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STUDIES ON THE ROLE OF METHIONINE IN PORCINE PANCREATIC PHOSPHOLIPASE A_2

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Summary

The unique methionine-15 residue located at the N-terminal site of iso- or β-phospholipase A_2 from porcine pancreas has been specifically carboxymethylated with iodoacetic acid.

The modification results in a complete inactivation of the enzymatic activity toward micellar and monomeric substrates.

Spectroscopic measurements reveled that the carboxymethylated protein still binds Ca^{2+} and monomeric substrates with comparable affinities as the native enzyme. The active site histidine-54 residue in the modified enzyme shows a reactivity toward the active site-directed irreversible inhibitor *p*-bromophenacylbromide which is identical to that of the native enzyme. The alkylated protein, however, has lost its ability to bind to lipid-water interfaces. Although circular dichroic spectra of the carboxymethylated enzyme display some changes in the tertiary structure as compared with the native enzyme, the α-helix content remains rather constant. It is concluded that carboxymethylation of methionine-15 destroys the interface recognition site but has only limited influence on the active site of the molecule. Therefore, it seems that methionine-15 is not involved in the catalytic events but that this residue is part of the interface recognition site which embraces the N-terminal hydrophobic part of the enzyme:

8 10 15
Ala-Leu-Trp-Gln-Phe-Arg-Ser-Met

Introduction

Phospholipase A_2 (EC 3.1.1.4) specifically catalyzes the hydrolysis of the fatty acid ester bond at the 2-position of 1,2-diacyl-*sn*-phosphoglycerides [1]. The pancreatic enzyme, which has an absolute requirement of Ca^{2+} for ac-

Abbreviation: Tos-Phe CH_2Cl, L-1-tosylamide-2-phenylethyl chloromethyl ketone.

tivity [2], is secreted by the porcine gland as a zymogen which can be converted by limited proteolysis by trypsin into the active enzyme [3]. Although porcine pancreatic phospholipase A_2 slowly degrades substrate molecules in monomeric solutions, the same substrate when present as an organized lipid-water interface is hydrolyzed much more efficiently [4]. The zymogen, however, although possessing about 50% of the activity of the enzyme toward monomeric substrate, does not show the increase in enzymatic activity when the substrate concentration passes the critical micellar concentration [5]. Upon activation of the precursor, a conformational change takes place [6], giving rise to the formation of an interface recognition site [5,7]. This site, present in the active enzyme and not in the zymogen, is required for a specific interaction with certain organized lipid-water interfaces. Recently, evidence has been reported that the N-terminal region of the active enzyme: Ala.[8] Leu.Trp.[10] Gln.Phe.Arg[13] is directly involved in this interface recognition site [8].

To extend further our present knowledge on the mechanism of action of pancreatic phospholipase A_2 and in particular its intriguing behaviour toward organized lipid-water interfaces the study of chemically modified enzymes can give valuable information. The present paper describes the results obtained by specific modification of the methionine residue located at position 15 in porcine pancreatic phospholipase A_2. This modification has been performed by using the recently described [9] iso- or β-phospholipase A_2, because this enzyme contains only one methionine residue (located at position 15) whereas the normal more abundantly occurring α-phospholipase A_2 possesses two methionine residues (located at positions 15 and 27, respectively).

Materials and Methods

Porcine pancreatic iso-phospholipase A_2 and its zymogen were obtained as described previously [9]. 1,2-Dihexanoyl- and 1,2-dioctanoyl-*sn*-glycero-3-phosphorylcholines were synthesized as described previously [4]. *n*-Alkylphosphorylcholines were prepared as described by Van Dam-Mieras et al. [8]. Tos-Phe CH_2Cl-treated trypsin was obtained from Serva (Germany). Iodoacetic acid was purchased from Merck (Germany) and recrystallized from ethanol before use. Iodoacetamide was obtained from Fluka A.G. (Switzerland). DEAE-cellulose (DE-52) was purchased from Whatman (England) and Sephadex G-25 fine from Pharmacia Fine Chemicals (Sweden) and prepared for use according to the manufacturer's recommendations.

Iodo-[2-^{14}C]acetic acid was obtained from the Radiochemical Centre (England).

All the other chemicals used were of the highest purity available.

