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Production of Hydroxyl Radicals and α-dicarbonyl Compounds Associated with Amadori Compound–Cu^{2+} Complex Degradation

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The chemical properties of Amadori compounds in the presence of transition metal ions were studied, using the analogs 1-deoxy-1-*n*-butylamino-D-fructose (DBF) and N^{α}-formyl-fructoselysine (fFL). The following characteristics were revealed: (a) DBF combined easily with Cu^{2+} (but no other transition metal ions) to form a DBF–Cu^{2+} complex in phosphate buffer, pH 7.4; (b) the complex was unstable, and degraded with the release of Cu^{+} during incubation at 37°C; (c) degradation of the complex was associated with the production of hydroxyl radicals by the Fenton reaction and α-dicarbonyl compounds by non-autoxidative degradation; and (d) properties of DBF were similar to those of fFL. The above properties were additionally observed in glycated poly-Lys (GPL). Our findings indicate a novel mechanism for the generation of hydroxyl radicals and α-dicarbonyl compounds from Amadori adducts in the presence of Cu^{2+}.

Keywords: Amadori compound–Cu^{2+} complex; Non-autoxidative degradation; Hydroxyl radicals; α-Dicarbonyl compounds

INTRODUCTION

Glycation occurs as the result of a reaction between glucose and the primary amino groups of a protein.[1,2] The initial product is a Schiff base derivative of the protein, followed by rearrangement to form a stable ketoamine adduct, designated "Amadori compound". Numerous *in vitro* studies indicate that the Amadori compound undergoes a further series of reactions (collectively described as the Maillard reaction), leading to the formation of hydroxyl radicals and highly reactive α-dicarbonyl compounds, which play an important role in the synthesis of advanced glycation end products (AGEs) implicated in the development of diabetic complications and aging.[3–6] It is generally believed that hydroxyl radicals and α-dicarbonyl compounds from Amadori compounds are produced by a Fenton reaction via superoxide[7–10] and the autoxidative degradation of sugar moieties,[11,12] respectively, in the presence of transition metal ions, such as Fe^{3+} or Cu^{2+}.[12–15] However, the detailed reaction mechanism of formation of these products remains to be elucidated. We demonstrate in this study that upon complexing with Cu^{2+}, 1-deoxy-1-*n*-butylamino-D-fructose (DBF) and N^{α}-formyl-fructoselysine (fFL) produce hydroxyl radicals by a Fenton reaction, and α-dicarbonyl compounds by non-autoxidative degradation. These reactions are additionally observed with glycated poly-Lys (GPL), which is commonly utilized as a model of glycated proteins *in vivo*.

MATERIALS AND METHODS

Chemicals

DBF was synthesized from *n*-butylamine and glucose, and obtained as monohydrochlorides in the form of needle crystals by the procedure of Kato.[16]

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Anal. Found: C, 44.21; H, 8.14; N, 5.13. Calculated for $C_{10}H_{21}NO_5 \cdot HCl$: C, 44.20; H, 8.16; N, 5.16%. The structure was confirmed by ^1H NMR spectrum (400 MHz, D_2O) using a Bruker DPX400 NMR spectrometer. δ; 4.06–3.91 (5H, m), 3.29 (2H, s), 3.11 (2H, t, $J = 7.8$ Hz), 1.69 (2H, tt, $J = 7.8, 7.4$ Hz), 1.39 (2H, tq, $J = 7.4, 7.4$ Hz), 0.93 (3H, t, $J = 7.4$ Hz). The fFL analog was synthesized from N^{α}-formyl-lysine[17] and glucose, using the procedure of Finot and Mauron;[18] the product was purified by chromatography on Dowex50W-X8 using the pyridine-acetate buffer described by Chiou *et al.*[19] GPL was synthesized as follows: a 0.1% solution of poly-Lys (hydrochloride, M_r, 22,800, Sigma) in 100 mM phosphate buffer, pH 7.4, containing 0.5 M glucose, 0.02% sodium azide and 1 mM diethylenetriamine-pentaacetic acid (DTPA) was incubated for 14 days at 37°C. Following dialysis against 100 mM phosphate buffer, pH 7.4, the resultant dialysate was equalized to the volume before dialysis with an ultrafiltration system, and used as "glycated poly-Lys" in subsequent experiments. The level of fructosamine in "glycated poly-Lys" was determined as 3.5 μmol/ml with a Fructosamine assay kit (Roche Diagnostic Co.). Catalase (EC. 1.11.1.6.) from bovine liver was purchased from Sigma Co. All other chemicals employed were of the best grade commercially available.

