Radical-Assisted Melanoidin Formation during Thermal Processing of Foods as well as under Physiological Conditions

Thomas Hofmann,*,† Wolf Bors,‡ and Kurt Stettmaier‡

Deutsche Forschungsanstalt für Lebensmittelchemie, Lichtenbergstrasse 4, D-85748 Garching, Germany, and Institut für Strahlenbiologie, GSF Forschungszentrum für Umwelt und Gesundheit, 85746 Neuherberg, Germany

Color-generating reactions of protein-bound lysine with carbohydrates were studied under thermal as well as under physiological conditions to gain insights into the role of protein/carbohydrate reactions in the formation of food melanoidins as well as nonenzymatic browning products in vivo. EPR spectroscopy of orange-brown melanoidins, which were isolated from heated aqueous solutions of bovine serum albumin and glycolaldehyde, revealed the protein-bound 1,4-bis(5-amino-5-carboxy-1-pentyl)pyrazinium radical cation (CROSSPY) as a previously unknown type of cross-linking amino acid leading to protein dimerization. To verify their formation in foods, wheat bread crust and roasted cocoa as well as coffee beans, showing elevated nonenzymatic browning, were investigated by EPR spectroscopy. An intense radical was detected, which, by comparison with the radical formed upon reaction bovine serum albumin with glycolaldehyde, was identified as the protein-bound CROSSPY. The radical-assisted protein oligomerization as well as the browning of bovine serum albumin in the presence of glycolaldehyde occurred also rapidly under physiological conditions, thereby suggesting CROSSPY formation to be probably involved also in nonenzymatic glycation reactions in vivo.

Keywords: *Melanoidin; nonenzymatic browning; lysine; pyrazinium radical cation; protein cross-link; protein glycation; Maillard reaction; CROSSPY*

INTRODUCTION

The Maillard reaction between reducing carbohydrates and compounds bearing an amino group is chiefly responsible for the development of desirable as well as undesirable colorations occurring, for example, during processing of foods. To prevent undesired browning during industrial production of foods such as condensed milk or dried fruits, these products were treated with sodium sulfite or sulfur dioxide. Extensive studies have been undertaken to understand the mode of action of sulfite ions as an inhibitor of the nonenzymatic browning (McWeeny et al., 1969; Knowles, 1971; Wedzicha et al., 1984; Kroh et al., 1989; Wedzicha and Kaputo, 1992; Noll, 1993). Because sulfite ions were found to block reactive advanced Maillard reaction products such as 3-deoxyosone by formation of 3-deoxy-4-sulfopentosulose (Wedzicha and Mc Weeny, 1974) and furan-2-carboxaldehyde by bisulfite adduct formation (Noll, 1993), these reactions were proposed to be responsible for the browning inhibition. It is as yet, however, not clear whether also other reaction intermediates might be blocked by sulfite ions, thereby inhibiting the browning formation.

On the contrary, in the production of thermally processed foods, such as coffee, malts, dark beer, bread crust, or breakfast cereals, the formation of high molecular weight colored products with molecular weights up to 100000 Da is desired due to their attractive brown color. This browning, their antioxidant activity (Kato et al., 1986; Yamaguchi, 1986), and their antimutagenic properties (Kim et al., 1986) initiated the extensive studies performed in the past 30 years to clarify the structures and formation of these so-called melanoidins from carbohydrates and amino compounds. The knowledge on the structure and the formation of these colored polymers is, however, still very poor.

Investigations relating the molecular weight distribution and the color potency of thermally treated solutions of glucose with amino acids and proteins, respectively, revealed that carbohydrate-induced protein oligomerization generated melanoidins very effectively (Hofmann, 1998a). In contrast, the reaction of glucose and amino acids did not produce high molecular weight colored compounds (Hofmann, 1998a).

Studies on the nonenzymatic browning of carbohydrate/amino acid mixtures evidenced that, besides ionic condensation reactions (Severin and Krönig, 1972; Ledl and Severin, 1978; Hofmann, 1997, 1998b–d), also mechanisms involving amine-assisted oxidative carbohydrate fragmentation (Hofmann et al., 1999) and free radical formation produce colored compounds in the Maillard reaction, prior to the Amadori rearrangement (Namiki and Hayashi, 1983; Hofmann et al., 1999). Because radicals were also measured in melanoidins (Mitsuda et al., 1965; Wu et al., 1987), similar reactions might participate in the formation of high molecular weight colored structures during the thermal processing of foods.

Studies in the past 15 years demonstrated that nonenzymatic browning reactions between carbohy-

* Author to whom correspondence should be addressed (telephone +49-89-289 14181; fax +49-89-289 14183; e-mail hofmann@dfa.leb.chemie.tu-muenchen.de).