Phospholipase A_2 activities were determined at 40°C using the titrimetric assay procedure with egg-yolk lipoproteins as substrates, as described previously by Nieuwenhuizen et al. [10] and with monomeric and micellar short-chain lecithins as described by de Haas et al. [4].

Protein concentrations were calculated from the absorbances at 280 nm with an $E_{1\,cm}$ (1%) of 13.0 for isophospholipase A_2 and its carboxymethylated derivative, and 12.3 for the zymogen.

Starch-gel electrophoresis at pH 5.0 and 5.5 (0.05 M sodium acetate) as de-

scribed by De Haas et al. [11] was used to check the purity of the various proteins.

Amino acid analyses were performed by the method of Spackman et al. [12] on a Beckman Unichrom amino acid analyzer equipped with a high-sensitivity attachment. Samples were hydrolyzed for 24 h at 110°C in evacuated, sealed tubes with 6 M HCl.

Cyanogen bromide cleavage in 70% trifluoroacetic acid was performed as described by Bargetzi et al. [13], and performic acid oxidation as described by Hirs [14].

Tryptic hydrolysis was performed at pH 8.0 and 37°C with 2% (w/w) Tos-Phe CH_2Cl-trypsin at a substrate concentration of 2 mg/ml. Tryptic peptides, isolated by filtration over Sephadex G-25 fine (10^{-3} M HCl), were further purified by descending chromatography (solvent system: *n*-butanol/acetic acid/water, 4 : 1 : 5, by vol) and high-voltage electrophoresis (3000 V; buffer system: pyridine/acetic acid/water, 25 : 1 : 475, by vol), both on Whatman-paper No. 1. Radioactive peptides were located with a Panax thin-layer chromatography-scanner-ratemeter (P7900A). Radioactivity of samples was determined in a Packard Tri-Carb liquid scintillation counter using a dioxan based scintilland; efficiencies were determined using the external standard [15].

The reaction with [^{14}C]-*p*-bromophenacylbromide was carried out as described previously by Volwerk et al. [15].

Ultraviolet difference spectroscopy and fluorimetric measurements were performed as described recently in detail by Pieterson et al. [16] and Van Dam-Mieras et al. [8].

Circular dichroic spectra were recorded at 17°C on a Cary 60 recording spectropolarimeter with a Model 6002 CD-attachement. Protein samples were dissolved in 0.01 M phosphate buffer (pH 7.5) at concentrations of approximately 0.02, 0.1 and 1 mg/ml using Cary cells of pathlengths 10, 2 and 1 mm, respectively. Spectra were recorded from 260 to 190 nm. Observed ellipiticity (θ_{obsd}) was converted to mean residue ellipiticity, [θ], by the following equation:

$$[\theta] = \frac{\theta_{obsd} \times (\text{average residue weight})}{10 \times (\text{path length in cm}) \times (\text{g/ml of protein})}$$

assuming a mean residue weight of 112.8. Percentage α-helix was calculated at 208 nm according to the method described by Greenfield and Fasman [17].

Results and Discussion

Most of the methods to modify methionine residues in proteins described in the past decade are based on selective oxidation or alkylation of the sulphur atom. Oxidation is generally performed by hydrogen peroxide [18—24], periodate [25—27] or trichloromethanesulphonylchloride [28], and alkylation by iodo- or bromoacetic acid and iodoacetamide [21,29—38]. In some particular cases, successful alkylation of methionine residues has been achieved by using active-site directed irreversible inhibitors [39—41].

The most attractive procedure for the modification of the unique methionine residue in β-phospholipase A_2 seems to be alkylation, because this reaction is reported to be very selective for methionine when performed at acidic pH.

