Studies on the Aerobic Photooxidation of Cysteine Using Riboflavin as a Sensitizer: Evidence for the Photogeneration of a Superoxide Anion and Hydrogen Peroxide

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The riboflavin sensitized oxidation of cysteine under an aerobic condition was investigated. The effects of various scavengers, such as superoxide dismutase, catalase, mannitol, sodium azide and potassium ferrocyanide (an electron donor), on the photooxidation were determined. A reaction mechanism involving the superoxide anion is proposed for the photooxidation of cysteine to cysteic acid.

Key words riboflavin; sensitizer; photooxidation; radicals; cysteine

Photosensitized reactions of sulphur containing amino acids have been studied as a method for the selective oxidation of these amino acids and also for understanding the effect of the photooxidation of these amino acid residues on the conformation and stability of proteins.[1-5]

Amongst the many sensitizers available, riboflavin (RF) is of special importance because of its widespread occurrence as a blue light receptor pigment[6,7] and as an integral component of flavin coenzymes.[8] Because of its presence in ocular tissue, RF-induced conformational changes in the microenvironment of thiol groups have received considerable attention.[9,10]

In this work, we have studied the RF-sensitized photooxidation of cysteine in the presence of oxygen. Photooxidation, sensitized by RF, may proceed by two different routes.[11,12] The radical mechanism involves the direct interaction of a triplet sensitizer with a substrate, ultimately generating various oxygen-containing radicals as intermediates (type I). In a type-II process, the reaction proceeds *via* singlet oxygen obtained by energy transfer from a triplet sensitizer to molecular oxygen. In order to determine the mechanism, the reaction has been studied in the presence of sodium azide (a quencher for singlet oxygen), superoxide dismutase, catalase, and mannitol (scavengers for superoxide, hydrogen peroxide, and hydroxyl radicals, respectively), and potassium ferrocyanide as an electron donor to RF.

MATERIALS AND METHODS

Riboflavin, superoxide dismutase (SOD), and catalase were obtained from Sigma Chemical Co.; L-cysteine, sodium azide, mannitol and potassium ferrocyanide were from SRL (India), Wilson Laboratory, S.D. Chem. Pvt. Ltd. and Sarabhai M. Chemicals Ltd., respectively. All the chemicals were used without further purification.

Aqueous solutions of RF (4×10^{-5} M) and cysteine (1×10^{-3} M) in the absence and in the presence of different quenchers and scavengers were irradiated with a 150 W, 24 V Phyllips Comptalux Tungsten lamp kept at a distance of 40 cm from the glass reaction cell. The progress of the reaction was followed by monitoring the concentration of cystein with time. The concentration of cysteine was determined by the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) method.[13] Unless otherwise stated the experiments were carried out at pH 3.7. All experiments were performed at 37°C. Water-saturated oxygen was bubbled through the solution continuously to keep the solution saturated with oxygen. For anaerobic reactions, the solution was deoxygenated by bubbling moist nitrogen gas for fifteen minutes prior to and during the irradiation. The formation of hydrogen peroxide was assayed spectrophotometrically with horseradish peroxidase[14] at 510 nm. For the determination of cystine and cysteic acid we have used the NTSB method[15] and ninhydrin method,[16] respectively. Absorbances were measured by an Elico ULTRA-SPEC Model-CL 54D spectrophotometer, and the fluorescence measurements were performed using a Perkin Elmer MPF-44B spectrofluorimeter.

