Identification of histidyl and cysteinyl residues essential for catalysis by 5'-nucleotidase

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Inactivation of both cytosolic 5'-nucleotidase and ecto-5'-nucleotidase by diethylpyrocarbonate indicated the presence of an essential histidyl residue which in the cytosolic enzyme was conclusively located at the active site. Inactivation by thiol reagents indicated the presence of an essential cysteinyl residue in both enzymes. The data suggest that both 5'-nucleotidases belong to a group of histidine phosphatases which also includes glucose-6-phosphatase and acid phosphatase. A working hypothesis for the catalytic mechanism of these enzymes is proposed.

5'-Nucleotidase *Diethylpyrocarbonate* *Histidine* *Cysteine* *Phosphatase* *Catalytic mechanism*

1. INTRODUCTION

The cytoplasmic 5'-nucleotidase is the rate-limiting enzyme controlling loss of cellular adenine nucleotides during energy depletion [1]. The activity of the enzyme also determines the rate of formation from cytosolic AMP of the regulatory metabolite adenosine [2,3]. Adenosine subsequently acts to ameliorate or reverse the energy imbalance [4]. The plasma membrane 5'-nucleotidase is an ecto-enzyme [5] which may participate in the repletion of intracellular purine nucleotides by facilitating the incorporation of the nucleoside moiety from extracellular AMP [6,7]. The ecto-enzyme may also generate adenosine from extracellular nucleotides [8].

We have demonstrated using enzyme kinetic studies [9] that the catalytic mechanism of the cytosolic 5'-nucleotidase involves a phosphorylated enzyme intermediate. We report here investigations using group specific reagents to identify the residues essential for catalysis by the cytosolic enzyme and to establish similarities with the ecto-5'-nucleotidase.

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2. EXPERIMENTAL

2.1. *Materials*

Diethylpyrocarbonate (DEPC), iodoacetamide, *N*-ethylmaleimide (NEM), 5'-*p*-fluorosulphonylbenzoyladenosine (5'FSBA), phenylmethylsulphonylfluoride (PMSF), *N*-acetylimidazole (NAI), methylmethanethiosulphonate (MMTS), and diisopropylfluorophosphate (DFP) were obtained from Sigma. Hydroxylamine was obtained from Aldrich and mercaptoethanol from BDH.

2.2. *Enzymes*

Rat liver cytosolic 5'-nucleotidase (spec. act. 4–6 units/mg protein) was prepared by an abbreviation of the procedure in [10] as described in [9]. Rat liver plasma membrane 5'-nucleotidase (spec. act. 36 units/mg) was prepared as in [11].

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2.3. *Conditions for inhibition and reactivation*

Cytosolic 5′-nucleotidase (final concentration 0.2–0.5 units/ml) was incubated with 5 mM Tes–NaOH, 25 mM sodium β-glycerophosphate (pH 7.4), 100 mM NaCl, 0.5 mM EDTA and inhibitors or reactivators at concentrations shown in the text. DEPC was dissolved at 20-times the final concentration in ethanol. 5′FSBA was dissolved to 8 mM in ethanol–dimethylformamide (1:1, v/v). In these cases control incubations containing vehicle but no inhibitor were conducted and the activity of inhibited or reactivated enzyme related to this control. Other inhibitors were added from aqueous solutions containing 50 mM sodium β-glycerophosphate (pH 7.4). Incubations were conducted for periods and at temperatures shown in the text and then terminated by dilution 10-fold into the assay medium. In the case of DEPC reactions were quenched with 10 mM (final concentration) imidazole–HCl (pH 7.4). Enzyme activity was determined as in [3].

Plasma membrane 5′-nucleotidase (final concentration 0.2–0.5 units/ml) was incubated with 10 mM Tes–NaOH (pH 7.4) 2 mM $MgCl_2$ and inhibitors or reactivators added from stock solutions dissolved in the same buffer. DEPC was added as mentioned above. Enzyme activity was determined as in [11].

3. RESULTS

3.1. *Inactivation of 5′-nucleotidase by DEPC*

The cytosolic and plasma membrane 5′-nucleotidases were both rapidly inactivated by DEPC (fig.1). The cytoplasmic enzyme was more susceptible. Inactivation of the cytosolic enzyme was initially a first-order process (fig.2a) and the apparent rate constant (k_i) was obtained at a series of DEPC concentrations. A plot [12] of $\log k_i$ *vs* log(concentration of DEPC) was linear with a slope of 0.84 ± 0.06 (mean ± SE, 5 degrees of freedom) (fig.2b). This value was close to unity and demonstrated that modification of a single residue inactivated the enzyme.