† Deutsche Forschungsanstalt für Lebensmittelchemie.

‡ Institut für Strahlenbiologie.

10.1021/jf980627p CCC: $18.00
Published on Web 01/22/1999

drates and amino acids or proteins, respectively, do not exclusively occur under elevated temperatures but also at 37 °C in living systems, for example, in the human body. Physiological Maillard reaction products, so-called advanced glycation end (AGE) products, were identified in various human tissues, in hemoglobin, or in lens proteins of the human eye, respectively, and their concentrations were found to correlate with age and severity of diabetes (Monnier, 1989; Bucala et al., 1992). Also, the nonenzymatic formation of colored compounds in vivo was reported, for example, with increasing age; the human lens accumulates cross-linked proteins as yellow-brown pigments, which are particulary pronounced in senile and brunescent cataracts (Monnier et al., 1981). EPR measurements and fractionation experiments with several rabbit tissues revealed that protein aggregates are associated with long-lived, immobilized radicals (Commoner et al., 1954). The radical species detected were, however, not characterized.

Because melanoidins in foods as well as in vivo nonenzymatic browning compounds were found to be associated with proteins and, in addition, free organic radicals were detected in such colored polymers, it might be possible that the ϵ-amino group of lysine side chains of proteins might take part in browning reactions involving free radical formation similar to those recently reported for the α-amino acid alanine (Namiki and Hayashi, 1983; Hofmann et al., 1999).

The objectives of the present investigation were, therefore, to study whether the lysine side chains of proteins are participating in radical-assisted nonenzymatic browning reactions during thermal processing of foods and to clarify whether these reactions can be inhibited by sulfite ions. Additional experiments performed under physiological conditions should give insights into possible nonenzymatic glycation reactions in vivo.

EXPERIMENTAL PROCEDURES

Chemicals. The following compounds were obtained commercially: N^{α}-acetyl-L-lysine, glucose, furan-2-carboxaldehyde, glyoxal (30% solution in water), glycolaldehyde (Aldrich, Steinheim, Germany); ascorbic acid, sodium sulfite (Merck, Darmstadt, Germany); bovine serum albumin (Sigma, Deistenhifen, Germany). The furan-2-carboxaldehyde was freshly distilled prior to use.

The water used for the preparation of the buffer solutions was stirred for 12 h with an ion-exchange resin (Amberlite MB-1; Merck) to remove adventitious metal ions.

Influence of the Sulfite Content on the Formation of Colored Compounds from Glucose and N^{α}-Acetyl-L-lysine. A solution of glucose (5 mmol) and N^{α}-acetyl-L-lysine (5 mmol) was heated in phosphate buffer (5 mL; 0.5 mmol/L; pH 7.0) in the presence of sodium sulfite (Figure 1) in closed vials for 20 min at 95 °C. After the mixtures had cooled to room temperature, the color intensity was determined by measuring the visible absorption at 420 nm.

Influence of the Sulfite Content on the Formation of Free Radicals from Glucose and N^{α}-Acetyl-L-lysine. A solution of glucose (5 mmol) and N^{α}-acetyl-L-lysine (5 mmol) was heated in phosphate buffer (5 mL; 0.5 mmol/L; pH 7.0) in the absence or presence of sodium sulfite (Table 1) in closed vials for 3 min at 95 °C. After the mixtures had cooled to room temperature, ascorbic acid (1 mmol) was added, and the mixtures were maintained for 10 min at room temperature and then were applied to the EPR cavity.

Detection of Free Radicals in Melanoidins Formed upon Thermal Treatment of Bovine Serum Albumin with Glycolaldehyde or Glyoxal, Respectively. A mixture of the carbonyl compound (1.25 mmol) and bovine serum albumin (0.05 mmol) was reacted in phosphate buffer (15 mL; 0.5 mmol/L; pH 7.0) at 95 °C for 5 min. After cooling, high molecular weight melanoidins were isolated by ultracentrifugation with a cutoff of 100000 Da (Centriplus, Amicon, Witten, Germany) as recently reported (Hofmann, 1998a). The retentate was suspended in water (10 mL) and analyzed by EPR spectroscopy. The reaction mixture containing glyoxal was incubated with ascorbic acid (1 mmol) for 10 min at room temperature and then analyzed again by EPR spectroscopy.

