Affinity labelling of 5'-nucleotidases with 5'-*p*-fluorosulphonylbenzoyl adenosine

Ursula STOCHAJ* and Hans Georg MANNHERZ

Institut für Anatomie und Zellbiologie, Robert-Koch-Strasse 5, 3550 Marburg, Federal Republic of Germany

5'-Nucleotidases play an important role in the metabolism of nucleosides; for example, the hydrolysis of AMP generates adenosine, which can modulate a variety of cellular functions. We have used the membrane-bound AMPase from chicken gizzard and a secreted form of these enzymes to analyse their modification by the substrate analogue 5'-*p*-fluorosulphonylbenzoyl adenosine (5'-FSBA). 5'-FSBA irreversibly inactivates 5'-nucleotidases by means of covalent modification of the proteins. ATP, a competitive inhibitor of chicken gizzard and snake-venom 5'-nucleotidase, abolished the inactivation by 5'-FSBA, demonstrating that the inactivation was due to the modification of amino acid residues essential for AMPase activity. We have synthesized radioactive 5'-FSBA, which was employed for the radiolabelling of chicken gizzard 5'-nucleotidase. Incorporation of radioactivity was completely abolished in the presence of ATP, which showed that 5'-FSBA acted by the selective modification of amino acid residues at the active site whereas other potential reactive residues of the protein were not attacked. Limited proteolysis of affinity-labelled chicken gizzard 5'-nucleotidase permitted the identification of digestion products containing the catalytic centre. Pseudo-first-order kinetics indicate that modification of a minimum of one amino acid side chain at the active centre is sufficient to result in inactivation of both chicken gizzard and snake-venom 5'-nucleotidases. Incorporation of the radioactive *p*-sulphonylbenzoyl adenosine moiety parallels the inactivation of 5'-nucleotidase by 5'-FSBA and further substantiated the idea that modification of one amino acid residue at the active centre results in loss of the AMPase activity.

INTRODUCTION

5'-Nucleotidase is a ubiquitous enzyme that mediates the hydrolysis of AMP. In view of the various effects exerted by adenosine these enzymes are of general interest. Membrane-bound, secreted as well as cytosolic forms of this enzyme can be obtained from a variety of a different tissues (Worku *et al.*, 1984; Fini *et al.*, 1985; Truong *et al.*, 1988). We have concentrated on the investigation of the membrane-bound AMPase present in chicken gizzard and the secreted form isolated from snake venom.

The membrane-bound AMPase of chicken gizzard is an ectoenzyme, i.e. its active centre faces the exterior of the cell. This enzyme is anchored to the plasma membrane via a phosphatidylinositol glycan, and cytoplasmic and membrane-spanning proteinaceous portions are lacking (Stochaj *et al.*, 1989a).

Detailed studies have analysed the kinetic properties of chicken gizzard 5'-nucleotidase (Dieckhoff *et al.*, 1986a) and have demonstrated that the AMPase plays an important role as membrane-bound receptor for components of the extracellular matrix (Dieckhoff *et al.*, 1986b; Stochaj *et al.*, 1989b).

The secreted 5'-nucleotidase of snake venom shares several properties with the membrane-bound enzyme, i.e. the susceptibility towards different competitive and non-competitive inhibitors as well as amino acid residues essential for AMP hydrolysis. Our present contribution describes the specific modification and radiolabelling

of chicken gizzard and snake-venom 5'-nucleotidases with the substrate analogue 5'-*p*-fluorosulphonylbenzoyl adenosine (5'-FSBA).

MATERIALS AND METHODS

Materials

5'-Nucleotidase from *Crotalus adamanteus* and *p*-fluorosulphonylbenzoyl chloride were obtained from Sigma, and ATP was from Pharma-Waldhof. [U-¹⁴C]Adenosine and [2-³H]adenosine were from Amersham-Buchler.

5'-*p*-Fluorosulphonylbenzoyl adenosine (5'-FSBA) was synthesized by the method of Colman *et al.* (1977), and [¹⁴C]5'-FSBA and [³H]5'-FSBA were prepared under identical conditions but with [U-¹⁴C]adenosine and [³H]adenosine respectively. The purity of 5'-FSBA was analysed by t.l.c. on silica-gel plates developed in butan-2-one/acetone/water (12:4:3, by vol.). Protein concentrations were determined with the Pierce BCA protein assay kit.

