Photochemistry and Photobiology, 2005, 81: 884–890

Reactions of Excited States of Phenoxazin-3-one Dyes with Amino Acids¶

M. L. Villegas[1], S. G. Bertolotti[2], C. M. Previtali[2] and M. V. Encinas*[1]

[1]Facultad de Química y Biología, Universidad de Santiago de Chile, Casilla 40, Correo 33, Santiago, Chile
[2]Departamento de Química Física, Universidad Nacional de Río Cuarto, Argentina

Received 12 October 2004; accepted 13 January 2005

ABSTRACT

The interaction with amino acids of the excited states of the N-oxide resazurin and its deoxygenation product resorufin, has been studied in aqueous solution at pH 7.5. Steady-state and time-resolved studies show that the fluorescence is quenched by amino acids. Complexation of the dyes in the ground state with aromatic amino acids was also observed. The singlet quenching is attributed to electron transfer from the amino acids to the excited dye based on the dependence of the bimolecular rate constants with the ionization potential of quenchers. Flash photolysis experiments allowed determination of the quenching rate constants for the triplet deactivation of dyes by several amino acids, as well as the characterization of the transients formed in the process. These data show that the triplet is also deactivated by an electron transfer process. However, the deactivation of the N-oxide dye by tryptophan can be described by a hydrogen atom transfer. The protolytic dissociation constants of the dye radical ions are reported. The irradiation of rezasurin in the presence of amino acids leads to deoxygenation of the dye to give resorufin. This process involves the triplet excited state of resazurin and is efficient only in the presence of amino acids containing the –SH group.

INTRODUCTION

Resazurin (I) is a heterocyclic N-oxide dye that is often used to study biological materials (1,2). Most of these applications are based on the oxygen atom transfer reaction with the dye as donor. The resazurin is reduced to the strongly fluorescent product resorufin (II) (3,4), which can be used as a target fluorescent probe. Frequently, these processes require the use of organic compounds or enzymes as catalyst (5–7), and in few cases the reaction has been photocatalyzed (8).

The photophysics of these phenoxazin-3-one dyes, resazurin and resorufin, has been much less explored. In a previous work we reported the photophysics and photochemical behavior of resazurin and resorufin in aqueous solutions (9). We demonstrated that the excited states of the N-oxide dye are deactivated by amines via electron transfer, but the deoxygenation to resorufin is efficient only in the presence of tertiary aliphatic amines. These results suggest that these dyes also could be reactive toward biological substrates. Thus, an understanding of the mechanism of these reactions is important for improving the different applications of the dyes. In this work we carried out a detailed study of the interaction of the excited states of the phenoxazine-3-one dyes with amino acids. Transient absorption spectra and protolytic dissociation constants of the radical ions formed in the irradiation of dyes in the presence of several amino acids are reported.

MATERIALS AND METHODS

Resazurin and resorufin were from Aldrich (Milwaukee, WI) and used as supplied. The amino acids were obtained from Sigma (Saint Louis, MO) and were used without further purification.

Dye solutions were purged with argon for 30 min before use. All experiments were carried out in aqueous solutions, and the pH of solutions was maintained by 30 mM phosphate buffer or adjusted by addition of NaOH or HCl, once all the other reagents were added. The photoreduction of the dyes by amino acids was studied spectrophotometrically in aqueous solution at pH 7.5. Resazurin samples were irradiated at 615 nm and resorufin samples at 572 nm with a Photon Technology International (Lawrenceville, NJ) irradiation system comprising a 150 W xenon lamp and a monochromator. The rate of the photoreaction was measured following the decrease of the absorbance of the dyes at different irradiation times in free-oxygen solutions. The photoreaction quantum yield was determined taking as reference Aberchrome 540 (10). Absorption spectra were determined on a Hewlett-Packard 6453E diode array spectrophotometer.

Steady-state fluorescence measurements were made using a Fluorolog-Spex spectrofluorimeter. Fluorescence lifetime measurements were performed with an Edinburgh Instruments (Edinburgh, Scotland) OB 900 time-correlated single photon counting fluorometer. The singlet quenching rate constants were measured by following the decrease of the fluorescence intensity or the emission decay elicited by the amino acid addition.

Transient absorption measurements were made using laser-flash photolysis equipment. The harmonic wavelengths of an Nd:YAG laser (532 nm, 25 mJ/pulse, 20 ns) were used for sample excitation. The signals from the monochromator/photomultiplier system were initially captured by a Hewlett-Packard 54504 digitizing oscilloscope and transferred to a computer for storage and analysis. Measurements were performed in samples subjected to continuous argon bubbling.