Detection of Free Radicals in Thermally Processed Foods. Wheat bread crumb was toasted using a kitchen-like toaster until the crust turned golden brown. After toasting, the crust was scraped off, ground, and analyzed by EPR spectroscopy. Sulfite-added bread was prepared by spraying the wheat bread crumb with an aqueous solution of sodium sulfite (20% in water) and drying at 35 °C, prior to the toasting process. Cocoa beans were roasted in an alumina block for 30 min at 160 °C and then ground in a mill. Roasted coffee was purchased from a local market. Milk was heated under reflux for 30 min, prior to EPR spectroscopy.

Free Radical Formation and Color Development in Solutions of Bovine Serum Albumin and Glycolaldehyde Incubated under Physiological Conditions. A solution of glycolaldehyde (2.5 mmol) and bovine serum albumin (0.1 mmol) in phosphate buffer (30 mL; 0.2 mol/L, pH 7.4) was incubated at 37 °C. At the times given in Figure 4, the color intensity was determined by measuring the visible absorption at 420 nm and the radical formation was monitored by EPR spectroscopy.

Free Radical Formation in Solutions of Bovine Serum Albumin and Glyoxal, Respectively, Incubated under Physiological Conditions in the Absence and in the Presence of Ascorbic Acid. A solution of glyoxal (2.5 mmol) and bovine serum albumin (0.1 mmol) in phosphate buffer (30 mL; 0.2 mol/L, pH 7.4) was incubated at 37 °C for 2 h and then analyzed by EPR spectroscopy. After additional incubation with ascorbic acid (2.5 mmol) for 10 min, the mixture was analyzed again by EPR spectroscopy.

EPR Spectroscopy. The EPR spectra were recorded on a Bruker ESP 300 spectrometer; the experimental parameters were as follows: modulation amplitude, 1 G; sweep rate, 2.4 Gs^{-1} at a frequency of 9.75 GHz and a gain of 1×10^4. All spectra were recorded at room temperature. Liquid samples were filled in an EPR flat cell; ground foods were filled in a glass tube.

Liquid Chromatography/Mass Spectrometry (LC/MS). An analytical HPLC column (Nucleosil 100-5C18, Macherey and Nagel, Dürren, Germany) was coupled to an LCQ-MS (Finnigan MAT GmbH, Bremen, Germany) using electrospray ionization (ESI). After injection of the sample (2.0 μL), analysis was performed using a gradient starting with a mixture (90:10, v/v) of acetonitrile and water and increasing the acetonitrile content to 100% within 15 min.

UV−Vis Spectrocopy. UV−Vis spectra were obtained by means of a U-2000 spectrometer (Colora Messtechnik GmbH, Lorch, Germany). To follow the color formation in the reaction mixtures, the visible absorption at 420 nm was determined in appropriate dilutions of the reaction mixtures.

RESULTS

Thermal treatment of neutral aqueous solutions of glucose and N^{α}-acetyl-L-lysine, which was chosen as suitable model substance to study Maillard reactions of lysine side chains of proteins, was recently reported to generate 1,4-bis[5-(acetylamino)-5-carboxy-1-pentyl]-pyrazinium radical cations (Hofmann et al., 1999) accompanied by intense browning formation. Studies on the mechanisms of radical formation revealed that glyoxal is formed as an early product in the Maillard reaction prior to radical formation. Reductones then initiate radical formation upon reduction of glyoxal and/or glyoxal imines into the effective radical precursor glycolaldehyde.

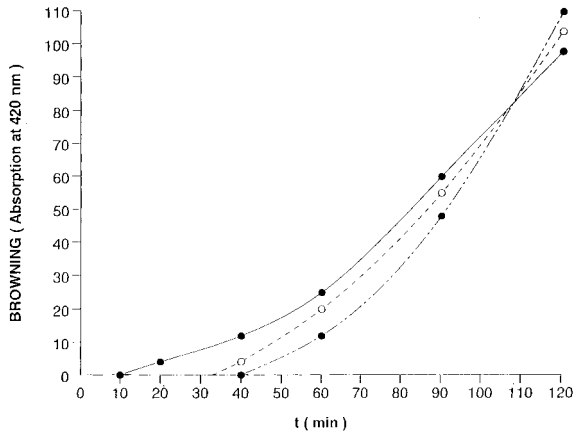

Figure 1. Influence of sodium sulfite (0%, —; 2%, - - -; 20%, — - - —) on the color formation from glucose and N^{α}-acetyl-L-lysine.

