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Spectroscopic studies of the protein–methylglyoxal adduct

(lysine/Schiff base/charge transfer/conductivity)

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ABSTRACT **Spectroscopic measurements are reported for the effects of pH, time, solvent, and chemical modification of arginine and lysine side chains on the reaction of proteins with methylglyoxal. The reaction responsible for the appearance of a brown coloration and increased submolecular electronic activity in the proteins involves the ϵ-amino groups of the lysine residues. It is concluded that the primary step in the reaction involves the formation of a Schiff base linkage between the lysine side chain and methylglyoxal. These findings reaffirm the concept that, by the formation of Schiff bases, aldehydes can act as electron acceptors in charge transfer interactions with proteins.**

Considerations (1, 2) of the possible biological role of aldehydes and ketones in acting as electron acceptors for proteins have led us to study the interactions of proteins with methylglyoxal. These studies have shown that when proteins such as casein, collagen, bovine serum albumin, and lysozyme react with methylglyoxal they assume a stable brown color and exhibit a greatly enhanced electronic conductivity and electron spin resonance activity compared with the normal (white) unreacted proteins (3–5).

Scintillation counts by W. M. Arnold and J. Behi using ^{14}C-labeled methylglyoxal (personal communication) and weight increase measurements have shown that after reaction with methylglyoxal for 5 days the resulting brown proteins contain on average 8–12% methylglyoxal on a dry weight basis. Amino acid analyses for the adducts of bovine serum albumin and of casein with methylglyoxal, performed by G. E. Siefring, Jr., at Northwestern University (personal communication) indicate that the arginine (≈100% reacted) and lysine residues (80–90% reacted) are the ones that react most strongly with methylglyoxal. We have blocked the arginine side chains of albumin with cyclohexanedione, using the procedure described by Smith (6), and this has produced no observable difference in the way the protein reacts with methylglyoxal to produce the brown color and electronic activity. However, when the ϵ-amino groups of the lysine side chains of albumin and casein have been reductively dimethylated by the method of Lin *et al.* (7), upon reaction with methylglyoxal the proteins remain white and do not exhibit enhanced electronic activity. These results indicate that the reaction of particular importance in these studies involves the protein lysine residues, and the following spectroscopic studies were made as a step toward understanding the nature of this reaction.

METHODS

Measurements were obtained by using a dual beam Beckman model 35 spectrophotometer and quartz sample cells (optical pathlength, 1 cm). For the spectra described here, 0.15 mM bovine serum albumin was allowed to interact with approximately 10 mM methylglyoxal in water at 297 ± 1 K; and the reference system consisted of an aqueous solution of methylglyoxal at the same concentration and pH as in the albumin/methylglyoxal sample. The bovine serum albumin (fraction V) was obtained from Sigma. The methylglyoxal (Aldrich) was twice distilled prior to use. Adjustment of pH was made by using NaOH or HCl, and no buffer solutions were utilized.