RESULTS AND DISCUSSION

The visible spectrum of resazurin in the pH range 7–12 consists of a strong band at 602 nm, while the visible transition of resorufin presents a maximum at 572 nm (9). These spectra did not show any change in the presence of aliphatic amino acids; thus ground-state complex can be disregarded. However, with the addition of tryptophan, a significant change in the absorption spectrum of the dyes was observed. The absorption band is red-shifted and broadened, a decrease of the absorbance and two well-defined isobestic

¶Posted on the website 18 January 2005
*To whom correspondence should be addressed: Universidad de Santiago de Chile, Facultad de Química y Biología, Casilla 40, Correo 33, Santiago, Chile. Fax: 56-2-6812108; e-mail: mencinas@lauca.usach.cl

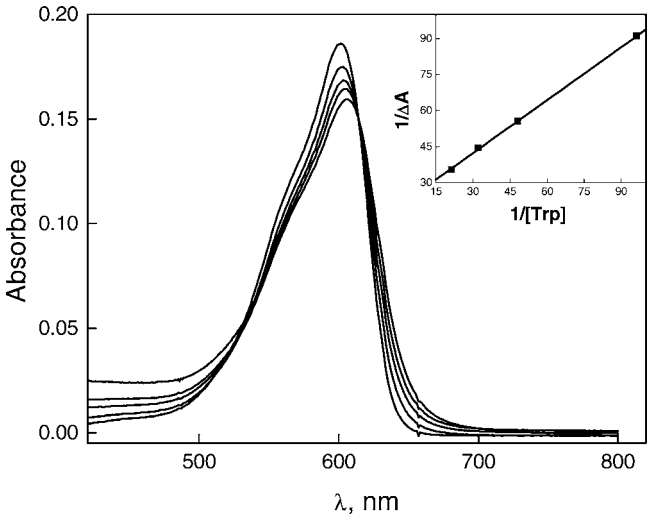


Figure 1. Absorption spectra of resazurin in water pH 7.5 at different concentrations of tryptophan. From the top: 0, 10, 21, 31 and 47 mM tryptophan. Inset: changes of dye absorption with the tryptophan concentration plotted according to the Benesi-Hildebrand equation.

points are observed (Fig. 1). These facts indicate the formation of a ground-state complex between the tryptophan and the dye.

Because of the low solubility of tyrosine, the possible complex formation with this amino acid and the dyes could not be investigated by spectroscopic methods. As analogs of tyrosine, two phenol derivatives were employed: 4-methoxyphenol and 4-ethylphenol; the latter could be used only in fluorescence-quenching experiments due to solubility limitations.

Association constants (K_S) were estimated from the changes in absorbance at a fixed wavelength using the Benesi-Hildebrand equation (11) and are collected in Table 1. These data show that the interaction of both dyes in the ground state with the tryptophan and phenolic compounds is similar, although the association constants of resazurin are slightly higher than those of resorufin. The higher electronic delocalization in the aromatic ring of the N-oxide could lead to a more favorable association with the aromatic amino acids. Larger differences arise when the comparison is between both amino acids. That is, the complex with phenol is weaker than with tryptophan. The difference could be explained in terms of the higher electronic delocalization on the indole ring compared to that of the benzene ring of the phenol leading to a stronger association. The interaction with tryptophan is slightly less favorable at high ionic strength, suggesting some contribution of charge transfer contribution to complex formation. On the other hand, the association of both dyes with phenol at high pH is very small, as expected from the negative charge on the phenoxide and on the dye.

Table 1. Association constants for the interaction of resazurin and resorufin with tryptophan and two substituted phenols in aqueous solution, pH 7.5

		K_S, M^{-1}	
Dye	Amino acids	Absorption	Fluorescence
Resazurin	tryptophan	28	35
	4-methoxyphenol	16	29
	4-methoxyphenol (pH 11)	<1	1
	4-ethylphenol	—	11
	4-ethylphenol (pH 11)	—	6
Resorufin	Tryptophan	22	29
	Tryptophan, NaCl, 1 M	16	24
	4-methoxyphenol	10	26
	4-methoxyphenol (pH 11)	<1	3
	4-ethylphenol	—	15
	4-ethylphenol (pH 11)	—	4

Table 2. Rate constants for the singlet quenching of dyes by amino acid, aqueous solution at pH 7.5

		$^1k_q \times 10^9$ M^{-1} s^{-1}	
Amino acid	E_{ox}, V	Resazurin	Resorufin
Tryptophan	1.02*	6.0	4.5
4-Ethylphenol	0.94*†	6.5	3.6
4-Ethylphenol (pH 11)	0.76*†	6.4	4.0
Methionine	1.34‡	4.3	3.2
Histidine	1.36§	2.8	2.4
Cysteine	—	Reaction	0.8
Cysteine (pH 9.5)	—	2.5	1.7
Phenylalanine	>2‖	0.8	0.9
Serine	—	0.3	0.34
Alanine	>2.2‖	0.3	0.12

*Redox potential *vs* NHE at pH 7 (12,13).
†Assumed to be the same as tyrosine.
‡From (14).
§From (15).
‖From (16).