DEPC can modify lysyl, histidyl, cysteinyl, seryl and tyrosyl residues of proteins [13]. Modification of histidine and serine is rapidly reversible by NH_2OH [13] whilst modification of tyrosine is more resistant to reversal [13]. Modification of lysine and cystine is irreversible by NH_2OH [13].

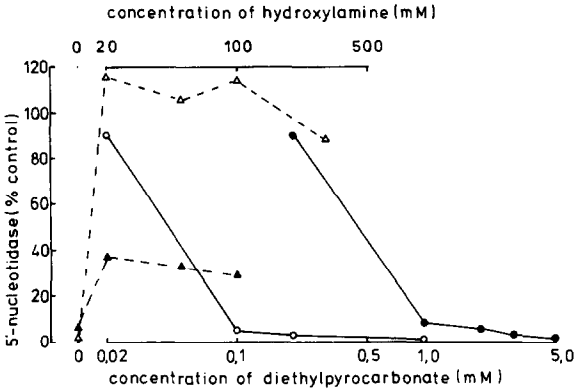

Fig.1. Inactivation and reactivation of 5′-nucleotidase. Cytosolic 5′-nucleotidase (○) or plasma membrane 5′-nucleotidase (●) was incubated with various concentrations of DEPC for 15 min at 23°C. The reaction was terminated with 10 mM imidazole–HCl (pH 7.4) and the remaining activity then determined. Cytosolic enzyme so treated with 0.2 mM DEPC (△) or plasma membrane enzyme treated with 1 mM DEPC (▲) was incubated for 1 h at 37°C with various concentrations of NH_2OH and then activity determined. Values are means from two experiments each conducted in triplicate.

The inhibition of the cytosolic 5′-nucleotidase by DEPC was readily and completely reversed by NH_2OH (fig.1). Reactivation of the plasma membrane enzyme was also readily achieved but was only partial. Reactivation was not improved by incubation with 100 mM NH_2OH for up to 3 h.

The ease of reactivation by NH_2OH argued against the participation of lysine, cysteine or tyrosine [13]. Further evidence against tyrosine was gained using NAI [14]. Incubation of cytosolic 5′-nucleotidase with 20 mM NAI at 37°C for 30 min followed by dialysis to remove NAI reduced enzyme activity to 63 ± 5% (results from 4 experiments). However, since no reactivation was achieved after incubation with 100 mM NH_2OH at 37°C for 1 h (final activity 50 ± 5%) the inactivation could not be ascribed to modification of tyrosine [14]. The plasma membrane 5′-nucleotidase may be iodinated without loss of enzymatic activity [15] again arguing against the presence of an active site tyrosyl residue.

Incubation of cytosolic 5′-nucleotidase with 1.25 mM DFP for 3 h at 37°C did not cause inhibition (final activity 98 ± 2% of control, 2 experiments). Incubation under the same conditions

