Photooxidation of Hypotaurine to Taurine in the Presence of Flavins

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Summary Irradiation of organic sulfinates such as hypotaurine and cysteine sulfonic acid in the presence of catalytic amounts of flavins led to the oxidation of its sulfinic groups (−SO₂H) to the corresponding sulfonylates. The process of hypotaurine oxidation in the presence of riboflavin, followed by absorbance decrease at 220 nm and by ion-exchange chromatography, showed a pseudo-first-order kinetics at pH 6.0. The k value depended linearly on flavin concentrations. The reaction rate was higher at acidic pHe. Although the reaction rate was not affected by the addition of superoxide dismutase or catalase, superoxide ions were supposed to be by-products of the reaction. The effectiveness of allyl alcohol as a scavenger pointed to a free-radical mechanism of the reaction. We propose a new reaction mechanism involving sulfinic radicals on this photochemical reaction.

Key Words photooxidation, flavin, hypotaurine, taurine, cysteine sulfonic acid

Naturally occurring sulfinates, such as hypotaurine and cysteine sulfonic acid, have been isolated, and their metabolic origin has been demonstrated (1–3). The synthesis of cysteine sulfonic acid from cysteine by cysteine dioxygenase [EC 1.13.11.20] has been demonstrated in vivo (4). Hypotaurine and homohypotaurine are the main oxygenation products by cysteamine dioxygenase [EC 1.13.11.19] of cysteamine and homocysteamine, respectively (5). Both enzymatic activities are present in animal tissues (6).
Sulfinates are chemically rather stable compounds. Hypotaurine readily converts to taurine in vivo (7–9). Hypotaurine-oxidizing activities have been reported in ox retina (10), in rat spleen (11) and other rat tissues (12, 13); the reported activities in the tissues are in the range of 2–2,000 nmol taurine formed per min per g protein. The presence of the enzyme involved in this reaction, however, has not yet been reconfirmed (14), and the significance of the enzymatic conversion is still unclear.

At physiological pH, hypotaurine is slowly oxidized to taurine by H₂O₂ (15). The UV irradiation brings about quantitative conversion of hypotaurine to taurine at neutral and acidic pH through a reaction mechanism involving sulfinyl radicals (16). In connection with these findings, the release of taurine from chick retina membrane reported by Pasantes-Morales et al. (17) is interesting.

Preliminary observations concerning the significant oxidation of organic sulfinates such as hypotaurine and cysteine sulfinic acid by visible light in the presence of flavins in vitro have been reported (18). This paper presents more detailed data about this system in vitro using various analytical approaches. A new reaction mechanism is proposed for this photochemical reaction.

MATERIALS AND METHODS

Taurine, hypotaurine, cysteine sulfinic acid, cysteic acid, riboflavin, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), nitro blue tetrazolium, allyl alcohol, and bovine liver catalase were from Sigma Chem. Co. (St. Louis, Mo., U.S.A.).

Homohypotaurine was prepared according to De Marco and Rinaldi (19). Homotaurine was a gift from Prof. C. De Marco. Bovine erythrocyte superoxide dismutase was a gift of Prof. L. Calabrese.

[^5]SHypotaurine was prepared from[^5]Scysteamine (Amersham International plc, Buckinghamshire, England; 10.2 mCi/mmol) according to the method of Scandurra et al. (20).

Incubation standard conditions were 7 × 10⁻⁴ M hypotaurine and 3.3 × 10⁻⁶ M riboflavin in 0.1 M phosphate buffer, pH 6.0. Irradiation was performed with a Long Wave Lamp, Model SL 3660 (Ultra-Violet Products Inc., South Pasadena, Ca, U.S.A.; 365 nm, with light intensity of 390 uW/cm² at 15.2 cm). Samples were irradiated in 3 ml quartz cuvettes (light path, 1 cm) at 3 cm or more distance from the lamp, at 20–25°C. Experiments in anaerobiosis were performed in Tunberg cells under nitrogen atmosphere. The irradiation was performed continuously and it was discontinued just for the time interval needed to record the absorbance values.