Singlet quenching

The fluorescence quenching of resazurin and resorufin in water at pH 7.5 by a series of amino acids was measured. The quenching occurs without change of the shape of the fluorescence spectrum, even at the highest concentrations of the amino acid used (0.1 M). Thus, no exciplex formation is indicated. Bimolecular rate constants were determined from the Stern-Volmer (SV) plots of I°/I or τ°/τ vs. amino acid concentration [Q] (Eq. 1):

$$I^\circ/I \text{ or } \tau^\circ/\tau = 1 + K_{SV}[Q] = 1 + {}^1k_q\tau^\circ[Q] \quad (1)$$

where I° and I stand for the fluorescence intensity, τ° and τ are the fluorescence lifetimes in the absence and the presence of amino acid, respectively and ^1k_q is the dynamic singlet quenching rate constant.

For aliphatic amino acids, the Stern-Volmer plots determined from the fluorescence intensity have a linear relationship, and τ°/τ data points lie on the same straight line within the experimental error. Therefore, static quenching is negligible in these cases. However, for the stronger quenchers, aromatic amino acids, steady-state plots do not coincide with those of τ°/τ and showed positive deviations. These results indicate the presence of static quenching in addition to dynamic quenching. The static Stern-Volmer constant can be calculated from the quenching of fluorescence intensity by (Eq. 2):

$$I^\circ/I = (1 + {}^1k_q\tau^\circ[Q])(1 + K_S[Q]) \quad (2)$$

where K_S is the association constant of a nonfluorescent 1:1 ground-state complex. Values for K_S determined using ^1k_q obtained from quenching of the fluorescence lifetimes are included in Table 1. These values are in satisfactory agreement with the association constant determined by absorption spectroscopy (Table 1).

Table 3. Triplet quenching rate constants by amino acids, in aqueous solutions pH 7.5

Amino acids	$^3k_q \times 10^7$ M^{-1} s^{-1}	
	Resazurin	Resorufin
Tryptophan	200	4.0
Indole-3-acetic acid	163	7.0
4-ethylphenol	26	6.3
4-ethylphenol (pH 11)	106	72
Histidine	0.4	1.0
Methionine	0.1	0.7
Cysteine	—	0.5
Arginine	0.1	0.3

Dynamic quenching efficiencies for the different amino acids, ^1k_q, are collected in Table 2. These values follow the same trend for both dyes. Effect of the ionic strength on the quenching efficiency can be disregarded because quenching experiments carried out in high ionic strength buffer did not change the ^1k_q values. Rate constants for the quenching by the more easily oxidized compounds tryptophan, protonated phenol and phenoxide, methionine and histidine approach a value close to the diffusional limit controlled process (6.5×10^9 M^{-1} s^{-1} [17]). The quenching rate constants are smaller for the amino acids that are more difficult to oxidize, such as cysteine, phenylalanine, serine and alanine. These results suggest an electron transfer mechanism for the singlet-quenching process. Furthermore, data obtained with cysteine ($pK_a = 8.3$ [18]) show that the quenching rate constant increases when the pH is raised from 7.5 to 9.5. This result agrees with an electron transfer mechanism in which the ^1k_q value increases when going from a higher redox potential of the protonated thiol to a lower redox potential of the thiolate form. In all cases, ^1k_q values are slightly higher for resazurin than for resorufin. This points to a higher reactivity of the excited dye involving the N-oxide moiety. Photo-induced electron transfer also has been proposed for the singlet quenching of acridine by sulfur-containing amino acids (19), and for the quenching of tirapazamine, an N-oxide compound, by tryptophan and tyrosine (20).

Triplet quenching

The irradiation of an aqueous solution of resazurin (pH 7.5) with 532-nm laser pulses yields a transient absorption in the region 650–800 nm, corresponding to the triplet spectrum. On the other hand, the absorption spectrum of resorufin showed the maximum at 700 nm corresponding to the triplet absorption (9). When amino acids were added to aqueous solutions of the dyes the absorption due to the triplet is quenched. The plots of decay rates of the triplet against concentrations of added compounds gave straight lines. Bimolecular triplet-quenching rate constants (^3k_q) for several amino acids determined from the slope of these plots are summarized in Table 3.

