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# Activation of Phospholipase $A_2$ by Long Chain Fatty Acyl Groups Involves a Novel Unstable Linkage

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**The acidic isoform of phospholipase $A_2$ from *Naja mossambica mossambica* was activated by treatment with a molar equivalent of oleoyl imidazolide. Modification of the protein was accompanied by 50% quenching of tryptophan fluorescence and a significant red shift. The $^3$H(9,10) labeled oleoyl residue was co-eluted with the enzyme during gel filtration in the presence of 20% 1-propanol or excess albumin, both of which remove free oleic acid from the enzyme. In contrast, the adduct was labile as to electrophoresis on SDS-PAGE and acid or alkali urea PAGE. The formation of a covalently linked adduct was demonstrated by electrospray mass spectrometry in the presence of 2% formic acid. No such adduct was formed by the phospholipase $A_2$ isoform from *Naja naja atra*, which differs in sequence from the *N. mossambica mossambica* isoform by seven residues including 2 histidine residues and 1 lysine residue. We conclude that oleoyl imidazolide activates the *N. mossambica mossambica* enzyme by forming an acyl adduct which is unstable as to protein denaturation. The magnitude of tryptophan fluorescence quenching indicates that the site of acylation lies in the sequence WWHF.**

**Key words: activation, acylation, electrospray mass spectrometry, phospholipase $A_2$, tryptophan fluorescence.**

Long-chain fatty acyl residues are frequently added post-translationally to proteins (*1–4*). They were first identified as stable amide adducts for N-terminal glycine residues and subsequently as relatively labile thioesters attached to cysteine residues (*5–7*). There is evidence that some members of the very broad class of phospholipase $A_2$ enzymes can undergo acylation of lysine side chain residues as a rare consequence of a catalytic reaction with either physiological or artificial substrates (*8–10*). It is clear that this modification does not lie on the normal reaction pathway because the derivatives are stable and prevent degradation of the protein. There is also evidence that acylation activates the enzyme, but the kinetics of generation of the activated state show that protein acylation is not tightly coupled to substrate hydrolysis (*9*) and must therefore represent a leaky reaction pathway. van der Weile and co-workers showed that pancreatic phospholipase $A_2$ can be activated through specific chemical acylation of two lysine residues, at least one of which is implicated in substrate binding (*11*, *12*). They presented a model in which the acyl residue acted as a hydrophobic anchor, penetrating the lipid surface and increasing the lytic power of the enzyme, though not its catalytic activity. We have shown that $PLA_2$ enzymes are strongly activated by free long-chain fatty acids (*13*) and further that for a sub-group of these enzymes, the effect of a fatty acid can be achieved by incubating the enzyme with a weakly activated long-chain fatty acid derivative. In contrast to fatty acid activation, which is instantaneous, only achieved at a relatively high concentration and obviated by the presence of a dilute organic solvent or albumin, activation by a fatty acid derivative (typically oleoyl imidazolide) is slow, achieved with a 1:1 molar ratio of reagent to protein and stable as to organic solvents or albumin. This is compelling evidence that activation of the enzyme is a consequence of protein acylation. It is also of interest that bee venom $PLA_2$ can be activated through limited fixation with glutaraldehyde in the presence, but not the absence of long chain fatty acids, and remains activated even after the fatty acid has been removed from the protein (*13*). The data suggest that the binding site for the fatty acid acyl group lies within the protein and that activation is, a least in part, due to a conformation change. In contrast to the known stable acyl-modification of proteins, a derivative formed through treatment of phospholipases with acyl imidazolides does not survive reduction and carboxymethylation, the mild preliminary procedures required before analytical degradation of the protein. In the present paper, we provide evidence that $PLA_2$ enzymes are activated through a novel unstable acylation.

## EXPERIMENTAL PROCEDURE

*Naja mossambica mossambica* and *Naja naja atra* venoms were purchased from Sigma Chemical, Dorset, UK. Bio-gel P-30 was from Bio Rad, and [$^3$H]oleic acid from Amersham Life Science, and all other chemicals were of analytical

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Abbreviations: $PLA_2$, phospholipase $A_2$, SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

grade. The acidic isoform from the venom of *N. mossambica mossambica* and the major isoform from the venom of *N. naja atra* of phospholipase $A_2$ enzymes were purified according to the protocol described previously (*14*, *15*). $PLA_2$ activation was determined by conductimetric estimation of the sublytic leakage of electrolytes from mouse erythrocytes (*14*).