Preparation of N_2- or O_2-saturated Buffer for Incubation

The buffer employed was 100 mM sodium phosphate, pH 7.4, unless otherwise indicated. N_2-saturated buffer was prepared by bubbling N_2 gas (purity ≥ 99.999%, Taiyo Toyo Sanso Co., Japan) into buffer cooled in ice water, until the dissolved oxygen content decreased to less than 0.02 ppm. O_2-saturated buffer was prepared by employing the above procedure with O_2 gas (purity ≥ 99.5%). The concentration of dissolved oxygen was determined with a D.O. meter-B-505 (Iijima Electronics Co., Japan).

Synthesis of the DBF–Cu^{2+} Complex

The DBF–Cu^{2+} complex was prepared by mixing DBF (10 mM) with $CuSO_4$ (100 μM) in phosphate buffer, pH 7.4, unless otherwise specified.

Degradation of the DBF–Cu^{+} Complex under various Conditions

Aliquots (2.99 ml) of N_2-saturated phosphate buffer containing DBF (10 mM) were pipetted into a quartz cell (filled with N_2) fitted with a self-sealing cap in a stream of nitrogen gas. After setting the temperature to 37°C, aliquots (10 μl) of N_2-saturated 30 mM $CuSO_4$ aqueous solution were added through the septum using a micro-syringe, mixed, and subsequently incubated in nitrogen for another 40 min without stirring. The concentration of the DBF–Cu^{2+} complex was monitored during incubation by measuring the decrease in A_{250} at 2 min intervals. The same mixture containing NBT (8.3 μM) was incubated under identical conditions, and reduced NBT formation was monitored by measuring the increase in A_{550}. Experiments were carried out in air- and O_2-saturated phosphate buffer, either in the absence or presence of catalase (140 units/ml). All reactions were performed without making a dead space in the cell.

Electron Paramagnetic Resonance (EPR) of the DBF–Cu^{2+} Complex

Aliquots (400 μl) of N_2-saturated phosphate buffer solution containing DBF–Cu^{2+} complex were pipetted into three quartz EPR sample tubes filled with N_2 in a stream of nitrogen gas, and immediately incubated under nitrogen without stirring for various time (5, 10, and 20 min) at 25°C. At indicated times, a sample tube was removed and the EPR spectrum was immediately recorded.

EPR spectra were measured using an electron paramagnetic resonance spectrometer (JES-RE1X, JEOL Co. Ltd., Tokyo, Japan). Measurement conditions were as follows: microwave frequency (9.04 GHz), microwave power (4.0 mW), time constant (0.1 ms), sweeptime (60 s), center field (330 mT), scan range (± 50 mT), modulation frequency (100 kHz), field modulation width (0.63 mT) and receive gain (× 500).