Inhibition of Radical Formation and Color Development by Sulfite Ions. In contrast to the accelerating effect of reductones, another reducing agent, the sulfite ion, is discussed in the literature as an inhibitor of the nonenzymatic browning. To gain insights into the role of sulfite in browning inhibition, aqueous solutions of glucose and N^{α}-acetyl-L-lysine were refluxed in the presence of various amounts of sodium sulfite and the time course of color formation was determined by measuring the absorption of the reaction mixtures at 420 nm. As outlined in Figure 1, after an induction period of 10 min, the color formation in the glucose/N^{α}-acetyl-L-lysine mixture, which was heated in the absence of sulfite, gradually increased with increasing the reaction time. Addition of 2% sodium sulfite led to a drastic delay of the induction period to ~30 min; however, as soon as the production of colored compounds started, their formation was significantly accelerated in comparison to the mixture without added sulfite. This effect was even more drastic when the sulfite content was increased to 20% (Figure 1). The higher sulfite concentration further increased the induction period to ~40 min, but, thereafter, the color formation was even more accelerated. These data showed that, under the reaction conditions applied, sulfite ions inhibited the nonenzymatic browning, especially in the early stage of the Maillard reaction.

Because glyoxal was found to be formed predominantly in the beginning of the Maillard reaction and was established as an effective color precursor (Hofmann et al., 1999), the rapid reaction of sulfite with free glyoxal and "hidden" glyoxal in Schiff bases, repectively, might probably be involved in the sulfite-induced delay in color formation in the initial phase of browning. The corresponding glyoxal−sulfite adduct might then not be reduced into the radical precursor glycolaldehyde, thus stalling the radical formation as well as browning formation. If this assumption is valid, then the reductone-induced radical production in a glucose/N^{α}-acetyl-L-lysine mixture should be inhibited in the presence of sodium sulfite. To confirm this, a mixture of glucose and N^{α}-acetyl-L-lysine was heated in the presence and absence of sodium sulfite, and after 3 min, prior to the end of the induction period, the reaction was stopped by rapid cooling. In both solutions radicals could not be observed by EPR spectroscopy, indicating that the mixtures were still in the induction period of radical formation. Addition of ascorbic acid to the sulfite-free mixture generated an intense signal of the pyrazinium radical cation (Table 1). On the contrary, in the mixture containing 2% sodium sulfite the free radical concentration was decreased to 49%, whereas in the presence of 20% sodium sulfite the radical was completely stalled. Also, reacting glyoxal/ascorbic acid or the radical precursor glycolaldehyde in the mixture with N^{α}-acetyl-L-lysine and 20% sodium sulfite completely suppressed radical formation (data not shown). Probably glycolaldehyde also reacts with sulfite to form the bisulfite adduct rather than with the amino acid to produce the radical cation. These results demonstrate that sulfite ions block the radical precursors glyoxal and glycolaldehyde and inhibit radical as well as browning formation already at the very early stage of the Maillard reaction.

Table 1. Influence of Sulfite Ions on the Formation of Free Radicals in the Glucose/N^{α}-Acetyl-L-lysine Mixture[a]

sulfite content (%)	rel radical intensity (%)
0	100
2	49
20	1

[a] The model experiment is detailed under Experimental Procedures.

Radical-Assisted Melanoidin Formation from Bovine Serum Albumin. It is well-known that melanoidins are formed from carbohydrates and amino compounds during thermal processing of foods such as roasting of cocoa and coffee or baking of bread. Because proteins were found to be effective melanoidin precursors and occur in foods in higher contents compared to the free amino acids, Hofmann (1988a) proposed that proteins might play a key role in melanoidin formation in processed foods. To maintain closer food-related conditions, we therefore studied whether lysine side chains of proteins might generate these free radicals but in protein-bound form instead. An aqueous solution of bovine serum albumin, which was chosen as a suitable model food protein, was heated in the presence of glycolaldehyde leading to a rapid browning of the reaction mixture. Because the expected pyrazinium radical cation formation should be accompanied by an oligomerization of the bovine serum albumin (MG = 67000 Da), we focused the identification experiments on the high molecular weight fraction. Fractionation of the reaction mixture by ultracentrifugation revealed orange-brown melanoidins with molecular weights >100000 Da, whereas the low molecular weight fraction (MG < 100000 Da) was colorless. This was well in line with recent findings that melanoidins were formed upon carbohydrate-induced protein oligomerization (Hofmann, 1998a). Analyzing the melanoidins by EPR spectroscopy revealed a single broad signal (Figure 2A). The lack of hyperfine structure and the g value of 2.0038, being close to the free electron spin value of 2.0023, indicated immobilized organic free radicals (Commoner et al., 1954; Steelink and Tollin, 1962; Pearce et al., 1997). Because the formation of 1,4-disubstituted pyrazinium radical cations is accompanied by an inter- and/or an intramolecular cross-linking of the protein, a strongly restricted rotation of the pyrazinium radical should result, thus inhibiting the resolution of the EPR signal. We therefore assumed the broad singlet to represent protein-bound 1,4-bis(5-amino-5-carboxy-1-pentyl)pyrazinium radical cations (CROSSPY), the structure of which is displayed in Figure 3.

