HIGH ENZYMATIC ACTIVITY OF SPECIFICALLY IODINATED BOVINE PHOSPHOLIPASE A_2

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1. Introduction

Phospholipase A_2 (EC 3.1.1.4) catalyses the hydrolysis of the fatty acid ester bond at the 2-position of 3-*sn*-phosphoglycerides [1]. The pancreatic enzyme occurs as a zymogen and both proteins show comparable, though low activity toward substrate present as a monomeric dispersion indicating that a functional active site is present already in the zymogen [2]. The activity of the enzyme, in contrast to that of the zymogen, increases tremendously when substrate is present in certain organized lipid-water interfaces like, e.g., micelles [2]. It has been proposed that the pancreatic phospholipase A_2 possesses an Interface Recognition Site (IRS) through which the enzyme binds to lipid-water interfaces and which is not present in the zymogen [2,3]. Most probably a conformational change occurs during this binding, giving rise to an optimization of the active site.

It has been demonstrated that the activity of the enzyme depends very much on the structure of the micellar lipid-water interface, the structure of which is determined for a given phospholipid and which cannot easily be varied [3]. Therefore an important tool to study the influence of interfacial properties on enzymatic activity is the use of the monolayer technique. In this technique only a very limited amount of the added enzyme will be present in the monolayer, the bulk of the enzyme remains freely dissolved in the large subphase. One attractive possibility for the accurate determination of the amount of enzyme present in the monolayer is the use of a highly radioactive enzyme. The amount of enzyme acting in the monolayer can then be measured by transporting the lipid monolayer plus enzyme to a clean subphase, followed by collection of the film [15]. In addition one is also able to determine the rate of adsorption or desorption of the enzyme to or from the monolayer.

In this report we describe the preparation of a highly radioactive, ^{125}I-labelled phospholipase A_2 using equivalent amounts of triiodide solution. In addition the identification of the site of labelling and the kinetic properties of the iodinated enzyme are described.

2. Materials and methods

(Pro)phospholipase A_2 was purified from bovine pancreas [4]. Phospholipase A_2 activities were routinely determined using the egg-yolk lipoprotein assay [5]. Kinetic measurements using micellar 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine and monomeric 2-*sn*-decanoyl-thio-1-ethyl phosphocholine were performed as described before [6,7]. Protein concentrations for (iodinated) phospholipase A_2 and their zymogens were calculated from the absorbance at 280 nm with an E_{1cm} (1%) of 12.5 and 12.1, respectively.

Sodium [^{125}I]iodide was obtained from the Radiochemical Centre, Amersham (England) in NaOH solution, free from reducing agent and was added to NaI_3 solution to give the required range of specific radioactivity (0.05×10^9 to 33.5×10^9 cpm/μmol). Radioactivity of samples was determined in a Searle Isocap 300 Liquid Scintillation System (Nuclear Chicago Division) in the presence of 10 ml of a liquid scintillation cocktail (Packard Instagel). Radioactive

peptides were located on paper with a thin-layer scanner (RTLS_1A, PANAX Equipment Ltd., Redhill, Surrey, England).

Trypsin was purchased from Serva (Germany), α-chymotrypsin (3-times crystallized) from Fluka A.G. (Switzerland) and carboxypeptidase Y from Pierce (USA). Before enzymatic digestion, the protein was reduced and cysteines were converted into thialamines with 2-bromoethyltrimethylammonium bromide [8]. Tryptic and chymotryptic digestions were carried out for 60 min and 30 min, respectively under conditions described before [9]. Carboxypeptidase Y digestion was done in 0.1 M pyridine-acetate (pH 6) by incubating 70 nmol of chymotryptic peptides with 6 μg of enzyme in a final volume of 100 μl for 16 h. Peptide mapping was done by first carrying out high-voltage electrophoresis on Whatmann no 3 MM paper in pyridine-acetate (0.4 M, pH 6.5) followed by chromatography in the system *n*-butanol–pyridine–acetic acid–water (60:40:12:48, by vol). Spots were located by staining with fluorescamine and scanning. Amino acid analysis were determined on a Technicon TSM Amino acid Analyser after hydrolysis of the isolated peptides in 5.8 N HCl for 24 or 48 h in evacuated tubes. Fluorescamine was obtained from Hoffmann-La Roche (Switzerland), 3-iodotyrosine and 3,5-diiodotyrosine from Sigma (USA).