DBF–Cu^{2+} Complex-induced Hydroxyl Radical Production

Hydroxyl radicals were detected with the benzoate-hydroxylate method.[20] Resulting hydroxybenzoates were extracted and determined by high-performance liquid chromatography (HPLC), using salicylate and other appropriate hydroxybenzoates as standards. Briefly, aliquots (3 ml) of phosphate buffer containing the DBF–Cu^{2+} complex and benzoate (1 mM) were incubated in a cell fitted with a self-sealing cap without stirring, as described above. After a 30 min incubation period, the entire reaction mixture was titrated to a pH of 2–3 using hydrochloric acid, followed by three extractions with equal volumes of diethylether. Organic solvents were combined and subsequently removed with a rotary evaporator. The remaining residue containing the resultant hydroxybenzoates was dissolved in 0.5 ml of 30% (v/v) acetonitrile aqueous solution and analyzed by HPLC. The same phosphate buffer containing the DBF–Cu^{2+} complex and benzoate was incubated in O_2-saturated phosphate buffer, either in the absence or presence of catalase (140 units/ml), and

in the presence of 1 mM ethylenediaminetetraacetic acid (EDTA). Hydroxybenzoate was determined by reversed-phase HPLC on a Tosoh-tsk gel ODS-80T column (4.6 mm × 150 mm). For chromatography, 25 μl aliquots of samples were loaded (unless otherwise specified) and eluted at 1 ml/min with a mixture of acetonitrile/deionized water (30:70, v/v) containing 0.01% (w/v) phosphoric acid. Eluted hydroxybenzoate was monitored at 290 nm.

Measuring the Effect of Catalase on DBF–Cu^{2+} Complex-induced Hydroxybenzoate Production

Aliquots (2.99 ml) or phosphate buffer containing DBF (10 mM) and benzoate (1 mM) were pipetted into five cells. After setting the temperature to 37°C, 30 mM $CuSO_4$ (10 μl) was added to each cell and incubated in open air for 40 min with vigorous stirring using a magnetic stirrer. At 10 min intervals, individual cells were removed and salicylate was extracted and determined by HPLC, as described above. The above mixture was additionally incubated in the presence of catalase (140 units/ml) and resultant salicylate concentrations were determined.

Detection of the DBF–Cu^{2+} Complex-induced α-dicarbonyl Compound

Aliquots (3.0 ml) of N_2-saturated phosphate buffer containing DBF–Cu^{2+} complex were pipetted into five cells (filled with N_2) fitted with a self-sealing cap, and incubated for 12 h at 37°C with vigorous stirring. At 3 h intervals, individual cells were removed and aliquots (300 μl) of the reaction mixture were immediately subjected to α-dicarbonyl compound measurement. Experiments were performed in open air with vigorous stirring, either in the absence or presence of DTPA (1 mM). The resultant α-dicarbonium compounds were measured using Girard-T reagent, as described by Mitchel and Birnboim.[21] Assays were performed at pH 2.9, and the reaction product was measured at 295 nm.

RESULTS AND DISCUSSION

Formation of the DBF–Cu^{2+} Complex

The ultraviolet absorption spectra of DBF, fFL and GPL in phosphate buffer, pH 7.4, were measured either in the absence or presence of Cu^{2+}, using a Shimadzu Spectrophotometer UV-120-02 at 20°C. As shown in Fig. 1, the spectrum of DBF alone did not reveal any characteristic absorption bands within the wavelength range observed (220–340 nm) (Fig. 1a), while Cu^{2+} alone displayed a weak absorption maximum (λ_{max}) at around 240 nm (Fig. 1c). Mixing of DBF with Cu^{2+} led to a shift in the λ_{max} of Cu^{2+} to 250 nm, accompanied by a corresponding increase in peak intensity (Fig. 1d). The absorbance at 250 nm increased linearly with increasing Cu^{2+} concentrations at fixed DBF (data not shown). Addition of EDTA to the mixture of DBF Cu^{2+} resulted in a novel absorption spectrum with a shoulder at around 270 nm, while λ_{max} was observed at 290 nm with DTPA (data not shown). The former spectrum closely resembled that of a mixture of EDTA and Cu^{2+}, indicating that DBF forms a complex with Cu^{2+}. However, the latter spectrum was clearly distinct from that of a mixture of DTPA and Cu^{2+}, suggesting the formation of a novel complex comprising DBF, Cu^{2+} and DTPA (data not shown). DBF did not complex with any other transition metals, judging from its combination spectra with other metal ions (data not shown). Both fFL and GPL similarly displayed an ability to form a complex with Cu^{2+} (Fig. 1e and g). The data presented in Fig. 2 reveal the pH dependence of complex formation. No complex was formed at pH 5, but increasing concentrations were observed at higher pH values. This probably indicates that the keto form of DBF at pH 5 is modified to endiol- and enaminol-DBF at increasing pH, and one or the other binds Cu^{2+} to form the DBF–Cu^{2+} complex. Significantly, complex formation was observed, even at the pH range of acidosis.

