

From the ratio of the intercept b and the slope m of the straight line of the plot $1/k_{exp}$ *versus* lysozyme concentration $(k_6 + k_7)$ can be calculated

$$\frac{b}{m} = \frac{k_5}{k_6 + k_7}\ .$$

In H_2O the sum of the constants becomes $(k_6 + k_7) = 4.4 \times 10^8\ \text{M}^{-1}\,\text{sec}^{-1}$ (maximal error $\pm 30\%$). Thus $k_7 = 4.1 \times 10^8\ \text{M}^{-1}\,\text{sec}^{-1}$ ($\pm 40\%$) can be calculated. In D_2O we obtained $(k_6 + k_7) = 6.4 \times 10^8\ \text{M}^{-1}\,\text{sec}^{-1}$ and $k_7 = 5.9 \times 10^8\ \text{M}^{-1}\,\text{sec}^{-1}$ respectively §. A kinetical isotopic effect could be responsible for the slight differences of the rate constants of the reactions between singlet oxygen and lysozyme in H_2O and D_2O. However, the effect does not exceed the uncertainty of the method very much.

* At this point we want to emphasize again that these uncertainties do not come from our photodynamic experiments but result above all from the data in the literature, which are necessary to calculate the kinetical constants. Under the assumption of either of these data, however, the error is no more than about 8%.

§ The corresponding values of Kepka and Grossweiner [3] for the eosin sensitized reaction are $(k_6 + k_7) = 1.9 \times 10^9\ \text{M}^{-1}\,\text{sec}^{-1}$ and $k_7 = 1.8 \times 10^9\ \text{M}^{-1}\,\text{sec}^{-1}$ in water. The physical quenching of singlet oxygen by lysozyme in D_2O has been measured by Matheson *et al.* [8]. The authors found a value of $k_7 = 1.5 \times 10^9\ \text{M}^{-1}\,\text{sec}^{-1}$ at pD 8.

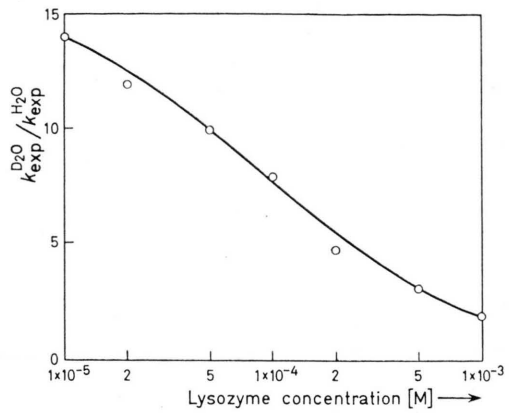

Fig. 7. $k_{exp}^{D_2O}/k_{exp}^{H_2O}$ in dependence of the lysozyme concentration.

While reaction (6) describes the photodynamic inactivation of the enzyme the question arises what kind of reaction could be responsible for step (7). Reaction (7) could be a simple physical quenching process of singlet oxygen by lysozyme (k_7^0) without any chemical modification of the protein. As pointed out by Young *et al.*[32] amines can be very good quenchers of singlet oxygen. Such a quenching process will probably not be influenced by reaction (6).

Alternatively singlet oxygen could react with amino acid side chains of lysozyme without changing its enzymatic activity (k_7^*).

In summary the following reactions between singlet oxygen and the protein could occur:

$$P + {}^1\Delta \xrightarrow{k_6} PO_2 \quad \text{(inactivation)} \qquad (6)$$

$$P_0 + {}^1\Delta \xrightarrow{k_7^0} P_0 + O_2 \quad \text{(physical quenching)} \qquad (7)$$

$$P + {}^1\Delta \xrightarrow{k_7^*} P' \quad \text{(chemical reaction without loss of enzymatic activity)} \qquad (7\,a)$$

$$P' + {}^1\Delta \xrightarrow{k_6} P'O_2 \quad \text{(inactivation)} \qquad (6\,a)$$

$$PO_2 + {}^1\Delta \xrightarrow{k_7^*} P'O_2 \qquad (7\,b)$$

under the assumption that both reactions (6) and (7) are independent of each other §§. The activity of P′ could be 100% or less.

