Acta Dermatovener (Stockholm) 54: 433-436, 1974

PARTICIPATION OF SINGLET STATE EXCITED OXYGEN IN PHOTOHEMOLYSIS INDUCED BY KYNURENIC ACID

Gunnar Swanbeck, Göran Wennersten and Robert Nilsson

From the Department of Dermatology, Karolinska sjukhuset, and the Department of Radiation Biology, University of Stockholm, Stockholm, Sweden

Abstract. The tryptophan metabolite kynurenic acid has been shown to be photohemolytically active. Since this effect is oxygen dependent and can be inhibited by beta-carotene, the primary oxidizing species involved could possibly be identical with singlet state excited oxygen. Under certain conditions, diagnostic use can be made of the strong influence of solvent on the lifetime of singlet oxygen. Thus, the decay of this excited molecule is slowed down in deuterium oxide by a factor of ten, compared with ordinary water, and the degree of photohemolysis in both types of solvent has therefore been compared. In D_2O the degree of photohemolysis was up to approximately ten times higher than in the corresponding protonated solvent. Histidine, a known quencher of singlet oxygen but which reacts comparatively slowly with the triplet states of several photosensitizers, effectively protects the red cells from photohemolysis. Finally, kynurenic acid was capable of inducing the sensitized oxidation of the singlet oxygen acceptor 1, 3-diphenylisobenzofuran. These findings strongly support the hypothesis that the photohemolysis observed in presence of kynurenic acid is mediated by singlet oxygen.

Kynurenic acid is an endogenously occurring tryptophan metabolite, which in earlier work proved to be photohemolytically active (18, 20). The photohemolysis is oxygen dependent and is inhibited by beta-carotene (20). Kynurenic acid could possibly function as an important photosensitizer in disorders of tryptophan metabolism, where metabolites are accumulated in the tissues, and the patients experience hypersensitivity towards light.

In view of the ability of beta-carotene to inhibit the photohemolytic reaction, it was tempting to speculate about the involvement of singlet state excited oxygen in this process (1, 7). In photodynamic action, two major primary mechanisms are found to occur: (*a*) Direct reaction beween sensitizer triplets and the oxidizable substrate, followed eventually by reaction with molecular oxygen, and (*b*) reaction between oxidizable substrates and singlet oxygen, which has been generated by energy transfer from the triplet state of various sensitizers according to the reaction sequence

(1) $^3S + {}^3O_2 \rightarrow {}^1S_0 + {}^1O_2$ (energy transfer)

(2) $^1O_2 \rightarrow {}^3O_2$ (decay)

(3) $^1O_2 + A \rightarrow {}^3O_2 + A$ or AO_2 (sum of physical quenching and chemical reaction)

where ^3S and $^1S_0$ denote excited-triplet and ground-singlet states of the sensitizer, $^1O_2$ is $^1\Delta_g$ excited singlet state oxygen, A is the oxidizable substrate and AO_2 is some oxidation product. Which pathway predominates is mainly dependent on the relative effectiveness of singlet oxygen generation (reaction 1) as compared with the rate of direct reaction between sensitizer triplets and oxidizable substrate:

(4) $^3S + A \rightarrow {}^1S_0 + A$ or products

Mixed mechanisms are, naturally, also conceivable.

The participation of singlet oxygen in various biological systems has previously been postulated on the basis of very inconclusive evidence (4, 17). Recently, however, we have developed diagnostic tests which make possible the demonstration of singlet oxygen. These tests are based on the large solvent deuterium effect on the lifetime of this excited molecule, as well as on the correlation between the lifetime of singlet oxygen and the intensity of the infrared absorption of the solvent (4, 6). Using these methods together with quenching experiments involving azide and kinetic spectroscopy, the involvement of singlet oxygen in the photosensitized oxidation of free amino acids and en-