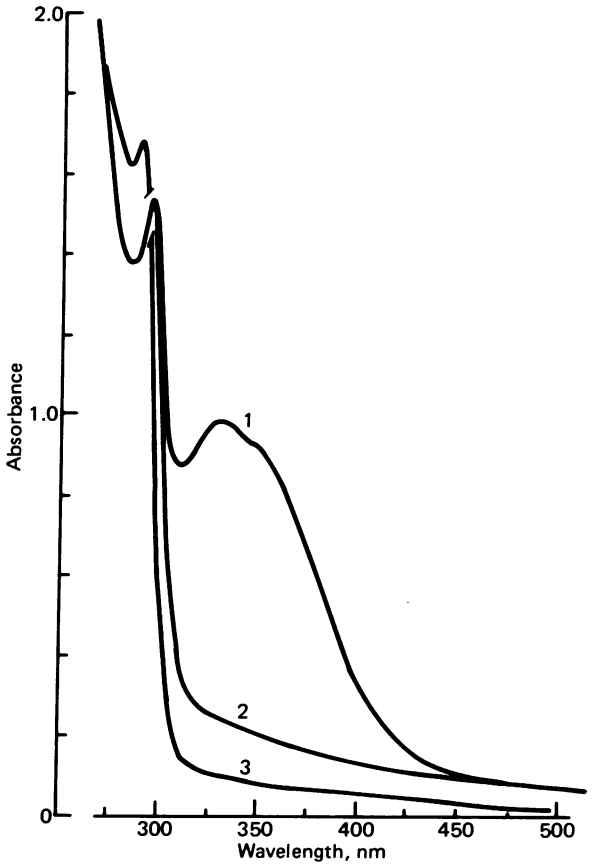

FIG. 1. Absorption spectra. Curves: 1, bovine serum albumin + methylglyoxal at pH 7, 1 hr 40 min; 2, *N,N*-dimethylalbumin + methylglyoxal at pH 7, 1 hr 50 min; 3, albumin + methylglyoxal at pH 4, 1 hr 45 min.

RESULTS AND DISCUSSION

Fig. 1 shows the absorption spectrum obtained after approximately 1¾ hr of reaction time for normal albumin and for albumin whose lysine groups had been reductively dimethylated by the method of Lin *et al.* (7). These results demonstrate that the presence of the ϵ-amino groups of the lysine residues is re-

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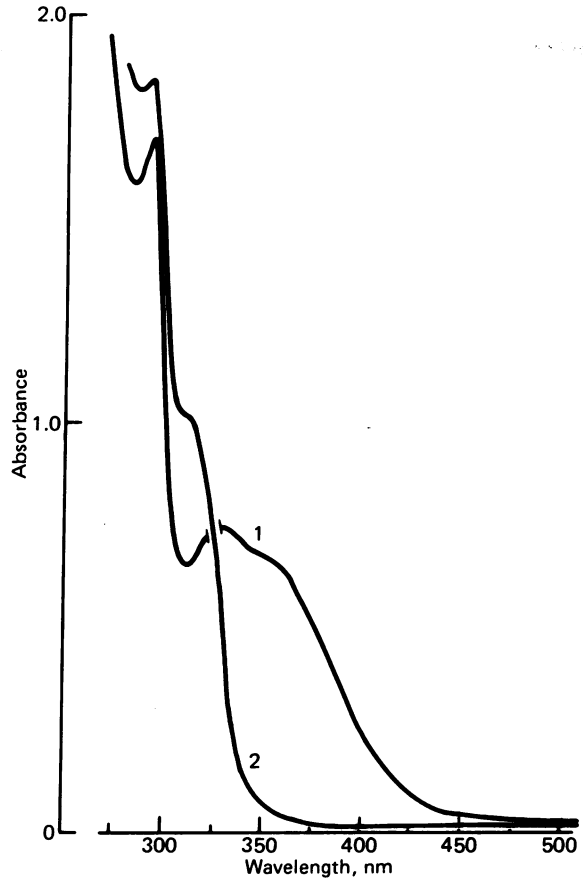

FIG. 2. Absorption spectra. Curves: 1, bovine serum albumin + methylglyoxal at pH 7, 1 hr 5 min; 2, albumin + methylglyoxal at pH 7, with $NaBH_4$ added at 1 hr 15 min.

