Vol. 202, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1994 Pages 10-16

HUNT FOR SINGLET OXYGEN UNDER *IN VIVO* CONDITIONS

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Received May 24, 1994

It is widely accepted that applying exogenous photosensitizers in living organism the photodynamic effect is mediated by singlet oxygen formed by energy transfer from excited sensitizer to oxygen present in the cells (Type II mechanism). Though in homogeneous solutions singlet oxygen was detected during sensitization, efforts to observe it *in vivo* failed, so far.
Kinetic considerations based on the assumption that triplet sensitizer molecules interact with (doublet) free radicals accumulated in the tissue as well as on literature data indicate that the steady state concentration of singlet oxygen can decrease by more than one order of magnitude at transition from homogeneous systems to in vivo conditions making it undetectable.

Recent interest in application of photosensitizers in biology resulting in photodynamic phenomena initiated thorough research toward the elucidation of the primary steps taking place after excitation of the sensitizer molecules. It is widely accepted that the excited sensitizer, as a rule in its triplet state, transfers its energy to oxygen present in the system, leading to singlet oxygen and this latter *mediates* the transformation of the light energy into chemical one (Type II mechanism) [1].

This mechanism has been supported by numerous experimental findings obtained in homogeneous environment using IR monitoring of singlet oxygen at 1269 nm. However, attempts to measure the appearance of singlet oxygen *in vivo* have failed, so far, and results are contradictory.

Thus, e.g. Parker detected IR signal in tissue in the presence of sensitizer, while Patterson *et al.* were unable to repeat these results, though used identical instrumentation [2].

Gorman and Rodgers in their excellent review [1] came to the conclusion that by further development of the detection technique (IR monitoring) and by increasing the radiative rate constant of

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deactivation of singlet oxygen, *in vivo* measurements could be solved. Patterson *et al.* suggested [2] three possible reasons for the failure to detect singlet oxygen *in vivo* : (i) the *yield* of singlet oxygen *in vivo* is lower than in homogeneous solutions and the photodynanic effect is due to some other mechanism; (ii) the oxygen concentration is lower *in vivo* (hypoxic conditions) and this results in the lower yield of singlet oxygen. The authors excluded this latter explanation since no luminescence was observed in surgically exposed and well oxigenated liver samples and finally (iii) *not* the yield is decreased but the rate of *consumption* of singlet oxygen is increased, possibly by some quenchers, in cellular environment.

Majority of the authors agree with this last view and several extremely low lifetime values are published for singlet oxygen *in vivo* to support it. Moan and Berg published an indirect method [3] based on the photodegradation of porphirins and estimated a lifetime between 0.01 - 0.04 μs for singlet oxygen *in vivo*.

We intend to show that the yield of singlet oxygen under certain *in vivo* conditions might be much lower compared to homogeneous solvents resulting in the very low *steady state* concentration of singlet oxygen.

KINETIC CONSIDERATION

Let us assume the following simplified mechanism for the primary steps of sensitization in homogeneous solvent:

$$S_o + h\nu \longrightarrow {}^1S^* \xrightarrow{ISC} {}^3S^* \qquad (1)$$

S is the sensitizer, ISC refers to intersystem crossing and the rate of process (1) can be given as $W = I_{abs}\,\Phi$ where I_{abs} and Φ are the light absorbed and the quantum yield of the formation of triplet sensitizer, respectively.

^3S undergoes quenching with oxygen molecules either resulting in singlet oxygen or other products (chemical reaction or catalyzed decay of ^3S) :

$$^3S^* + {}^3O_2(^3\Sigma_g^-) \longrightarrow S_o + {}^1O_2(^1\Delta_g) \qquad (2)$$

$$^3S^* + {}^3O_2 \longrightarrow \text{products} \qquad (3)$$

Singlet oxygen either decays to its ground state (radiative and non-radiative transitions):

$$^1O_2 \longrightarrow {}^3O_2 \qquad (4)$$

or reacts with a biomolecule or any other molecule, (A), present in the solution :