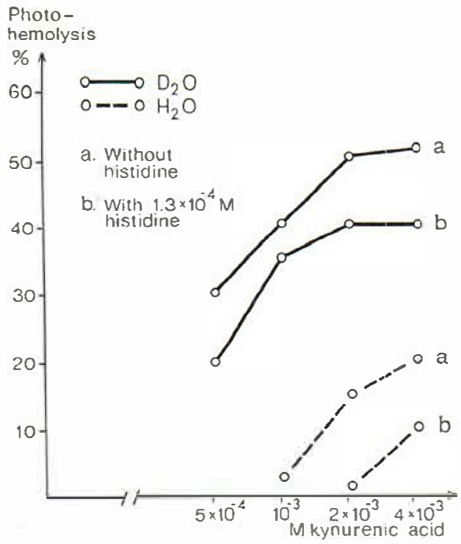

Fig. 1. The degree of photohemolysis as a function of the concentration of kynurenic acid. The scale on the abscissa is logarithmic. ——, experiments with D_2O; – – – –, with H_2O as solvents. Curves indicated by (*a*) without addition of histidine, and by (*b*) with 1.3×10^{-4} M (2 mg%) histidine added.

zymes could for the first time be conclusively demonstrated (6, 8, 9, 10). For singlet oxygen reactions which occur in aqueous solution, a tenfold increase in efficiency of photo-oxidation of amino acids and proteins is obtained by replacing H_2O by D_2O as solvent, depending on a corresponding increase in singlet oxygen lifetime from 2 μsec to about 20 μsec (6, 9, 10). These findings have been confirmed in other laboratories (5, 13). The use of the deuterium effect for identification of singlet oxygen has the great advantage that it does not require addition of foreign components to the solvent, which might react with the excited molecules formed or with the reaction products, nor is there any change in most properties of the solvent (6).

It should be emphasised, however, that large deuterium effects on reaction rates for processes involving singlet oxygen will be observed only if the singlet oxygen acceptor is able to intercept a small fraction of the total amount of singlet oxygen formed in the protonated solvent, i.e. singlet oxygen disappears mainly by reaction 2 in this medium.

In the present study, the effect of substituting D_2O for H_2O on the photohemolytic effect of kynurenic acid was investigated.

In aqueous solution, histidine acts as an efficient quencher of singlet oxygen. Although being less efficient than beta-carotene in this respect, histidine has the advantage of being more readily soluble in hydroxylic solvents and of having a low quenching rate on triplets of several photosensitizers (8). The effect of this amino acid on the photohemolytic activity of kynurenic acid has therefore been investigated.

MATERIAL AND METHODS

The experiments were performed with the photohemolysis technique described by Peterka et al. (12), modified by Kahn & Fleishaker (2, 3) and earlier used by us (18, 19, 20).

Erythrocytes were obtained from healthy human adults, and only blood group O Rh+ was used.

Kynurenic acid in final concentrations varying from 4.8×10^{-4} M to 4.2×10^{-3} M in 0.02 M phosphate buffer, pH 7.4, containing 0.14 M NaCl based either on H_2O or D_2O (99.8%, AB Atomenergi, Studsvik, Sweden) was used.

Packed human red blood cells were washed three times in physiological saline and 0.1 ml was then added to 10 ml of the buffered solutions mentioned above, and poured into 2 mm quartz cuvettes. The H_2O content of red cell suspensions with D_2O as a solvent, was calculated to be less than 1%. Controls were incubated in the dark at 37°C.

Test suspensions were exposed to longwave ultraviolet light after about 10 min, which was considered sufficient time for equilibration of the two types of water. The ultraviolet source was a Black-Ray B-100 A lamp (Ultra-Violet Products Inc.) giving a longwave ultraviolet radiation ranging from 350 to 380 nm, with its intensity maximum at 366 nm. Test suspensions were irradiated at a distance of 12 cm from the lamp in the quartz cuvettes placed against a black background. The intensity of the lamp at this distance was 6.5 mW/cm², measured with a Hewlett & Packard Radiant Flux meter. The exposure time was 1.2×10^3 sec. After irradiation the test suspensions were centrifuged at 2 000 rpm and the optical density of the supernatant fluid at 540 nm was determined on a Beckman DB Spectrophotometer, after an incubation time of 2 hours at 37°C. Results were compared with the dark control and with a total hemolysis control (2, 3, 19). Results were expressed in percent relative to the 100% hemolysed specimen.

The effect of histidine in final concentrations of 1.3×10^{-4} M and 3.2×10^{-4} M was investigated under the same experimental conditions.