One-electron Transfer from DBF to Cu^{2+} within the DBF–Cu^{2+} Complex

As illustrated in Fig. 3, when the DBF–Cu^{2+} complex was incubated in N_2 gas without stirring, concentration levels immediately began to deplete, and

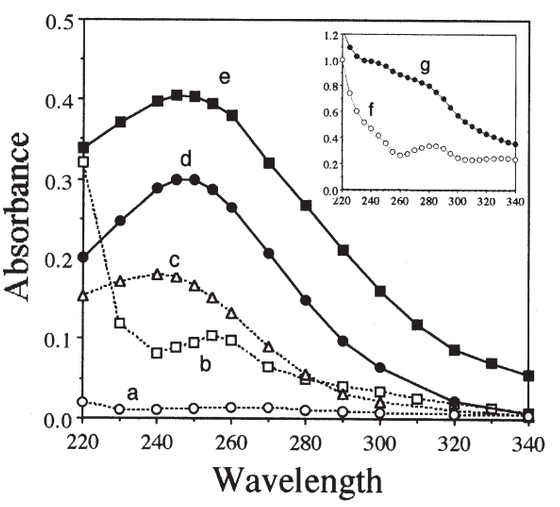

FIGURE 1 Ultraviolet absorption spectra of the DBF–Cu^{2+} complex (d) and related compounds in the 100 mM phosphate buffer, pH 7.4. (a) DBF (10 mM) alone: (b) fFL (10 mM) alone; (c) $CuSO_4$ (100 μM) alone; (d) a + c; (e) b + c; (f) GPL alone; (g) f + c. The GPL solution was prepared by diluting the "glycated poly-Lys" (described in "Materials and Methods" section) three times in 100 mM phosphate buffer (pH 7.4).

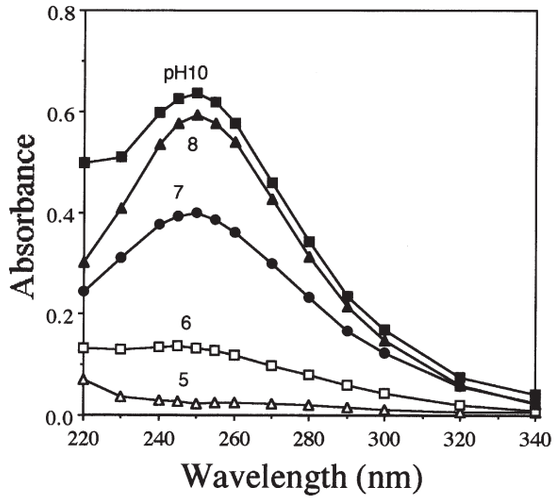

FIGURE 2 Effect of pH on the formation of the DBF–Cu^{2+} complex. Deionized solution containing DBF (10 mM) and $CuSO_4$ (100 μM) was prepared, and the pH adjusted to the indicated levels by titration with sodium hydroxide aqueous solution.