To ascertain this assumption, in a first experiment, we heated the aqueous glycolaldehyde/bovine serum

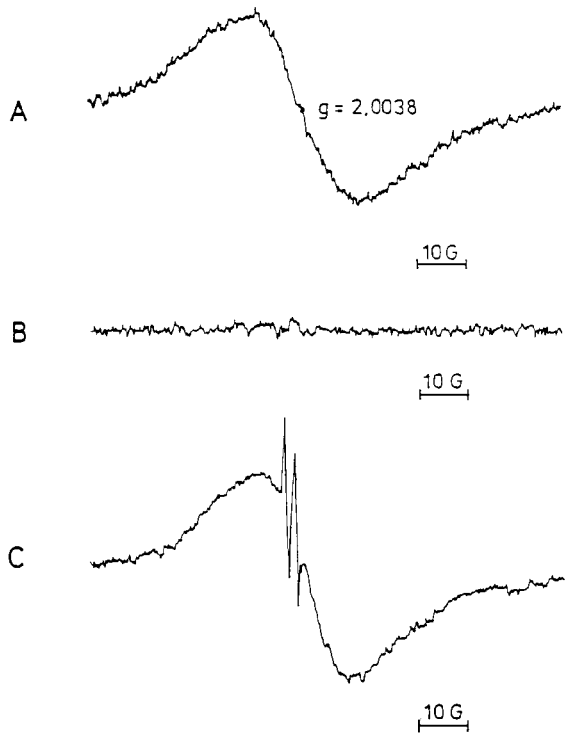

Figure 2. EPR signal of (A) a heated glycolaldehyde/bovine serum albumin solution, (B) a heated glycolaldehyde/bovine serum albumin mixture containing 20% sodium sulfite, and (C) a heated glyoxal/bovine serum albumin solution incubated with ascorbic acid.

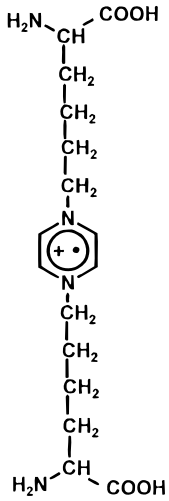

Figure 3. Structure of protein-cross-linking amino acid 1,4-bis(5-amino-5-carboxy-1-pentyl)pyrazinium radical cation (CROSSPY).

albumin mixture in the presence of 20% sodium sulfite. In contrast to the sulfite-free mixture (Figure 2A), addition of sulfite completely inhibited the radical formation in the glycolaldehyde/bovine serum albumin mixture (Figure 2B). These data were well in line with the sulfite-induced suppression of pyrazinium radical cation formation, which was found in the mixture of glycolaldehyde and N^{α}-acetyl-L-lysine.

Because preliminary studies showed that thermal treatment of glyoxal/N_{α}-acetyl-L-lysine mixtures did not generate the radical cation (Hofmann et al., 1999), in a second experiment, an aqueous mixture of bovine serum albumin and glyoxal was thermally treated and then analyzed by EPR spectrosopy. No radicals could be detected, being well in line with the lack of radical formation in the glyoxal/N^{α}-acetyl-L-lysine solution. Addition of ascorbic acid to the thermally pretreated glyoxal/bovine serum albumin mixture, however, generated the same broad singlet (Figure 2C) with a g value of 2.0038 as detected before in the mixture (Figure 2A). These data corroborate well our findings that reductones are able to initiate the radical cation formation in the presence of amines by a reduction of glyoxal and/or glyoxal imines. In addition to the broad signal, a doublet with a splitting constant of 1.8 G was detected (Figure 2C). This additional radical could be identified as the ascorbate radical (Bors et al., 1997), thereby demonstrating that the reduction of the glyoxal and/or glyoxal imine is achieved by a single-electron transfer.

The results of the experiments with bovine serum albumin were well in line with those found for N^{α}-acetyl-L-lysine and confirmed that CROSSPY, a previously unknown cross-link amino acid, might be participating in protein oligomerization involved in melanoidin formation.