RESULTS

The RF sensitized photooxidation of cysteine has been found to follow apparent zero order kinetics with re-

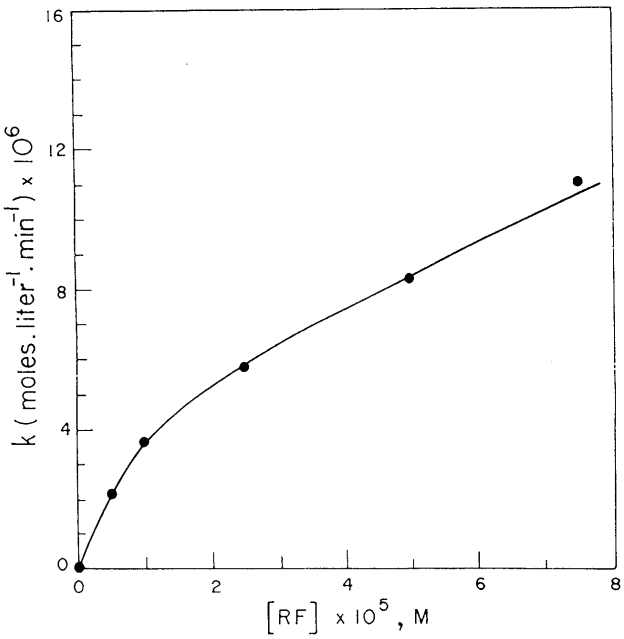

Fig. 1. Variation of the Rate Constant for the Photooxidation of Cysteine (1.2×10^{-3} M) with RF Concentration at 310 K and pH 3.7

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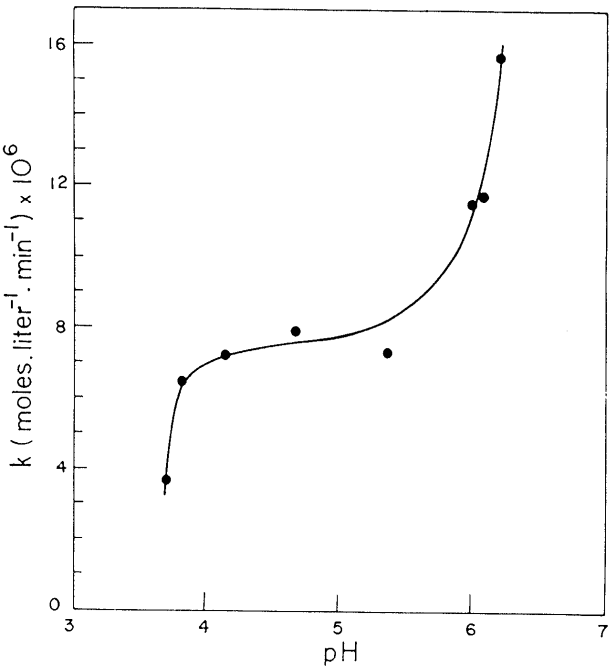

Fig. 2. Variation of the Rate Constant for the RF (3×10^{-5} M) Sensitized Photooxidation of Cysteine (10^{-3} M) as a Function of pH at 310 °K

Table 1. Effect of Various Scavengers on the Rate of Aerobic Photooxidation of Cysteine Sensitized by Riboflavin

Scavenger added	% of reaction[a]	Remarks
Control	100	—
SOD (10 units/ml)	53	Scavenger for $O_2^{\cdot-}$ radical
NaN_3 (1.0 mM)	173	$^1O_2$ quencher
Catalase (30 units/ml)	104	H_2O_2 scavenger
Mannitol (10 mM)	100	Trap for ·OH radical
$K_4[Fe(CN)_6]$ (0.5 mM)	500	Electron donor

a) % of reaction relative to control. Results are accurate within ±5%.

spect to cysteine, but the rate of photooxidation depends on the concentration of RF, as shown in Fig. 1. The photoreaction does not occur in the absence of oxygen. It has been found that cysteine has very little effect on the fluorescence of RF ($k_{sv}=2.1\ M^{-1}$) or on the bleaching of RF, which is known to proceed primarily *via* the singlet state of RF.[17] It has been observed that the rate of photooxidation depends on the pH of the reaction medium, as shown in Fig. 2. Simultaneous quantitative analysis of the photolyzed reaction mixture indicated that only cysteic acid was produced in this reaction. A small amount of hydrogen peroxide (10^{-4} M, after 2 h irradiation) was also detected in the reaction mixture. The generation of a superoxide anion ($O_2^{\cdot-}$) in the photolytic solution has been indirectly confirmed by the inhibitory effect of SOD. The effects of different scavengers are summarized in Table 1.

