SINGLET OXYGEN QUENCHERS AND THE PHOTODYNAMIC INACTIVATION OF E. COLI RIBOSOMES BY METHYLENE BLUE*

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Received April 13,1977

SUMMARY E. coli ribosomes are readily photoinactivated by methylene blue in the presence of air. A variety of singlet oxygen quenchers like NaN_3, 2,5-dimethylfuran, hydroquinone and ascorbic acid provide about 60% protection against this photoinactivation indicating that a major mechanism of ribosome inactivation proceeds through the formation of singlet oxygen, with small contributions (<40%) from other mechanisms. The singlet oxygen quenchers, 1,4-diazabicyclo [2.2.2] octane and triethylamine give unexpected results, in that they show no protection against photoinactivation.

INTRODUCTION It is well known that in the presence of dyes, O_2 and light, many of the biologically important molecules undergo oxidation (1). The photoreactions of a number of dyes have been studied intensively for many years not only because of their intrinsic interest to chemists, but also because of the biological implications of sensitized photoxidation or photodynamic action (1,2). It has been convincingly argued that singlet oxygen ($^1O_2$) plays a central role in dye-photosensitized oxidations (3,4). Several amino acids, enzymes, and guanosine have been shown (5,6) to be susceptible to damage by $^1O_2$. However, there have been suggestions of alternate mechanisms of photodynamic action (5, 7-10). We have examined photoinactivation of ribosomes by a number of dyes and in this report discuss the results with methylene blue in terms of possible mechanisms.

EXPERIMENTAL E. coli ribosomes were prepared as previously described (11). Irradiation of the ribosome samples containing 10 µg/ml methylene blue was carried out at 590 nm using a 500 watt, high pressure mercury lamp. A 1/4 meter

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* Part of this work was presented at the VII Intl. Congr. Photobiol. in Rome, September, 1976.

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Abbreviations: DABCO, 1,4-diazabicyclo [2.2.2] octane; $^1O_2$, singlet oxygen; O_2^-, superoxide anion.

ISSN 0006-291X

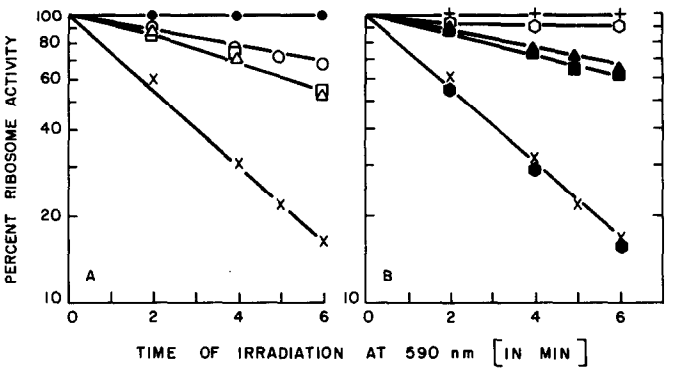

Fig. 1. Methylene blue sensitized photoinactivation of ribosomes and the effect of protectors. Ribosome samples in A, X——X methylene blue; ●——● irradiated control without methylene blue; △——△ 0.05 M NaN_3; □——□ 5 mM 2,5-dimethylfuran; ○——○ 5 mM 2,5-dimethylfuran + 0.15 M NaN_3 and in B, X——X methylene blue; +——+ unirradiated control with methylene blue; ○——○ N_2 purged; ▲——▲ 0.15 M NaN_3; ■——■ 20 mM 2,5-dimethylfuran; ●——● 2 mM DABCO (or 2 mM triethylamine).

Jarrell-Ash Czerni-Turner monochromator with Corning filter 3-66, was used to obtain monochromatic light (2 mm slits). Air from the samples was removed by purging with N_2 for 30 min before irradiation. Assay of the ribosomes was carried out by polyU directed polyphenylalanine synthesis by a modified procedure of Söll (12) and will be detailed elsewhere.

RESULTS Figure 1A shows the inactivation curve for ribosomes as a function of time when irradiated in air, in the presence of methylene blue. Under the conditions of irradiation the ribosomes show no loss of activity in the absence of methylene blue indicating that the photoinactivation is solely due to photoexcitation of methylene blue (Figure 1A). The unirradiated controls in which the ribosomes containing methylene blue are kept under identical conditions also show no loss of activity (Figure 1B).