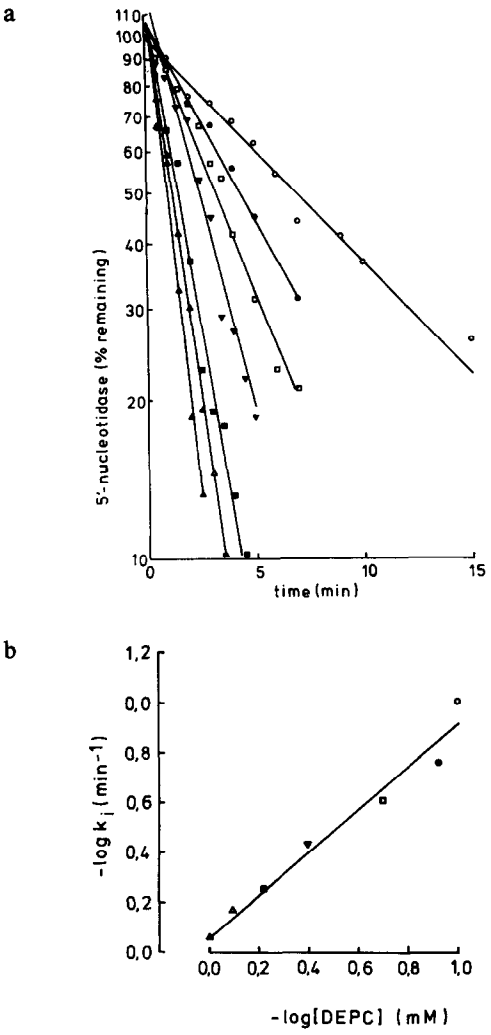

Fig.2. Kinetics of inactivation of cytosolic 5′-nucleotidase by DEPC. (a) Cytosolic 5′-nucleotidase was incubated at 23°C with 0.1 mM (○), 0.12 mM (●), 0.2 mM (□), 0.4 mM (▼), 0.6 mM (■), 0.8 mM (△) and 1.0 mM (▲) DEPC. At the times shown the reaction was quenched with 10 mM imidazole–HCl (pH 7.4) and enzymatic activity determined. Values are means of two experiments each conducted in triplicate. (b) The slopes of the lines in (a) were used to determine the first-order rate constant k_i.

with 4 mM PMSF also failed to inhibit the cytosolic 5′-nucleotidase (final activity 104 ± 4%, 3 experiments) or the plasma membrane 5′-nucleotidase (final activity 106 ± 4%, 2 experiments). These experiments demonstrated that 5′-nucleotidase is not a serine esterase [16].

In summary, the results eliminated all but histidyl residues as possible sites for reaction with DEPC.

3.2. *Protection against inactivation by DEPC*

Inactivation of the cytosolic 5′-nucleotidase with 0.2 mM DEPC for 15 min at 23°C was reduced from 99 ± 1% to 75 ± 9% in the presence of 5 mM IMP but was not reduced in the presence of 70 mM inosine. Inhibition was reduced to 12 ± 1% in the presence of 3 mM IMP plus 70 mM inosine (results of 4 experiments). Time-course studies (fig.3) showed that the first-order rate constant for inactivation by 0.2 mM DEPC was reduced by 3 mM IMP and 70 mM inosine from 0.198 to 0.010 min^{-1} while that for inactivation by 1 mM DEPC was reduced from 0.792 to 0.038 min^{-1}. The synergistic effect of inosine and IMP indicated that they did not protect the enzyme by reacting with DEPC.

Inactivation of the plasma membrane 5′-nucleotidase with 1 mM DEPC at 23°C for 15 min was reduced from 91 ± 1% to 77 ± 1% in the presence of 2 mM AMP and to 75 ± 2% in the presence of the competitive inhibitor ATP (2 mM).

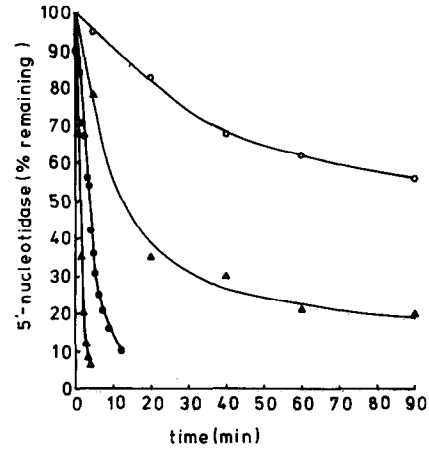

Fig.3. Protection of cytosolic 5′-nucleotidase from inhibition by DEPC. Cytosolic 5′-nucleotidase was incubated with 0.2 mM DEPC (○, ●) or 1 mM DEPC (△, ▲) either in the presence (○, △) or absence (●, ▲) of 3 mM IMP and 70 mM inosine for 15 min at 37°C. Reaction was terminated with 10 mM imidazole–HCl (pH 7.4) and then enzyme activity was determined. Values are means from two experiments each performed in triplicate.