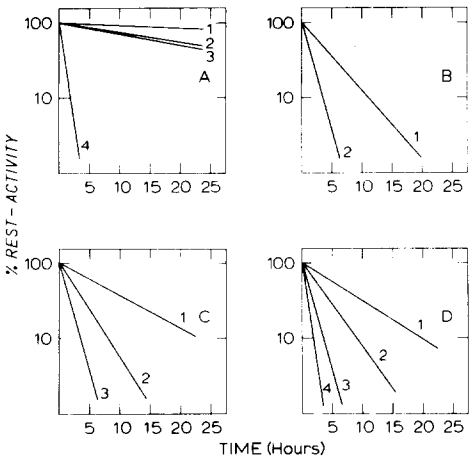


Fig. 1. Loss of β-phospholipase A_2 activity as a function of time with iodoacetic acid and iodoacetamide under various conditions. All experiments were performed in 0.2 M sodium formate/formic acid buffer (pH 3) with protein concentrations of 71.4 μM. A. Effect of medium (50°C): 1, without iodoacetic acid, in the presence of 8 M urea; 2, 500-fold molar excess of iodoacetic acid; 3, 500-fold molar excess of iodoacetic acid in the presence of 2% sodium dodecyl sulphate; 4, 500-fold molar excess of iodoacetic acid in the presence of 8 M urea. B. Effect of alkylating reagent (45°C): 500-fold molar excess of reagent, 8 M urea. 1, iodoacetamide; 2, iodoacetic acid. C. Effect of concentration of iodoacetic acid (45°C), 8 M urea. 1, 100-fold, 2, 250-fold, 3, 500-fold molar excess. D. Effect of temperature. 500-fold molar excess of iodoacetic acid, 8 M urea. 1, 25°C; 2, 37°C; 3, 45°C; 4, 50°C.

Inactivation by iodoacetic acid

Alkylation reactions of β-phospholipase A_2 were performed essentially as described by Wallis [29]. Fig. 1 shows the loss of β-phospholipase A_2 activity as a function of time upon incubation of the enzyme with iodoacetic acid under various conditions. From Fig. 1A, it is obvious that there is almost no decrease in enzymatic activity upon treatment of the enzyme at pH 3.0 and 50°C with a 500-fold molar excess of iodoacetic acid in the absence (curve 2) or in the presence of 2% sodium dodecyl sulphate (curve 3). However, when the medium contains 8 M urea, a rapid loss of enzymatic activity is observed (Fig. 1A, curve 4). Apparently, the presence of 8 M urea is required to make the Met residue accessible to this modification. Almost no loss of activity was observed after 24 h when the alkylating agent was omitted. (Fig. 1A, curve 1). β-Phospholipase A_2 is also inactivated by alkylation with iodoacetamide, although the rate of inactivation is slower by a factor 3 as compared to that of iodoacetic acid under identical conditions (cf. Fig. 1B). The loss of β-phospholipase A_2 activity as a function of time upon incubation of the enzyme with different molar excesses of iodoacetic acid (Fig. 1C) gives straight lines through 90% inactivation. This indicates that the reaction follows pseudo first-order kinetics and that inactivation does not involve sequential modification of groups with different reactivities. As can be seen from Fig. 1D, the rate of inactivation is dependent on the temperature. Below pH 4, the rate of inactivation was found to be independent of the pH of the medium.

Preparation of ^{14}C-labeled β-phospholipase A_2

β-Phospholipase A_2 (1 mg/ml) was incubated with a 500-fold molar excess of ^{14}C-labeled iodoacetic acid at 45°C in 0.2 M sodium formate/formic acid (pH 3.0) containing 8 M urea. After 7 h, the reaction mixture was dialyzed exhaustively at 0°C and lyophilized. The protein was purified by chromatography on a DEAE-cellulose column equilibrated with $5 \cdot 10^{-3}$ M sodium acetate (pH 6.3) and developed with a linear salt gradient reaching 0.12 M NaCl in the same buffer. Fractions containing the carboxymethylated protein were pooled, dialyzed for 24 h and lyophilized. Gel electrophoresis at pH 5.0 of the purified carboxymethylated protein showed the presence of only one band.

Identification of the site of modification

The carboxymethylated protein could no longer be split by cyanogen bromide, indicating that the unique methionine residue has been modified. This was confirmed by the absence of methionine sulfone in the acid hydrolyzate of the carboxymethylated enzyme after performic acid oxidation. This latter procedure is known to convert methionine into its sulfone derivative, whereas carboxymethylated methionine is transformed into homoserine, homoserine lactone, methionine, homocystine and *S*-carboxymethylhomocysteine [42]. Amino acid analysis of the acid hydrolyzate of performic acid oxidized, carboxymethylated protein revealed the presence of homoserine lactone while homoserine and *S*-carboxymethylhomocysteine emerged from the long column at the position of glutamic acid and glycine, respectively.

