On the Participation of Singlet Oxygen in the Acridine Orange Sensitized Photoinactivation of Lysozyme

HARTMUT SCHMIDT and PETER ROSENKRANZ

Institut für physikalische Biochemie der Universität Frankfurt a. M.

(Z. Naturforsch. **27 b**, 1436—1437 [1972]; received August 11, 1972)

Photodynamic effect, singlet oxygen, lysozyme, acridine orange

In a great many cases the participation of singlet oxygen in sensitized photooxigenation processes in solution has been stated [1]. Singlet oxygen has sometimes also been assumed to be the important intermediate in photodynamic reactions [2]. Unfortunatly in most instances a direct proof by spectroscopic methods is practically impossible because of its low emission quantum yield in solution. Therefore evidence has frequently only been produced by more or less specific chemical reactions and by the interpretation of kinetical data, especially of quenching reactions [3].

Recently MERKEL, NILSSON and KEARNS [4] demonstrated that the lifetime of singlet oxygen is 20 μsec in D_2O as compared with 2 μsec in water. Consequently a tenfold increase of the rate constant of a photooxidation process involving singlet oxygen is expected in changing the solvent from water to D_2O. Of course this is right only if quenching processes of the singlet oxygen can be neglected and if the behavior of the reactants is unaffected by deuteration of the solvent.

Thus the authors [5] deduced that singlet oxygen has to be involved in the methylene blue sensitized photooxidation of the amino acids tryptophane, histidine and methionine.

We used this method to investigate the mechanism of the photosensitized inactivation of lysozyme with acridine orange as sensitizer. In all experiments the photodynamic inactivation followed first-order kinetics within the experimental error.

Fig. 1 shows that the ratio of the experimental rate constants of D_2O to H_2O is dependent on the protein concentration. At low lysozyme concentrations the value of the ratio is about 14 and decreases with increasing protein concentration. This decrease is to be expected because at higher protein concentrations second-order reactions between the protein and singlet oxygen dominate over the first-order decay of the excited oxygen.

With the common assumptions the kinetics of the inactivation of lysozyme can be described in the simplest case by the following scheme:

$$F_0 \xrightarrow{I_{abs}} {}^1F \rightarrow F_0$$

$$^1F \rightarrow {}^3F \qquad \varphi_T = 0.10\,^5 \qquad (1)$$

$$^3F \rightarrow F_0 \qquad k_2 = 4\cdot 10^3\ \mathrm{sec}^{-1}\,^6 \qquad (2)$$

Requests for reprints should be sent to Dr. H. SCHMIDT, Institut für physikalische Biochemie der Universität, *D-6000 Frankfurt a. M.*, Sandhofstr. 3.

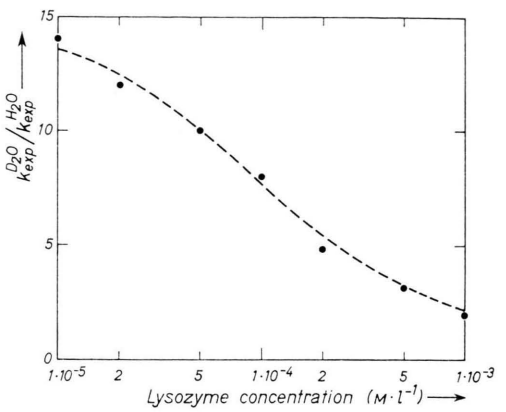

Fig. 1. Ratio of the pseudo first-order rate constants of D_2O to H_2O of the photodynamic reaction between lysozyme and acridine orange in dependence upon the protein concentration. Dye Concentration: $1\cdot 10^{-4}$ M in Sörensen buffer pH 5.9 at 20 °C. The solutions were air saturated. Illumination were carried out with a xenon high pressure lamp XBO 450 (Osram) and a interference filter PIL 492 nm (Schott & Gen.). — — — calculated with $1/k_5 = 30$ μsec (in D_2O).