To investigate whether the singlet oxygen acceptor 1,3-diphenylisobenzofuran was photo-oxidized in the presence of kynurenic acid, the following experiment was performed.

Silica gel particles (60–200 mesh) were coated with sensitizer by evaporation of solvent from a solution of kynurenic acid in ethanol mixed with silica gel in a rotary evaporator to give a coating corresponding to an amount of about 30 mg of sensitizer per gram of silica gel (11). This coating was found to be insoluble in several non-polar media. Photo-oxidation of 1,3-diphenylisobenzofuran (Aldrich Chemical Co.: 2×10^{-5} M) was carried out by irradiation with light from a 1 000 W xenon-lamp, focused by thick glass lenses, in chloroform to which the coated silica gel was added.

RESULTS

A light dose that gives a significant hemolysis with H_2O as solvent and a subtotal hemolysis with D_2O has been chosen.

The results are summarized in Fig. 1 where the percentage of hemolysis is given as function of the concentration of kynurenic acid. Obviously the photohemolytic activity is considerably higher in D_2O than in H_2O. Thus, at the lowest concentration of kynurenic acid used, 30% hemolysis is observed in the deuterated solvent when the corresponding degree of photohemolysis in ordinary water is insignificant. The difference in photohemolytic activity is approximately 10-fold in the lower range of the concentrations used, but levels off towards higher concentrations of the sensitizer. In the presence of 1.3×10^{-4} M histidine a certain inhibition of the photohemolysis is observed in D_2O as well as in H_2O. 3.2×10^{-4} M of the amino acid gives total inhibition in both solvents.

In a heterogeneous system consisting of silica gel particles coated with kynurenic acid suspended in chloroform containing the singlet oxygen acceptor 1,3-diphenylisobenzofuran (11) the tryptophan metabolite was found to promote the photo-oxidation of the furan derivative when irradiated with long-wave UV light.

DISCUSSION

Photohemolysis is a reproducible phenomenon which can be used at least for semiquantitative analysis, when it is carried out under standardized conditions. The degree of photohemolysis, up to about 60%, according to the method used in our laboratory, is proportional to the light dose given and to the logarithm of the concentration of the photosensitizer. However, it is difficult to compare the efficacy of two sensitizers quantitatively with accuracy.

The data presented in Fig. 1 indicate that by using D_2O as solvent instead of H_2O the photohemolytic activity of kynurenic acid increases by a factor of 3–10. In the case when D_2O is used as solvent we have estimated that the contamination with H_2O is less than 1%. Under these conditions the expected increase in lifetime of singlet oxygen would be around ten-fold, giving a corresponding increase in photohemolysis, provided that the rate of chemical reaction between singlet oxygen and oxidizable components of the erythrocyte cell membrane is slower than the rate of spontaneous relaxation of singlet oxygen to the ground triplet state. Such an effect is indeed observed at the lower concentrations of the sensitizer. The decreasing deuterium effect observed at the higher sensitizer concentrations may be ascribed to the fact that the extensive photohemolysis observed in D_2O at these levels approaches its maximum, and is no longer proportional to the number of primary chemical events. The results obtained are therefore compatible with the hypothesis that singlet oxygen is involved in the photodynamic hemolysis sensitized by kynurenic acid.

The protection of the erythrocytes against hemolysis in presence of histidine is in agreement with kinetic data previously obtained for singlet oxygen quenching by flash photolysis and pulsed laser photolysis (8, 9).

The following facts about the photohemolytic effect of kynurenic acid makes it very likely that singlet oxygen is involved in the primary, photo-oxidative attack on the cell membrane, namely its oxygen dependence, its suppression by beta-carotene (20) and histidine (9), the pronounced deuterium solvent effect as well as its ability to induce photo-oxidation of the singlet oxygen acceptor 1,3-diphenylisobenzofuran (6, 9).

Possible targets in the red cell membrane for the oxidative effect of singlet oxygen are unsaturated fatty acids, cholesterol and the amino acids in the proteins. The findings by Schothorst et al. (14, 15, 16) may indicate that an oxidation of some amino acids is the primary event. This seems reasonable, since the proteins rather than the lipids of the red blood cell membrane are exposed to the surrounding water solution.