Radical-Assisted Melanoidin Formation in Thermally Processed Foods. The identification of CROSSPY as an intermediate in the formation of model melanoidins prompted us to investigate whether such radicals also exist in food melanoidins. Light-colored wheat bread crumb and the dark browned crust of toasted wheat bread crumb were ground, and the powders obtained were analyzed by EPR spectroscopy. A broad singlet was detected in the brown crust, showing the identical g value of 2.0038 as found for CROSSPY in the heated mixture of bovine serum albumin and glycolaldehyde (Figure 2A). In contrast, in the ground crumb, showing no significant nonenzymatic browning, radicals were lacking. These data were well in line with the results obtained from the model experiments of glycolaldehyde and N^{α}-acetyl-L-lysine or bovine serum albumin, respectively, and showed that CROSSPY is probably associated with browning development. This was further evidenced by the detection of the same type of radical (g value = 2.0038) in dark brown roasted cocoa as well as in coffee beans and by the lack of this radical in heated milk, which did not show any brown colorization.

To further ascertain our assumption, wheat bread crumb was sprayed with a sodium sulfite solution (20% in water), dried at 35 °C, and then toasted. Compared with the toasted sulfite-free bread crumb, in the toasted sulfite-containing material the browning was completely suppressed and the radical formation was completely blocked (data not shown). Because the radical detected in the toasted bread crumb as well as in roasted cocoa and coffee beans showed the same g value as the radical observed in the bovine serum albumin/glycolaldehyde mixture and, in addition, sulfite showed the same inhibitory effect on browning and radical formation as in the model systems, we propose that the protein cross-link CROSSPY might be involved in the formation of food melanoidins, for example, in bread crust. It is an interesting finding that the existence of CROSSPY in the dark brown wheat bread crust and its lack in the light-colored wheat bread crumb is well correlated with the high amounts of the CROSSPY precursors glyoxal (48840 μg/kg) and glycolaldehyde (2700 μg/kg), which were very recently found in bread crust, and their low

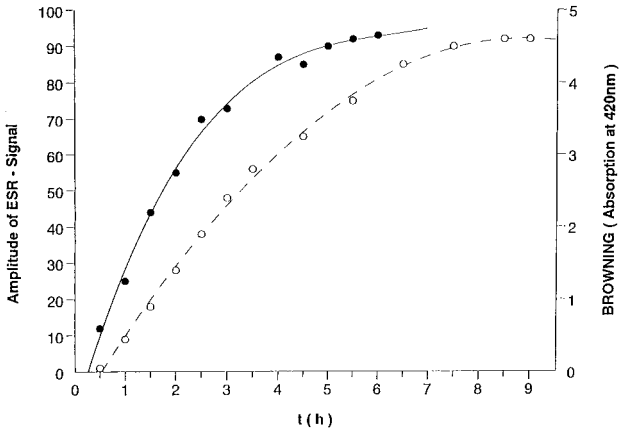

Figure 4. Time course of radical formation (—) and color development (- - -) in an aqueous solution of glycolaldehyde and bovine serum albumin incubated under physiological conditions.

amounts detectable in bread crumb (glyoxal, 2100 μg/kg; glycolaldehyde, < 10 μg/kg) (Hofmann, unpublished data).

CROSSPY Formation and Color Development under Physiological Conditions. The reaction of proteins and carbohydrates was proposed to be responsible also for the formation of nonenzymatic browning products in vivo; for example, the yellow-brown pigments in human eye lens are postulated to originate from Maillard-mediated reactions of the protein side chains (Monnier et al., 1981). Several carbohydrate degradation products have been reported to be potentially involved in posttranslational protein modifications in vivo; for example, glyoxal and glycolaldehyde were found to form imines upon reaction with lysine residues (Glomb and Monnier, 1995). However, these C-2 imines cannot explain the yellow-brown pigments formed, for example, in cataracts.

Recent studies revealed glycolaldehyde as the most effective carbohydrate degradation product in generating colored products when reacted with amino acids (Hofmann et al., 1999). In addition, the present investigation showed this C-2 compound as the precursor of the protein cross-linking pyrazinium radical cations. This encouraged us to study whether such radical-assisted reactions and browning development from proteins and glycolaldehyde might occur also under physiological conditions.

Solutions of bovine serum albumin and glycolaldehyde were, therefore, incubated at 37 °C at pH 7.4, and over a period of 9 h the color development was followed by measuring absorption at 420 nm. The data, outlined in Figure 4, showed that after an induction period of only 0.5 h, yellow-brown compounds were formed even under physiological conditions. With increasing incubation time, colorization became more intense, approaching a maximum value after 9 h. By monitoring the radical formation by EPR spectroscopy, the broad singlet of a radical exhibiting the *g* value of 2.0038 was detected as soon as the mixture was incubated. With increasing incubation time, the radical concentration increased rapidly within the first 4 h and then asymptotically approached a maximum value after 6 h. These data, obtained under physiological conditions, were well in line with those found for the color development and radical formation in the glycolaldehyde/amine mixtures at elevated temperatures (Hofmann et al., 1999). On the basis of these findings, the 1,4-bis(5-amino-5-carboxy-1-pentyl)pyrazinium radical cation might participate in protein cross-linking and nonenzymatic browning also under in vivo conditions.