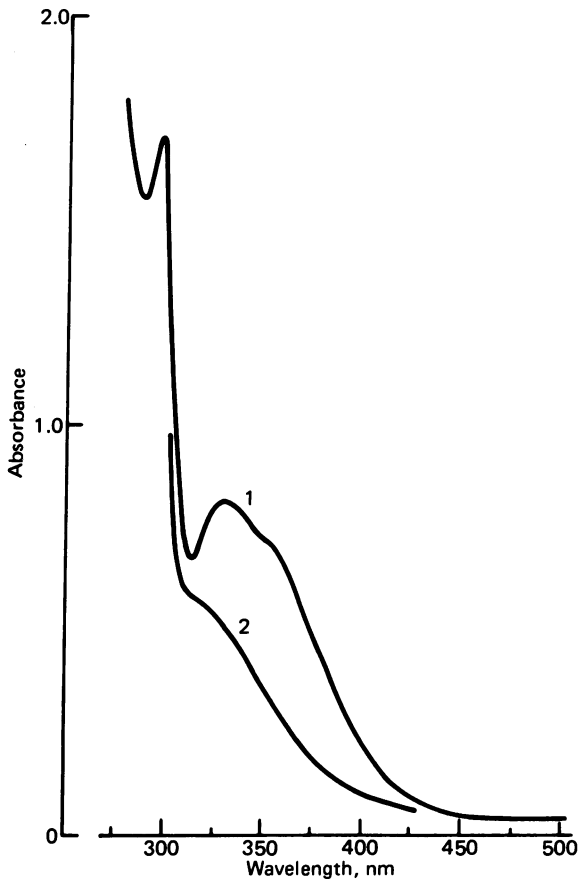

FIG. 3. Absorption spectra. Curves: 1, bovine serum albumin + methylglyoxal at pH 7, 1 hr 25 min; 2, reaction at pH 2.

quired to produce the color reaction with methylglyoxal. A similar conclusion has been drawn from measurements on normal casein (Fisher) and *N,N*-dimethylcasein. When the arginine side chains of albumin were modified with cyclohexanedione as described by Smith (6), the reaction with methylglyoxal still produced an absorption peak around 328 nm and a shoulder around 350 nm. The reaction with normal casein, lysozyme (hen egg white, Sigma) and poly(L-lysine) hydrobromide (type V, Sigma) produced the main absorption peak around 328 nm but the shoulder at 350 nm was absent. The reaction is dependent on pH, with the reaction rate decreasing when the pH is below 7. This effect is shown in Fig. 1: after a reaction time of 1¾ hr at pH 4 there was no evidence of the appearance of the 328-nm absorption peak. This result indicates that protonation of the lysine ϵ-amino group retards the reaction.

The yellow color produced by the protein–methylglyoxal interaction was found to be bleached by the addition of sodium borohydride (Fig. 2). The yellow color disappeared slowly when the pH of the reaction mixture was adjusted from pH 7 to pH 3 or below; the associated change in the absorption characteristic is shown in Fig. 3. The rate of appearance of the absorption

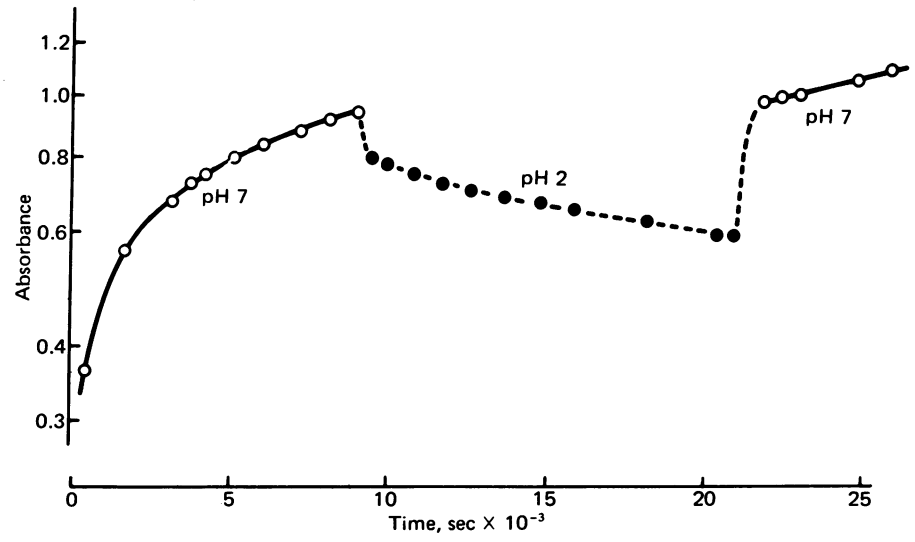

FIG. 4. Changes of absorbance of bovine serum albumin/methylglyoxal mixture with time as a function of pH; details are given in the text.