The corresponding differential equations have been solved simultaneously by analog computation using a quasi-stationary singlet oxygen concentration of

$$\cdot [{}^1\Delta] = A(P_0) \cdot \{k_5 + k_6([P(t)] + [P'(t)]) + k_7^*([P(t)] + [PO_2(t)]) + k_7^0[P_0]\}^{-1}$$

§§ Therefore the same values of k_7^* in reaction (7 a) and (7 b) as well as k_6 in (6) and (6 a) can be assumed.

with A from Eqn (1). For the calculations we used the kinetical constants evaluated in the previous sections and varied k_6 and respectively k_7^* or k_7^0.

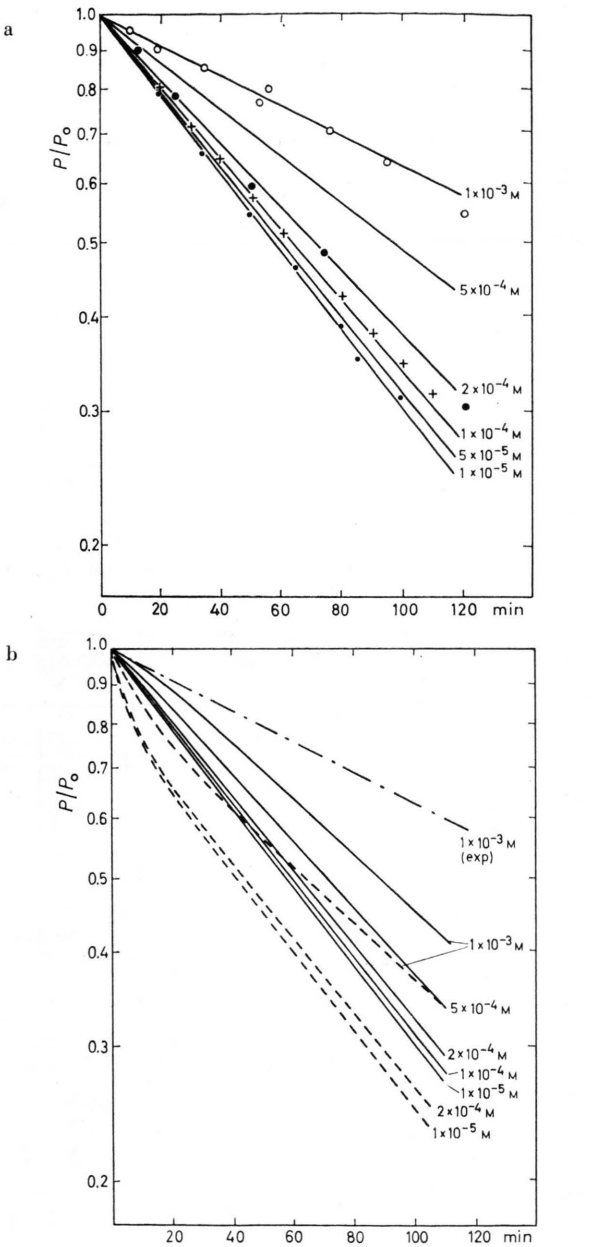

Fig. 8. Decrease of the enzymatic activity with time of exposure (492 nm). Dependence of the kinetics upon the lysozyme concentration. Solvent: phosphate buffer pH 5.9 (H_2O, air saturated), acridine orange 1×10^{-4} M. Lysozyme $= 1 \times 10^{-3}$ (○), 2×10^{-4} M (●), 1×10^{-4} M (×), 1×10^{-5} M (●). Analog computation: (a) $k_6 = 3.3 \times 10^7$ M^{-1} sec^{-1}, $k_7^0 = 4.0 \times 10^8$ M^{-1} sec^{-1}, $k_7^* = 0$ (solid lines); (b) $k_6 = 3.3 \times 10^7$ M^{-1} sec^{-1}, $k_7^0 = 0$, $k_7^* = 4.0 \times 10^8$ M^{-1} sec^{-1}, activity of P′ (see text) = 100% (solid lines), 80% (dashed lines).

