Free Radicals, Malonaldehyde and Protein Damage in Lipid-Protein Systems

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ABSTRACT

The free radical concentration in lipid-protein mixtures with a low moisture content was monitored both during and after the time that the lipid actively absorbed oxygen. The data show that, in dry systems, the decay in radical content is followed by a rise in malonaldehyde protein fluorescence. Analysis of amino acid content at two distinctly different periods in the reaction substantiate the hypothesis that radicals and not aldehydes are a major cause of protein damage.

INTRODUCTION

In studies of lipid-protein nucleotide interaction, a number of investigations have shown that polyunsaturated fatty acids undergoing oxidation can induce extensive changes in enzymes, proteins and nucleotides (1-8). In some of these studies, radicals derived from oxidizing lipid have been implicated as the chief cause of damage. It is therefore of some importance to consider lipid oxidation, radicals and their involvement in biological systems in detail. Although there is ample evidence that lipid oxidation is a chain reaction involving free radical intermediates, limitations in instrumental sensitivity make it most unlikely that radicals derived from lipid can be readily characterized in native biological material or in any system high in moisture content (1).

Free radicals are a chief cause of the observed damage, i.e., destruction of amino acids of proteins or enzymes, as well as polymerization or depolymerization of nucleotides. However, aldehydes or other nonradical lipid oxidation products may also be capable of causing damage to such biopolymers. The purpose of this investigation was to ascertain which mechanism, radical or nonradical, dominates in systems containing protein in close association with oxidizing lipid.

EXPERIMENTAL PROCEDURES

Electron Paramagnetic Resonance of Dry Biological Systems

To detect and study radicals and radical lifetime, solid state systems were used because, in these, line widths are not too broad and the lipid-derived radical steady state concentration is high enough to make detection by electron paramagnetic resonance (EPR) an easy matter. In contrast to the study of radicals in solution, however, EPR of many solids is complicated by the fact that the observed EPR spectrum will consist of a superposition of absorbancies arising from all orientations of immobilized radicals. The worst situation would be one in which anisotropies in both the g-value and hyperfine splitting would spread the EPR absorption line over hundreds of gauss. If this were to happen, the line would probably be so badly broadened that observation would be almost impossible. Fortunately, with most biological samples, organs, tissue, etc., such extreme broadening is not encountered. In fact, the absorptions are, for the most part, fairly narrow, on the order of 3-11 gauss.

Lipids, Proteins and Test Mixtures

The fatty acid mixture used in all studies consisted of approximately 75% (by weight) of C22:6 and approximately 25% C20:5. Rockfish myofibrilliar protein, freeze dried and extracted with isopropyl alcohol and hexane, and then dried in vacuum, was supplied by the NMFS Technological Laboratory in Seattle. Bovine serum albumin (BSA; lyophillized) was obtained from the Sigma Chemical Corporation.

Lipid-protein test mixtures were prepared merely by stirring together lipid and dry protein (1:2 w/w) for 1-2 min. Except for the buffering capacity of protein itself, no attempt was made to control the pH of the lipid-protein mixtures. Samples were allowed to oxidize under oxygen at 37 C and sampling was begun 2 hr after mixing. All samples for EPR study were analyzed at room temperature as described previously (9). Amino acid analyses were performed on hydrolysates (conventional 16 hr hydrolysis in HCl in sealed tubes) by the National Center for Fish Protein Concentrate, College Park, Maryland, using an amino acid analyzer.

Chloroform-methanol (2:1 v/v) extracts of solid reaction products were scanned for fluorescence using an Aminco-Bowman spectrophotofluorimeter equipped with a solid state blank substract photometer.