Spectrophotometric kinetics and analyses were carried out with a Beckman Acta III. Amino acid analysis was performed with standard procedures on an Optica amino acid analyzer (Milano, Italy).

Descending paper chromatograms on 3 × 31 cm Whatman n. 4 paper (Kent, England) were developed with water-saturated phenol. Radioactive spots were located and evaluated quantitatively by a model 7201 Packard radiochromatogram.
RESULTS

Solutions of $7 \times 10^{-4} \text{M}$ hypotaurine, irradiated at pH 6.0 in the presence of $3.3 \times 10^{-6} \text{M}$ riboflavin under the conditions described in the METHODS section, was nearly quantitatively oxidized to taurine within 1h (Fig. 1). Paper chromatography of the reaction mixture using $[^{35}\text{S}]$hypotaurine was performed after various reaction times and showed the presence of only one new radioactive ninhydrin-positive spot beside hypotaurine ($R_f$ 0.65). This new radioactive spot had $R_f$ 0.40 and was identified as taurine by cochromatography with authentic samples of $[^{35}\text{S}]$taurine ($R_f$ 0.40). Amino acid analysis of the same reaction mixture after 1h of reaction showed the presence of hypotaurine and taurine, confirming the results obtained by paper chromatography.

Hypotaurine was not appreciably oxidized in the dark (<2%) or when riboflavin was omitted (<5%).

Photooxidation performed at various acidic pH's (pH 1.0-5.0) gave qualitatively the same results as those obtained at pH 6.0, showing that only taurine is formed. In the neutral and alkaline range, however, (between pH 7.0 and 10.0) at least one new $[^{35}\text{S}]$ninhydrin-negative compound ($R_f$ 0.15) was formed, but its presence was not detected in blanks. So it might arise from the photooxidation of the amino group of hypotaurine.

Homohypotaurine or cysteine sulfinic acid irradiated at pH 6.0 in the presence of $3.3 \times 10^{-6} \text{M}$ riboflavin were quantitatively oxidized to homotaurine or cysteic acid, respectively. These two sulfonates were identified, after completion of the reaction, either by amino acid analysis or by paper chromatography.

Kinetics of hypotaurine disappearance in the irradiated solutions has been studied by measuring absorbance decrease at 220 nm. At this wavelength, sulfonates like hypotaurine, unlike sulfonates, show a strong absorption (15). Hypotaurine disappearance (oxidation rate) followed pseudo-first-order kinetics with rate constant $k = 3 \times 10^{4} \text{M}^{-1}\text{min}^{-1}$ under standard conditions (Fig. 1). The oxidation rate was a linear function of riboflavin concentration up to $2 \times 10^{-5} \text{M}$. This linear relationship was no longer retained at higher riboflavin concentrations, probably due to its aggregation or dimerization (21).

The initial rate of photooxidation of hypotaurine ($7 \times 10^{-4} \text{M}$) in the presence of $6.6 \times 10^{-6} \text{M}$ riboflavin in 0.1 M phosphate buffer, pH 6.0, was studied as a function of the light intensities, by irradiating the hypotaurine solutions in the presence of riboflavin from various distances. The plot of the rate of hypotaurine disappearance versus the light intensities gave a straight line (data not shown).

Kinetics of hypotaurine oxidation in the presence of FMN resembles that in the presence of riboflavin. FAD had a lower catalytic activity, about 10% activity of the other flavins such as FMN and riboflavin.