peak and the effect of changing the pH also were investigated, and a typical result is shown in Fig. 4. In this case the initial reaction of albumin with methylglyoxal at pH 7 was monitored by measurement of the absorption peak at 330 nm. After the pH was adjusted to 2, the disappearance of the yellow color was monitored by observing the change in the absorbance at 342 nm. On later readjustment to pH 7, the yellow color rapidly reappeared and the absorbance at 330 nm was found to equal that when the pH was altered from pH 7 to pH 2. If adjustment to pH 2 had resulted in a reversal of the reaction, through hydrolysis for example, then the reattainment of the absorption peak should have occurred at the original reaction rate. The decrease in absorbance at pH 2 most likely resulted from a protonation effect.

The protein–methylglyoxal samples used for the spectral measurements were prepared in 90% methanol, and for this reason the absorption spectra have also been studied in methanol/water solutions. With increasing methanol concentration the rate of the color reaction increased and the absorption peak progressively moved to ≈336 nm at 90% methanol. The total spectrum also broadened out to extend further into longer wavelengths. These results indicate that the reaction producing the brown protein–methylglyoxal adduct in 90% methanol is the same as that which produces the yellow color in water.

CONCLUSIONS

These studies confirm that the primary step by which methylglyoxal reacts with proteins to produce a brown coloration and increased electronic activity involves a reaction with the accessible lysine side chains of the protein. Furthermore, we consider that the spectroscopic data described here are consistent with the concept that this reaction involves the formation of a Schiff base (—HC=N—) linkage involving the aldehydic carbonyl group of methylglyoxal and the ϵ-amino group of a lysine side chain. The following observed features support this conclusion.

(*i*) A λ_{max} value of 328 nm is not unreasonable for a resonance-stabilized $n \rightarrow \pi^*$ transition for a Schiff base that is linked to a ketonic carbonyl group of methylglyoxal.

(*ii*) The 8-nm red shift of λ_{max} that is observed when the solvent is changed from water to 90% methanol is consistent with the absorption being due to an $n \rightarrow \pi^*$ transition.

(*iii*) The result shown in Fig. 1 (curve 3) is consistent with protonation of the lysine ϵ-amino groups' inhibiting the formation of Schiff bases.

(*iv*) The bleaching effect of $NaBH_4$ shown in Fig. 2 is consistent with hydrogenation of the (—HC=N—) chromophore to give (—CH_2—NH—).

(*v*) The result in Fig. 3 is consistent with the protonation of the Schiff base preventing the nitrogen lone-pair electrons undergoing $n \rightarrow \pi^*$ transitions.

(*vi*) The alternative to the Schiff base formation is a base-catalyzed Michael addition of the methylglyoxal to the lysine side chain, but in this case no chromophore group would be produced.

(*vii*) Lysine is known to catalyze the polymerization of methylglyoxal (8). However, the amino acid analyses, weight change, and ^{14}C measurements indicate that polymeric methylglyoxal can only form a small component of the protein–methylglyoxal adduct.

We hope that the presence of Schiff base linkages to the ϵ-amino groups of the lysine side chains can also be confirmed by nuclear magnetic resonance measurements. Such confirmation would highlight the relevance of recent calculations (9) which show that such a Schiff base represents a good electron acceptor capable of forming charge transfer interactions with neighboring peptide units. Such interactions possibly give rise to the observed (3–5) electron spin resonance, dielectric, and electronic properties of the protein–methylglyoxal adducts.

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