3. Results

Iodination of phospholipase A_2 or its zymogen, at a concentration of 5–10 mg/ml, was performed in 0.05 M glycine–KCl buffer (pH 9.2), at 0°C by slowly adding 1 equivalent of 3 mM iodine dissolved in 9 mM potassium iodide with stirring. After 30 min [10–12] the protein was dialyzed against distilled water. To remove traces of iodide the proteins were chromatographed on CM-cellulose under conditions as described before [4]. The modified proteins eluted as single symmetrical peaks. Using this procedure one atom of iodine was incorporated per mole of protein. The number of iodine atoms incorporated per mole of phospholipase could be raised by using increasing amounts of iodine and the resulting proteins still showed considerable activity in the egg-yolk test. These enzyme were not further investigated since it could be expected that these proteins are heterogeneous with respect to the localization of iodine.

Peptide mapping was performed to localize the radioactive iodinated peptide in the protein containing one iodine atom per mole of protein. The thialaminated protein was digested first with trypsin followed by a brief incubation with chymotrypsin. As shown in fig.1 three radioactive spots could be detected. Peptide A, containing 72% of the radioactivity gave correct amino acid analyses for the peptide Val^{63} Leu Val Asp Asn Pro Tyr^{69}; peptide B, corresponding to 21% of the radioactivity analysed as Val^{65} Asp Asn Pro Tyr^{69} (for numbering see reference 9). The radioactive spot C which did not react with fluorescamine and does not correspond to a peptide accounted for 6% of eluted radioactivity. The chemical nature of the iodinated residue was

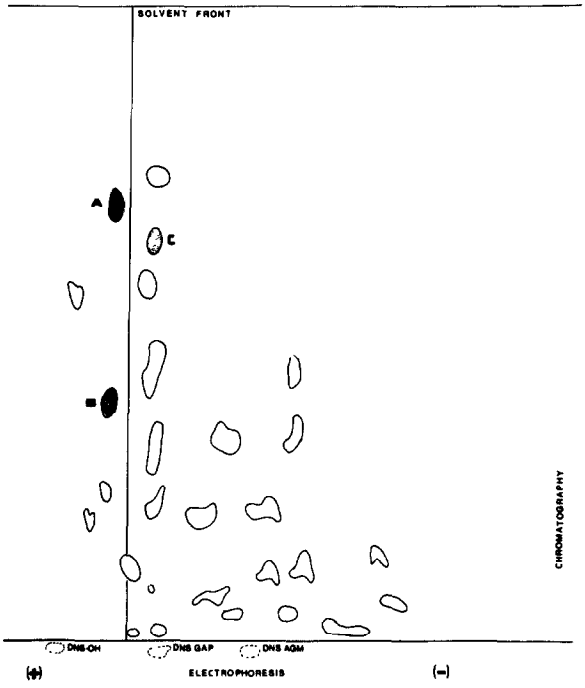

Fig.1. Peptide mapping of tryptic/chymotryptic digest of thialaminated [^{125}I]phospholipase A_2. Electrophoresis in 0.4 M pyridine-acetate pH 6.5, chromatography in *n*-butanol–pyridine–acetic acid–water (60:40:12:48, by vol). ○, fluorescamine-positive spots; ●, ^{125}I-containing spots. DNSOH, dimethyl naphthyl sulphonic acid, DNS-Gap, dimethyl naphthyl sulphonyl-guanidino amino propionic acid; DNS-Agm, dimethyl naphthylsulphonyl agmatine.