The effect of pH on the photooxidative disappearance of hypotaurine (as
Fig. 1. Rate of photooxidation of hypotaurine in the presence of riboflavin. Oxidation of hypotaurine was monitored spectrophotometrically at 220 nm. Pseudo-first-order plot: \(7 \times 10^{-4} \text{M}\) hypotaurine in 0.1 M phosphate buffer, pH 6.0; riboflavin (from lower to higher rate, respectively) 1.65 \(\times\) 10\(^{-7}\), 4.95 \(\times\) 10\(^{-7}\), 8.25 \(\times\) 10\(^{-7}\), 1.65 \(\times\) 10\(^{-6}\), and 3.3 \(\times\) 10\(^{-6}\) M. In the inset: a plot of pseudo-first-order rate \((k)\) against riboflavin concentration. \(A_{\text{to}}\) = absorbance at 220 nm before irradiation; \(A_{t}\) = absorbance at 220 nm after time \(t\) of irradiation.

followed at 220 nm) is shown in Fig. 2. The reaction followed pseudo-first-order kinetics over the pH range from 1.0 to 10.0. At every pH tested, the rate of photooxidation decreased in the order: homohypotaurine > hypotaurine > cysteine sulfinic acid.

Figure 3 illustrates spectral changes between 230 and 500 nm of a catalytic amount of riboflavin when irradiated in the presence of hypotaurine in water. Figure 3 curves A and B show, respectively, the riboflavin spectra in the presence of hypotaurine before and after 1-h irradiation. An irreversible fading of the solution was observed. A stable flavin derivative was formed, with spectral features similar to lumiflavin, which is probably a stable adduct between a photoexcited riboflavin and a hypotaurine oxidation by-product intermediate formed during the reaction. In fully anaerobic conditions, the spectrum rapidly bleached (Fig. 3, curve C) and the oxidation of hypotaurine did not take place, as seen either by no decrease of absorbance at 220 nm or by no radioactive taurine formation by paper chromatography. Restoring aerobic conditions did not reverse the photobleaching of the flavin spectrum, suggesting that an irreversible process took place in anaerobiosis.

Photooxidation of hypotaurine (\(7 \times 10^{-4} \text{M}\)) by riboflavin (3.3 \(\times\) 10\(^{-6}\) M) at pH 7.0 was also performed under aerobic conditions in the presence of 0.1 mM nitro
Fig. 2. Effect of pH on the rate of photooxidation of hypotaurine in the presence of riboflavin (followed at 220 nm). Pseudo-first-order rate constants ($k$) were plotted against pH. Hypotaurine ($7 \times 10^{-4}$ M) and riboflavin ($3.3 \times 10^{-6}$ M) in 0.1 M, $10^{-2}$ M, $10^{-3}$ M HCl, and 0.01 M acetate buffer (pH 4.5 and 5.0), and 0.01 M phosphate buffer (pH 6.0, 7.0, 7.6, and 8.0), and 0.01 M carbonate buffer (pH 10) were irradiated respectively. The irradiation was performed continuously and it was interrupted for just the time needed to record absorption changes.

Fig. 3. Spectral changes during irradiation of riboflavin in the presence of hypotaurine in water. Riboflavin ($8 \times 10^{-6}$ M) and hypotaurine ($1.66 \times 10^{-3}$ M) were irradiated continuously in water. Curve A: zero time; curve B: after 1-h irradiation in the presence of oxygen; curve C: after 10-min irradiation under nitrogen.

blue tetrazolium, which was reduced to the insoluble formazan by superoxide ions (22). A fast reduction of the dye is observed at 560 nm (data not shown). The pseudo-first-order rate constant of the reduction ($k = 0.05$ min$^{-1}$) was similar to the
Table 1. Effect of superoxide dismutase, catalase, and anaerobiosis on hypotaurine oxidation rate and $^{35}$S]taurine formation.
Hypotaurine ($7 \times 10^{-4}$ M, 1 mCi/mmol) and riboflavin ($3.3 \times 10^{-6}$ M) were irradiated in 0.1 M phosphate buffer, pH 7.0 at 25°C, in a 3 ml quartz cuvette at 3 cm distance from the U.V. lamp. Irradiation was performed continuously and it was interrupted for just the time needed to record absorbance values. The final concentration of superoxide dismutase or catalase was $10^{-6}$ M. Initial reaction rate of hypotaurine oxidation ($v_0$) was estimated from the absorption decrease at 220 nm.