$$^1O_2 + A \longrightarrow \text{products} \qquad (5)$$

$$^1O_2 + A \longrightarrow {}^3O_2 + A \qquad (6)$$

and process (5) leads to the photodynamic effect. It is suggested that under *in vivo* conditions there exist a species B, not present in homogeneous solutions, which reacts both with triplet sensitizers and singlet oxygen :

$$^3S^* + B \longrightarrow {}^1S + \text{products} \qquad (7)$$

$$^1O_2 + B \longrightarrow {}^3O_2 + \text{products} \qquad (8)$$

and process (7) competes with process (5) Thus, in homogeneous solutions the overall reaction can be represented by processes (1)-(6) while the same for *in vivo* conditions are (1) - (8).
Under continuous illumination the steady state treatment can be applied for ^3S and for $^1O_2$ and consequently it can be deduced that the ratio of the steady state concentration of singlet oxygen in solution to the same *in vivo* can be given by (11) :

$$R \equiv \frac{[^1O_2]_s}{[^1O_2]_v} = \left(1 + \frac{k_7[B]}{(k_2+k_3)[O_2]_v} \right) \left(1 + \frac{k_8[B]}{k_4 + k_A[A]} \right) \equiv F_1 \times F_2 \qquad (9)$$

where indices "s" and "v" refer to the steady state concentrations in homogeneous solutions and *in vivo* conditions, respectively. while $k_A = k_5+k_6$; F_1 and F_2 are the factors in the brackets. Equation (9) leads to unity if [B] = 0, and $R > 1$ at any concentration of B.

THE NATURE OF SPECIES B

A significantly larger **R** compared to unity is expected if B reacts with the triplet sensitizer in a fast process decreasing the rate of (2), the rate of (8) is considerable and $[O_2]_v$ is small.

Recent studies [4-8] of chemical systems of excited photosensitizer molecules and free radicals generated *in situ* indicated, that triplet - doublet interactions proceed with a diffusion controlled rate constant.

Based on these results we have suggested earlier [9] that such processes might contribute considerably to the photodynamic action in biological systems with the participation of *bioradicals (species B)* accumulated *in vivo*. This suggestion, called modified type one (MTO) mechanism, in contrast to Type I mechanism which assumes that radicals formed from the excited *sensitizer* mediate the overall effect, depends on the steady state concentration of the bioradicals *in the tissue* under study.

Although the formation of endogenous free radicals accumulated *in vivo*, has been followed for more than three decades [10-12] data with respect to their steady state concentrations are scarce. It has been established, however, that these concentrations increase to high values during the early stages of deseases (e.g. tumour, inflammation, etc...)

QUANTITATIVE EVALUATION OF EQUATION (9)

Value of k_7. Unfortunately, available data, so far, refer to homogeneous solutions.

Hoytink suggested already in 1968 [13] that radical ions are excellent quenchers of triplet states. According to Levin and Szwartz [4] the rate constant of the reaction between tetracene and tetracene radical ions is 6.6×10^9 M^{-1} s^{-1} while Hiratsuka *et al.* published for the same process between triplet naphthalene and benzophenol ketyl radical $(7-8)\times10^9$ $M^{-1}s^{-1}$ [5].

Tatikolov, Kuzmin and Singer [6] and Seret *et al.* [7] carried out experiments in micellar media to avoid triplet-triplet interactions and radical formation from the sensitizer. Former authors found strong decrease of triplet lifetime of sensitizers in the presence of radicals, while the latter established that both photochemical and photophysical processes participate in the quenching.

Kawai *et al.* suggested [8] that the reaction proceeds *via* an encounter complex and by twofold mechanism : energy transfer from the triplet to the radical or by enhanced intersystem crossing of the triplet, though electron transfer can not be excluded.

Our measurements [14] with 6 various *stable* free radicals yielded values for $k_7 \sim 2x10^9$ $M^{-1}s^{-1}$, for *transient* radicals, however, an average $8x10^9$ $M^{-1}s^{-1}$ is very likely.