The rate constants obtained for resorufin are much lower than those corresponding to the diffusion control, and follow the expected trend from the oxidation potentials for an electron transfer–quenching reaction. Tryptophan and 4-ethylphenol are the more efficient quenchers. The quenching efficiency increases for the phenol derivative when the pH is raised to 11, which is in agreement with the higher electron donor ability of the phenoxide with respect to the protonated phenol. An almost negligible quenching was observed for amino acids of higher oxidation potential (*i.e.* histidine, methionine, arginine and cysteine). In these cases only an upper limit could be estimated for the rate constant. Similar trends with the structure of the amino acid have been reported for quenching efficiency of several electronically triplet-excited compounds (21–23) and radical cations (24). On the other hand, rate constants for the triplet-quenching of the N-oxide dye resazurin by amino acids are also those expected from the oxidation potential of the quencher with the exception of the indolic compounds. This behavior indicates that the quenching of the excited triplet of resazurin involves an electron transfer processes. Data in Table 3 also show that rate constants for triplet-quenching are lower than those of the singlet quenching, which is in agreement with that expected for a triplet process.

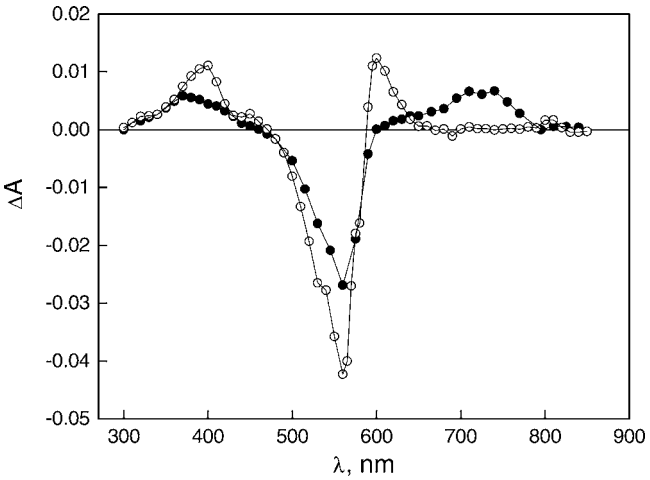


Figure 2. Transient absorption spectra of resorufin in the presence of 8 mM tryptophan recorded at 40 μs after the laser pulse, in water at (●) pH 7.5; and (○) pH 11.

The transient absorption spectrum of resorufin in the presence of tryptophan at pH 7.5 is shown in Fig. 2. Two main absorptions with maxima at 370 and 720 nm can be observed. However, the spectrum in the presence of tryptophan is dependent on the pH. At pH 11 the spectrum shows maxima at 400 and 600 nm (Fig. 2). This spectrum is similar to that previously found for the quenching of the dye by triethanolamine at pH > 9.5 and is assigned to the resorufin dianion radical, $D^{-\bullet}$ (9).

The absorption at 400 and 720 nm as a function of pH is presented in Fig. 3. The titration curve, despite the error at 720 nm due to the low absorbance values at both wavelengths, shows an inflection point at pH = 9.1. The spectrum at low pH is assigned to the protonated radical anion ($DH^{-\bullet}$) formed by the electron transfer from the amino acid to the triplet of the dye followed by a fast in-cage protonation of the dye dianion radical $D^{-\bullet}$ (Eq. 3):

$$(D^-)^* + Trp \rightarrow D^{-\bullet} + Trp^{+\bullet} \rightarrow DH^{-\bullet} + Trp(-H)^{\bullet} \quad (3)$$

At pH > 9, the radical anion $DH^{-\bullet}$ undergoes a fast deprotonation (Eq. 4), producing the dianion radical:

$$DH^{-\bullet} + OH^- \rightarrow D^{-\bullet} + H_2O \quad (4)$$

Figure 4 shows the transient absorption of resorufin in the presence of 4-ethylphenol at pH 11. This spectrum coincides with that obtained when resorufin is irradiated in the presence of triethanolamine (9) or in the presence of tryptophan at pH 11 (Fig. 3) where the signals at 400 and 600 nm correspond to the deprotonated anion radical of the dye. This behavior demonstrates that the triplet-quenching of resorufin by the 4-substituted