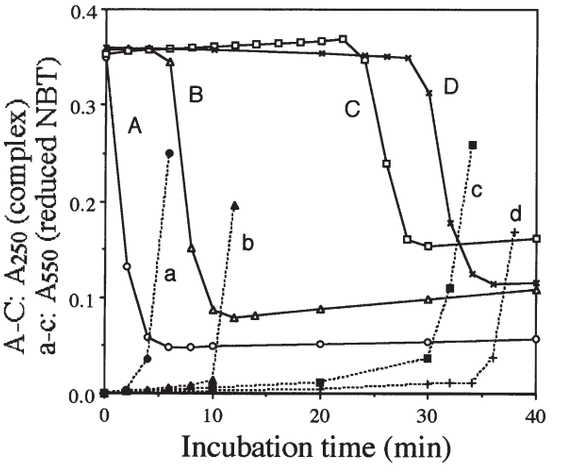

FIGURE 3 Degradation of the DBF–Cu^{2+} complex associated with NBT reduction under various conditions. Degradation of the complex (A–D) and reduction of NBT (a–d): in N_2 gas, (A, a); air, (B, b), and O_2 gas in the (C, c) absence or (D, d) presence of catalase (140 units/ml).

disappeared within the first 5 min of incubation (Fig. 3A), while in air and O_2 gas, this decrease in complex concentration was observed after a lag time of ~5–7 and ~20–25 min, respectively, following the onset of incubation (Fig. 3B and C). The extent of each lag time was extended by the addition of catalase (140 units/ml) (Fig. 3D, data in air not shown). Experiments performed in the presence of NBT resulted in the formation of reduced NBT associated with DBF–Cu^{2+} complex depletion in all cases (Fig. 3a–c). From these results, we hypothesize that the DBF moiety of the complex transfers one of its electrons to Cu^{2+}, thereby forming Cu^{+} and a DBF radical (see Fig. 8). NBT is subsequently reduced by released Cu^{+}.[22] This proposal is in agreement with the one-electron transfer theory in Amadori compound–transition metal ion systems.[10,14,23]

Appearance of the lag time (Fig. 3B and C) suggests that released Cu^{+} immediately reacts with dissolved oxygen, consequently forming superoxide and Cu^{2+}, which instantly binds free DBF to re-assemble the DBF–Cu^{2+} complex (see Fig. 8). Since this process is repeated until all dissolved oxygen is consumed, a certain level of complex concentration is maintained, and reduction of NBT by Cu^{+} does not occur over this period of time (Fig. 3b and c). The length of the lag time therefore appears to reflect the level of dissolved oxygen. Addition of catalase induces an extension in lag time, suggesting that hydrogen peroxide derived from superoxide by disproportionation is degraded to water and oxygen by the enzyme, which provides continual aeration until the dissolved oxygen is completely consumed (Fig. 3D and d) (see Fig. 8). This theory is supported by a number of results. As shown in Fig. 3C, the absorbance at 250 nm gradually increased until the initiation of complex loss, while absorbance levels remained constant in the presence of catalase (Fig. 3D). These data indicate that hydrogen peroxide, which absorbs at around 240 nm[24] is formed in the absence of catalase, but does not accumulate in the presence of the enzyme (140 units/ml). In all cases, production of reduced NBT commenced slowly, following the initiation of complex loss. This observation was largely explained by the oxidation of Cu^{+} by a Cu^{+}-oxidizable substance (X) produced concomitantly with the

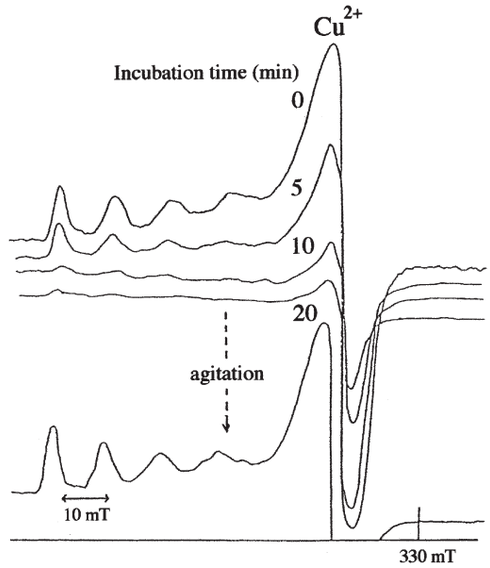

FIGURE 4 EPR spectrum of Cu^{2+} of the DBF–Cu^{2+} complex. The peak height of the EPR spectrum of Cu^{2+} of the DBF–Cu^{2+} complex was measured during incubation in N_2 gas at 25°C.

