EVIDENCE FOR AN ESSENTIAL METHIONINE RESIDUE IN LIPOXYGENASE

Rina ZAKUT, Shlomo GROSSMAN, Alex PINSKY and Meir WILCHEK
Department of Life Sciences, Bar-Ilan University, Ramat-Gan, and Department of Biophysics, The Weizmann Institute of Science, Rehovot, Israel

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1. Introduction

Soybean lipoxygenase (linoleate: O_2 oxidoreductase, EC 1.13.11.12) catalyzes the hydroperoxidation of polysaturated fatty acids interrupted by cis-methylene [1]. The enzyme was first obtained by Theorell [2] who reported that lipoxygenase did not require a metal or other prosthetic group, since it was not inhibited by compounds that bind to prosthetic groups or metals. Stevens et al. [3] purified the enzyme to homogeneity and found that on treatment with guanidine hydrochloride the protein dissociates into two identical subunits of 54 000 mol. wt each. Recently it was reported that the enzyme indeed contains one atom of iron per molecule and that the metal is essential for enzymatic activity [4–6]. In order to define the functional amino acid residues in the active center of lipoxygenase, we have undertaken chemical modification studies of this enzyme.

Reactive compounds structurally related to substrates or specific inhibitors of enzymes have been used in many investigations for the study of active sites [7]. In favorable cases such reagents enter the active sites of the enzymes and react specifically with nearby amino acid residues. If partial or complete loss of activity occurs, the involvement of the modified residue in the catalytic process of the enzyme can often be inferred.

In the present study iodoacetic acid has been used to probe the active site of lipoxygenase. Iodoacetic acid is not a substrate analog; nevertheless it inhibits this enzyme by binding specifically to a methionine residue at or near the active site.

2. Materials and methods

2.1. *Preparation*

[*S-Carboxymethyl*-^{14}C]methionine was prepared according to Gundlach et al. [8,9], using [^{14}C]iodoacetic acid. [^{14}C]iodoacetic acid was a product of Amersham Lab.

[*Carboxymethyl*-^{14}C]histidine was prepared according to Crestfield et al. [10] using [^{14}C]iodoacetic acid.

Purified preparation of soybean lipoxygenase according to Grossman et al. [11] was used in this study.

2.2. *Enzyme activity*

Lipoxygenase activity was determined spectrophotometrically (Coleman-spectrophotometer 124 D-Double Beam) by following the formation of conjugated dienes at 234 nm according to Ben-Aziz et al. [12]. The substrate used was linoleate at a concentration of 6×10^{-4} M in 0.2 M phosphate buffer, pH 6.5.

2.3. *Protein determination*

Protein concentration was determined by measuring the absorption at 280 nm assuming that 1.0 A_{280} is equivalent to a concentration of 1 mg/ml protein.

2.4. *Reaction with iodoacetic acid*

The alkylating agents were added to the buffered enzyme solution at 25°C in the dark and samples were taken at different times. In order to identify the modified amino acids, lipoxygenase at a concentration of 1.5×10^{-6} M was reacted with 10^{-3} M [^{14}C]iodo-

acetic acid (1.5 μCi) in 0.05 M sodium acetate buffer, pH 3.5, and was incubated for 8 h. The alkylated enzyme was dialyzed against distilled water, changing the dialyzing medium several times until no counts were detected.

The dialyzed enzyme was lyophilized and then hydrolyzed by 6N HCl at 105°C for 24 h. A sample of the hydrolyzate was applied to an amino acid analyzer connected to a flow cell to count radioactivity. Another sample was determined by high voltage paper electrophoresis, at pH 3.5, 3000 V for 60 min. After drying the paper, strips were cut and the radioactivity was counted.

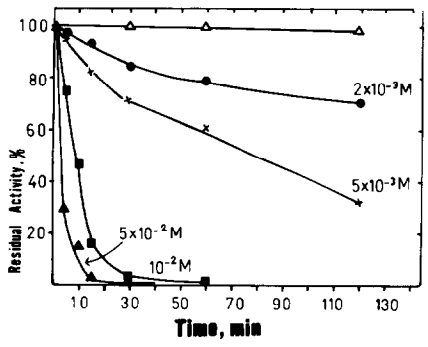

Fig.2. Rate of inactivation of lipoxygenase by iodoacetic acid. The oxidation of linoleate by $1.5.10^{-6}$ M lipoxygenase was followed at pH 3.5 using 0.05 M sodium acetate buffer. (△——△) control.

3. Results

3.1. *Inhibition of lipoxygenase by iodoacetic acid*

The alkylation of nucleophilic amino acid residues by iodoacetate is pH dependent. Therefore lipoxygenase was incubated at different pH values for 2 h. The pH profile of the inhibition of lipoxygenase by iodoacetate and iodoacetamide is shown in fig.1. From this figure it can be seen that complete inactivation of the enzyme is obtained at pH 3.5. Under the same conditions no inhibition was observed with iodoacetamide, indicating that the carboxyl group of iodoacetic acid is essential for the interaction.

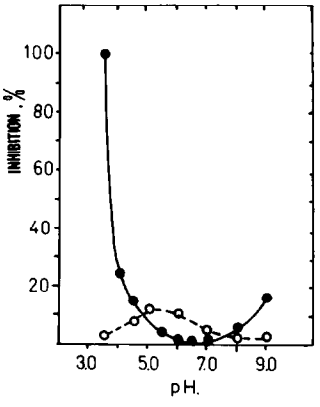

Fig.1. pH profile of the alkylation and inhibition of soybean lipoxygenase by iodoacetate (•——•) and iodoacetamide (o——o). Concentration of 10^{-2} M of the inhibitors were added to $1.5.10^{-6}$ M of the enzyme and after 2 h incubation at 25°C the oxidation of linoleate by the treated enzyme was followed. 0.05 M buffer concentrations of the following buffers were used: Sodium acetate pH 3.0–5.5; Sodium phosphate pH 6.0–7.0; Tris–HCl pH 8.0–9.0.