5'-Nucleotidase from *C. adamanteus* was purified by affinity chromatography on AMP-Sepharose. Preparation of chicken gizzard 5'-nucleotidase and determination of the AMPase activity followed the procedures of Dieckhoff *et al.* (1985). The purified proteins were routinely analysed by electrophoretic separation in polyacrylamide gels in the presence of SDS followed by silver staining, which gave single bands for both snake-venom and chicken gizzard 5'-nucleotidases.

Abbreviations used: 5'-FSBA, 5'-*p*-fluorosulphonylbenzoyl adenosine; SBA moiety, *p*-sulphonylbenzoyl adenosine moiety.

* To whom correspondence should be sent, at present address: Department of Biology, Princeton University, Princeton, NJ 08544, U.S.A.

Radioiodination of detergent-solubilized chicken gizzard 5'-nucleotidase

This followed the procedure of Stochaj *et al.* (1989b).

Incubation of 5'-nucleotidase with 5'-FSBA

Chicken gizzard 5'-nucleotidase was incubated with 5'-FSBA in buffer A (5 mM-Hepes/NaOH buffer, pH 7.4, containing 0.1 mM-CaCl₂, 0.2 mM-NaN₃ and 0.1% Triton X-100) unless otherwise indicated. Snake-venom 5'-nucleotidase was treated identically but with omission of detergent in all buffers used. 5'-FSBA and *p*-fluorosulphonylbenzoyl chloride were dissolved in ethanol/dimethylformamide (1:1, v/v). Before addition to 5'-nucleotidase the pH of *p*-fluorosulphonylbenzoyl chloride was adjusted to 7.4. Appropriate controls were run in parallel in the presence of identical concentrations of solvent but without addition of the chemical agent.

Radiolabelling of chicken gizzard 5'-nucleotidase

5'-Nucleotidase was incubated with 0.56 mM-[¹⁴C]5'-FSBA in the absence and in the presence of 50 μ M-ATP for 3 h at 30 °C. Non-incorporated radioactivity was removed by dialysis against buffer A. Electrophoretic separation was carried out in polyacrylamide gels in the presence of SDS (Lugtenberg *et al.*, 1975). After electrophoresis gels were soaked for 1 h in distilled water, dried and exposed to Kodak X-AR5 films.

Determination of the number of modified amino acid residues at the active site

Since only limited quantities of the purified AMPases can be obtained, we used pseudo-first-order kinetics to correlate the minimum number of amino acid residues modified to the inactivation of 5'-nucleotidase. The number of amino acid residues modified at the active site was determined from the plot of $\log(1000/t_{1/2})$ versus $\log[\text{modifier}]$ according to Levy *et al.* (1963).

In addition, we employed [³H]5'-FSBA to correlate the amount of radioactivity incorporated into 5'-nucleotidase with the decrease in AMPase activity. Towards this aim samples were incubated with the radioactive 5'-FSBA followed by precipitation of snake-venom 5'-nucleotidase with acetone (20-fold volume of the sample) in the presence of 1 mg of cytochrome *c*. Denatured protein was collected by suction on Whatman GF/D filters, which were then washed extensively with ethanol to remove non-incorporated [³H]5'-FSBA. The presence of Triton X-100 in incubation mixtures of chicken gizzard 5'-nucleotidase interfered with quantitative precipitation of the AMPase. Therefore most of the non-incorporated radioactivity was removed by dialysis against buffer A followed by determination of the residual AMPase activity and radiolabelling of 5'-nucleotidase. To correct for the presence of minor amounts of non-incorporated [³H]5'-FSBA that was still present after dialysis controls were treated in parallel with [³H]5'-FSBA in the presence of 50 μ M-ATP. These samples fully retained their AMPase activity. Radioactivity recovered in the controls was subtracted from the determinations for inactivated 5'-nucleotidase.

Proteolytic digestion of chicken gizzard 5'-nucleotidase with clostripain

This was carried out in buffer A containing 1 mM-CaCl₂, 1 mM-dithiothreitol and 100 μ g of cytochrome *c* /

ml as carrier. After 1 h incubation at 37 °C the digestion was terminated by addition of EDTA to 5 mM final concentration, and this was followed by electrophoretic separation of the digestion products.

RESULTS AND DISCUSSION

Our present studies describe the application of a substrate analogue for specific inactivation and radiolabelling of two forms of 5'-nucleotidase: the membrane-bound ectoenzyme purified from chicken gizzard and the secreted protein prepared from snake venom.