More conclusive evidence that the unique methionine residue has been selectively carboxymethylated was obtained by analysis of the ^{14}C-labelled carboxymethylated protein. The completely inactivated enzyme had a specific radioactivity almost equal to that of the [^{14}C]iodoacetic acid used, indicating the incorporation of one carboxymethyl moiety per mol of enzyme. Elution of a tryptic digest of the carboxymethylated protein over Sephadex G-25 (10^{-3} M HCl) revealed one major fraction containing approximately 70% of the radioactivity applied to the column. The peptide material present in this peak was further purified by high voltage electrophoresis and chromatography on paper. A radioactive peptide was obtained which had the following amino acid composition: $Lys_{1.0}$, $HomoSerlacton_{0.3}$, $Ser_{1.0}$, $HomoSer_{0.2}$, $Met_{0.1}$ and $Ile_{1.0}$. The only tryptic peptide from β-phospholipase A_2 compatible with this amino acid composition is [9]:

14 15 16 17
Ser-Met-Ile-Lys

It can therefore be concluded that the reaction of iodoacetic acid with pancreatic β-phospholipase A_2 results in the modification of the unique methionine-15 residue.

Properties of methionine-15 carboxymethylated β-phospholipase A_2

The carboxymethylated β-phospholipase A_2 has lost all its enzymatic activity towards micellar substrates using egg-yolk lecithin or 3-*sn*-dioctanoyllecithin. Moreover, no enzymatic activity of the modified enzyme toward

monomeric substrate, e.g. 3-*sn*-di-hexanoyllecithin could be detected. Although these observations could be explained by assuming that methionine-15 is an active site residue, this seems highly improbable. It is more reasonable to attribute the complete loss of enzymatic activity to an effect of the carboxymethylated methionine residue on the binding properties for substrate and Ca^{2+}.

As has been recently described in detail, direct binding studies can conveniently be performed by spectroscopic procedures using *n*-alkylphosphorylcholines as substrate analogs [8]. These compounds, which are not hydrolyzed by phospholipase A_2 give similar results as 1-acyl lysolecithin or as the competitive inhibitor D-lecithin. For the interaction with micelles we used *n*-hexadecylphosphorylcholine, which has an extremely low critical micellar concentration value (0.01 mM), while interactions with monomers were conducted with *n*-dodecylphosphorylcholine below its critical micellar concentration (1.1 mM). The interaction of native β-phospholipase A_2 with micellar *n*-hexadecylphosphorylcholine produces an ultraviolet difference spectrum as shown in Fig. 2. (upper 2 curves), which is characteristic for tryptophan and tyrosine perturbations [8]. The carboxymethylated β-phospholipase A_2, however, does not produce a difference spectrum upon addition of *n*-hexadecylphosphorylcholine (Fig. 2, lower 2 curves), indicating that the modified enzyme is no longer able to bind micellar interfaces.

This finding was confirmed by studying the protein-lipid/water interaction by measurement of the intrinsic fluorescence of the unique tryptophan residue in the protein. Amino acid analysis of the carboxymethylated β-phospholipase