Free [$^3$H]oleic acid was separated from $PLA_2$ by gel filtration on a column (1 × 16 cm) of Bio-gel P-30 equilibrated with 10 mM triethanolamine buffer, pH 8.0, containing 20% aqueous 1-propanol. $PLA_2$ (100 μg) was incubated in 100 μl of 10 mM triethanolamine buffer, pH 8.0, in the presence of a molar equivalent of either [$^3$H]oleic acid or [$^3$H]oleoyl imidazolide for 2 h. The sample was then applied to the gel filtration column in either the presence or absence of an 8-fold molar excess of albumin. Elution was performed at 0.1 ml/min and 0.5 ml fractions were collected. The enzyme, albumin and fatty acid were eluted with the same solution, and all fractions were assayed for radioactivity and for $PLA_2$ activity. Acid/urea and basic/urea gels were prepared as described (*14*).

Fluorescence measurements were made at 25°C with a Perkin-Elmer LS 50 spectrofluorimeter.

For mass spectrometry, the acidic isoform of phospholipase $A_2$ was activated (*14*) and then dialysed extensively against 3 changes of 2 litres of water using a 12,000 Da molecular mass cut-off dialysis membrane (Sigma Chemical). $PLA_2$ samples, both native and activated (1 mg/ml), were prepared in 2% aqueous formic acid and analysed with a Micromass Platform II electrospray mass spectrometer.

## RESULTS

The venom $PLA_2$–catalysed hydrolysis of purified egg phosphatidyl choline in 20% 1-propanol shows sigmoidal progress curves which are, in most cases, rendered hyperbolic by >100 μM oleic acid (concentration ratio, 1:100). In the case of bee venom enzyme or the acidic isoform from *N. mossambica mossambica*, equivalent activation is also achieved by preincubation of the enzyme with a 1:1 molar ratio of oleoyl imidazolide, but not with free oleic acid (Fig. 1). The oleoyl imidazolide modified enzyme is highly lytic for erythrocytes in the presence of albumin at a concentration that totally prevent activation of free fatty acids, thus providing a sensitive and specific assay for the presumed modification. Gel filtration in 20% aqueous 1-propanol was then performed to compare the strengths of association of [$^3$H]oleic acid and [$^3$H]oleoyl imidazolide with the enzyme from *N. mossambica mossambica* venom, and the results confirmed that the oleoyl moiety derived from imidazolide (added to the enzyme in a 1:1 molar equivalent amount) is much more strongly associated with the protein than an equivalent concentration of the free acid. The activation exhibited the progressive nature of a chemical reaction and the modified protein was stable as to dilute organic solvents and also to albumin, which was necessary for expression of lytic activity in the erythrocyte leakage assay. Radiolabelling experiments involving [$^3$H]oleic acid showed that free oleic acid could be separated from the native enzyme by gel filtration in 20% aqueous 1-propanol (Fig. 2A). However, when the enzyme was activated with [$^3$H]oleoyl imidazolide the activity was co-eluted with the major peak of radioactivity in 20% 1-propanol (Fig. 2B), and only a minor fraction of the radioactivity co-migrated with albumin when it was included with the protein sample (Fig. 2D). These experiments confirmed that the native enzyme exhibited low affinity for free fatty acids, but the oleoyl residue became strongly bound when the enzyme was activated with oleoyl imidazolide, thus lending support to possible acylation of the protein. In this study we performed SDS-PAGE and variants of urea-PAGE which exhibit higher resolving power than SDS-PAGE for these enzymes (*16*), and with which enzymic activity can be recovered in high yields. We have shown elsewhere that acid-urea PAGE, which detects the addition of a single acetyl group to a enzyme, does not resolve the native and oleoyl imidazolide–activated forms of the *N. mossambica mossambica* isoform. The basal enzymic activity of the normal and treated enzymes could be recovered in high yields from gel slices after either acid-urea or alkaline-urea PAGE (*14*). However, the activation recovery was poor and the ratio of lytic to hydrolytic activity was very similar for the treated and untreated enzymes. In contrast, electrophoresis of the enzyme activated by [$^3$H]oleoyl imidazolide showed conclusively that the bulk of the radiolabel was recovered as free oleic acid. Thus, in the acid-urea gel, the major peak of radioactivity was found near the origin and a negligible amount co-migrated with the protein band (Fig. 3b). Under alkaline-urea conditions the major peak of radioactivity was found at the electrophoresis front, well separated from the enzymic activity (Fig. 3a). Similarly, on SDS-PAGE the radioactivity was found at the electrophoresis front and well clear of the major protein band (Fig. 3c). The fact that the peak of radioactivity was extremely sharp in both the latter cases indicated that the residue was released from