Treatment of 5'-nucleotidase with the adenine nucleotide analogue 5'-FSBA

5'-FSBA has been used to identify polypeptides binding adenine nucleotides (Bennett *et al.*, 1978; Ferrer *et al.*, 1987). It is suspected to modify covalently several classes of amino acid side chains, including serine, tyrosine, lysine and histidine residues (Colman *et al.*, 1977). Incubation of 5'-nucleotidase with this agent irreversibly inactivated both the membrane-bound and the secreted enzymes. As shown for the membrane-bound enzyme (Fig. 1), the inactivation increased with time and was dependent on the concentration of 5'-FSBA (see also Fig. 5). Under identical conditions the AMPase activity of controls was not affected (Fig. 1).

Competitive inhibitors abolish the inactivation of 5'-nucleotidase by 5'-FSBA

To analyse whether 5'-FSBA modifies amino acid residues located at the active site we tested the inactivation of 5'-nucleotidases in the presence of different competitive inhibitors. Concentrations of ADP and [α , β -methylene]ADP required for efficient protection against modification strongly interfered with the determination of the AMPase activity by the optical assay used in our studies. Therefore ATP was employed to demonstrate that 5'-FSBA specifically attacks the active site of chicken

Fig. 1. Inactivation of chicken gizzard 5'-nucleotidase by 5'-FSBA

Plasma-membrane 5'-nucleotidase was incubated in the presence of 50 μ M-ATP (▲), 0.56 mM-5'-FSBA (■) or both 50 μ M-ATP and 0.56 mM-5'-FSBA (●) at 30 °C. The incubation was carried out in buffer A, pH 7.4, as detailed in the Materials and methods section. At the indicated times samples were diluted 40-fold for measurement of the residual AMPase activity.

Fig. 2. Protection by ATP against the inactivation of chicken gizzard 5'-nucleotidase and snake-venom 5'-nucleotidase by 5'-FSBA

Chicken gizzard 5'-nucleotidase (●) and snake-venom 5'-nucleotidase (▲) were incubated for 1 h with 1 mM-5'-FSBA at 30 °C in buffer A, pH 7.4. The incubation mixtures contained ATP at the final concentrations indicated. Controls were incubated with omission of 5'-FSBA but with identical concentrations of ATP and solvent. The results shown in the Figure are means of duplicate determinations.

gizzard and snake-venom 5'-nucleotidase. As shown in Figs. 1 and 2, addition of 50 μM-ATP to the incubation mixture resulted in optimal protection against inactivation. The protection by ATP was almost complete, which demonstrates that the modification of amino acid residues present at the active site results in inactivation of the AMPases.

Both chicken gizzard and snake-venom 5'-nucleotidases could be modified by 5'-FSBA, although the secreted enzyme was less susceptible to this inactivation (see Figs. 2 and 4). However, increased concentrations of 5'-FSBA gave results comparable with those for the chicken gizzard enzyme, as shown in Fig. 1. Similar results were obtained for another secreted 5'-nucleotidase, from bull seminal plasma, whose inactivation was comparable with the snake-venom AMPase (U. Stochaj, unpublished work).

As shown in Fig. 1, the inactivation of 5'-nucleotidase by 5'-FSBA represents a rather slow process, which could reflect diminished binding of this modifier at the catalytic centre owing to the presence of its bulky benzoyl moiety.

To analyse the modification of 5'-nucleotidase further we tested the effect of *p*-fluorosulphonylbenzoyl chloride, a precursor for the synthesis of 5'-FSBA, on the AMPase activity. Incubation of either enzyme with *p*-fluorosulphonylbenzoyl chloride up to 5 mM hardly affected the AMPase activity (approx. 5% inhibition).

This demonstrates that the inactivation of chicken gizzard and snake-venom 5'-nucleotidases requires the adenosine moiety of 5'-FSBA. The results indicate that inactivation of the 5'-nucleotidases depends on the structural similarity of 5'-FSBA to the enzyme's substrate AMP.