degradation of the DBF–Cu^{+} complex (see below and Fig. 8). All these properties of DBF were essentially comparable to those of fFL and GPL (data not shown).

The conversion of the Cu^{2+} of the complex to Cu^{+} during incubation was directly confirmed by EPR. On incubation in N_2 gas, the peak height of the EPR spectrum decreased with time, in proportion to Cu^{+} concentrations of the DBF–Cu^{2+} complex (Fig. 4). This observation further supports our theory of a one-electron transfer from DBF to Cu^{2+} within the complex. As shown in Fig. 4, the decrease in peak height of the EPR spectrum of Cu^{2+} of the DBF–Cu^{2+} complex is reversed and nearly restored to initial levels in response to agitation in a stream of nitrogen, suggesting the accumulation of an unknown Cu^{+}-oxidizable substance (X) that is produced concomitantly with the non-autoxidative degradation of the DBF radical. Released Cu^{+} was oxidized by this product in response to agitation, followed by the re-synthesis of DBF–Cu^{2+} complex by binding to free DBF (see Fig. 8). Ahmed *et al.* previously observed the degradation of fFL in phosphate buffer in the absence of added Cu^{2+}.[25] This was possibly due to the presence of trace amounts of the metal ion in phosphate buffer.

DBF–Cu^{2+} Complex-induced Hydroxyl Radical Production

When the DBF–Cu^{2+} complex was incubated with benzoate without stirring in phosphate buffer, at least three types of hydroxybenzoate, i.e. salicylate, 3-hydroxybenzoate and 4-hydroxybenzoate, were formed (see Fig. 5 for details). On the basis of these data, the total amount of salicylate (nmol) in each 30 min reaction mixture (3 ml) was calculated as follows: 5.4 ± 0.3 in air, 13.0 ± 0.1 in oxygen, and 3.5 ± 0.3 in oxygen in the presence of catalase (140 units/ml). Hydroxylation of benzoate was