Concentrations [B]. Our measurements revealed [15] that e.g. in P-388 tumour of mice at early stages of cancer the *total* concentration of free radicals (measured without spin trapping, in frozen samples) reaches average values of about $\sim 10^{-5}$ spin M. After further development of the tumour, however, these values sharply decrease.

Values of $k_2 + k_3$. Moan *et al.* referred to[16] a value of $1.6x10^9$ $M^{-1}s^{-1}$ while Redmond and Braslavsky gave[17] $\sim 2x10^9$ emphasizing that it is approximately one ninth of that for diffusion controlled process. However, as suggested earlier [1] this constant is some one order of magnitude lower *in vivo*. Since no exact value is available for *in vivo* conditions, an average $\sim (5\text{-}8)x10^8$ $M^{-1}s^{-1}$ is acceptable.

Concentrations $[O_2]_v$. According to Moan and Sommers $[O_2]_v \sim 7x10^{-5}$ M in tissues of normal vascularity [18] but e.g. in tumours it might be significantly lower. Earlier works [19] indicate concentrations in tumour being $\sim 10^{-5}$ M.

Values of k_8. According to our recent measurements [14] in agreement with literature data, the corresponding value in case of stable free radicals as reactant is in the range $(1\text{-}10)x10^6$ $M^{-1}s^{-1}$ depending on the structure of the latter.

Value of k_4. The decay rate constant of singlet oxygen, including radiative and non-radiative decay, in various solvents is tabulated by Wilkinson and Brummel [20] giving $k_4 \sim 10^5$ s^{-1} while Scurlogh and Ogilby [21] referred to $(2\text{-}5)x10^4$ s^{-1}.

Values of $k_5 + k_6$. Apart from special singlet oxygen quenchers the rate constants for quenching by possible target molecules might vary in a wide range between 10^3 to 10^8 $M^{-1}s^{-1}$ compiled for hundreds of compounds [20]. According to Baker and Kanofsky [22] proteins are the main quenchers in cells having a quenching rate constant $\sim 10^4$ $(g/L)^{-1}s^{-1}$.

Concentration [A]. Since the actual target molecules "attacked" by singlet oxygen in the living cells are not known, so far, only rough estimation can be carried out. E.g. the total concentration of histidin, tryptophan and methionin present in proteins [16] is $\sim 10^{-2}$ M, that is, $(k_5+k_6)[A] \sim 10^6 s^{-1}$. (These data indicate that the target

molecules *in vivo* can not compete with oxygen in quenching triplet sensitizers and therefore the process $^3S + A$ has not been incorporated into the mechanism).

Above data lead to the following evaluation of equation (9):

- since $1 >> k_8[B]/\{k_4+(k_5+k_6)[A]\}$, the factor $F_2 \sim 1$
- in tumor cells for factor F_1, taking into account the uncertainties of the rate constants and concentrations, we obtain :

$$1 < F_1 < 20$$

shifted toward the higher values at early stages of cancer where. as a rule, application of sensitizers is more efficient. (See e.g. Photodynamic Therapy).

- Steady state concentration of singlet oxygen can decrease by more than one order of magnitude *in vivo* compared to homogeneous solutions, due to the presence of free radicals.

POSSIBLE EXPLANATION OF CONTRADICTIONS OF LITERATURE DATA

In case of IR measurements [1-2] the detection of singlet oxygen *in vivo* depends on the actual *biological conditions* determining the concentrations of bioradicals (e.g. stage of the disease, hypoxic status, pretreatment of the biological sample causing scavenging of free radicals, etc...) and leads to different results even for *apparently* identical systems.

With respect to measurements of singlet oxygen e.g. by photodegradation of the sensitizer [3] it has been shown by us [23], applying chemiluminescence technique, that free radicals attack the sensitizer already in their ground state resulting in the degradation.

CONCLUSIONS

Symbolically, if the hunt for singlet oxygen *in vivo* is unsuccessful, it does not mean necessarily that the "shot-gun" is not sophisticated or the fellow hunters are more skillful, but possibly the proliferation of the target has decreased due to environmental changes.

Acknowledgments: The author is grateful to the National Scientific Research Foundation for the financial help and to Dr. I.Nemes for helpful discussions.

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