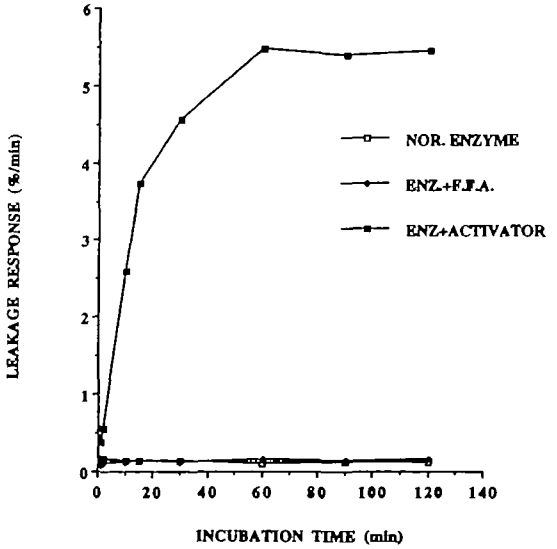


Fig. 1. **Activation of phospholipase $A_2$.** Purified phospholipase $A_2$ (100 μg) was incubated with a molar equivalent of either free oleic acid (FFA) or oleoyl imidazolide (activator) in 10 mM triethanolamine buffer, pH 8.0, for two hours at 37°C. Two-microliter samples were withdrawn from three Eppendorf tubes (one control and two treated) at different times and activation was examined by conductimetric analysis of mouse erythrocytes in the presence of 10 μM bovine serum albumin as described under "MATERIALS AND METHODS."

the protein very early in the procedure.

We then attempted to determine the effects of fatty acids and imidazolides on the protein conformation by means of fluorescence measurements because of possible involvement of the sequence WWHF in the *N. mossambica mossambica* enzyme. The fluorescence measurements were carried out to see if activation perturbed the tryptophan environment. Firstly, emission spectra of the normal and activated enzymes were compared; it is clear (Fig. 4A) that on activation there is a significant decrease in fluorescence intensity and a marked red shift. Unexpectedly part of the decrease was found to be due to the acetone used as the solvent for the activator. In contrast, acetonitrile had no effect on the fluorescence emission (not shown), and when it was used as the solvent for the imidazolides the decrease and red shift effects were still observed. In order to determine the specificity of the activator (oleoyl imidazolide) the acidic isoform of $PLA_2$ was treated with different molar concentrations of dimethyl maleic anhydride, and then the fluorescence spectra of the native and activated forms were compared; however, in this case there was an increase in the fluorescence intensity and a very slight blue shift (Fig. 4B). No changes in the fluorescence emission were observed when the enzyme was treated with short chain fatty acids or imidazolide derivatives (not shown). Furthermore, in order to characterize the nature of the group which binds the activator, an extensive kinetic study of pH-dependent activation was carried out. Firstly, we studied the rate of activation over the pH range of 6–9 using bis-tris or borate buffers, measuring the ability of the enzyme to increase the rate of erythrocyte leakage. Secondly, we examined the effect of pH on the rate of change in tryptophan fluorescence. Activation was very slow below pH 6.0, but in both experiments a 2–3-fold increase in the rate was observed between pH 7 and 8. However, the rate decreased very rapidly above pH 8 and activation was virtually indetectable at pH 9.0. This is the classic behaviour expected for the environment of two ionising groups, one in its protonated form and one in its non-protonated form.

