Mechanism of the reaction catalysed by cytosolic 5'-nucleotidase/phosphotransferase: formation of a phosphorylated intermediate

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Cytosolic 5'-nucleotidase preferentially catalysing the hydrolysis of IMP, GMP and their deoxy derivatives, and endowed with phosphotransferase activity, was purified from calf thymus and its reaction mechanism was studied. In the presence of [³²P]IMP, ATP and MgCl₂, a covalent enzyme-phosphate intermediate was trapped by mixing with an SDS solution. Heat or acid treatment

of the enzyme before incubation with radiolabelled substrate prevented formation of the intermediate. Furthermore, on the basis of studies on the kinetic parameters of the enzyme as function of pH, and of experiments on thiol oxidation and photo-oxidation, we suggest the involvement of cysteine and histidine residue(s) in the reaction mechanism.

INTRODUCTION

Cytosolic 5'-nucleotidase (EC 3.1.3.5), which preferentially hydrolyses IMP, is a widespread enzyme that shares with other phosphohydrolases the ability to transfer the phosphate to a suitable acceptor [1]. The occurrence of the phosphotransferase reaction *in vivo* has been hypothesized and indicated as being responsible for the phosphorylation of several anti-viral or anti-tumour purine pro-drugs [2,3]. Both the phosphohydrolase and phosphotransferase activities of the enzyme may be regulated by several compounds. Our previous results indicate that, in the presence of a high energy charge and at physiological pH and phosphate concentration, the catabolic or interconverting activity of the enzyme purified from calf thymus depends only on the availability of its substrates. In fact, under such conditions, if a nucleoside substrate is present, the enzyme acts exclusively as a phosphotransferase [4]. It has been hypothesized by several authors that the reaction mechanism proceeds through the formation of an enzyme-phosphate intermediate, as for many phosphate-transferring enzymes. This hypothesis is based on kinetic evidence such as the inhibition exerted by inosine (a good phosphate acceptor) on phosphate evolution during IMP hydrolysis, but it has not yet been directly demonstrated [5,6]. In order to understand the role of cytosolic 5'-nucleotidase and to elucidate its reaction mechanism, the isolation and identification of the enzyme-phosphate intermediate is of great importance.

The formation of a phosphorylated intermediate has been demonstrated for low-molecular-mass acid phosphatases which can hydrolyse the tyrosine-phosphate bond in proteins, by using a ³²P-labelled substrate and trapping the intermediate in an SDS mixture followed by electrophoresis and autoradiography [7,8]. Here we have utilized a similar method to show the formation of an enzyme-phosphate complex as an intermediate in the hydrolysis of [³²P]IMP catalysed by cytosolic 5'-nucleotidase purified from calf thymus. Furthermore, we have studied the effect of pH on enzyme photo-oxidation and the effect of thiol reagents on enzyme activity, in order to formulate hypotheses on the role in enzyme catalysis of amino acid residues that have been indicated to participate in the reaction mechanisms of some phosphotransferases [7–9].

EXPERIMENTAL

Materials

[8-¹⁴C]inosine (57 mCi/mmol) was purchased from Sigma Chemical Co. Polyethyleneimine-cellulose precoated thin layer plastic sheets (0.1 mm thick) were obtained from Merck, and were prewashed once with 10 % NaCl and three times with deionized water before use. DE-81 chromatographic paper was from Whatman. Nucleosides, nucleotides, AMP deaminase (EC 3.5.4.4) from rabbit muscle (0.3 mg/ml; 85 units/mg) and Protein A coupled to peroxidase were purchased from Sigma. [³²P]IMP was obtained from [³²P]AMP (3 Ci/mmol) purchased from Amersham, by the following procedure: 0.66 mM [³²P]AMP, 4.75 mM AMP, 100 mM KCl and 1 unit of AMP deaminase were incubated for 2 h at 37 °C, heated for 10 min at 80 °C and then stored at –20 °C. Under these conditions a complete transformation of [³²P]AMP into [³²P]IMP was obtained, as verified by TLC on polyethyleneimine-cellulose developed with 1.4 M LiCl. Autoradiography film was Hyperfilm-max from Amersham. Rabbit anti-(goose IgG) was purchased from Nihon Biotest Laboratories, Inc. (Kokubunji, Japan). All other chemicals were of reagent grade. All solutions were prepared with Milli-Q water.