When the ribosomes containing methylene blue are irradiated in an atmosphere of N_2, only 5-10% photoinactivation is seen (Figure 1B). In the presence of methylene blue and 0.05 M NaN_3, a known $^1O_2$ quencher (13), provided partial protection amounting to 40-50% (Figure 1A). However, a higher concentration of NaN_3 (0.15 M, Figure 1B) provided only small additional protection (~10%), suggesting that inactivation of ribosomes by $^1O_2$ amounts to about 60% of the total photoinactivation observed. The remaining inactivation must therefore,

TABLE I Effect of Protector Concentration on Inactivation of Ribosomes by Methylene Blue.

PROTECTOR		Unirrd Control	Irrad	Unirrd Control	Irrad	Unirrd Control	Irrad
None		0	55*				
NaN_3	Conc.	0.05M		0.15M			
	% Inact.	0	31	6	24		
Triethylamine	Conc.	2mM					
	% Inact.	0	54				
DABCO	Conc.	2mM		0.02M		0.1M	
	% Inact.	0	58	0	80	25	96
Superoxide Dismutase	Conc./ml	1 µg		10 µg		50 µg	
	% Inact.	0	53	0	55	0	53
NaN_3 + 0.6 mM Ascorbic Acid	Conc.			0.15M			
	% Inact.			10	23		
Isopropanol	Conc.	0.01M		0.1M			
	% Inact.	0	54	0	55		

* Ribosome samples containing methylene blue, but without any of the protectors were also irradiated with each run described in this table. The values of all inactivations have been normalized to 55% inactivation by methylene blue alone.

proceed via a different mechanism(s) as will be discussed later. To further assess the participation of $^1O_2$ in this system, we have used several other known $^1O_2$ quenchers such as DABCO (14), 2,5-dimethylfuran (15), triethylamine (16), ascorbic acid (17) and hydroquinone (18). The latter two are also free radical scavengers (19,20) as will be discussed later.

The protection by 2,5-dimethylfuran at 5 mM (Figure 1A) and 20 mM (Figure 1B) parallels that seen with NaN_3 at 0.05 and 0.15 M concentration respectively, indicating that the two quenchers protect against $^1O_2$, to a similar extent.

Results with DABCO and triethylamine however, are not as expected. A concentration of 2 mM of DABCO or triethylamine provided no protection of

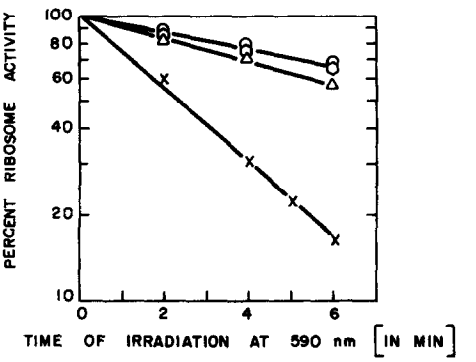

Fig. 2. Effect of protectors on the methylene blue sensitized photoinactivation of ribosomes, X——X methylene blue; △——△ 0.6 mM ascorbic acid; ○——○ 30 mM hydroquinone; ○——○ 30 mM hydroquinone + 20 mM 2,5-dimethylfuran.

ribosomes against photoinactivation by methylene blue (Figure 1B). Higher concentrations of DABCO (0.02 and 0.1 M) actually showed an increase in ribosome photoinactivation (Table I). We are not certain why these $^1O_2$ quenchers do not provide protection in this system and why at higher concentrations DABCO causes sensitization. It would seem that the observed result is the net effect of simultaneous $^1O_2$ quenching and sensitization. Whether the sensitization is due to DABCO itself or due to an impurity in it, is not known. Spectrophotometrically, no absorption bands attributable to an impurity were found in the region 400-700 nm, in 1 M solution of DABCO.

Homann and Gaffron (17) have argued that ascorbic acid quenches $^1O_2$. In our system, we have found (Figure 2) that ascorbic acid at a concentration of 0.6 mM provides protection close to that seen with NaN_3 and 2,5-dimethylfuran at high concentrations. No additive effect in the total protection was seen when ribosomes were irradiated in the presence of ascorbic acid and NaN_3 (Table I).