We now attempted to characterize the adduct by electrospray mass spectrometry to determine whether or not an acyl adduct was formed; we obtained a clear-cut result. The mass of native $PLA_2$ was found, on electrospray mass spectrometry, to be 13,204.6 ± 0.77 Da (calculated 13,204.86 Da after loss of 14 hydrogens on the formation of 7 disulfide bonds, confirming the identity and purity of the acidic isoform). After activation with oleoyl imidazolide, only one major species with a mass of 13,468.4 ± 1.27 Da was ob-

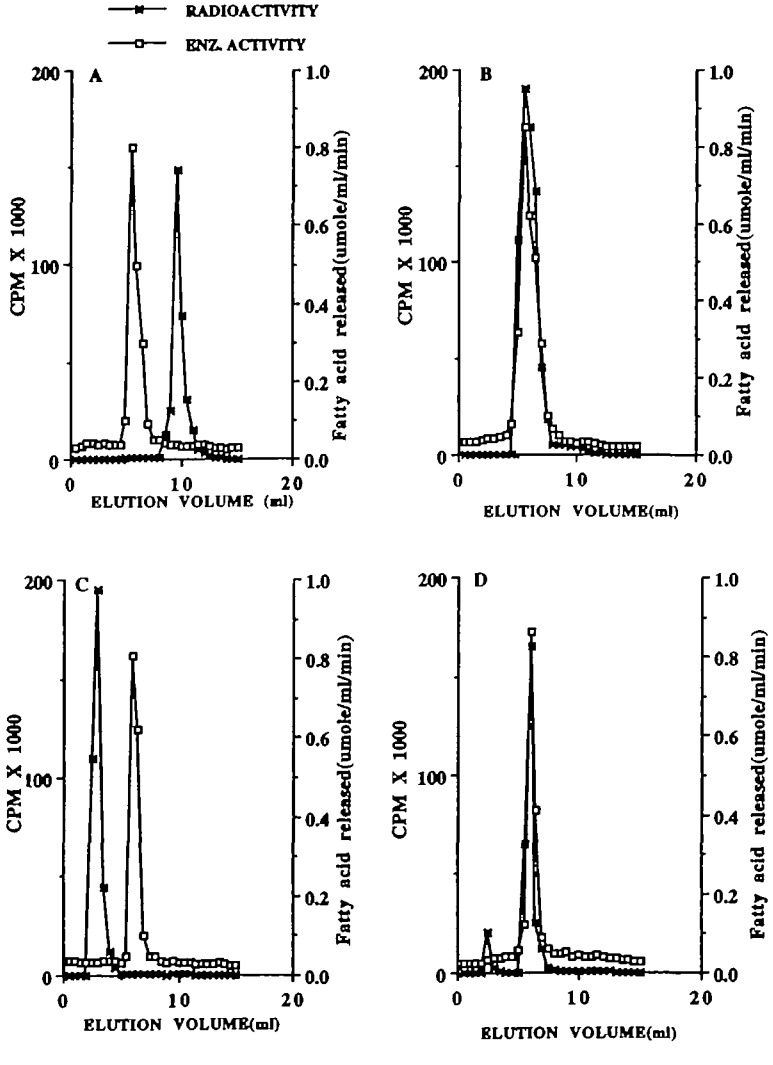


Fig. 2 **Gel filtration chromatography of native and activated $PLA_2$.** Purified $PLA_2$ (100 μg) was incubated with a molar equivalent of [$^3$H]oleic acid and [$^3$H]oleoyl imidazolide as described under "MATERIALS AND METHODS." (A) Enzyme incubated with [$^3$H]oleic acid. (B) Enzyme incubated with [$^3$H]oleoyl imidazolide. (C) Enzyme incubated with [$^3$H]oleic acid, and an 8-fold molar excess of albumin was added before application to the column. (D) Enzyme incubated with [$^3$H]oleoyl imidazolide, and an 8-fold molar excess of albumin was added before application to the column.