Table 1

Effect of thiol reagents on 5'-nucleotidase

Enzyme	Inhibitor	Concentration (mM)	Residual activity (%)
Plasma membrane 5'-nucleotidase	iodoacetamide	15	4 ± 1
	NEM	15	83 ± 1
	cysteine	15	63 ± 7
	$HgCl_2$	5	12 ± 1
Cytosolic 5'-nucleotidase	iodoacetamide	15	92 ± 6
	NEM	12	95 ± 8
	$HgCl_2$	2	0.3 ± 0.2
	5'FSBA	1.25	60 ± 20
	MMTS	10	95 ± 4
Carboxyethylated cytosolic 5'-nucleotidase	iodoacetamide	15	33 ± 1
	NEM	15	72 ± 3
	5'FSBA	1.25	9 ± 4
	MMTS	10	46 ± 3

Cytosolic or plasma membrane 5'-nucleotidase was incubated with inhibitors for 15 min at 37°C before dilution by 10-fold into assay medium for measurement of enzymatic activity. Carboxyethylated cytosolic 5'-nucleotidase was produced by incubation with 0.2 mM DEPC for 15 min at 23°C followed by quenching with 10 mM imidazole–HCl (pH 7.4). After incubation with inhibitors the enzyme was reactivated with 50 mM NH_2OH–HCl (pH 7.4) for 1 h at 37°C and activity determined. This condition completely reactivated the enzyme not treated with thiol reagents (fig.1). Nonetheless, carboxyethylated-reactivated enzyme served as a control for these experiments. During use of 5'FSBA 75 ± 5% inactivation was caused by the vehicle alone (results from 4 experiments). Thus vehicle treated controls were used for these experiments.

Each result is the mean (± SE) for at least 3 separate experiments

Adenosine (5 mM) did not enhance the protection conferred by AMP.

3.3. *Reaction with thiol reagents*

The plasma membrane 5'-nucleotidase was inhibited by iodoacetamide, NEM, cysteine and $HgCl_2$ (table 1) all of which react with thiol groups [17]. The inactivation on exposure to 15 mM iodoacetamide at 37°C for 15 min (96 ± 1%) was reduced to 53 ± 1% by 5 mM ATP but was not reduced by 5 mM AMP.

The cytosolic 5'-nucleotidase was not inhibited by thiol reagents except $HgCl_2$ (table 1). However, when the enzyme was treated first with DEPC and then with thiol reagents, the enzyme could no longer be fully reactivated by NH_2OH (table 1). When reactivation of the enzyme treated with DEPC and the thiol reagent 5'FSBA [18] was attempted with 50 mM NH_2OH only 9% of activity was recovered (table 1). This was improved to 60 ± 10% if reactivation was conducted with 50 mM NH_2OH plus 10 mM mercaptoethanol. Thus reaction of the cytosolic 5'-nucleotidase with DEPC exposed a susceptible cysteinyl residue.

4. DISCUSSION

4.1. *Essential residues of 5'-nucleotidase*

Inactivation of both cytosolic and plasma membrane 5'-nucleotidase by DEPC and its ready reversal by NH_2OH suggested the presence of an essential histidyl or seryl residue. Failure to inhibit the enzyme with inhibitors of serine esterases indicated that the residue modified by DEPC was a histidyl residue. Partial reactivation of the plasma membrane 5'-nucleotidase could be explained by

the presence of a susceptible cysteinyl or lysyl residue in addition to the histidyl residue. The experiments with thiol reagents indicated that the plasma membrane (but not the cytosolic) 5'-nucleotidase possesses an accessible cysteinyl residue. Modification of this residue by the relatively high concentration of DEPC needed might explain the irreversible loss of activity. The cytosolic 5'-nucleotidase possesses an essential cysteinyl residue which was revealed only after reaction of the enzyme with low concentrations of DEPC.

4.2. *Location of the essential residues*

The pH-activity profile of the cytosolic 5'-nucleotidase is consistent with the participation of both histidyl and cysteinyl residues in the catalytic mechanism [19]. A combination of IMP and inosine protected the cytosolic 5'-nucleotidase from inhibition by DEPC. These ligands induce the formation of an enzyme–phosphate–inosine complex [9] locating the susceptible histidyl residue at the active site. The poor protection conferred by IMP alone demonstrated that the phosphorylated enzyme remained reactive to DEPC (possibly at the unphosphorylated nitrogen of the histidine ring) unless access to the active site was sterically hindered by inosine.

The inactivation of plasma membrane 5'-nucleotidase was only slightly decelerated in the presence of substrate. The failure of adenosine to inhibit plasma membrane 5'-nucleotidase non-competitively [20,21] or to promote nucleoside exchange [9,22] indicates that it has a low affinity for any postulated enzyme–phosphate intermediate. Therefore, additional protection of the enzyme from attack by DEPC was not expected. In addition, DEPC may act at a second site. Thus location of the susceptible histidine to the active site of the plasma membrane 5'-nucleotidase was suggestive but not conclusive.