Purification of 5'-nucleotidase

The enzyme was purified approx. 1000-fold from calf thymus as previously described [4], by a procedure comprising seven purification steps. The final yield was 16 %, and the specific activity was 6 units/mg, evaluated as phosphotransferase activity with the radiochemical assay.

Enzyme assays

Phosphotransferase activity was determined by a radiochemical assay in the presence of [8-¹⁴C]inosine as substrate, as previously described [6]. Alternatively, both the hydrolase and phosphotransferase activities of the enzyme were determined by a stopped method in which substrates and products were separated using a

Abbreviations used: DTT, dithiothreitol; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); PVDF, poly(vinylidene difluoride).

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capillary electrophoresis apparatus [10]. The mixture for determination of the kinetic constants for deoxy-GMP hydrolysis was as follows: 0.3 $\mu\text{g/ml}$ purified enzyme preparation, deoxy-GMP ranging between 0.09 mM and 1.6 mM, 4.5 mM 2,3-bisphosphoglycerate, 20 mM MgCl_2 , 1 mM dithiothreitol (DTT) and 200 mM Tris/HCl buffer (pH 7.0–8.8) or 200 mM 3,3-dimethylglutarate buffer (pH 5.0–7.5). After 0, 10, 20 and 30 min of incubation at 37 °C, 45 μl of the mixture was withdrawn, added to 75 μl of ice-cold methanol and kept at –20 °C overnight. The aliquots were centrifuged in a Microfuge and the supernatants were dried under vacuum, resuspended in water and analysed by electrophoresis in a capillary electrophoresis apparatus Beckman P/ACE system 2100 as previously described [10]. A similar procedure was applied for determination of the rate of inosine phosphorylation as function of pH. In this case the mixture was as follows: 0.3 $\mu\text{g/ml}$ enzyme preparation, 5 mM deoxy-GMP, 0.8 mM inosine, 4.5 mM 2,3-bisphosphoglycerate, 20 mM MgCl_2 , 1 mM DTT and 200 mM Tris/HCl buffer (pH 7.0–8.8) or 200 mM 3,3-dimethylglutarate buffer (pH 5.0–7.5). The values of K_m and $V_{\max}$ were determined by computer-assisted analysis. One unit of enzyme activity is the amount of enzyme that catalyses the conversion of 1 μmol of substrate per min under our experimental conditions.

Specific antiserum

Goose anti-(pig lung cytosolic 5'-nucleotidase) serum was produced as previously described [11].

Immunotitration

Quantitative immunotitration of cytosolic 5'-nucleotidase/phosphotransferase was performed as described previously, using goose anti-(pig lung cytosolic 5'-nucleotidase) and rabbit anti-(goose IgG) [11]. Briefly, 3 m-units of phosphotransferase activity in crude extract or purified preparation were incubated with variable amounts of specific antiserum in a final volume of 50 μl . After a 2 h incubation at 37 °C, 10 μl of rabbit anti-(goose IgG) was added. After 30 min at room temperature, samples were centrifuged in a Microfuge and the supernatant was used for phosphotransferase assays as previously described [6]. Controls incubated under the same condition as the samples, but in the absence of primary and secondary antibodies, indicated that the procedure caused a decrease in enzyme activity of about 50 %.

Phosphoenzyme covalent intermediate trapping

A 3 μg sample of enzyme preparation was incubated for 30 s at 37 °C with 2 mM [^{32}P]IMP (3 Ci/ mmol), 20 mM MgCl_2 , 4.5 mM ATP, 1 mM DTT, 10 mM Tris/HCl buffer, pH 7.4, in a final volume of 24 μl and then added to 6 μl of a mixture containing 20 % glycerol and 4 % SDS in 125 mM Tris/HCl, pH 7.4. The samples were loaded on a 9 % polyacrylamide/SDS gel and electrophoresis was performed according to Laemmli [12]. When specified, the proteins were transblotted on to a poly(vinylidene difluoride) (PVDF) membrane for 2 h at 200 mA. ^{32}P -labelled proteins were identified by autoradiography. The PVDF membrane was immunostained by successive incubation with specific antiserum (diluted 1:20000), rabbit anti-(goose IgG) (1:1500) and peroxidase-conjugated Protein A (1:500).