| Hypotaurine oxidation rate ($v_0$) at 220 nm | $^{35}$S]Taurine formation after 30 min irradiation |
|------------------------------------------|-----------------------------------------------|
| Standard conditions at pH 7.0            | 0.062                                          |
| + superoxide dismutase                   | 0.062                                          |
| + catalase                               | 0.060                                          |
| under nitrogen                           | 0.005                                          |

Table 2. Effect of allyl alcohol on hypotaurine disappearance and products formation.
$^{35}$S]hypotaurine ($7 \times 10^{-4}$ M, 1 mCi/mmol) and riboflavin ($3.3 \times 10^{-6}$ M) were irradiated for 60 min in standard conditions as described in METHODS. The final concentration of allyl alcohol was 0.5 mM. Separation of products (taurine and compound X) from hypotaurine was performed by paper chromatography. See METHODS for details. Compound X was tentatively identified as the addition product between sulfinic radical and allyl alcohol.

| Hypotaurine$^a$ | Taurine$^b$ | Compound X$^b$ |
|-----------------|-------------|----------------|
| Standard conditions at pH 7.0 | 18% | 82% | — |
| + allyl alcohol  | 24% | 44% | 32% |
| + allyl alcohol in the dark | 95% | 5% | — |

$^a$Percentage of residual hypotaurine; $^b$percentage of taurine or compound X formed.

value obtained with the oxidation of hypotaurine, as followed at 220 nm (Fig. 2). No variation of the absorbance at 560 nm was observed in solutions shielded from irradiation during 120-min incubation. A slow reduction of nitro blue tetrazolium was observed in solutions irradiated in the absence of hypotaurine. This may be related to the slow reduction of riboflavin occurring in the absence of electron donors like hypotaurine (2J). Superoxide dismutase ($10^{-6}$ M) completely inhibited the reduction of nitro blue tetrazolium. On the contrary, the oxidation rate of hypotaurine as followed at 220 nm and the formation of $^{35}$S]taurine from $^{35}$S]-hypotaurine are completely unaffected either by $10^{-6}$ M superoxide dismutase or by
HYPOTAURINE PHOTOOXIDATION

10^{-6} M catalase at pH 7.0 (Table 1) or at pH 6.0 (data not shown).

Irradiation of [35S]hypotaurine at pH 6.0 was also performed in the presence of 3.3 \times 10^{-6} M riboflavin and 0.5 mM allyl alcohol used as a radical scavenger (23). Paper chromatography after 60-min incubation showed the presence of three radioactive compounds: hypotaurine (R_f 0.65), taurine (R_f 0.40), and a ninhydrin-positive compound (compound X) with R_f 0.9 were detected by scanning the paper for radioactivity. Compound X was positive to the KCl-HCl test (24) for sulfones and sulfonates. Table 2 shows quantitative evaluation of the three compounds. Allyl alcohol inhibited taurine formation without affecting hypotaurine disappearance.

DISCUSSION

Riboflavin catalyzed the photooxidative conversion of sulfinites to sulfonates. The reaction can be monitored by different methods. The disappearance of sulfinites was monitored at 220 nm, where sulfinic compounds have a maximum absorbance. The appearance of sulfonates was also followed either by ion-exchange chromatography with an amino acid analyzer or by paper-chromatographic separation of radioactive products. Kinetics studies were performed mainly by measuring absorbance at 220 nm, and occasionally checked with the other two chromatographic methods.

The pH dependance of the photooxidation rate and chromatographic data for product determination show the following.

i) In the pH range above 7.0 the oxidation process was not as simple as that at pH below 7.0. More than one product was formed, probably by deamination or decarboxylation processes of hypotaurine.

ii) The reactive species seem to involve undissociated sulfinic acid and neutral flavin at pH below 4. These findings are in accordance with the reported pH-dependance of photochemical reaction of amino acid compounds (25) but are in contrast with the photooxidation of dimethylglycine and sarcosine, which is rapid at pH above 7.8 but is negligible at neutral or acidic pH (26).

iii) The presence of electronegative groups in the sulfinate, like cysteine sulfinic acid, lowers the susceptibility to photooxidation of the sulfinate group.

