The Reaction of an Autoxidized Lipid with Proteins

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Abstract

Evidence is presented which indicates that an interaction occurs between proteins and an autoxidizing unsaturated lipid. Using a model system approach, it has been established that two purified proteins (gelatin and insulin) are chemically modified in the presence of an autoxidizing lipid, methyl linoleate.

The insulin-methyl linoleate interaction has been studied chromatographically after acid and alkaline hydrolysis, and also by using the Sanger end group analysis method. The data indicate that lipid intermediates react with the ε-amino group of lysine, and also with phenylalanine and glycine, the N-terminal amino groups of insulin.

Hydrogen fluoride solubility and enzyme hydrolysis determinations indicate that the autoxidation products of methyl linoleate interact with protein to produce new chemical entities through cross-linking.

Introduction

An ever increasing number and variety of dehydrated foods are being developed for both human and animal consumption. Many of these contain lipids which become oxidized upon removal of water from the finished product (1).

The autoxidation of unsaturated lipids produces several active chemical intermediates: free radicals, hydroperoxides, oxiranes, and carbonyl compounds (2). These highly reactive intermediates, in turn, are capable of entering into reactions with one or more of the functional groups within protein molecules (3). As a consequence, one would expect a variety of complex interactions to occur between proteins and the degradation products of autoxidizing lipids under suitable reaction conditions, and in most instances the effect on food items could be undesirable.

The present study was designed to determine whether autoxidizing lipids do form addition products with proteins in the absence of water. A simplified model system approach has been employed with both gelatin and insulin serving as receptors for such intermediates. The assumption was made that the most likely interaction between the model protein and the lipid would involve either an alkylation or a cross-linkage of free amino groups; our approach was so tailored.

When protein alteration occurs in the presence of autoxidizing lipid a change in hydrolysis rate with proteolytic enzymes may be used as an index of such reactions. For this reason a trypsin assay technique was adapted to the problem.

Because the enzyme approach does not yield exact information concerning either the chemical nature of the interaction or the sites of reaction within the protein, two specific techniques were employed.

In order to identify the sites of lipid interactions with protein amino groups, the 1-fluoro-2,4-dinitrobenzene (FDNB) reagent of Sanger (4) can be used indirectly. Native insulin, for example, contains two N-terminal amino groups (phenylalanine and glycine) and one free ε-amino group (lysine) which react with FDNB. If, however, any of these amino groups are involved in an interaction with lipid intermediates, subsequent reaction with FDNB will not yield the usual yellow colored dinitrophenyl derivative. Therefore, by indirection, the sites of lipid-protein interactions for amino groups can be determined.

Solubility of the reacted proteins was also determined in anhydrous hydrogen fluoride (HF). This test has been used to differentiate cross-linked proteins from their denatured or alkylated counterparts because the former are insoluble while the latter are soluble in this reagent (5).

Materials and Methods

Materials

Pigskin gelatin (312 Bloom) obtained from the Grayslake Gelatin Co., Grayslake, Ill., was used in the preparation of model foams. Commercially available crystalline zinc insulin in acid solution (500 units/ml) obtained from Eli Lilly & Co., Indianapolis, Ind., was used for end group marking studies.

Reagents used in the hydrolysis and chromatographic phases of this study were obtained from Mallinckrodt Chemical Works, St. Louis, Mo. Amino acid controls and 1-fluoro-2,4-dinitrobenzene were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, while hydrogen fluoride (HF) was obtained from Matheson Co., Joliet, Ill. Methyl linoleate (ML) having an iodine value of 172 was obtained from Hormel Institute, Austin, Minn. This high purity lipid was used throughout as a model lipid.

Methods

Gelatin-ML Foam Preparation. Five-gram samples of gelatin were dissolved in 100 ml. of distilled water at 45–50C; 1.0 g of ML was added and the resulting reaction mixture was whipped to a stiff foam in a Sunbeam mixer. The foams were freeze-dried and the dehydrated materials were autoxidized in either air or nitrogen for five days at 50C. After autoxidation the foams were shredded in a Waring blender with ethanol-benzene azeotrope. The resulting slurries were poured into thimbles and Soxhlet extracted with the azeotrope for 16 hr to remove residual lipid. As a final step in preparation for enzymatic hydrolysis, the defatted foams were further dried overnight in vacuum desiccators.