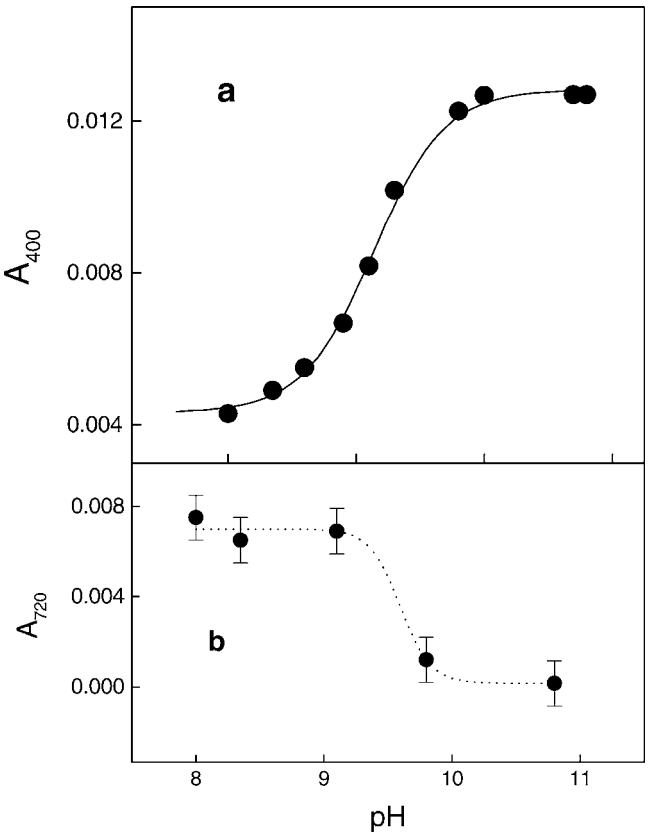


Figure 3. pH titration curve of the transient absorbance measured at 40 μs after the laser pulse obtained on excitation of resorufin in the presence of 8 mM tryptophan at (a) 400 nm and (b) 720 nm. Solid line: calculation with the pK value 9.1.

phenolate involves the one electron reduction of the dye, according to (Eq. 5):

$$(D^-)^* + PhO^- \rightarrow D^{=\bullet} + PhO^\bullet \quad (5)$$

On the other hand, as can be seen in Table 3, the resazurin triplet-quenching rate constants by tryptophan and indole-3-acetic acid are one order of magnitude higher than that found for 4-ethylphenol,

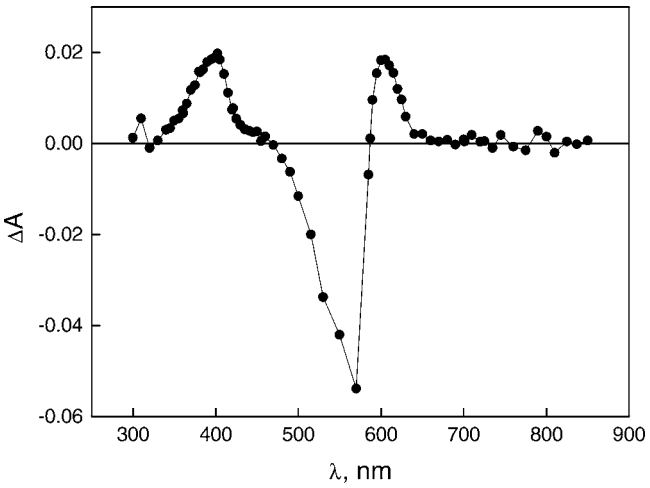


Figure 4. Transient absorption spectrum of resorufin at 40 μs after the laser pulse in the presence of 6 mM 4-ethylphenol in water pH 11.

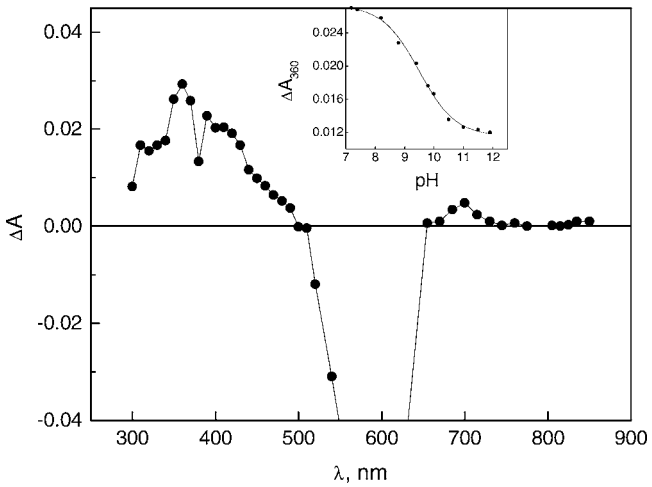


Figure 5. Transient absorption spectrum of resazufin at 20 μs after the laser pulse in the presence of 6 mM tryptophan in water at pH 11. Inset: pH titration curve of the transient absorbance at 360 nm measured at 40 μs after the laser pulse.