Inactivation of 5'-nucleotidases by 5'-FSBA depends on pH

In the pH range 6.0–9.0 5'-FSBA is reported to be reasonably stable, whereas more acidic or alkaline conditions result in hydrolysis of the compound. We

Fig. 3. pH-dependent inactivation of 5'-nucleotidases by 5'-FSBA

Chicken gizzard 5'-nucleotidase (●) and snake-venom 5'-nucleotidase (▲) were incubated with 0.56 mM-5'-FSBA for 1 h at 30 °C. The incubation was carried out at different pH values as indicated. Controls were incubated under identical conditions in the presence of solvent. Results shown in the Figure are means of triplicate determinations.

further characterized the inactivation of 5'-nucleotidase by analysing the pH-dependence of this reaction between pH 6.0 and 9.0 (Fig. 3). Both chicken gizzard and snake-venom 5'-nucleotidases could be efficiently inactivated under alkaline conditions. This is in accordance with the assumption that unprotonated forms of amino acid residues, such as histidine, are attacked by 5'-FSBA (Colman *et al.*, 1977). In addition, we found enhanced inactivation even under acidic conditions, whereas loss of enzymic activity was minimal at neutral pH. The efficient inactivation of 5'-nucleotidases at pH 6.0 might reflect the modification of another class of amino acid side chains involved in catalysis. Both chicken gizzard and snake-venom 5'-nucleotidases carry essential carboxylate groups with apparent *pK* values around 7 (results not shown). In their protonated form these carboxylate groups might represent a target site for the electrophilic attack mediated by 5'-FSBA. The inactivation by 5'-FSBA under acidic as well as basic conditions was decreased in the presence of ATP (results not shown). This demonstrates that, although different types of amino acid residues might be attacked at acidic or basic pH, 5'-FSBA specifically modifies side chains localized at the active site. Therefore the requirements for an affinity label, i.e. specific attack at a well-defined target site such as the catalytic centre, are fulfilled for 5'-FSBA under both acidic as well as basic conditions.

Radiolabelling of the active centre of 5'-nucleotidase with radioactive 5'-FSBA

5'-FSBA can be synthesized as a radioactive compound by the use of [¹⁴C]- or [³H]-adenosine. These agents were employed for selective radiolabelling of the active centre of chicken gizzard 5'-nucleotidase. Treatment of chicken gizzard 5'-nucleotidase with [¹⁴C]5'-FSBA led to radiolabelling of the 79000-*M_r* enzyme (Fig. 4, lane 2). Incorporation of radioactivity was not detected if the reaction mixture contained the competitive inhibitor ATP (Fig. 4, lane 1). The lack of radiolabelling in the presence of ATP demonstrates the specific attack of 5'-FSBA at the active centre. Obviously, potential reactive amino

Fig. 4. Radiolabelling of chicken gizzard 5'-nucleotidase with [^{14}C]5'-FSBA

Chicken gizzard 5'-nucleotidase was incubated with [^{14}C]5'-FSBA in the presence (lane 1) or in the absence (lane 2) of 50 μM -ATP. The incubation was carried out for 3 h at 30 $^{\circ}\text{C}$ in buffer A, pH 7.4, as described in the Materials and methods section. Equal amounts of protein were separated in parallel. Positions of marker proteins are indicated at the left-hand side.

acid side chains located distant from the catalytic site were not modified by this agent. From these data we conclude that the substrate analogue 5'-FSBA selectively radiolabels the active centre of 5'-nucleotidases.

Determination of the number of amino acid residues modified by 5'-FSBA at the active site

Owing to the limiting amounts of highly purified 5'-nucleotidases that can be prepared for modification with 5'-FSBA, we used pseudo-first-order kinetics as one approach to determine the minimum number of amino acid residues modified at the catalytic centre. For both chicken gizzard and snake-venom 5'-nucleotidases the plot of $\log(1000/t_{1/2})$ versus $\log[5'\text{-FSBA}]$ gave a slope of 0.7, indicating the covalent modification of one amino acid side chain per active site (Fig. 5). In addition, we have determined more directly the number of amino acid residues modified at the active site by the use of ^3H -labelled 5'-FSBA.

The amount of 5'-nucleotidase inactivated was correlated to the incorporation of the [^3H]SBA moiety. As shown in Table 1, data obtained for chicken gizzard and snake-venom 5'-nucleotidases are close to 1 mol of [^3H]SBA moiety incorporated/mol of enzyme inactivated, which is in accordance with the idea that modification of a single amino acid side chain at the catalytic centre results in loss of the AMPase activity.