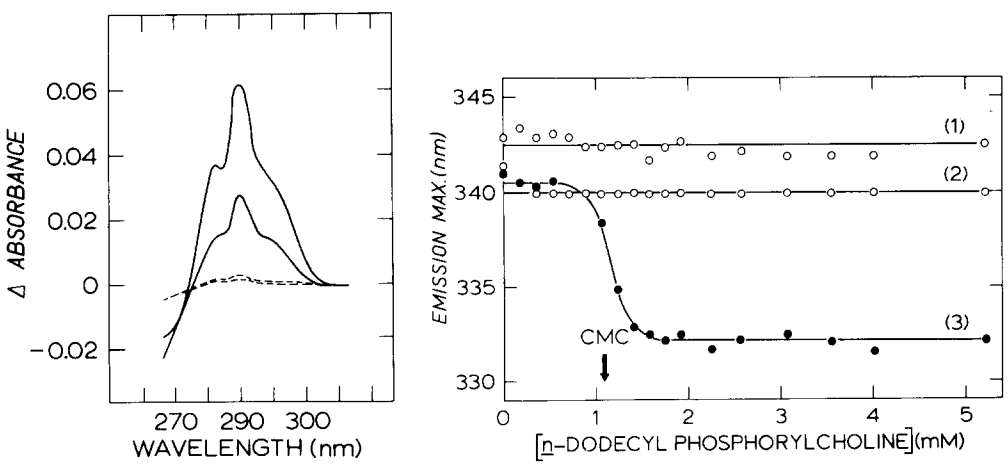


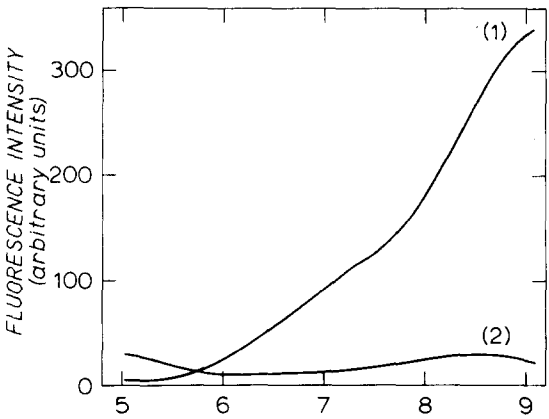
Fig. 2. Ultraviolet difference spectra produced by the interaction of β-phospholipase A_2 (———) and its carboxymethylated derivative (- - - - - -) with *n*-hexadecylphosphorylcholine. Conditions: 0.05 M sodium acetate, 0.05 M $CaCl_2$, 0.1 M NaCl, pH 6.0, 50 μM protein, 0.3 and 1.0 mM *n*-hexadecylphosphorylcholine (25°C).

Fig. 3. Dependence of emission maxima of carboxymethylated β-phospholipase A_2 (1), β-prophospholipase A_2 (2) and β-phospholipase A_2 (3) upon addition of increasing amounts of *n*-dodecylphosphorylcholine. Conditions: 0.05 M sodium acetate, 0.05 M $CaCl_2$, 0.1 M NaCl, pH 6.0, 15 μm protein (25°C). CMC stands for critical micellar concentration.

A_2 in the presence of 4% of thioglycollic acid as described by Matsubara and Sasaki [43] showed the presence of one residue of tryptophan/protein molecule, indicating that the iodoacetic acid has no effect on the single tryptophan residue. Fig. 3 shows the emission maxima of β-phospholipase A_2, its zymogen and the carboxymethylated enzyme as a function of the *n*-dodecylphosphorylcholine concentration. In the monomeric region, the emission maximum of all 3 proteins remains constant which has previously been interpreted that the microenvironment of tryptophan is not perturbed upon monomer binding [8]. When increasing the *n*-dodecylphosphorylcholine concentration above the critical micellar concentration the emission maximum for the native enzyme-micelle complex shifts from about 342 to 332 nm as a result of the interaction with the micellar interface. This blue shift of the emission maximum indicates that the environment of the tryptophan residue becomes more hydrophobic, probably because the tryptophan residue is buried in the lipid-H_2O interface [8]. In contrast to the active enzyme, the carboxymethylated protein just as the zymogen does not show this characteristic blue shift upon passing the critical micellar concentration (Fig. 3) and therefore these proteins do not bind to micellar interfaces.

Apparently, the minor modification of methionine-15 by carboxymethylation has destroyed the interface recognition site required for interaction with lipid-water interfaces. As shown previously [5,8], a functionally active interface recognition site is connected with the presence of a salt bridge between the α-NH_3^+ group of alanine-8 and a buried carboxylate group. The disrupture of this salt bridge in β-phospholipase A_2 can be followed by measuring the fluoresence intensity as a function of pH as shown in Fig. 4, curve 1. This figure strongly indicates that at least two amino acid side chains abolish or diminish the quenching of tryptophan fluorescence by neutralizing positive charges in its environment. These latter two amino acid residues have recently been identified as histidine-54 ($pK \approx 6.6$) and alanine-8 ($pK \approx 8.3$) [44]. From a comparison of curve 2 (Fig. 4) for the carboxymethylated β-phospholipase A_2 with curve 1 for the native enzyme, it can be concluded that notwithstanding the fact that the carboxymethylated β-phospholipase A_2 has a free α-NH_3^+ group, its microenvironment is quite different from that of the α-NH_3^+ group in the native enzyme. Most probably, the carboxymethylated β-phospholipase A_2 is devoid of the internal salt bridge.