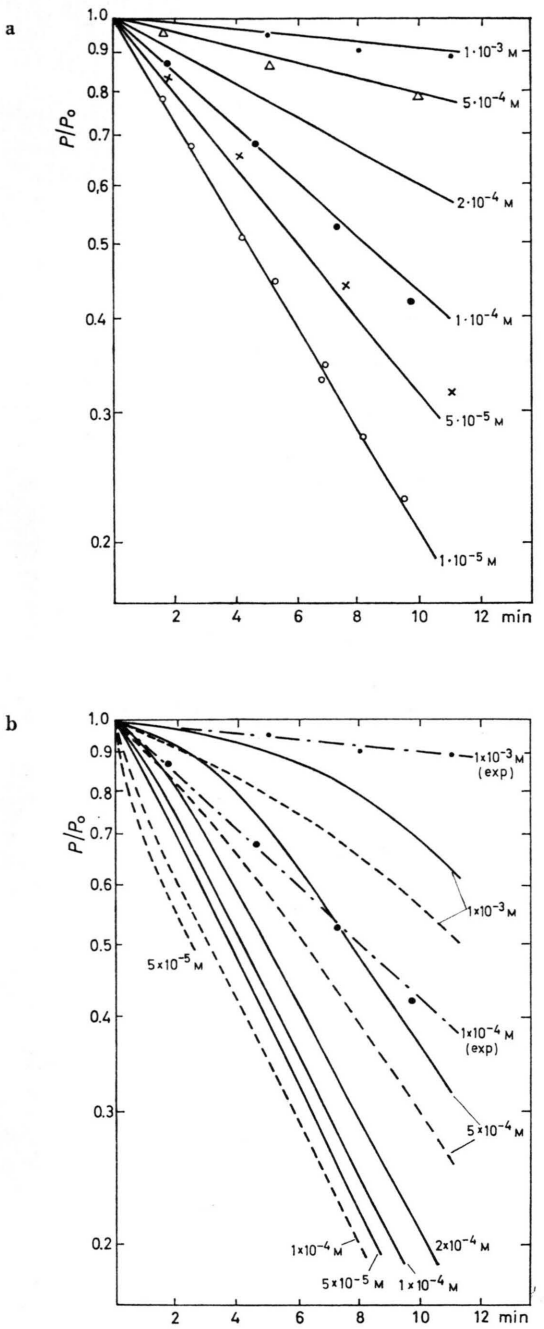

Fig. 9. Decrease of the enzymatic activity with time of exposure (492 nm). Dependence of the kinetics upon the lysozyme concentration. Solvent: phosphate buffer pD 5.9 (D_2O, air saturated) acridine orange 1×10^{-4} M. Lysozyme = 1×10^{-3} M (●), 5×10^{-4} M (△), 1×10^{-4} M (●), 5×10^{-5} M (×), 1×10^{-5} M (○). Analog computation: (a) $k_6 = 3.9 \times 10^7\ M^{-1}\ sec^{-1}$, $k_7^0 = 4.2 \times 10^8\ M^{-1}\ sec^{-1}$, $k_7^* = 0$ (solid lines), (b) $k_6 = 3.9 \times 10^7\ M^{-1}\ sec^{-1}$, $k_7^0 = 0$, $k_7^* = 4.2 \times 10^8\ M^{-1}\ sec^{-1}$ activity of P′ = 100% (solid lines), 80% (dashed lines).

The results are reproduced in Figs 8 and 9. The best fit of the curves could be obtained with $k_6 = (3.3 \pm 0.5) \times 10^7\ M^{-1}\ sec^{-1}$ and $k_7^0 = (4.0 \pm 0.5) \times 10^8\ M^{-1}\ sec^{-1}$ in H_2O (Fig. 8 a) or $k_6 = (3.9 \pm 0.5) \times 10^7\ M^{-1}\ sec^{-1}$ and $k_7^0 = (4.2 \pm 0.5) \times 10^8\ M^{-1}\ sec^{-1}$ in D_2O (Fig. 9 a), $k_7^* < 1 \times 10^8\ M^{-1}\ sec^{-1}$. The numerical values of k_6 and k_7^0 agree well with those obtained for k_6 and k_7 respectively by our graphical evaluation.