despite the similar oxidation potentials of these compounds. For resorufin, the rate constants are similar, which is in line with that expected from an electron transfer process. This would suggest a different quenching mechanism for the resazurin, probably involving a specific interaction of the N-oxide group with the indole ring in the case of the resazurin. To get more information on this mechanism we measured the triplet-quenching by indole and N-methylindole in water/ethanol (1/1) mixture. Rate constants of 3×10^8 M^{-1} s^{-1} and 3×10^7 M^{-1} s^{-1} were obtained for indole and 1-methylindole, respectively. That ^3k_q is one order of magnitude lower for the 1-methyl substituted compound, despite their similar oxidation potential (25), indicates the importance of the indolic H-atom in the quenching of the N-oxide compound. A plausible mechanism could be a direct hydrogen atom transfer from the NH moiety of indole to give the protonated anion radical of the dye and the tryptophan neutral radical, (Eq. 6):

$$(D^-)^* + Trp \rightarrow DH^{-\bullet} + Trp(-H)^\bullet \quad (6)$$

The oxygen atom on the N-oxide group is probably the protonation site, as reported by Shi and Platz (26) for the triplet-excited state of nitroquinoline N-oxides.

The quenching process given by (Eq. 6) is reinforced by the transient absorption spectrum of resazurin in the presence of tryptophan at pH 7.5 (Fig. 5). The spectrum shows a maximum at 360 nm and a weak absorption at 700 nm, which can be assigned to the protonated radical anion of the dye. Similarly, to that described for resorufin, these maxima are dependent on the pH. The absorption at 360 nm decreases with increasing pH and, at the same time, a new maximum appears at 410 nm. The inset in Fig. 5 shows the pH dependence of the absorption at 360 nm. From the inflection point a pK value of 9.4 is obtained that is almost the same as that obtained for resorufin, and corresponds to the deprotonation of the anion radical, $DH^{-\bullet}$.

Photoreactions with amino acids

The photochemical deoxygenation of N-oxides mediated by electron donors has been described for some heterocyclic N-oxides (27). These data indicate that the characteristics of the process are very dependent on the structure of the N-oxide and the electron

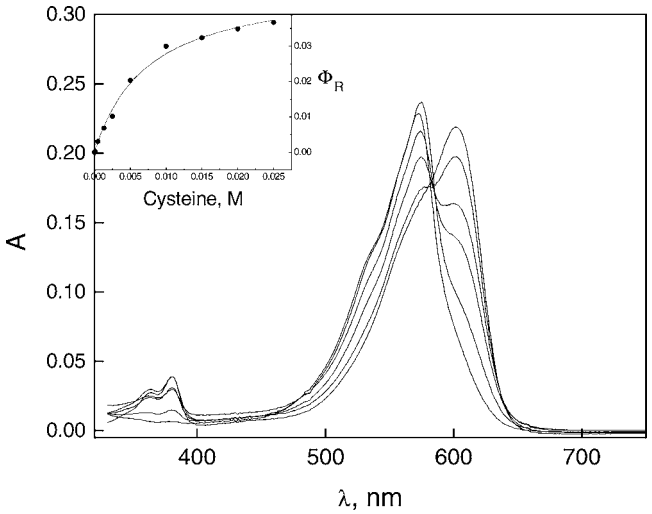


Figure 6. Effect of the irradiation time on the absorption spectrum of resazurin in the presence of 20 mM cysteine irradiated at 615 nm in aqueous solution at pH 7.5. Time from the top: 0, 1.5, 6.5, 15, 31 and 42 min. Inset: effect of cysteine concentration on the photoreaction quantum yield of resazurin; solid line corresponds to the fitting in (Eq. 7).

donor employed. We found that irradiation at 615 nm of resazurin in water at pH 7.5 in the presence of cysteine changes the solution from blue to pink. This reaction is not observed when the dye is irradiated alone. The absorption spectra at different irradiation times are shown in Fig. 6. A new product with a maximum at 572 nm is formed, and a well-defined isosbestic point is observed at 582 nm. The final spectrum corresponds to the deoxygenated dye, resorufin, being the only product even at longer irradiation time. The photoreaction quantum yield (Φ_R) was determined from the plot of the initial rate of absorbance decrease at 615 nm, where the photoproduct does not absorb. Values for Φ_R increase with the amino acid concentration reaching a maximum value at 0.02 M (Fig. 6). Until this thiol concentration was reached the deoxygenation of the dye did not occur in dark. The photoreaction quantum yield at the maximum was 0.033. Of interest, this process occurs only in the presence of cysteine. The initial bleaching rates for other amino acids such tryptophan, tyrosine and methionine were very slow.