Spectral changes during photooxidation give some more insights about the reaction mechanism. Smith and Metzler showed that the spectrum of riboflavin, irradiated with visible light under anaerobic conditions in the absence of any electron donors, rapidly have faded and the addition of oxygen to the system has not completely restored the original spectrum (27). They identified fragments of riboflavin side chain as its oxidation products, together with lumiflavin or lumichrome (28). Radda and Calvin showed that the electrons for the photoreduction of the riboflavin core are derived from the side chain of the flavin by an intramolecular rearrangement (21). Prolonged irradiation under aerobic conditions in the presence of sulfinites (in this paper) also led to a slow irreversible bleaching of the flavin after a short time lag (Fig. 3, curve B). Spectral evidence does not allow
Fig. 4. A reaction scheme proposed for the photooxidation of sulfinates in the presence of flavins. $\text{HO}_2^\cdot$, hydroperoxy radical; $\text{O}_2^\cdot$, superoxide anion; Flavins t, excited triplet state of flavins; $\text{H}^\cdot$, hydrogen radical; Flavins $\text{H}^\cdot$, semiquinone radicals of flavins; R-$\text{SO}_2^\cdot$, sulfinic radicals; R-$\text{SO}_3^-$, sulfonate compounds.

the univocal identification of the faded products. It is likely that the irreversible loss of photocatalytic activity of riboflavin is due to the formation of bleached products. The products might be formed during the reaction between semiquinone free radical flavin (flavin $\text{H}^\cdot$) and a reactive intermediate ($-\text{SO}_2^\cdot$) which accumulates during the photooxidation of sulfinates. The reaction might occur at an appreciable rate when a sufficient steady state concentration of both compounds was attained during the reaction.

Experiments in this paper with nitro blue tetrazolium and with superoxide dismutase indicate that $\text{O}_2^\cdot$ was produced during photooxidation of hypotaurine. However, the oxidation rate of hypotaurine was unaffected by the addition of either superoxide dismutase or catalase, thus suggesting that superoxide ions or hydrogen peroxide were not directly involved in the oxidation of sulfinates but they might be formed as by-products of the reaction.

A minimal reaction scheme in the presence of oxygen and water, consistent with all the above experimental data, is shown in Fig. 4. The light-excited triplet state of flavin (flavin t) (29) may be reduced in a one-electron step by the sulfinate (R-$\text{SO}_2\text{H}$) to a semiquinone free radical intermediate (flavin $\text{H}^\cdot$) as has been demonstrated for other flavin-mediated photooxidations (29). The semiquinone may be reoxidized by triplet oxygen to basal flavin with production of hydroperoxy radical (30, 31). The involvement of sulfinic radicals (R-$\text{SO}_2^\cdot$) as primary oxidation products (with we proposed in this photooxidation reaction) was supported by the following evidence: free radicals of sulfur-containing compounds (R-$\text{SO}_2^\cdot$) were efficiently scavenged by compounds having olefinic double bonds in its molecule, the addition of which led to the formation of an anti-Markovnikov product, as

*J. Nutr. Sci. Vitaminol.*
reported by Caspari and Granzow (23). The addition of allyl alcohol scavenged sulfinic radicals and gave a product (compound X) which was tentatively identified as propanolaminoethansulfone \((\text{HOCH}_2\cdot\text{CH}_2\cdot\text{SO}_2\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{NH}_2)\). Expectedly the addition of allyl alcohol inhibited the taurine formation without affecting hypotaurine disappearance (Table 2). So we proposed that sulfinic radicals \((\text{R-SO}_2\cdot)\) intermediately formed in the sulfinate photooxidation in the presence of riboflavin could interact with water and was converted to sulfonates \((\text{R-SO}_3\cdot\text{H})\) and radicals \((\text{H}^\cdot)\). This mechanism can be supposed in analogy to reactions postulated for thiol radicals (32).

In conclusion, the photooxidation of hypotaurine in the presence of flavins may be physiologically important and play a significant role in the taurine formation in photosensitive flavin-rich tissues such as the retina. Taurine is absolutely required for retina function and development (33).

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