Only if step (7) is a physical quenching process the enzymatic activity decreases by a pseudo-first-order rate process (Figs 8 a and 9 a). When $k_7^* < 1 \times 10^8\ M^{-1}\ sec^{-1}$ the kinetics is only little influenced by additional chemical reactions with singlet oxygen leading to photooxidation products with an enzymatic activity of nearly 100%. On the contrary if step (7) would be a chemical reaction ($k_7^0 \ll k_7^*$) leading to enzymatically active photoproducts at high protein concentrations (especially in D_2O) the kinetics would deviate strongly from a first-order rate process (Figs 8 b and 9 b). This is in contradiction to our experimental results.

Our kinetical analysis shows that independent photosensitized chemical reactions even faster than the actual photodynamic inactivation can additionally occur without visible influence on the kinetics of the desactivation.

8. Quenching experiments with azide ions

NaN_3 at relatively high concentrations (5×10^{-2} M) prevents completely the photodynamic inactivation of lysozyme (acridine orange concentration between 2×10^{-5} and 8×10^{-4} M). Azide is a specific agent of quenching singlet oxygen. Compared with this effect quenching of the dye triplet state by NaN_3 can be neglected [39].

At lower azide concentrations (between 1×10^{-4} and 5×10^{-3} M) there is a linear relation between the reciprocal quantum yield q of the photodynamic effect and the concentration of the quencher (Fig. 10) according to the following equation

$$\frac{1}{q} = \frac{1}{q_0}\left(1 + \frac{k_{N_3^-}}{(k_6 + k_7)\,[P] + k_5}\,[N_3^-]\right)$$

with q_0 = quantum yield in absence of azide. $k_{N_3^-}$ is the rate constant responsible for the quenching reaction between singlet oxygen and N_3^-.

With a lysozyme concentration of 1×10^{-4} M, $(k_6 + k_7) = 4.4 \times 10^8\ M^{-1}\ sec^{-1}$, $k_5 = 5 \times 10^5\ sec^{-1}$ and $q_0 = 3.1 \times 10^{-4}$, $k_{N_3^-} = 7.8 \times 10^8\ M^{-1}\ sec^{-1}$ can

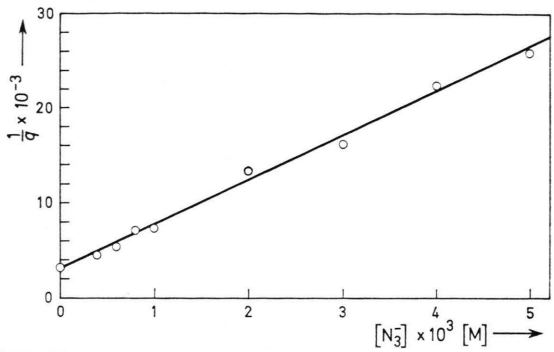

Fig. 10. Quantum yield of the photodynamic reaction in dependence of the azide concentration. Lysozym 1×10^{-4} M, acridine orange 1×10^{-4} M, phosphate buffer pH 5.9 (air saturated).

be calculated. (The corresponding constant for the methylene blue sensitized photooxidation of DPBF in methanol is found to be $2.2 \times 10^8\ M^{-1}\ sec^{-1}$ [39].)

The complete suppression of the photodynamic reaction by azide ions gives evidence that the acridine orange sensitized desactivation of lysozyme is exclusively caused by singlet oxygen. The corresponding reaction with eosin [3] cannot be quenched totally by N_3^-. Obviously in this case additional radical reactions take place.

Radicals are formed also during the illumination of acridine orange in presence as well as in absence of lysozyme [40]. But they do not seem to be important in respect to the photodynamic desactivation of the enzyme.