In one series of tests, standard gelatin foams were prepared as detailed above, except that 1.4 g of sodium bisulfite were dissolved in gelatin-ML solution to act as an aldehyde trap.

Trypsin Assay of Gelatin-ML Foams. Samples of defatted foams (0.5 g) were mixed with 59 ml of Sørensen's buffer (pH 7.7) containing 10 mg of Difco trypsin (1:250) in 125 ml. Erlenmeyer flasks. Each sample was set up in duplicate with one reaction mixture precipitated immediately upon mixing with 20 ml of 20% trichloroacetic acid (TCA). The second flask was shaken for 20 hr at 38C and then precipitated with 20 ml of 20% TCA. Both samples were filtered

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TABLE I

Liberation of Alpha-Amino Nitrogen in Gelatin-ML Foams After Autoxidation and Trypsin Hydrolysis

Gelatin foam	g ML/5 g gelatin	Mg of N_2 lib./g gelatin (ave.[a])	% Reduction by hydrolysis
Control	0.	28.8	0
Experimental (in air)	1.0	22.4	−22.2
Experimental (in N_2)	1.0	28.0	− 2.8
Experimental (with $NaHSO_3$)	1.0	28.1	− 2.1

[a] Significant at the 5% level based on an average of six analyses.

and washed with 5% TCA into 100 ml volumetric flasks The filtrates were used for α-amino nitrogen determinations with the standard Van Slyke apparatus.

Preparation of Insulin-ML Samples. Three-milliliter aliquots (estimated to contain 60 mg of insulin) of Lilly U-500 zinc insulin solution were placed in interjoint boiling flasks. The amount of 50 μl of ML was added to the flasks designated as experimentals, while nothing was added to the controls. Both control and experimental samples were shell-frozen and freeze-dried. The dried samples were oxygenated, stoppered, and placed in an oven at 50C. At the end of five days the samples were removed from the oven and both control and autoxidized insulins were ether extracted before further treatment with chemical agents.

Hydrolysis of Insulin. Control and experimental insulins, after ether extraction, were hydrolyzed with 10 ml of either 6N HCl or 14% $Ba(OH)_2$ by refluxing for 20 hr. The hydrolysates were then prepared for paper chromatographic analysis according to the method of Block et al. (6).

Paper Chromatographic Technique. Control and experimental hydrolysates were carefully spotted on Whatman No. 1 paper and dried. The paper was treated with ammonia vapors and placed in the Chromatocab. Butanol:water:acetic acid (250:250:60) was used as the developing solvent (6). Color development was accomplished by spraying with ninhydrin (0.2%) in acetone, and duplicate chromatograms were dipped in 0.2% isatin in acetone containing 4% acetic acid.

N-Terminal Amino Group Determinations. Control and lipid oxidized insulins were reacted with FDNB to tag the free N-terminal and ϵ-amino groups of the protein according to the method of Sanger (4). The resulting dinitrophenyl (DNP)-insulins were hydrolyzed with 6N HCl for 6 hr and then extracted with ether to separate amino acids and DNP derivatives. Both the ether and water phases were evaporated in vacuo and prepared for chromatographic analysis. The water phase was developed in butanol:water:acetic acid as described above and the ether-soluble derivatives were developed in benzene: 1% aqueous acetic acid (1:1) (Ref. 7).

Hydrogen Fluoride Solubility Test. The technique of Katz (8) as modified by Bjorksten et al. (5) was used as a qualitative test for cross-linking of both insulin and gelatin after reaction with autoxidizing ML as described earlier.