The rate of inactivation of the enzyme is dependent on the incubation time as well as on the concentration of iodoacetic acid. It can be seen from fig.2 that at 10^{-2} M iodoacetic acid, linoleate oxidation by lipoxygenase is more than 90% inhibited after 30 min incubation. The amount of iodoacetate bound to the enzyme was determined using [2-^{14}C]iodoacetic acid. On complete inhibition of the enzyme about 1.2 mol iodoacetate was bound per mole of enzyme, implying that approximately one amino acid residue combined with the reagent.

The finding that the optimum pH for inhibition by iodoacetic acid is about 3.5 indicates that a histidine, methionine or a carboxylic group may have reacted. In order to test which of the above-mentioned residues was modified, lipoxygenase was alkylated with [^{14}C] iodoacetic acid. After dialysis and lyophilization the enzyme was hydrolyzed and its amino acid composition was determined by an amino acid analyzer, with an attached flow cell to measure radioactivity. The radioactivity was detected in the region where proline is usually eluted. This region corresponds with the elution patterns of *N-carboxymethyl*-histidine and *S-carboxymethyl*-homocysteine, a derivative of methionine. In order to distinguish between these two possibilities, high voltage paper electrophoresis was performed and the migration of the various compounds was compared. It was found that the main peak of radioactivity migrated together with

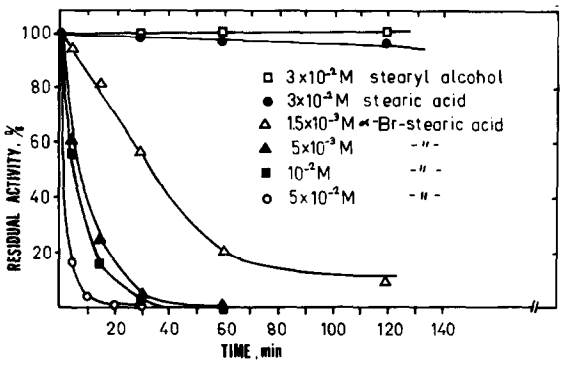

Fig.3. Rate of inactivation of lipoxygenase by α-bromostearic acid, stearyl alcohol and stearic acid. The enzyme concentration was $1.5.10^{-6}$ M and the activity was followed as described in Materials and methods.

S-carboxymethyl-homocysteine, which indicates that methionine was modified.

3.2. *Inhibition of lipoxygenase by α-bromostearic acid*

Since iodoacetic acid is not a substrate analog we decided to check the inhibition of the enzyme with an analog of stearic acids which is a competitive inhibitor of the enzyme. α-Bromostearic acid was found to inhibit the enzyme irreversibly at pH values between 3.5–9. The rate of inhibition is shown in fig.3. At a concentration of 5×10^{-3} M the α-bromostearic acid caused 95% inhibition of the lipoxygenase activity after 30 min, whereas no inhibition was observed with stearic acid or stearyl alcohol. The rate of inhibition of the enzyme with α-bromostearic acid was faster than with iodoacetic acid and required ten times less reagent. The amino acid modified was not established since we could not prepare α-bromostearic acid.

4. Discussion

The mechanism of action of lipoxygenase is not yet known. Recent work has shown that iron is involved in the enzymatic activity [4–6]. The data described in this work provide some further information on the active site of the enzyme and the contribution of individual amino acid side chains to the enzymatic activity. Using ^{14}C-labelled iodoacetic acid we were able to demonstrate that one methionine residue out of 17 residues was modified per protein with concurrent loss of activity. One possible explanation implicates methionine directly in the catalytic process and its participation in the free radical mechanism suggested by Dolev [13,14]. It is also possible that a methionyl residue functions as a nucleophilic moiety and thus interferes in the production of free radicals.

Another alternative is that alkylation of methionine may alter structures other than those in the active sites, and the resulting modified enzyme may change its conformation to a sufficient, although small, degree such that the active site structure is disrupted. However, the one-to-one relationship between the degree of inactivation and the extent of modification of methionine indicates strongly that methionine is involved directly in the catalytic process or acts by sterically blocking the active site.

It is clear from fig.1 that the carboxylic group in the alkylating reagent is essential for the modification of the enzyme, since iodoacetamide has no effect. It may indicate that the carboxylic group, analogous to the same group in the natural substrate (linoleic acid), has special affinity to the enzyme. This finding contradicts some of the conclusions drawn by Mitsuda et al. [15], that the hydrophobic fraction of the substrate is essential for the oxidation by lipoxygenase and that the nature of the polar group is less important. Our observations, however, are in agreement with the results obtained by Allen [16], who claims that the substitution of the polar group of the substrate reduces lipoxygenase activity.

The data presented in fig.3 demonstrate that α-bromostearic acid is an affinity label for lipoxygenase and inhibits it by covalent binding. Neither the amino acid residue in the protein (to which the inhibitor was bound), nor the ratio of binding could be determined, since it was impossible to obtain this inhibitor radioactively labeled. We assume that a methionyl residue is also alkylated by this reagent, in a manner similar to that observed with iodoacetic acid.

This study suggests that a single methionyl residue is involved in the activity of soybean lipoxygenase. The exact location of this methionine residue will be possible only after sequencing the protein.

References

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