9. pH-dependence

The pH-dependence of the photodynamic reaction (Fig. 11) is complicated. The measurements have been carried out in HCl/KCl buffer (pH 0.8 – 3.5), acetic acid/NaAc (pH 4.1 – 5.0), phosphate buffer (pH 5.9 – 7.6) and NH_4Cl/NH_4OH (pH 8.3 – 8.9). The ionic strength of the buffer solutions in every experiment was 0.2 [14].

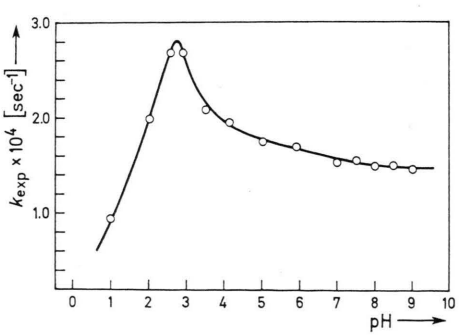

Fig. 11. pH dependence of k_{exp}. Lysozyme 1×10^{-4} M, acridine orange 1×10^{-4} M, ionic strength 0.2, air saturated solution.

The increase of k_{exp} at low pH values corresponds to the increase of the ESR signal of acridine orange in its lowest triplet state [17] at about 90 °K. Probably this effect has to be attributed mainly to a second protonation of the acridine orange cation. In its ground state the pK value of this process is 0.4 [15]. Therefore it has to be assumed that the basicity of the dye in its excited triplet state increases so that the pK value becomes about 2. The double protonated dye species does not seem to be able to excite oxygen to its singlet state as efficiently as the single protonated cation.

It is more difficult to explain the decrease of the experimental rate constant with increasing pH values above pH 3. The enzymatic activity of lysozyme has its maximum at about pH 5.5 – 6 [41]. Within the interesting pH range the state of ionization of Asp 52 and Asp 101 (involved in the enzymatic reaction) changes. Also the pH-dependence of the aggregation of lysozyme [42] has to be taken into consideration at higher pH values. The aggregation involves probably the enzymatic center, which could be protected against photodynamic reactions in this way.

10. Dye dependence and dye bleaching

The kinetics of the photodynamic desactivation of lysozyme depends on the dye concentration and on the excitation wavelength in a complicated manner (Fig. 12). We want to interprete the results in a subsequent communication.

At high protein concentrations the photodynamic reaction stops when the enzymatic activity of the

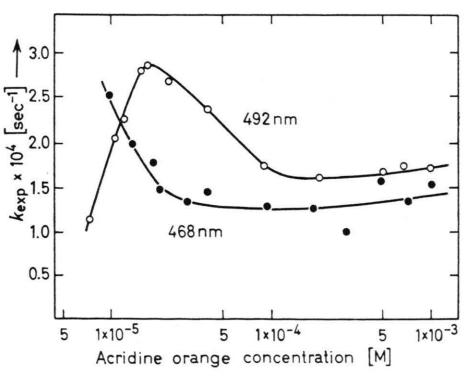

Fig. 12. k_{exp} in dependence on the dye concentration (lysozyme 1×10^{-4} M, phosphate buffer pH 5.9, air saturated). Illumination PIL 492 nm (○), PIL 468 nm (●).

solution is decreased to about 10% of its initial value. The desactivation goes on when fresh dye is added and stops finally at an activity of about 2 – 3%. Although the dye is bleached to some extent during the photodynamic reaction photofading of the dye cannot be solely responsible for this effect as proved by absorption spectroscopy. We are not able to explain the results at this time.

No photobleaching of the dye is observed in absence of oxygen under conditions comparable with the photodynamic experiments. The oxygen dependence of the dye bleaching is similar to the corresponding behavior of the photodynamic reaction. Dye bleaching depends on the lysozyme concentration. The kinetics becomes slower with increasing protein concentration. Assuming the photobleaching goes via the dye triplet state this result is in agreement with the triplet quenching by the enzyme as described in section 4.

At low protein concentrations the bleaching reaction is faster by a factor of 2 in D_2O compared with H_2O as solvent. It cannot be influenced by addition of azide ions. Thus it seems that singlet oxygen as well as radicals are responsible for the bleaching of the dye during the photodynamic reaction.

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