Photoinactivation of the enzyme

The photoinactivation of 5'-nucleotidase was studied between pH 6.0 and 8.7, using Methylene Blue as photosensitizer. Before irradiation, the enzyme was extensively dialysed against 20 mM

Tris/HCl buffer, pH 7.4, to remove DTT. Samples containing 70 m-units/ml enzyme, 0.15 mM Methylene Blue and 200 mM Tris/HCl buffer (pH 7.0–8.8) or 200 mM 3,3-dimethylglutarate buffer (pH 5.0–7.5) were exposed in a glass ice-cold bath to the light of a 100 W tungsten bulb at a distance of 15 cm. After 6 min of irradiation, aliquots of 5 μl were transferred to 50 μl of an assay mixture containing 1.4 mM [$8\text{-}^{14}\text{C}$]inosine (7000 d.p.m./nmol), 4.5 mM ATP, 20 mM MgCl_2 , 1 mM DTT and 200 mM Tris/HCl buffer, pH 7.4, and the residual phosphotransferase activity was measured by radiochemical assay [6]. Residual activity was also measured both after irradiation of the enzyme in the absence of Methylene Blue (light control) and after incubation of the enzyme/Methylene Blue mixture in the dark (dark control).

Other methods

Protein was determined by the Coomassie Blue binding assay [13] using BSA as standard.

RESULTS

Enzyme–phosphate intermediate trapping

[^{32}P]IMP was used for the phosphoenzyme covalent intermediate trapping experiments. The trapping of the phosphoenzyme intermediate was performed by the addition of an SDS solution after a brief incubation of the enzyme preparation with [^{32}P]IMP, MgCl_2 and ATP. The phosphorylated proteins were revealed either by autoradiography of an SDS/polyacrylamide gel or by autoradiography of a PVDF membrane after blotting of an SDS/polyacrylamide gel. For control experiments, the enzyme was inactivated either by boiling for 5 min or by exposure at pH 4 before the trapping experiment. To identify the protein corresponding to cytosolic 5'-nucleotidase we used a specific antiserum, which was formerly utilized to determine the concentration of the enzyme in crude extracts of several cells and organs by quantitative immunotitration [11]. As shown in Figure 1, the antiserum precipitated the phosphotransferase activity of

Figure 1 Immunoprecipitation of calf thymus phosphotransferase activity with a specific antiserum

Crude extract (●) or purified enzyme preparation (○) was mixed with various amount of antiserum and immunoprecipitation was carried out as described in the text. Phosphotransferase activity was determined as indicated in the Experimental section, with 1.4 mM [$8\text{-}^{14}\text{C}$]inosine as substrate in the presence of 2 mM IMP, 4.5 mM ATP and 20 mM MgCl_2 .

Figure 2 SDS/PAGE and immunoblotting of the purified enzyme preparation

Lane 1: 0.15 μ g of enzyme preparation was analysed on an SDS/9%-polyacrylamide gel and stained for protein. Lane 2: same as lane 1 after Western blotting on a PVDF membrane and immunostaining. The molecular masses of the following marker proteins are indicated: BSA (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa) and carbonic anhydrase (29 kDa).

Figure 3 Phosphoenzyme covalent intermediate trapping

A 3 μ g sample of purified enzyme preparation was incubated with [32 P]IMP for 30 s, and the phosphoenzyme was trapped by rapid mixing with 4% SDS as described in the text. Phosphorylated proteins were revealed by autoradiography of an SDS/polyacrylamide gel after electrophoresis. Purified enzyme preparation before (lane 1) and after (lane 2) affinity chromatography on ADP-agarose, and purified enzyme preparation boiled for 5 min before trapping (lane 3), were used as samples. The molecular masses (kDa) of the same marker proteins as in Figure 2 are indicated.

cytosolic 5'-nucleotidase both in the crude extract and in the purified enzyme preparation. In both cases, the same amount of antiserum was necessary to precipitate the enzyme activity.