Besides glycolaldehyde, glyoxal is also discussed as a cross-linker in vivo via the formation of C-2-lysyl imines (Glomb and Monnier, 1995). Because model experiments recently (Hofmann et al., 1999) indicated the formation of glycolaldehyde and alkylaminoacetaldehyde upon reductone-mediated reduction of glyoxal and glyoxal imine, it might be possible that under physiological conditions ascorbic acid reduces these C-2 imines into the protein-bound 2-lysylacetaldehyde, which then leads to radical formation and color development. Because Glomb and Monnier (1995) showed the formation of glyoxal imines after incubation of bovine serum albumin with glyoxal under physiological conditions (pH 7.4, 37 °C), we repeated this experiment. After incubation of bovine serum albumin with glyoxal for 2 h at 37 °C, no radicals could be observed by EPR spectroscopy. Addition of ascorbic acid, however, rapidly produced the radical cation as well as colored substances under physiological conditions. These data indicate that CROSSPY might be also formed in vivo by a ascorbic acid-assisted reaction of glyoxal with proteins.

The data, obtained under physiological conditions in vitro, provide new insights into the nonenzymatic browning reaction in living systems; however, additional studies in vivo are now necessary to prove the significance of the proposed radical cation formation as a key reaction step in protein cross-linking and color formation.

Conclusions. Detailed information on the chemical species involved in the complex reaction pathways leading to nonenzymatic browning might prove useful in accelerating the desired color formation during thermal processing of certain foods, such as baking of bread, and also in preventing an undesired colorization, for example, during long-term storage of foods. Investigating these reactions under physiological conditions gives insights into mechanisms that might be involved in protein glycation in living systems. Results, obtained thereof, might offer possibilities to counteract nonenzymatic browning in vivo, for example, in aging processes or in diabetes.

ACKNOWLEDGMENT

We thank the Institute of Food Chemistry, Technical University of Munich, for the LC/MS analyses. We are grateful to Mr. J. Stein for his excellent technical assistance.

LITERATURE CITED

Bors, W.; Michel, Ch.; Stettmaier, K. The interaction of flavonoids and ascorbate as studied by EPR spectroscopy. *J. Magn. Reson. Anal.* **1997**, *3*, 149−154.

Bucala, R.; Vlassara, H.; Cerami, A. Advanced glycation end products. In *Post-translational Modifications of Proteins*; Harding, J. J., Crabbe, M. J. C., Eds.; CRC Press: Boca Raton, FL, 1992; pp 54−79.

Commoner, B.; Townsend, J.; Pake, G. E. Free radicals in biological materials. *Nature* **1954**, *9*, 689−691.

Glomb, M. A.; Monnier, V. M. Mechanism of protein modification by glyoxal and glycolaldehyde, reactive intermediates of the Maillard reaction. *J. Biol. Chem.* **1995**, *270*, 10017−10026.

Hofmann, T. Determination of the chemical structure of novel colored 1H-pyrrol-3(2H)-one derivatives formed by Maillard-type reactions. *Helv. Chim. Acta* **1997**, *80*, 1843−1856.

Hofmann, T. Studies on the relationship between the molecular weight and the color potency of fractions obtained by thermal treatment of glucose/amino acid and glucose/protein solutions by using ultracentrifugation and color dilution techniques. *J. Agric. Food Chem.* **1998a**, *46*, 3891−3895.

Hofmann, T. Characterization of the chemical structure of novel colored Maillard reaction products from furan-2-carboxaldehyde and amino acids. *J. Agric. Food Chem.* **1998b**, *46*, 932−940.

Hofmann, T. Application of site specific [^{13}C] enrichment and ^{13}C NMR spectroscopy for the elucidation of the formation pathway leading to a red colored 1*H*-pyrrol-3(2*H*)-one during the Maillard reaction of furan-2-carboxaldehyde and L-alanine. *J. Agric. Food Chem.* **1998c**, *46*, 941−945.

Hofmann, T. Identification of novel colored compounds containing pyrrole and pyrrolinone structures formed by Maillard reactions of pentoses and primary amino acids. *J. Agric. Food Chem.* **1998d**, *46*, 3902−3911.

Hofmann, T.; Bors, W.; Stettmaier, K. Studies on radical intermediates in the early stage of the nonenzymatic browning reaction of carbohydrates and amino acids. *J. Agric. Food Chem.* **1999**, *47*, 379−390.

Kato, H.; Naguchi, G.; Fujimaki, M. Reductone contents of nondialyzable melanoidins. *Agric. Biol. Chem.* **1968**, *32*, 916−919.