$$^3F + O_2 \rightarrow F_0 + O_2 \qquad k_3 \ll k_4 \qquad (3)$$

$$^3F + O_2 \rightarrow F_0 + {}^1\Delta \qquad k_4 = 2.5\cdot 10^8\ \mathrm{l\cdot M^{-1}\,sec^{-1}} \qquad (4)$$

$$^1\Delta \rightarrow O_2 \qquad 1/k_5 = 2\ \mu\mathrm{sec\ (water)}\,^4 = 20\ \mu\mathrm{sec\ (D_2O)}\,^4 \qquad (5)$$

$$P + {}^1\Delta \rightarrow PO_2 \qquad k_6 = 1.6\cdot 10^7\ \mathrm{l\cdot M^{-1}\,sec^{-1}} \qquad (6)$$

$$P + {}^1\Delta \rightarrow P + O_2 \qquad k_7 = 3.5\cdot 10^8\ \mathrm{l\cdot M^{-1}\,sec^{-1}}, \qquad (7)$$

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 lysozyme, PO_2 inactivated lysozyme and φ_T the quantum yield of (1).

Under quasi-stationary conditions for the triplet state of the dye and for the singlet state of the oxygen these equations lead to the following expression of the experimental pseudo-first-order rate constant of the lysozyme inactivation:

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

Actually we found a linear relation between the experimental rate constant and the number of the absorbed light quanta I_{abs} and also between the reciprocal experimental rate constant and the protein concentration and the inverse oxygen concentration respectively.

With a quantum yield of $\varphi_T = 0.10\,^5$, $k_2 = 4\cdot 10^3$ $\mathrm{sec}^{-1}\,^6$ and $1/k_5 = 2$ μsec [4] we calculated $k_4 = 2.5\cdot 10^8$ $\mathrm{l\cdot M^{-1}\,sec^{-1}}$, $k_6 = 1.6\cdot 10^7\ \mathrm{l\cdot M^{-1}\,sec^{-1}}$ and $k_7 = 3.5\cdot 10^8$ $\mathrm{l\cdot M^{-1}\,sec^{-1}}$. k_3 could not be determined; it has to be very much smaller than k_4. All these values came from the measurements in aqueous buffer solutions.

We got the same values for k_6 and k_7 as in water from our measurements in D_2O under the assumption that the lifetime of singlet oxygen in D_2O is 30 μsec instead of 20 μsec calculated by MERKEL, NILSSON and

KEARNS [4]. With 20 μsec we would get $2.2 \cdot 10^7\,l \cdot M^{-1}$ sec^{-1} and $4.8 \cdot 10^8\,l \cdot M^{-1}\,sec^{-1}$ for k_6 and k_7 respectively.

We think that singlet oxygen reacts with a tryptophane side chain at the active center of the enzyme. This is supported by the fact that parallel to the lysozyme inactivation a decrease of the tryptophane fluorescence intensity is observed without any change of the maximum of the fluorescence spectrum. A similiar fluorescence behavior has also been found by HOPKINS and SPIKES [8] during the photodynamic reaction between lysozyme and methylene blue and Eosin Y respectively.

This work was supported by the Deutsche Forschungsgemeinschaft. The authors wish to thank Professor J. STAUFF for the continuous encouragement of their work.

[1] K. GOLLNICK, in: "Adv. Photochem." **Vol. 6** (Edts. W. A. NOYES, JR., G. S. HAMMOND, and J. N. PITTS, JR.), Intersci. Publ. 1968.
[2] J. D. SPIKES and M. L. MACKNIGHT, Ann. New York Acad. Sci. **171**, 149 [1970].
[3] D. R. KEARNS, Chem. Rev. **71**, 395 [1971].
[4] P. B. MERKEL, R. NILSSON, and D. R. KEARNS, J. Amer. Chem. Soc. **94**, 1030 [1972].
[5] R. NILSSON, P. B. MERKEL, and D. R. KEARNS, Photochem. Photobiol. **16**, 117 [1972].
[6] B. SOEP, A. KELLMANN, M. MARTIN, and L. LINDQVIST, Chem. Phys. Letters **13**, 241 [1972].
[7] G. BLAUER and H. LINSCHITZ, J. chem. Physics **33**, 937 [1960].
[8] T. R. HOPKINS and J. D. SPIKES, Photochem. Photobiol. **12**, 175 [1970].