Discussion and Results

Gelatin-ML Reactions

The interreaction of autoxidized lipid with proteins can be demonstrated by the liberation of nitrogen through an enzyme approach. In a protein, such as gelatin, the hydrolysis rate with trypsin is fairly constant. An alteration of the protein, whether it be in the form of alkylation, denaturation, or cross-linkage, will alter the rate of enzyme action. If, after incubation with autoxidizing ML, a measurable change in hydrolysis rate (either higher or lower) is found, it may be taken as an indication of protein-lipid interaction. In general, alkylated and denatured proteins are more easily attacked by proteolytic enzymes than their native counterparts (9). Cross-linked proteins, on the other hand, resist hydrolysis by proteolytic enzymes (10).

The results in Table I show the influence of the autoxidizing ML on the digestibility of gelatin foams with trypsin.

It may be noted that gelatin-ML foams after 5 days' incubation in air at 50C become considerably resistant to hydrolysis by trypsin. The data also indicate that the interaction requires oxygen, since little inhibition of hydrolysis was noted in foams incubated under nitrogen. The addition of $NaHSO_3$ to model foams effectively inhibits the interaction between gelatin and autoxidizing ML. Since $NaHSO_3$ forms an addition product with aldehydes it is quite likely that lipid-derived carbonyls are preferentially complexed with this salt and the protein is thus unaltered.

Insulin-ML Reactions

In preliminary studies of the insulin interreaction with autoxidizing ML, a biological assay method was used to follow the course of the reaction. It was observed in previous work that insulin loses biological activity and becomes quite insoluble in acids as autoxidation proceeds (11). However, when insolubilized material was hydrolyzed in 6N HCl and compared chromatographically with control insulin, the amino acid compositions were found to be identical. Chromatographic comparisons of control and experimental (insolubilized) alkaline hydrolysates, on the other hand, revealed that appreciable amounts of lysine are lost as a result of autoxidation. Since amino acid-aldehyde complexes are acid-unstable but base-stable under reflux, and amino acid-epoxy complexes are acid-stable but base-unstable, the results of the hydrolysis studies suggested that lipid-derived aldehydes were reacting with the protein.

Hydrolyses, either acid or basic, are rather severe methods and as such may break down many of the amino acid-aldehyde complexes. Because of this it was decided to attack the problem indirectly by using the FDNB technique.

In the Sanger technique the free amino and hydroxyl groups of the protein are reacted with FDNB. The resulting bright yellow N-dinitrophenyl (DNP)-amino acid derivatives are relatively stable in strong acids, are ether-soluble with the exception of DNP-lysine, and are easily identified chromatographically after acid hydrolysis. The reaction is shown below:

$$R\text{–}NH_2 + FDNB \longrightarrow RNH(DNP) \xrightarrow[\text{Reflux}]{HCl}$$

DNP–amino acids + amino acids

If pure insulin is reacted with FDNB, acid hydrolyzed, then ether extracted to remove DNP derivatives, and finally paper chromatographed, no spot is found for lysine in the aqueous phase, although a bright yellow spot for ϵ-DNP-lysine is seen. Likewise, appreciably less phenylalanine and glycine are found than in pure insulin hydrolysates. On the other hand, insulin which has been in intimate contact with the autoxidizing ML prior to FDNB treatment will not react with the reagent since the reaction sites have already re-

acted with the oxidation products. Subsequent hydrolysis in strong mineral acid then will liberate free amino acids as follows:

$$\text{Protein–NH–CRHOH} + \text{FDNB} \xrightarrow{\text{HCl}} \underset{\text{(amino acids)}}{\text{R–NH}_2} + \text{dinitrophenol} + \text{RCHO}$$

Therefore, by indirection, under relatively mild conditions the sites of ML interaction within the protein molecule may be determined by comparing the missing amino acids on the control samples with those present in the autoxidized material.

Chromatographic separation of the amino acids in the aqueous phase of acid hydrolyzed DNP-insulins clearly indicated that lysine was primarily involved in the insulin-ML interaction. Lysine was clearly evident in the experimental sample but not in the control. It was also noted that in the experimental sample no water-soluble DNP derivative was found, while in the controls a very sharp bright yellow spot was found at Rf = 0.70. This spot was cut from the chromatogram, hydrolyzed with $Ba(OH)_2$ to regenerate the parent amino acid, and was identified chromatographically as lysine. ϵ-DNP-lysine was then synthesized and its Rf value was determined in the partitioning solvents mentioned above. The synthetic derivative gave a bright yellow spot Rf = 0.70, thus confirming the involvement of the ϵ-amino group of lysine in lipid-protein interactions.