Table 1
Kinetic constants of bovine (iodinated) phospholipase A_2 and (iodinated) prophospholipase A_2

Protein	V_{max} μmol/min/mg protein	K_m mM
Phospholipase A_2	2100[a]	2.1[a]
Iodinated phospholipase A_2	2000[a] (2000)	0.53[a]
Prophospholipase A_2	0.340[b]	3.4[b]
Iodinated prophospholipase A_2	0.380[b]	4.0[b]

[a] Measured at 25°C using 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine (1.5–15 mM) as a substrate in the presence of 5 mM sodium acetate, 50 mM calcium chloride and 100 mM sodium chloride at pH 6.0

[b] Measured at 25°C using 1-decanoyl thioethyl phosphocholine (0.25–2.5 mM) as a substrate in the presence of 200 mM sodium acetate, 100 mM calcium chloride, 100 mM sodium chloride at pH 6.0

determined after carboxypeptidase Y digestion of the whole chymotryptic digest. The products were identified by peptide mapping. The spot corresponding to authentic monoiodotyrosine (R_F = 0.60) contained 58 times more radioactivity than the spot corresponding to authentic diiodotyrosine (R_F = 0.68). Therefore, it was concluded that monoiodinated (pro)phospholipase A_2 is a homogeneous protein in which tyrosine[69] is iodinated.

The kinetic properties of iodinated phospholipase A_2 were measured using the micellar substrate 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine at a fixed $CaCl_2$ concentration of 50 mM which is sufficiently high to saturate the enzyme with Ca^{2+}. As shown in table 1 iodination does not markedly influence V_{max} whereas K_m was lowered by a factor 4. The data shown in table 1 for the monomeric substrate indicate that iodination does not markedly influence the properties of the active centre. Thus iodination gives rise to an enzyme with increased affinity to interfaces and this strongly suggests that Tyr^{69} is part of the interfacial recognition site.

4. Discussion

Reaction of bovine pancreatic phospholipase A_2 and its zymogen with an equimolar quantity of iodine results in the specific monoiodination of Tyr^{69}*. The specific activity of the iodinate enzyme increased 2-fold as compared to the native enzyme, when assayed in the routine egg-yolk assay.

Upon further characterization of the kinetic properties of the iodinated zymogen toward monomeric substrate it turned out that both V_{max} and K_m values do not change significantly (table 1). Because it has been shown that the zymogen possesses a comparable functional active site as the native enzyme it can be concluded that iodination of Tyr^{69} has no effect on the catalytic process. The results obtained from kinetic experiments with iodinated phospholipase A_2 using micelles of 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine showed that iodination has almost no measurable effect on V_{max} but that the K_m decreased 4-fold. Since the value of K_m reflects two different affinities: (i) the binding of the enzyme to the micellar surface and (ii) the binding of monomeric substrate to active centre [3] we found it of interest to compare also the kinetic properties of prophospholipase A_2 and iodinated prophospholipase A_2, which do not bind to interfaces. These measurements showed that there is no major influence of the iodina-

* Similarly the porcine (pro)phospholipase A_2 can be monoiodinated, but in addition to Tyr^{69} also Tyr^{123} was partially modified

tion on the properties of the active centre and apparently the only effect of monoiodination of Tyr^{69} in the enzyme is an increased affinity toward lipid-water interfaces.

It has to be mentioned that also upon treatment of bovine phospholipase A_2 with tetranitromethane only Tyr^{69} is nitrated (H. Meijer et al., manuscript in preparation). From these latter studies it could be concluded that in addition to the previously found hydrophobic N-terminal sequence Ala^1-Met^8 [13,14] also Tyr^{69} is part of the IRS. Most probably the iodination of Tyr^{69} therefore reinforces the binding of the enzyme to lipid-water interfaces. In any case monoiodination only slightly improves binding of the enzyme to lipid-water interfaces and it seems justified to use the iodinated phospholipases A_2 in monolayer studies. Although some of the results obtained have been described already [15] a more quantitative comparison of monoiodinated pancreatic phospholipase A_2 with other radioactively labelled phospholipases will be published elsewhere (F. Pattus, et al., manuscript in preparation).

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