ATP protected the plasma membrane 5'-nucleotidase from inactivation by iodoacetamide implying the presence of an active site thiol group. ATP is a competitive inhibitor of this 5'-nucleotidase [20,21] and the enzyme does not display allosteric properties suggesting that ATP and substrates compete at the active site. However, substrate alone did not protect the enzyme. Whilst this might be due to the smaller size of AMP, it is not possible to rule out that ATP caused a conformational change or protected a residue outside the active site. Likewise, it is possible that reaction of the cytosolic 5'-nucleotidase with DEPC relieved an intramolecular interaction between histidine and cysteine at the active site. On the other hand carboxyethylation may have caused a gross conformational change which unmasked a cysteinyl residue.

4.3. *Comparison with other phosphatases*

A phospho-enzyme intermediate of the cytosolic 5'-nucleotidase was detected by observing nucleoside exchange and from the kinetics of inhibition by product, inosine [9]. Similar evidence has been presented for glucose-6-phosphatase [23]. Plasma membrane 5'-nucleotidase was reported in [20] to catalyse a low rate of nucleoside exchange but nucleoside exchange was undetectable in [9,22]. Nucleoside exchange may be unfavourable with the ecto-enzyme owing to the low affinity of product for the putative phospho-enzyme intermediate or due to its rapid hydrolysis. The turnover number of the ecto-enzyme is approx. 20-times that of the cytosolic 5'-nucleotidase [10,11].

Both acid phosphatase [24] and glucose-6-phosphatase [25] can be phosphorylated by substrate on a histidyl residue. These enzymes also possess essential cysteinyl residues [26,27] although in neither case was inactivation by thiol reagents completely prevented by substrate. In contrast, alkaline phosphatase is a serine esterase which is inhibitable by DFP [16]. Our results suggest that the cytosolic and the ecto-5'-nucleotidase belong to a group of histidine phosphatases. It will be interesting to study the evolutionary relation between these enzymes.

4.4. *Catalytic mechanism*

A working hypothesis for the catalytic mechanism of the cytosolic 5'-nucleotidase is shown in fig.4. Nucleophilic attack by histidine on IMP leads to an enzyme phosphate intermediate. The high pK_a of the 5'-OH of inosine implies that it would be a very poor leaving group unless aided by a general acid. Likewise, the uncatalysed hydrolysis of histidine phosphate is slow [28] implying the need for a general base to activate water or indeed inosine when it participates in the ex-

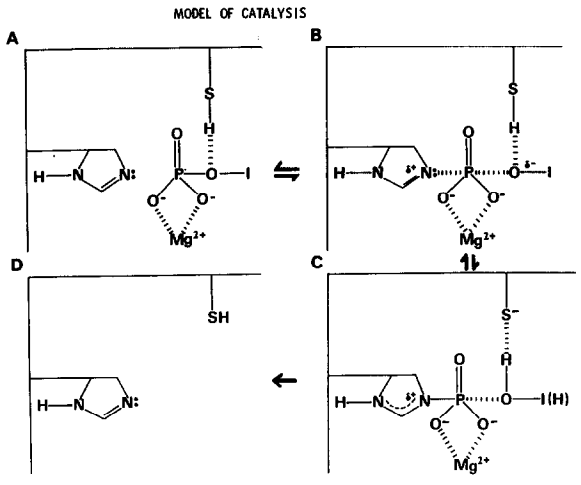

Fig.4. A working hypothesis for the catalytic mechanism of cytosolic 5′-nucleotidase. The working hypothesis attempts to account for: (1) the formation of a phosphoenzyme intermediate; (2) participation of an active site histidine; (3) the need for general acid/base catalysis, a function which cysteine might perform; (4) the ability of Mg^{2+} to activate the enzyme [10]. (A) The Michaelis complex of cytosolic 5-nucleotidase and IMP. (B) Acid catalysed rupture of the bond between the inosine and phosphate moieties whilst establishing a partial covalent bond with the active site histidine. (C) Base catalysed collapse of the phosphorylated enzyme intermediate by water (HOH) or inosine. (D) The free enzyme.

change reaction. This mechanism may be applicable to the other histidine phosphatases.

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