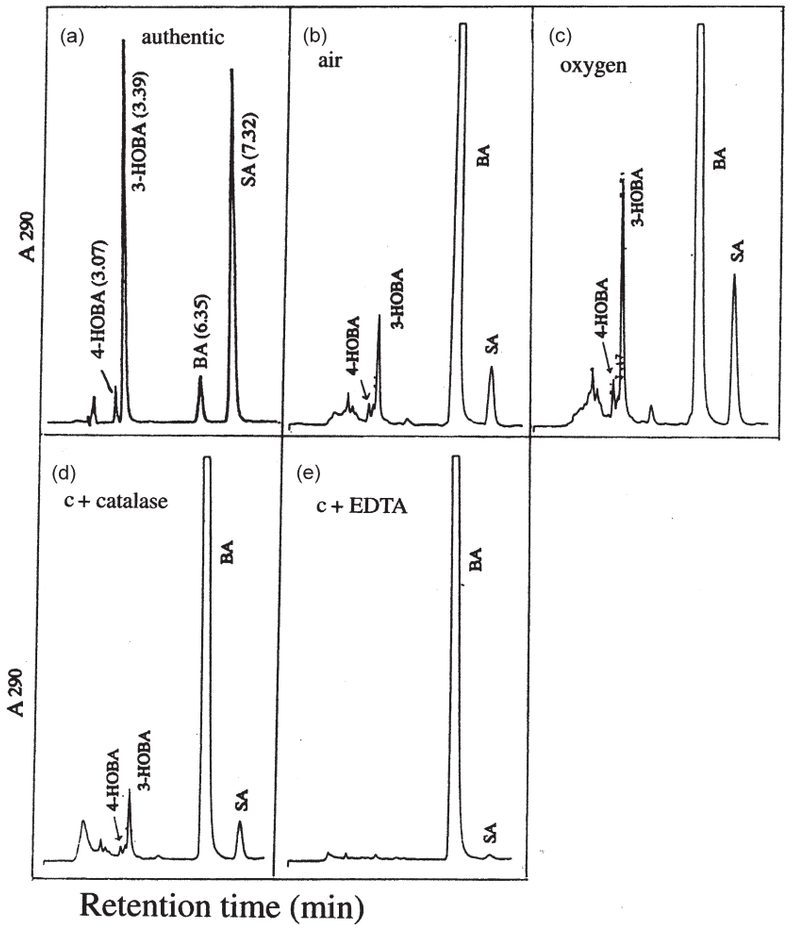

FIGURE 5 DBF–Cu^{2+} complex-induced benzoate hydroxylation under various conditions. a, Authentic: 4-hydroxybenzoate (4-HOBA), 3-hydroxybenzoate (3-HOBA), benzoate (BA) and salicylate (SA). Numbers in parentheses represent retention times (min) and the area under each peak corresponds to the amount of each hydroxybenzoate (2.5 nmol). Samples were incubated without stirring. All experiments were performed in triplicate and the results of a typical separation pattern of hydroxybenzoates by HPLC are presented in each case.

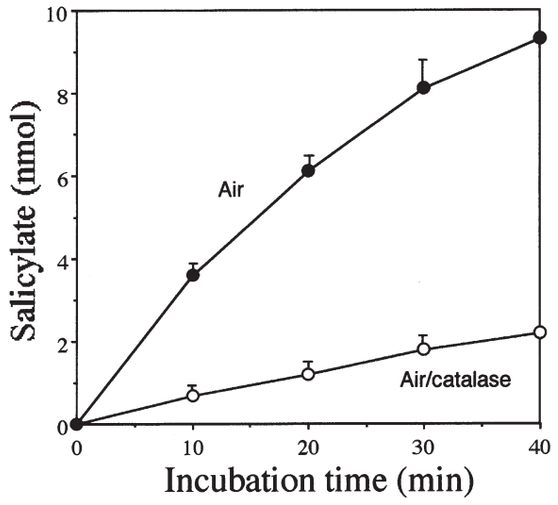

FIGURE 6 Effect of catalase on DBF–Cu^{2+} complex-induced benzoate hydroxylation. Samples were incubated under open air in the (•) absence or (○) presence of catalase (140 units/ml). All experiments were performed in triplicate. Data are represented as means ± SD.

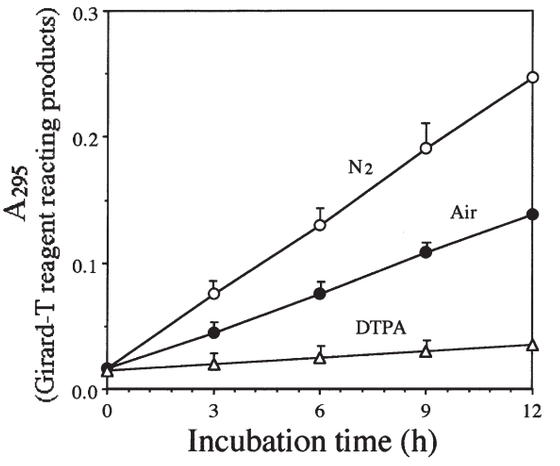

FIGURE 7 Time-course of the DBF–Cu^{2+} complex-induced α-dicarbonyl compound formation. Experiments were performed in N_2 gas and an open air, either in the absence or presence of DTPA (1 mM). All experiments were performed in triplicate. Data are presented as means ± SD.