ACKNOWLEDGEMENTS

This investigation was supported by the Swedish Medical Research Council (project 4226) and by the Swedish Atomic Research Council.

REFERENCES

1. Foote, C. S., Denny, R. W., Weaver, L., Chang, Y. & Peters, J.: Quenching of singlet oxygen. Ann N Y Acad Sci *171:* 139, 1970.
2. Kahn, G. & Fleishaker, B.: I. Red blood cell hemolysis by photosensitizing compounds. J Invest Derm *56:* 85, 1971.
3. — II. Evaluation of phototoxicity of salicylanilides and similar compounds by photohemolysis. J Invest Derm *56:* 91, 1971.

4. Kearns, D. R.: Physical and chemical properties of singlet molecular oxygen. Chem Rev *71:* 395, 1971.
5. Kepka, A. G. & Grossweiner, L. E.: Photodynamic inactivation of lysozyme by eosin. Photochem Photobiol *18:* 49, 1973.
6. Merkel, P. B., Nilsson, R. & Kearns, D. R.: Deuterium effects on singlet oxygen lifetimes in solutions. A new test of singlet oxygen reactions. J Am Chem Soc *94:* 1030, 1972.
7. Merkel, P. B. & Kearns, D. R.: Radiationless decay of singlet molecular oxygen in solution. An experimental and theoretical study of electronic-to-vibrational energy transfer. J Am Chem Soc *94:* 7244, 1972.
8. Nilsson, R., Merkel, P. B. & Kearns, D. R.: Kinetic properties of the triplet states of methylene blue and other photosensitizing dyes. Photochem Photobiol *16:* 109, 1972.
9. — Unambiguous evidence for the participation of singlet oxygen in photodynamic oxidation of amino acids. Photochem Photobiol *16:* 117, 1972.
10. Nilsson, R. & Kearns, D. R.: A remarkable deuterium effect on the rate of photosensitized oxidation of alcohol dehydrogenase and trypsin. Photochem Photobiol *17:* 65, 1973.
11. — Some useful heterogeneous systems for photosensitized generation of singlet oxygen. Photochem Photobiol *19:* 181, 1974.
12. Peterka, E. S., Runge, W. J. & Fusaro, R. M.: Erythropoietic protoporphyria. III: Photohemolysis. Arch Dermatol *94:* 282, 1966.
13. Schmidt, H. & Rosenkranz, P.: On the participation of singlet oxygen in the acridine orange sensitized photoinactivation of lysozyme. Z Naturforsch (B) *27:* 1436, 1972.
14. Schothorst, A. A., van Steveninck, J., Went, L. N. & Suurmond, D.: Protoporphyrin-induced photohemolysis in protoporphyria and in normal red blood cells. Clin Chim Acta *28:* 41, 1970.
15. — Metabolic aspects of the photodynamic effect of protoporphyrin in protoporphyria and in normal red blood cells. Clin Chim Acta *33:* 207, 1971.
16. — Photodynamic damage of the erythrocyte membrane caused by protoporphyrin in protoporphyria and in normal red blood cells. Clin Chim Acta *39:* 161, 1972.
17. Spikes, J. D. & Livingston, R.: The molecular biology of photodynamic action: Sensitized photoautoxidation in biological systems. *In* Advances in Radiation Biology, Vol. 3 (ed. L. G. Augenstein, R. Mason & M. Zelle), pp. 29–121, Academic Press, New York, 1969.
18. Swanbeck, G. & Wennersten, G.: Evidence for kynurenic acid as a possible photosensitizer in actinic reticuloid. Acta Dermatovener (Stockholm) *53:* 109, 1973.
19. — Effect of beta-carotene on photohemolysis. Acta Dermatovener (Stockholm) *53:* 283, 1973.
20. — Photohemolytic activity of tryptophan and phenylalanine metabolites. Acta Dermatovener (Stockholm) *54:* 99, 1974.

Received May 16, 1974

G. Swanbeck, M.D.
Department of Dermatology
Karolinska sjukhuset
S-104 01 Stockholm 60
Sweden