Assuming a value of 8×10^8 M^{-1} s^{-1} for the rate constant of the resazurin singlet-quenching by cysteine, at the maximum concentration used in these experiments (0.025 M), less than 3% of the resazurin singlets are quenched by the amino acid. Higher thiol concentrations could not be employed due to the dark reaction. Therefore, it may be safely assumed that the reaction proceeds only from the triplet state, and the photodeoxygenation quantum yield (Φ_R) can be related to the amino acid concentration by (Eq. 7):

$$\Phi_R = \Phi^\circ_T \frac{{}^3k_q[Aa]}{{}^3k_q[Aa] + (\tau_T^0)^{-1}} \alpha_T \qquad (7)$$

where α_T represents the fraction of triplets that leads to the deoxygenation reaction and Φ°_T is the triplet quantum yield. The experimental data for photoreduction yields at different cysteine concentrations could be fitted to (Eq. 7) with values of 5×10^6 M^{-1} s^{-1} and 0.60 for ${}^3k_q$ and α_T, respectively (Fig. 6). This correlation confirms that the photodeoxygenation reaction arises from the interaction of the triplet state of the dye with the thiol. Of interest, 60% of this interaction leads to the chemical reaction. The estimated ${}^3k_q$ value follows the same trend with the structure of amino acid than that obtained in the quenching of resorufin triplet, and reflects the electron donor ability of the quencher.

Table 4. Photoreaction quantum yields for the irradiation of resazurin in the presence of electron donor compounds (aqueous solution, pH 7.5)

	Φ_R	
Electron donor*	Resazurin	Resorufin
EDTA	0.013	—
$Fe(CN)_6^{4-}$	<0.001	—
Cysteine (pH 4)	0.01	0.0015
Cysteine (pH 7.2)	0.033	0.0084
Cysteine (pH 9.5)	0.0045	0.001
Glutathione	0.012	—
Methionine†	0.002	—

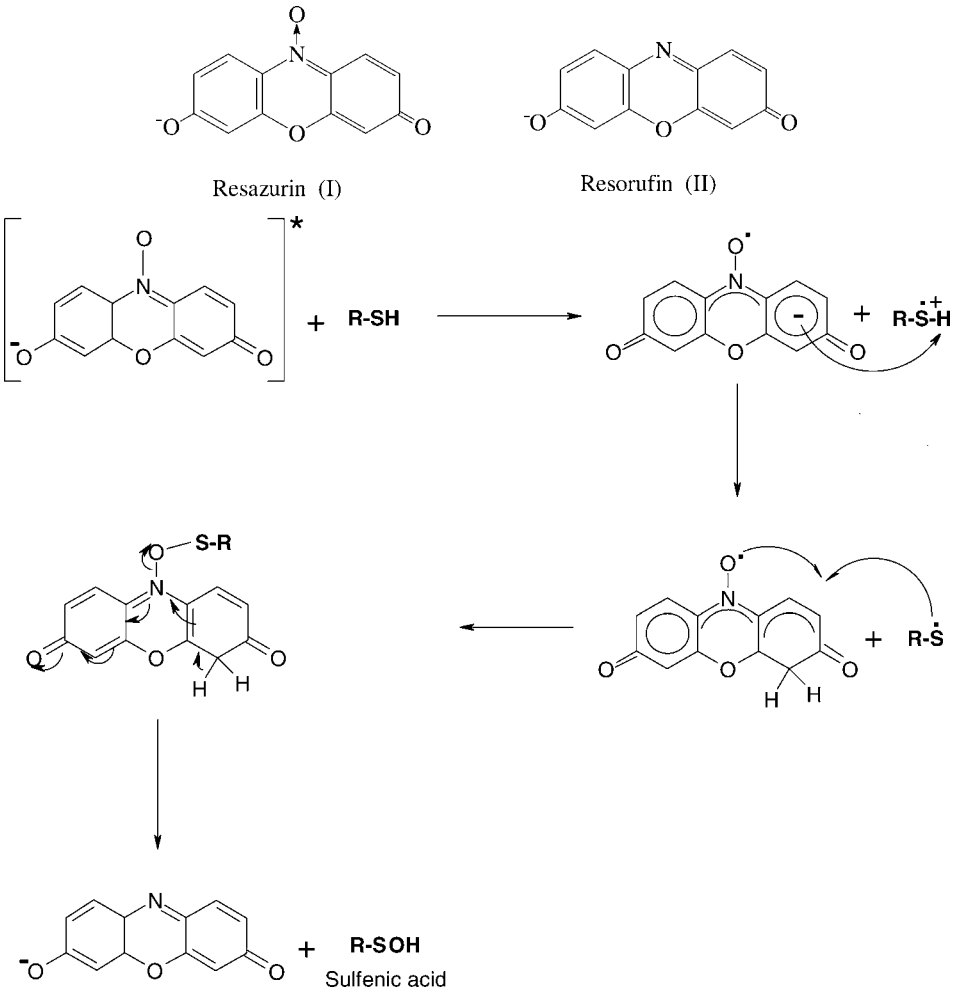
*20 mM.
†30 mM.