almost completely prevented by the addition of EDTA (Fig. 5e). This result, along with the above observation that the complex is not formed in the presence of EDTA, suggests that hydroxyl radicals are produced via the DBF–Cu^{2+} complex. As shown in Fig. 5d, hydroxylation of benzoate was not completely inhibited by the addition of catalase (140 units/ml) (by ca. 75% inhibition). To determine whether similar incomplete inhibition occurs, another experiment was performed under aerobic conditions with vigorous stirring, either in the absence or presence of catalase, and the resultant salicylate was analyzed by HPLC at 10 min intervals over a period of 40 min (Fig. 6). As expected, formation of salicylate increased linearly with incubation time. However, a ca. 75% inhibition of salicylate formation was noted over the time-course of the experiment in the presence of catalase, compared with no added catalase. As shown in Fig. 3D, no accumulation of hydrogen peroxide in the reaction mixture was observed when catalase (140 units/ml) was added prior to incubation. Hence, hydroxylation of benzoate observed in Figs. 5d and 6 may be due to hydroxyl radical formation by a non-Fenton reaction. However, the mechanism of formation of this product by the non-Fenton reaction is yet to be clarified. Incubation of fFL or GPL with Cu^{2+} under the same conditions also led to the formation of hydroxyl radicals (data not shown).

DBF–Cu^{2+} Complex-induced α-dicarbonyl Compound Formation by Non-autoxidative Degradation

Figure 7 shows that α-dicarbonyl compounds were additionally produced during incubation of the DBF–Cu^{2+} complex in both aerobic and anaerobic conditions. Moreover, these products were not formed in the presence of DTPA as well as EDTA. The data suggest that α-dicarbonyl compounds are formed by non-autoxidative degradation of the DBF radical derived form the DBF–Cu^{2+} complex. The production of α-dicarbonyl compounds was consistently lower in aerobic conditions. This is largely accounted for by the oxidation of the aldehyde group of α-dicarbonium to carboxylic acid, which leads to a decrease in Girard-T reagent reactive-compounds. GPL and fFL displayed analogous properties to DBF in the presence of added Cu^{2+}.

Based on the above results, a possible pathway for the production of hydroxyl radicals, α-dicarbonium compounds and Cu^{+} -oxidizable substance (X) from DBF in the presence of Cu^{2+} is summarized in Fig. 8.

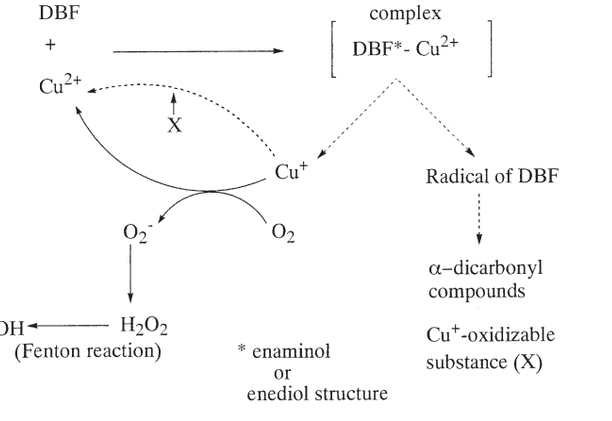

FIGURE 8 Possible pathway for the production of hydroxyl radicals, α-dicarbonium compounds and a Cu^{2+}-oxidizable substance (X) from DBF in the presence of Cu^{2+}. The reactions shown in the arrow of the dotted line occur in both aerobic and anaerobic conditions.

We conclude that the chemical properties of fFL and GPL in the presence of Cu^{2+} are substantially similar to those of those of the DBF–Cu^{2+} complex. Thus, the reaction mechanisms described in this study are applicable to Amadori compounds synthesized in glycated proteins *in vivo*, which form a complex with Cu^{2+}.

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