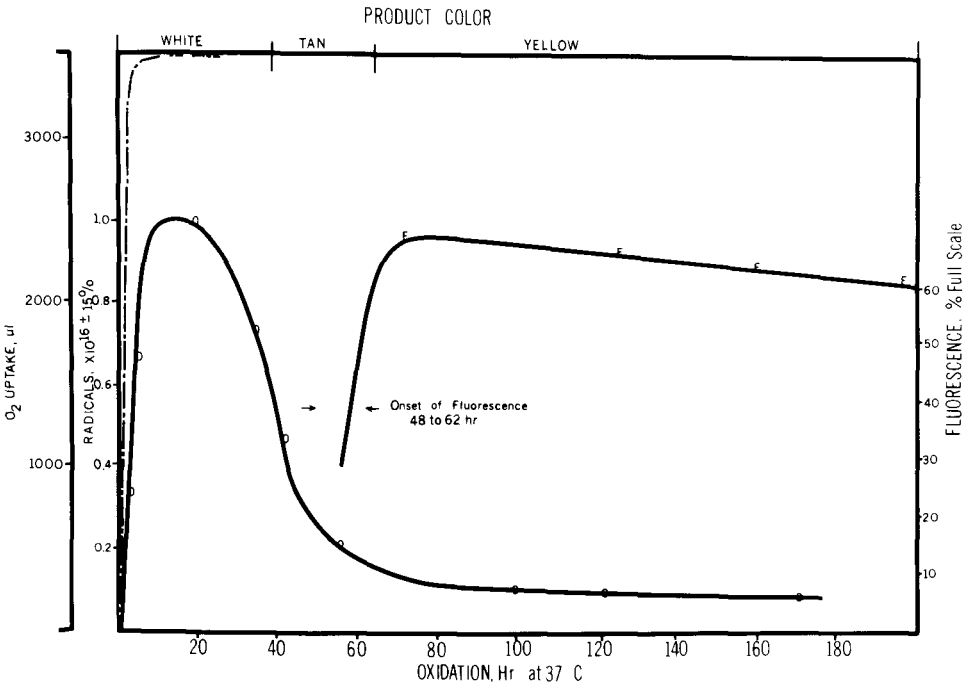

FIG. 1. Radical content, Schiff base fluorescence and product color vs. time of lipid oxidation in bovine serum albumin - C22:6. F, fluorescence; O, radical content; —— - —— -, oxygen uptake. Measurable oxidation, as monitored by oxygen uptake, ceased rather abruptly at about 8 hr under an excess of oxygen. Ratio of lipid-protein, 1:2 w/w.

Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Eastman Chemicals), in separate tests, were included in some lipid-protein mixtures in order to obtain information concerning the effects of these two popular antioxidants. They were added to protein along with lipid at a level amounting to twice the amount of lipid (on a mole basis).

RESULTS AND DISCUSSION

In a previous study (9) it was shown that, in addition to a dominant central EPR signal in the g=2 region (a central resonance not directly associated with lipid oxidation), there appeared, in those protein samples containing oxidizing or oxidized lipid, one and sometimes two additional peaks downfield from the central resonance. The nature of these lipid signals has been discussed. Since radicals could be immobilized, yet slowly decay (unpublished work by the author has recently shown that the rate of decay is dependent on the amount of lipid present and on the type of protein), the hypothesis that radicals would give way to fluorescence as malonaldehyde reaction products accumulate was tested. Indeed, the data of Figure 1 for lipid-BSA, show a rapid accumulation of radicals during the time that lipid actively absorbs oxygen. For the first day or two, chloroform-methanol extracts (no added antioxidant), were devoid of spectral characteristics indicative of malonaldehyde-amino acid interaction (formation of N,N'-disubstituted 1-amino-3-iminopropene fluorescence: $\lambda max_{excitation}$=390 mμ, $\lambda_{fluorescence}$ = 470-480 mμ). Figure 2 shows the relation between length of oxidation and specific fluorescence. Also, as shown in Figure 1, characteristic iminopropene fluorescence is detected in the very same time period that there is a marked decrease in observable radical content. When iminopropene fluorescence did appear, fluorescence intensity did not change significantly but remained more or less constant throughout the remainder of the examination (7-10 days). Myofibrilliar protein in place of BSA gave similar results. The greatest difference was in the somewhat shortened time required to produce measurable fluorescence.