Chromatographic separations of the ether extracts of DNP-insulin hydrolysates were also studied. These derivatives were spotted on paper and then chromatographed with benzene:1% acetic acid solution. This solvent separates DNP-glycine (Rf = 0.07) from DNP-phenylalanine (Rf = 0.70). Both DNP-amino acid derivatives were detected in the insulin controls, but not in the experimentals (lipid oxidized), thereby indicating that N-terminal amino acids were also involved in the protein-lipid interaction.

Since the reactions of lipid intermediates with proteins may be pH-dependent, it was necessary to conduct the end group analyses in both acid (pH2–3) and alkali (pH~9). Although it was expected that such widely diverse conditions would considerably affect the N-terminal lipid-protein interaction, the results obtained under alkaline conditions were quite similar to those obtained from acid-solubilized insulin.

In addition to free amino groups, insulin contains an appreciable number of carboxyl, phenolic, guanido, imidazole, amido, hydroxyl and disulfide groups, any one of which could potentially interreact with products of autoxidized lipid. Chromatograms of insulin hydrolysates, however, did not indicate that any stable derivatives involving these groups had resulted during autoxidation. Although time did not permit a complete examination of all reactive groups, attempts were made to obtain data on the possible involvement of the carboxyl and guanidine groups of insulin since known methods may be applied to such study.

The Bradbury modification (12) of the Akabori hydrazinolysis method (13) was employed to determine whether or not the C-terminal residues of acid or alkaline insulins react with ML intermediates. Hydrazinolysates of both acid and alkaline insulin samples were found to contain the C-terminal amino acids in free form when chromatographed on paper. This indicated that the C-terminal groups are not important sites in the insulin-ML interaction under the autoxidizing conditions employed.

The qualitative Sakaguchi reaction (14) used as a measure of guanidine interaction did not indicate a chemical alteration of this group; results from autoxidation for both acid- and alkaline-derived insulins gave positive reactions for unaltered guanidine groups. The results, therefore, suggest that the guanidine group of arginine is not involved in the insulin-ML interaction.

Because of the rather restricted reaction conditions used in the insulin studies, an apparent absence of reaction with active hydrogen groups, aside from that of the free amino groups, is not surprising. Aldehyde reactions with hydroxyl, thiol and other hydrogen groups, for example, would normally be studied in aqueous solution rather than in anhydrous systems such as those used here (15). Quite likely the presence of water and prooxidants would influence the types of interaction within a protein molecule.

Solubility Studies Using Anhydrous Hydrogen Fluoride

The results in both the trypsin assay and insulin studies suggested that lipid-derived aldehydes covalently cross-link gelatin under the test conditions. In cross-linking reactions the molecular weight of the protein greatly increases and the resulting polymer is generally quite insoluble in the usual protein solvents. Because of this insolubility it is not possible to use classical methods to determine increases in molecular weight as a proof that cross-linkage has occurred. It has been shown, however, that anhydrous hydrogen fluoride dissolves a large number of native and acylated proteins while their firmly cross-linked counterparts do not dissolve (5). By indirection, therefore, insolubility of a protein in HF can be used as a test for covalent cross-linkage.

Both insulin and gelatin were found to be readily soluble in the solvent, while after reaction with autoxidizing ML they were quite insoluble. On the basis of the trypsin hydrolysis and HF solubility tests it was concluded that, in the autoxidation of ML, reactive intermediates arise which insolubilize proteins via a cross-linking reaction.

ACKNOWLEDGMENT

This paper reports on research studies undertaken in cooperation with the U.S. Army Natick Laboratories, and has been assigned number 2202 in the series of papers approved for publication. The conclusions contained in this report are those of the authors, and are not to be construed as reflecting the views of the Department of Defense.

O. K. Behrens of Eli Lilly & Co. provided us with crystalline zinc insulin.

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[Received January 25, 1965—Accepted April 8, 1965]