The bleaching of resazurin was also carried out in the presence of reduced glutathione. These results follow similar characteristics to those obtained with cysteine. The photobleaching quantum yield increases with the glutathione concentration reaching a constant value at 0.02 M. However, the maximum photodeoxygenation quantum yield is two times lower than that found for cysteine. That the other two amino acids that constitute the glutathione peptide, glycine and glutamine, do not react with the N-oxide indicates that the reaction is located at the –SH group in the tripeptide. The ${}^3k_q$ was estimated to be 3×10^6 M^{-1} s^{-1} that is similar to that found for cysteine. Prütz (6) has reported that reduction of rezasurin by glutathione proceeds with high efficiency when it is promoted by sulfanes and selenite. The results described in this work show that this reaction is also efficiently promoted by light, which introduces a useful method for detecting glutathione in biological systems.

An electron transfer mechanism for the photodeoxygenation of heterocyclic N-oxides in the presence of N,N′-dimethylaniline was proposed by Sako *et al.* (28), who suggested that the oxygen loss takes place through an intermediate derived from the recombination of the radicals formed by proton transfer after the initial electron transfer step. A similar mechanism has been proposed in the photoreaction of resazurin with aliphatic tertiary amines (9). The same mechanism is probably involved in the reaction with cysteine. The low dissociation energy of the –SH makes the H atom transfer a highly favorable process. This is also consistent with the fact that methionine, in which the cysteine $-CH_2SH$ group has been replaced by $-CH_2SCH_3$ does not catalyze the deoxygenation of the N-oxide.

To obtain further information on the photodeoxygenation mechanism, the bleaching of resazurin was studied in the presence of ethylenediamine tetraacetic acid (EDTA) and $Fe(CN)_6^{4-}$, compounds with very low oxidation potentials. Photoreaction quantum yields are shown in Table 4. These data show that the photodeoxygenation reaction is efficient only with EDTA, which contains labile H atoms.

A mechanism compatible with these results is given in Scheme 1. The photodeoxygenation derived from the radicals produced after the H atom transfer was also confirmed by experiments carried out in the presence of cysteine at different pH values (Table 4). The photodeoxygenation yields in the presence of cysteine were highly dependent on the pH. The pK_a of cysteine is 8.3 (18), hence at pH 9.5 a negligible reaction between the protonated thiol and the



Scheme 1.

resazurin triplet can be expected, which is in agreement with the low photodeoxygenation yield at pH 9.5. This indicates that the reaction proceeds from the radical pair formed after the H atom transfer from the thiol. A low value of Φ_R was also found at pH 4.0. Laser-flash photolysis experiments carried out at this pH showed only the transient absorption of the protonated triplet dye. Then, the lower Φ_R value obtained at pH 4.0 shows a low reactivity for the protonated triplet dye. The photoproduct of the resazurin deoxygenation, the resorufin, also undergoes photodecomposition in the presence of cysteine. However, in this case the only spectral change in the visible spectrum is the bleaching of the dye. This indicates that the photoreaction involves the rupture of the dye ring. Tryptophan, phenol or histidine do not lead to photoconsumption of the dye. The photoreaction quantum yield at total triplet-quenching by cysteine was 0.0084 at pH 7.5, and is an order of magnitude lower at pH 4 and at pH 9.5. This indicates that the protonated thiol is the reactive species toward the deprotonated form of the dye.

Acknowledgements—This work was supported by FONDECYT (1030003 and 7040156).

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