Hydroquinone is both a $^1O_2$ quencher (18) and a free radical scavenger (19); it has been used as an antioxidant in free radical initiated oxidation reactions. However, in our system (Figure 2) at 0.03 M, hydroquinone provided as much protection as NaN_3 or 2,5-dimethylfuran at 0.15 M and 0.02 M respectively (Figure 1B). A mixture of hydroquinone (30 mM) and 2,5-dimethylfuran (20 mM) provided no additive effect (Figure 2).

Superoxide dismutase which should quench O_2^- (21,22), when used in concentrations of 1-50 µg/ml did not show any protection (Table I). Isopropanol, which is known to react with free radicals, also provided no protection against photoinactivation of ribosomes by methylene blue.

DISCUSSION We have found methylene blue to be a good photosensitizer of ribosomes *in vitro*, which is consistent with the work reported by Garvin et al. (23). The various possible mechanisms through which photodynamic action by dyes is brought about have already been discussed in the literature (7,24,25). Five main mechanisms may be applicable in our system, as follows:

1. $MB + O_2 \xrightarrow{h\nu} {}^1O_2 \xrightarrow{RIB}$ Inactive RIB

2. $MB + O_2 \xrightarrow{h\nu} O_2^- \xrightarrow{RIB}$ Inactive RIB

3. $MB + RIB \xrightarrow{h\nu}$ Free Radicals $\xrightarrow{O_2}$ Inactive RIB

4. $MB + RIB \xrightarrow{h\nu}$ MB-RIB (complex) $\xrightarrow{O_2}$ Inactive RIB

5. $MB + RIB \longrightarrow$ MB-RIB (ground state complex) $\xrightarrow[O_2]{h\nu}$ Inactive RIB

In reactions 1-5, MB and RIB represent methylene blue and ribosomes, respectively.

The ${}^1O_2$ mechanism (reaction 1) seems to be the most important, since with various ${}^1O_2$ quenchers, ribosomes are protected up to 60% of the total photoinactivation. Since no additive effect is found when some of the ${}^1O_2$ quenchers were used together (e.g. NaN_3 + 2,5-dimethylfuran, NaN_3 + ascorbic acid and hydroquinone + 2,5-dimethylfuran), we conclude that all of these quenchers are acting by one and the same mechanism, namely protection against ribosome inactivation by ${}^1O_2$ produced in the system. It is possible that the actual contribution to inactivation by ${}^1O_2$ is greater than the results with ${}^1O_2$ quenchers indicate. If methylene blue complexes with ribosomes (reaction 5), then the ${}^1O_2$ formed on irradiation is likely to react with the ribosomes within the solvent cage, before diffusing away - analogous to the self-quenching in

other systems (26). Such inactivation is not likely to be protected by the $^1O_2$ quenchers at the concentrations used in our system.

The extent to which O_2^- is formed in the dye-sensitized reactions (reaction 2), is not known but is most likely quite small (24,25). We have tested the possibility of the involvement of O_2^- by the use of superoxide dismutase (21,22) which was found to have no effect in our system. Thus a role of O_2^- in ribosome inactivation, is unlikely, although a small contribution within the solvent cage (as discussed above) may go undetected.

Free radical formation on irradiation of dyes (reaction 3) has been suggested by many authors (5,7,8) and demonstrated by others (9,10). However, the molecular nature of the free radicals formed and their reactivity is unknown (5). Any free radicals formed on the ribosomes will add on O_2 to form peroxy radicals leading to damage. Several of the additives we have used, namely isopropanol, ascorbic acid (20) and hydroquinone (19), are known to react with free radicals. But in our system, their addition affected only the mechanism involving $^1O_2$. Therefore, the extent and the nature of the free radical mechanism remains obscure.

The mechanism involving dye and ribosome complex formation by either reaction 4 or 5 could also be operative in our system. The use of relatively high concentration of dye in our system, is likely to favour the reaction 5. Further work is needed to establish the role of these mechanisms.

In conclusion, O_2 is required in most (90-95%) of the methylene blue sensitized photoinactivation of ribosomes and the most important mechanism is through the formation of $^1O_2$. Inactivation due to O_2^- is not important. The rest of the inactivation is through either free radical formation followed by peroxidation, or oxidation of the ribosome-dye complex formed, or both.

ACKNOWLEDGEMENTS - The authors would like to thank Dr. Ajit Singh for helpful discussions during the course of this work and Dr. A. Petkau for discussion on the use of superoxide dismutase.

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