Radiolabelling with 5'-FSBA permits the identification of proteolytic fragments containing the active site

Since we were particularly interested in studying the possible domain structure of chicken gizzard 5'-nucleotidase, we took advantage of the selective radiolabelling

Fig. 5. Determination of the minimum number of amino acid residues modified at the active site of 5'-nucleotidases by 5'-FSBA

Chicken gizzard 5'-nucleotidase (●) and snake-venom 5'-nucleotidase (▲) were incubated with different concentrations of 5'-FSBA. Data obtained from these pseudo-first-order kinetics were further processed in accordance with Levy *et al.* (1963). Slopes obtained from the plot of $\log(1000/t_{1/2})$ versus $\log[5'\text{-FSBA}]$ gave a minimum number of 0.7 amino acid residue modified at the catalytic centre of both chicken gizzard 5'-nucleotidase and snake-venom 5'-nucleotidase.

Table 1. Correlation of loss of the AMPase activity of chicken gizzard 5'-nucleotidase and snake-venom 5'-nucleotidase by [^3H]5'-FSBA to the incorporation of the [^3H]SBA moiety

(a) Chicken gizzard 5'-nucleotidase was incubated with 42 μM -[^3H]5'-FSBA at 30 $^{\circ}\text{C}$, resulting in the amount of inactivated enzyme depicted in Table 1. Radiolabel incorporated was determined as detailed in the Materials and methods section. (b) Snake-venom 5'-nucleotidase was treated with 186 μM -[^3H]5'-FSBA, leading to the concentration of inactivated AMPase indicated in Table 1. Samples of the incubation mixture were subjected to precipitation with acetone as described in the Materials and methods section.

AMPase inactivated (pmol/ml)	[^3H]SBA moiety incorporated (pmol/ml)	Radiolabelling/inactivation ratio (pmol/pmol)
(a) Chicken gizzard 5'-nucleotidase		
56	74	1.3
106	128	1.2
(b) Snake-venom 5'-nucleotidase		
680	620	0.9
840	1160	1.4
1100	1240	1.1

introduced by [^{14}C]5'-FSBA. Radiolabelled 5'-nucleotidase carrying the [^{14}C]SBA moiety was subjected to limited proteolysis. In parallel, radioiodinated 5'-nucleotidase was digested under identical conditions to enable

Fig. 6. Digestion of [^{14}C]SBA-labelled and radioiodinated chicken gizzard 5'-nucleotidase with clostripain

Chicken gizzard 5'-nucleotidase was treated with [^{14}C]5'-FSBA (lane 1) or radioiodinated (lane 2) as described in the Materials and methods section. Samples were incubated with clostripain (see the Materials and methods section), followed by electrophoretic separation of the digestion products. Dried gels were subjected to autoradiography. Positions of marker proteins are indicated at the left-hand side.

analysis of the production of additional proteolytic products. As shown in Fig. 6, treatment of chicken gizzard 5'-nucleotidase with clostripain produced a 45000- M_r ^{14}C -labelled fragment, whereas other polypeptides carrying [^{14}C]SBA were not detected (Fig. 6, lane 1). In contrast, radioiodinated 5'-nucleotidase gave additional labelled fragments of M_r about 40000 and 35000, which did not carry the affinity label (Fig. 6, lane 2). Thus we conclude that the 45000- M_r product of the chicken gizzard AMPase contains the essential amino acid residues of the catalytic centre that are lacking in the 40000- M_r and 35000- M_r proteins. Digestion of the 5'-nucleotidase with other proteinases, such as trypsin, chymotrypsin and thermolysin, gave similar results, i.e. only a restricted number of fragments obtained for the radioiodinated enzyme were labelled with the [^{14}C]SBA moiety. Additional analyses have shown that [^{14}C]SBA-labelled fragments do not contain the intact C-terminus of chicken gizzard 5'-nucleotidase and probably originate from the N-terminal half of the molecule (U. Stochaj, unpublished work). The results described in the present

paper further demonstrate the specificity of the modification by [^{14}C]5'-FSBA, i.e. only fragments containing the active site were recovered as ^{14}C -labelled peptides, whereas additional fragments were discovered with other procedures.

Taken together, affinity labelling of 5'-nucleotidases or other enzymes hydrolysing or binding adenosine nucleotides with 5'-FSBA can be used to investigate the catalytic centres or nucleotide-binding sites of these proteins. The selective radiolabelling described above will provide us with tools with which to analyse further the structural organization of 5'-nucleotidases and to identify domains carrying the active site.

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