The interaction of carboxymethylated β-phospholipase A_2 with monomeric substrate was investigated by ultraviolet difference spectroscopy. Upon titrating a solution of carboxymethylated β-phospholipase A_2 with increasing amounts of the substrate analog *n*-dodecylphosphorylcholine, difference spectra with a saturation character are produced. These spectra, which are identical to those obtained upon similar titration of β-phospholipase A_2, have recently been demonstrated to be due predominantly to tyrosine perturbations [8]. To quantitate the observed protein-lipid interaction the Δ-absorbances of the positive absorption peaks at 288 nm were plotted in a double reciprocal way as a function of lipid concentration (Figure 5). From these experiments it can be concluded that the carboxymethylated β-phospholipase A_2 is still able to bind monomeric substrate, although the affinity is less by a factor 2.5, as compared to native β-phospholipase A_2.



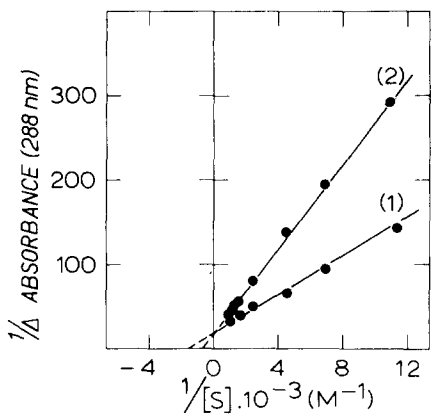


Fig. 4. Effect of pH on fluorescence intensity of (1) β-phospholipase A_2 and (2) carboxymethylated β-phospholipase A_2. Conditions: 1 mM Tris, 0.1 M NaCl, 15 μM protein, 25°C. Intensities were measured at the emission maxima.

Fig. 5. Lineweaver-Burke plots for interaction of (1) β-phospholipase A_2 and (2) carboxymethylated β-phospholipase A_2 with *n*-dodecylphosphorylcholine (S) obtained from ultraviolet difference spectroscopy. Conditions: 0.05 M sodium acetate, 0.05 M $CaCl_2$, 0.1 M NaCl, pH 6.0, 50 μM protein (25°C).

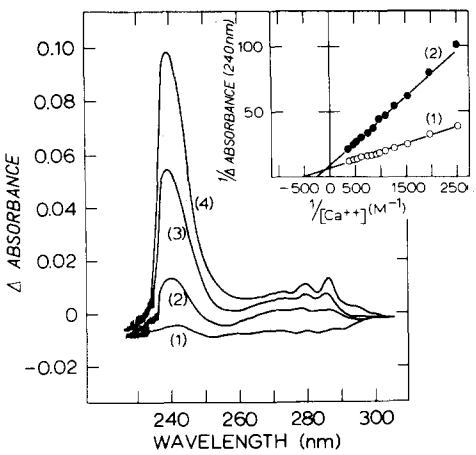
With ultraviolet difference spectroscopy it was also established that the carboxymethylated β-phospholipase A_2 is still able to bind Ca^{2+} (Fig. 6). The absorption at 240 nm was taken as a measure for this effect [16]. The dissociation constant for the carboxymethylated β-phospholipase $A_2 \cdot Ca^{2+}$ complex calculated from a double reciprocal plot (Fig. 6, inset) of Δ-absorbance vs. $[Ca^{2+}]$, was found to be twice as high as with the native enzyme.