DISCUSSION

Photooxidation was not observed in the absence of oxygen, so it may be surmised that the reaction proceeds through one or more reactive oxygen species as intermediates. Sodium azide, a well-known quencher of singlet oxygen,[18] is often used to detect the involvement of singlet oxygen as an intermediate. However, Table 1 shows that the rate of the photooxidation of cysteine in the presence of sodium azide increases instead of decreasing. Thus, this observation indicates that the RF-sensitized photooxidation of cysteine may not follow a type-II mechanism. Moreover, the detection of hydrogen peroxide in the reaction mixture, together with the evidence of $O_2^{\cdot-}$ formation during the reaction, suggest that the reaction takes place predominantly by a type-I mechanism.

Although relatively small amounts of hydrogen peroxide have been detected during photolysis, catalase, a scavenger of hydrogen peroxide, has no effect on the photochemical reaction rate. This suggests that photodynamically generated hydrogen peroxide does not take part in the photooxidation of cysteine. The participation of a highly reactive hydroxyl radical (·OH) as an intermediate may be ruled out because mannitol, a scavenger of ·OH, has no effect on the reaction rate.

The RF sensitized photooxidation of various substrates has been well documented.[19-21] Laser flash photolysis experiments[22] have revealed that a flavin semiquinone radical is formed in the presence of cysteine at pH 4.1, but virtually no flavin semiquinone radical was detected in the absence of amino acids. These earlier observations are in accordance with our result that the rate of photooxidation of cysteine dramatically increases in the presence of a well-known electron donor like $K_4[Fe(CN)_6]$. The RF semiquinone radical may react with oxygen molecules, yielding a superioxide anion which, by dismutation, produces hydrogen peroxide. It is event from the inhibitory effect of SOD that $O_2^{\cdot-}$ is the dominant reactive species in the photooxidation of cysteine. Thus, taking all these in consideration, the following reaction scheme may be proposed.

$$RF \rightarrow {}^1RF \quad (1)$$

$${}^1RF \rightarrow {}^3RF \quad (2)$$

$${}^3RF \rightarrow RF \quad (3)$$

$${}^3RF + Cys \rightarrow RF^{\cdot-} + Cys^+ \quad (4)$$

$$RF^{\cdot-} + O_2 \rightarrow RF + O_2^{\cdot-} \quad (5)$$

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (6)$$

$$Cys^+ + O_2^{\cdot-} \rightarrow \text{cysteic acid} \quad (7)$$

Where RF, ^1RF, ^3RF, $RF^{\cdot-}$ and Cys^+, denote the ground state, singlet state, triplet state, semiquinone radical of riboflavin and cysteine radical, respectively.

Our observation that the increase in the rate of photooxidation followed a sigmoidal function with pH (Fig. 2) matches the effect of pH on the quenching of flavin triplets by cysteine (this process leads to the formation of a semiquinone radical), reported by Heelis *et al.*[22] in their laser flash photolysis study.

Another aspect of our study is the increase in the rate of photooxidation of cysteine in presence of sodium azide. Heelis *et al.*[2] reported that a flavin semiquinone was observed in the presence of sodium azide and that the triplet quenching constant of flavin by sodium azide is much greater than that of cysteine. Thus, the accelerating effect of sodium azide may be due to the increased rate of formation of semiquinone radicals. In this connection,

it may be noted that sodium azide forms a change transfer complex with riboflavin, even in the ground state.[23]

Therefore, we conclude that the RF-senisitized photooxidation of cysteine in the presence of oxygen takes place primarily by a type-I mechanism and that the photooxidation of cysteine is mediated by a superoxide anion.

Acknowledgements B. Sarkar thanks CSIR, New Delhi, for financial support. We thank Sigma Chemical Co. (U.S.A.) for the free gift of riboflavin and superoxide dismutase. We also thank RSIC, Calcutta, for fluorescence measurements.

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