In an attempt to facilitate analyses, fluorescence of solid reaction mixtures themselves was investigated. However, it has been our experience, and that of others, that solid samples, however appealing they may be for direct fluorescence measurement, are not satis-

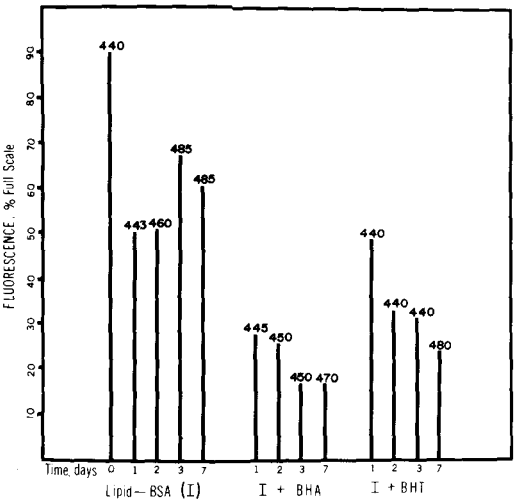

FIG. 2. Effect of added antioxidant on retardation of Schiff base formation. Numbers at tops of columns are fluorescent wave length maxima for chloroform-methanol extracts excited at 390 mμ. BSA, bovine serum albumin; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene.

factory. Thus all fluorescence data was obtained using chloroform-methanol extracts. (Tappel, for instance, points out that this solvent appears to be the appropriate mixture for selective removal of malonaldehyde-amino reaction products.)

When BHT or BHA were present (Fig. 2), the onset of fluorescence was delayed and it was not until the seventh day that iminopropenes could be detected. Although fluorescence did occur eventually, the fluorescence intensity was less than that for samples devoid of antioxidants. Although not shown in the figures, BHT or BHA, though effective in reducing onset of fluorescence, did not completely suppress the EPR lipid signal. BHT-treated material gave a weak recorded resonance after a few hours of oxidation and the signal was still detectable at 12 hr. Only a trace of EPR signal was ever detected in BHA-treated samples, an observation that correlates with a somewhat less intense fluorescence noted for BHA data in Figure 2. The eventual formation of fluorescence in antioxidant-treated material can only mean that a low but undetectable steady state concentration of radicals is presented.

Amino acid analyses are significant for they correlate well with time course observations of trapped radicals and fluorescence. With BSA for instance, a 14 hr period represents the approximate midpoint in time at which radical content is maximum. On the other hand, a 72 hr period represents a time at which radicals have decayed appreciably but fluorescence is maximum. Although all amino acids suffered damage, its extent was not as great as that observed in previous studies of protein-lipid-aqueous buffer systems. Losses ranged from 20% to 40% for the 14 hr period. Methionine, cystine, tyrosine, alanine and lysine suffered 30%, 40%, 34%, 27% and 22% losses, respectively. However, in the next 58 hr, the losses for these same amino acids were only 15%, 18%, 15%, 15% and 10%, respectively.

Compared to samples consisting of lipid-protein-aqueous emulsions, an overall lowering in the losses of amino acids is probably due in part to lipid polymerization with subsequent loss, as reactant, of the thin lipid layers deposited on proteins. However, this hypothesis cannot be entirely correct because fluorescence occurs at a time considerably displaced from the cessation of uptake of oxygen.

The data indicate that major losses in amino acids occur rather early in the oxidation. Some of the loss at a later period may not be due to aldehydes but may still be the result of a small but detectable radical content. Although Chio and Tappel (6,7) have only recently given a thorough account of malonaldehyde-protein/enzyme interaction, their data do not provide information on the significance of aldehydes in the chain of events which transpire as lipid oxidation progresses first to the free radical state and then to malonaldehyde and other nonradical reactants. The results of the present study, therefore, further strengthen the hypothesis first introduced by Roubal and Tappal (1,3) that radical attack and not aldehyde attack on protein is predominately responsible for damage to proteins.

Although the present study indicates that food grade antioxidants appear to act as inhibitors of lipid signals, that is, trapped radicals, additional research will be required to clarify the role and mode of action of antioxidants in foods exhibiting EPR signals.

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