From the above results, it seems evident that specific modification of methionine-15 in pancreatic β-phospholipase A_2 completely destroys the interface recognition site. Therefore, one might suppose that this latter site, which has been shown to involve the N-terminal sequence [8]:

8 13
Ala-Leu-Trp-Gln-Phe-Arg

should be extended to residue 15. On the other hand, upon carboxymethylation of methionine-15, the enzyme also looses its capacity to catalyze the hydrolysis of monomeric substrates. This could indicate that the rather rough conditions necessary to modify methionine-15 have caused gross conformational changes in the overall secondary and tertiary structure of the protein *. Therefore circular dichroic measurements on the native and carboxymethylated enzyme were performed (Fig. 7, curve 1 and 3, respectively). As can be seen from Fig. 7, carboxymethylation of methionine-15 gives rise indeed to a somewhat different CD-spectrum. Calculation, however, of the percentage α-helix according to the method of Greenfield and Fasman [17] using the mean

* Although the three-dimensional crystal structure of the active enzyme is not yet known, the 3 Å X-ray structure of the zymogen of porcine pancreatic α-phospholipase A_2 reveals that methionine-15 is deeply buried in the hydrophobic interior of the molecule (J. Drenth and J. Zwart-Vessius, personal communication).



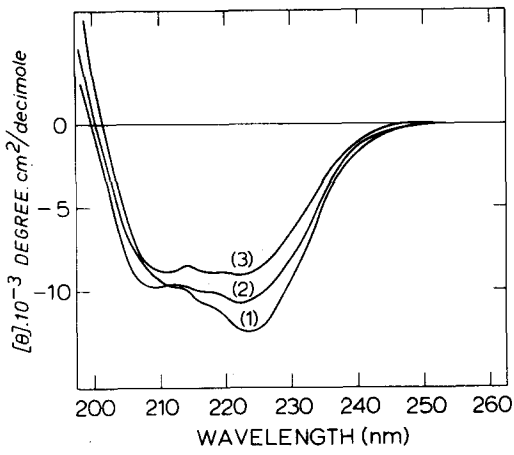


Fig. 6. Ultraviolet difference spectra produced by the interaction of carboxymethylated β-phospholipase A_2 with increasing amounts of Ca^{2+}. Conditions: 0.05 M sodium acetate, 0.1 M NaCl, pH 6.0, 50 μM protein (25°C). 1, 0.4 mM Ca^{2+}; 2, 0.8 mM Ca^{2+}; 3, 2.4 mM Ca^{2+}; 4, 6.0 mM Ca^{2+}. Inset: Lineweaver-Burke plot of (1) β-phospholipase A_2 and (2) its carboxymethylated derivative with Ca^{2+}, obtained from ultraviolet difference spectra.

Fig. 7. Circular dichroic spectra of (1) β-phospholipase A_2 (2) β-prophospholipase A_2 and (3) carboxymethylated β-phospholipase A_2. Conditions: see experimental part.

residue ellipticity at 208 nm yields very similar values for β-phospholipase A_2 and its carboxymethylated derivative: 15,5% and 14,5%, respectively. *

For the β-prophospholipase A_2 (Fig. 7, curve 2) the percentage α-helix was found to be 19 using the same method of calculation at 208 nm.

Moreover, the very similar affinity constants of β-phospholipase A_2 before and after carboxymethylation for monomeric substrate and Ca^{2+} (cf. Fig. 5 and 6, respectively) strongly suggest that the overall structure of the protein has not been extensively changed by the modification reaction.

Finally it could be shown, that the active site residue histidine-54 in native β-phospholipase A_2 and in the carboxymethylated protein reacts with the same velocity with the active site directed inhibitor [^{14}C]-*p*-bromophenacyl bromide [15] and in both cases about one mol of the labeled haloketone is incorporated per mol protein. Therefore we conclude that the described methionine modification reaction, which completely disturbs the interface recognition site, has only a very limited influence on other parts of the protein molecule. It is attractive to suppose that methionine-15 located in the rather hydrophobic N-terminal part of the protein, plays a role in the enzyme-interface binding and that the introduction of the negatively charged carboxymethyl group on methionine-15 impedes the formation of a functionally active inter-

* The previously reported [45] α-helix percentages for porcine pancreatic α-phospholipase A_2 and its zymogen (50 and 55%, respectively) have been derived from the negative CD minima at 222 nm and are probably overestimated. Taking into account the ellipticities at 208 nm an α-helix content of about 35% can be calculated, being more in agreement with the recent X-ray results (J. Drenth and J. Zwart-Vessius, personal communication).

face recognition site. Further work using other specifically methionine modifying agents is in progress to define the function of this very conservative amino acid residue in pancreatic phospholipase A_2.

Acknowledgments

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