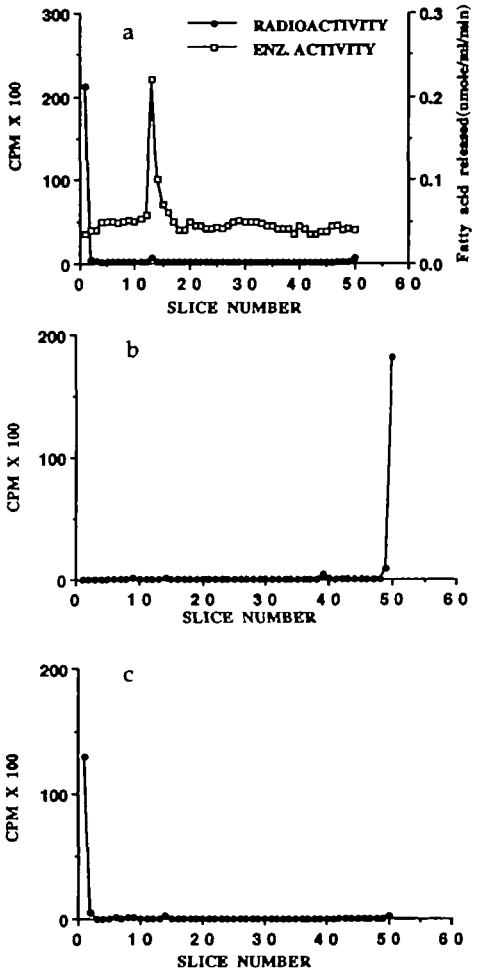


Fig. 3 **Gel electrophoresis of activated $PLA_2$.** Activated $PLA_2$ obtained from the gel filtration column (Fig. 2A) was run in all three types of gel on two lanes, *i.e.* experimental and guide lanes. Each experimental lane was cut into 1–2 mm slices starting from the dye front and the enzyme was extracted as described under "MATERIALS AND METHODS." Radioactivity was determined in all cases, but in case of the basic/urea gel enzymic activity was also determined. (a) Basic/urea gel, slice number 13 indicates the position of the enzyme. (b) Acid/urea gel, slice number 39 indicates the position of the enzyme. (c) 10 % SDS gel, slice number 14 indicates the position of the enzyme.

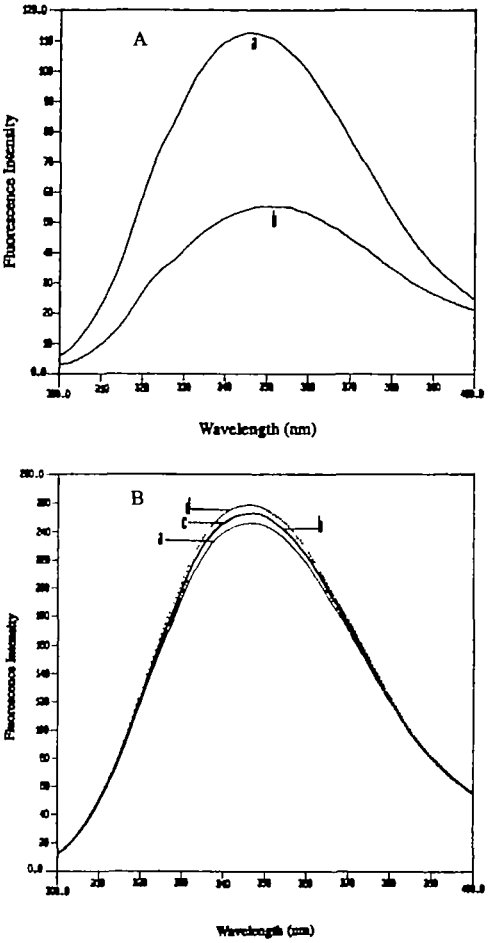


Fig 4 **Fluorescence spectroscopy of native and activated $PLA_2$.** (A) The acidic isoform of $PLA_2$ (0.1 mg/ml) was treated with one molar equivalent (8 μM) of oleoyl imidazolide and then fluorescence measurements were made at 25°C over the range of 300–400 nm. Samples were excited at 290 nm (a) Native enzyme, and (b) treated enzyme. (B) The acidic isoform of PLA2 (0 1 mg/ml) in 10 mM borate buffer, pH 8 0, was divided into four equal fractions, three of which were treated with different molar concentrations of dimethyl maleic anhydride. Fluorescence spectra of native $PLA_2$ (a), and the enzyme treated with one (b), two (c), and three (d) molar equivalents were recorded over the range of 300–400 nm Samples were excited at 290 nm

served. The mass difference between the native (Fig. 5A) and activated $PLA_2$ (Fig. 5B) is 263.8 ± 1, which is consistent with the addition of a single oleoyl residue (265 Da *cf.* oleic acid 282 Da).