Isolation of (—)-Manool from the Liverwort, *Jungermannia torticalyx* [1]

A. MATSUO, M. NAKAYAMA, J. ONO, and S. HAYASHI

Department of Chemistry, Faculty of Science, Hiroshima University, Hiroshima, Japan

(Z. Naturforsch. **27 b**, 1437—1438 [1972]; received May 23/Juli 3, 1972)

(−)-Manool, optical antipode, liverwort, *Jungermannia*

Owing to the chemical and biological interests, constituents of liverworts *(Hepaticae)* which form an unique group in the plant kingdom together with mosses *(Musci)* have been investigated actively in our and other laboratories, and several novel sesquiterpenoids, bazzanene [2], chiloscyphone [3], pinguisone [4], bazzanenol [5], miliol [6] and δ-cuparenol [7], have been isolated up to date. As to the isolation of diterpenoids, however, there are no reports except the isolation of (−)-16α-hydroxykaurane from *Anthelia julacea* and *A. juratzkana* [8]. We now report the isolation of a diterpene alcohol, (−)-manool, from *Jungermannia torticalyx* Steph. which is a leafy liverwort belonging to the Jungermanniaceae family.

The liverwort was extracted with hexane to obtain a dark green viscous oil which showed two spots at R_F 0.55 and 0.30 in thin layer chromatography using silica gel and a mixed solvent of hexane-ethyl acetate (4 : 1). The detection of the spots was carried out by heating the plate after spraying a mixed acid of sulfuric acid and nitric acid (95 : 5). Both the components were isolated by eluting through a silica gel column with the same solvent.

The first crystalline component (R_F 0.55) has a melting point of 52−53° and a composition of $C_{20}H_{34}O$ (M^+ 290), and furnished a 3,5-dinitrobenzoate (m.p. 94−95°). IR ($\nu_{max}^{CCl_4}$ 6300, 3450, 3075, 1650, 1415, 1395, 1375, 1210, 1045, 1000, 930 and 900 cm^{-1}), NMR ($\delta_{ppm}^{CCl_4}$ 0.65, 0.78, 0.86 and 1.18, each 3 H, s; 4.34 and 4.48, each 1 H, s; 4.94, 5.11 and 5.85, each 1 H, q) and mass (molecular ion at m/e 290 and base ion at m/e 137) spectra of this compound were identical with those of (+)-manool [9].

From the above evidence, however, it could not necessarily be concluded that the diterpene alcohol is (+)-manool, because 13-epimanool has also been reported to show the same spectra [10]. In order to obtain further evidences against the identification, melting points and specific rotations of this alcohol and its 3,5-dinitrobenzoate are compared with the data reported on (+)-manool and 13-epimanool (shown in Table I) [10, 11]. Excepting the fact that the signs of the rotation are opposite, both the melting points and the specific rotations are in good agreement with those of (+)-manool. But they are not with 13-epimanool.

Table I. Comparison of melting points and optical rotations [10, 11].

(−)-Manool from the liverwort m.p. 52−53°	$[\alpha]_D$	−33°
3,5-dinibrobenzoate m.p. 94−95°	$[\alpha]_D$	−10°
Authentic (+)-manool m.p. 53°	$[\alpha]_D$	+33°
3,5-dinitrobenzoate m.p. 95−96°	$[\alpha]_D$	+ 8°
Authentic 13-epimanool m.p. 36.5−38.5°	$[\alpha]_D$	+51°
3,5-dinitrobenzoate m.p. 116.5−118.5°	$[\alpha]_D$	+33°

Thus, the alcohol isolated from *Jungermannia torticalyx* in the present work was determined to be (−)-manool which is the optical antipode of (+)-manool isolated formerly from *Dacrydium* and *Cupressus* [12]. This is the first case of isolating (−)-manool from the nature.

Requests for reprints should be sent to Dr. A. MATSUO, Department of Chemistry, Faculty of Science, Hiroshima University, *Hiroshima* (Japan).