Kim, S. B.; Hayase, F.; Kazo, H. Desmutagenic effects of melanoidins against amino acid and protein pyrolyzates. In *Amino-Carbonyl Reactions in Food and Biological Systems*; Fujimaki, M., Namiki, M., Kato, H., Eds.; Kodansha: Tokyo, 1986; pp 383−392.

Knowles, M. E. Inhibition of non-enzymatic browning of sulfite: identification of sulfonated products. *Chem. Ind.* **1971**, 910.

Kroh, L.; Zeise, S.; Stösser, R.; Westphal, G. Studies on the Maillard reaction—radical inhibition of the non-enzymatic browning by sulfur compounds (in German). *Z. Lebensm. Unters. Forsch.* **1989**, *188*, 115−117.

Ledl, F.; Severin, Th. Non-enzymatic browning reactions of pentoses and amines (in German). *Z. Lebensm. Unters. Forsch.* **1978**, *167*, 410−413.

Ledl, F.; Fritsch, G.; Severin, Th. Methylene-reductinic acid, a novel reductone from glucose (in German). *Z. Lebensm. Unters. Forsch.* **1982**, *175*, 208−210.

Ledl, F.; Fritsch, G.; Hiebl, J.; Severin, Th. Detection of reductones and aminoreductones with methyl hydrazine (in German). *Z. Lebensm. Unters. Forsch.* **1983**, *176*, 294−297.

McWeeny, D. J.; Biltcliffe, D. O.; Powell, R. C. T.; Spark, A. A. The Maillard reaction and its inhibition by sulfite. *J. Food Sci.* **1969**, *34*, 641−645.

Mitsuda, H.; Yasamuto, K.; Yokoyama, K. Studies on the free radical in amino-carbonyl reaction. *Agric. Biol. Chem.* **1965**, *29*, 751−756.

Monnier, V. M. Toward a Maillard reaction theory of aging. In *The Maillard Reaction in Aging, Diabetes, and Nutrition*; Baynes, J. W., Monnier, V. M., Eds.; Liss: New York, 1989; pp 1−22.

Monnier, V. M.; Stevens, V. J.; Cerami, A. Maillard reactions involving proteins and carbohydrates in vivo: relevance to diabetes Mellitus and Aging. In *Maillard Reactions in Foods—Chemical, Physiological and Technological Aspects*; Eriksson, C., Eds.; Pergamon Press: London, U.K., 1981; pp 315−327

Namiki, M.; Hayashi, T. A new mechanism of the Maillard reaction involving sugar fragmentation and free radical formation. *ACS Symp. Ser.* **1983**, *No. 215*, pp 21−46.

Noll, P. Reductones as reactive intermediates in the Maillard reaction (in German). Ph.D., University of Stuttgart, Germany, 1993.

Pearce, R. B.; Edwards, P. P.; Green, T. L.; Anderson, A.; Fischer, B. J.; Carpenter, T. A.; Hall, L. D. Immobilized long-lived free radicals at the host-pathogen interface in sycamore (*Acer pseudoplatanus* L.). *Physiol. Mol. Plant Pathol.* **1997**, *50*, 371−390.

Severin, Th.; Krönig, U. Structur of a colored compound from pentoses (in German). *Chem. Mikrobiol. Technol. Lebensm.* **1972**, *1*, 156−157.

Steelink, C.; Tollin, G. Stable free radicals in soil humic acid. *Biochim. Biophys. Acta* **1962**, *59*, 25−34.

Wedzicha, B. L.; Kaputo, M. T. Melanoidins from glucose and glycine: composition, characteristics and reactivity towards sulfite ion. *Food Chem.* **1992**, *43*, 359−367.

Wedzicha, B. L.; McWeeny, D. J. Nonenzymatic browning reactions of ascorbic acid and their inhibition: the production of 3-deoxy-4-sulfopentosulose in mixtures of ascorbic acid, glycine and bisulfite ion. *J. Sci. Food Agric.* **1974**, *25*, 577−587.

Wu, C. H., Russell, G.; Powrie, W. D. Paramagnetic behavior of model system melanoidins. *J. Food Sci.* **1987**, *52*, 813−816.

Yamaguchi, N. Antioxidative activity of the oxidation products prepared from melanoidins. In *Amino-Carbonyl Reactions in Food and Biological Systems*; Fujimaki, M., Namiki, M., Kato, H., Eds.; Kodansha: Tokyo, 1986; pp 291−299.

Received for review June 9, 1998. Revised manuscript received November 3, 1998. Accepted November 12, 1998.

JF980627P