## DISCUSSION

The $PLA_2$ enzymes from *N. naja atra* and the acidic isoform from *N. mossambica mossambica* differ by only 7 residues; most significantly, Q10 becomes H10, D20 becomes H20, and S33 becomes K33. Both enzymes are activated as to the hydrolysis of long chain phosphatidyl choline derivatives in dilute organic solvents by the long chain fatty acid reaction products. This behaviour is typical of almost all venom $PLA_2$ enzymes. Furthermore, a long-chain fatty acid binding site can be titrated by measuring the reduction in Trp fluorescence. In the case of the *N. mossambica mossambica* enzyme, the reagent oleoyl imidazolide reacts relatively slowly with the enzyme (half-life, *ca.* 5 min.) to activate it in a number of assays. This reaction is accompanied by an even larger decrease in Trp fluorescence than reflects free fatty acid binding. Furthermore, it takes place with a 1:1 molar equivalent of reagent as to protein, and the changes generated are stable as to dilute organic solvents and high concentrations of albumin, which remove all traces of free fatty acids. From this and other evidence we have postulated that all of these enzymes contain an allosteric long-chain fatty acid activation site, but that the *N. mossambica mossambica* isoform and several other such enzymes possess a modified fatty acid binding site which is capable of being acylated by activated fatty acid derivatives, most notably long-chain imidazolides.

Full characterization of the phenomenon requires that

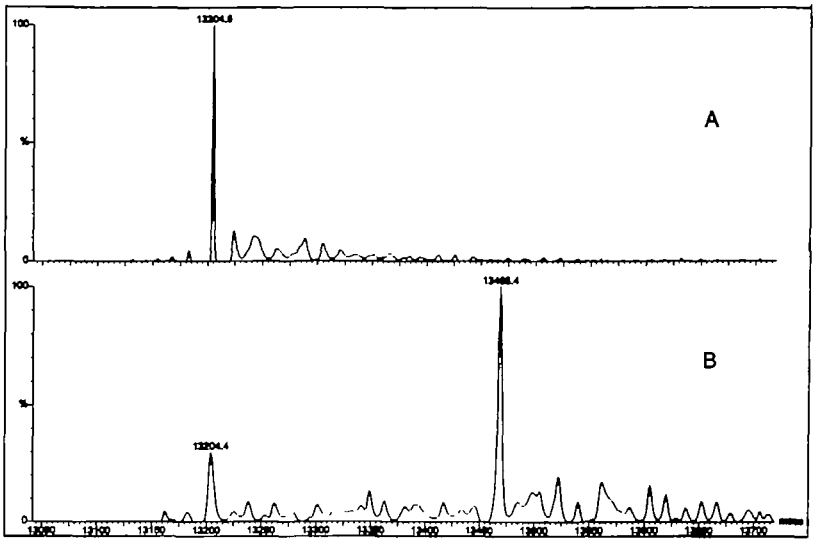


Fig. 5. **Electrospray mass spectra of native and activated phospholipase $A_2$.** The acidic isoform of phospholipase $A_2$ was activated as described under "MATERIALS AND METHODS," and analysed by electrospray mass spectrometry in aqueous 2% formic acid. (A) Native enzyme and (B) activated enzyme

the site of acylation of the protein is known, and this information has not been forthcoming, because although the activated enzyme is very stable under near physiological conditions it loses the oleoyl group very rapidly under the denaturing conditions for all types of electrophoresis tested. (It should also be emphasised that this modification is stable as to prolonged catalytic activity of the enzyme and does not therefore lie on the reaction pathway). This has precluded any direct attempt to determine the activation site. The present work shows, for the first time, that a covalent adduct is formed and that its mass (13,204.6 ± 0.77 Da becomes 13,468.4 ± 1.27 Da) corresponds to the addition of an oleoyl group, and not the unchanged reagent or free oleic acid. It also constitutes very convincing evidence of the instability of the adduct.

Of the protein side chains susceptible to fatty acylation, primary amino-groups and hydroxyl groups give stable adducts. Attention is therefore being directed to histidine or carboxylate side chains which could give unstable adducts. Comparison of the structures of the *N. naja atra* and *N. mossambica mossambica* enzymes strongly indicates that histidine side chains may be involved. Short chain *N*-acyl histidine derivatives are very unstable as to hydrolysis, but long-chain compounds are apparently protected through micelle formation. In the case of a single modified histidine side chain it is reasonable to suppose that the adduct would be very unstable if unfolded, but stable in a folded configuration. However, because of the instability of the adduct as to denaturation, proof will require confirmation by means of X-ray or NMR methods.

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