The electrophoretic pattern of the final enzyme preparation showed the presence of two major polypeptide species of molecular mass 59 and 54 kDa which, as shown by immunoblotting analysis, cross-reacted with the specific antiserum (Figure 2). The addition of protease inhibitors to the buffers used to isolate the enzyme did not change the electrophoretic pattern. The results of the enzyme-phosphate trapping experiments using active cytosolic 5'-nucleotidase indicated that the same two polypeptides were phosphorylated (Figure 3, lanes 1 and 2; Figure 4, lane 1). When we treated the enzyme preparation by exposing it to either high temperature or low pH, these radioactive bands were not detected, and a new radioactive band corresponding to a higher molecular mass appeared (Figure 3, lane 3; Figure 4, lanes 2 and 3), which was not cross-reactive with the specific antiserum (Figure 4, lanes 5 and 6). The bands corresponding to 59 and

Figure 4 Identification of radiolabelled cytosolic 5'-nucleotidase with a specific antiserum

The purified enzyme was incubated with [32 P]IMP and treated as in Figure 3. After SDS/PAGE and Western blotting, the PVDF membrane was exposed against an autoradiography film. Lane 1, 11 μ g of purified enzyme preparation; lane 2, same as lane 1, boiled for 5 min at 100 $^{\circ}$ C before trapping; lane 3, same as lane 1, treated at pH 4 before trapping. After exposing the PVDF membrane against an autoradiography film, the same membrane was immunostained using antibodies against cytosolic 5'-nucleotidase. Lanes 4, 5 and 6 correspond to the lanes 1, 2 and 3 respectively after immunostaining. The molecular masses (kDa) of the same marker proteins listed in Figure 2 are indicated.

54 kDa were still detected in denatured preparations by immunostaining (Figure 4, lanes 5 and 6).

Photoinactivation of the enzyme, and effects of thiol reagents on enzyme activity

The pH-dependence of the photoinactivation of cytosolic 5'-nucleotidase in the presence of Methylene Blue is indicated in Figure 5. The enzyme was effectively inactivated at pH 7–8, and was protected from photoinactivation by the addition of its substrate IMP.

Calf thymus cytosolic 5'-nucleotidase was sensitive to thiol reagents. In fact, the enzyme loses half of its activity within 2 days at -75° C in the absence of reducing agents (1 mM DTT) and is inhibited by 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB).

Figure 5 Photoinactivation of cytosolic 5'-nucleotidase

After 6 min of exposure to the light of a 100 W tungsten bulb, in the presence of Methylene Blue and with (●) or without (○) 2 mM IMP at different pH values, the residual phosphotransferase activity was measured as described in the text. Controls: Δ , irradiated in the absence of Methylene Blue; $\square$, incubated in the presence of Methylene Blue in the dark.

Figure 6 Inactivation of the phosphotransferase activity by DTNB

The enzyme was incubated with 0.3 mM DTNB either alone (○), or in the presence of 2 mM IMP (□) or 4.5 mM ATP (△). After 75 min of incubation, 5 mM DTT was added to the incubation mixture, and phosphotransferase activity was measured as described in the text. Closed symbols refer to the activity of the enzyme incubated under similar conditions but in the absence of DTNB.

In the presence of 0.3 mM DTNB we observed complete inactivation of the enzyme within 1 h (Figure 6). The effect was reversed by 5 mM DTT. The presence of a substrate (IMP) or an

allosteric activator (ATP) did not affect the DTNB-induced inactivation.

Effect of pH on kinetic parameters

In the course of our studies on cytosolic 5'-nucleotidase/nucleoside phosphotransferase purified from calf thymus, we became aware that the pH optimum of the enzyme acting as a phosphohydrolase was different from that of the enzyme acting as a phosphotransferase [10]. To investigate the molecular basis of this difference in pH optima, we determined the kinetic parameters of the enzyme as a function of pH. The pH-dependence of the $V_{\max}$ for the reaction of deoxy-GMP hydrolysis showed a pK_a of 8, which indicates a dissociation process between enzyme and substrate in which an ionizable group is involved (Figure 7A). Two additional pK_a s of 6.4 and 7.2 were observed from the plot of pK_m against pH (Figure 7B) which are related to ionizable groups either of the free enzyme or of the free substrate. We measured the rate of IMP formation in the presence of a subsaturating concentration of inosine as phosphate acceptor, and a saturating concentration of deoxy-GMP as phosphate donor, as a function of pH (Figure 7C). Two pK_a values of 6.4 and 7.8 were observed from the plot of $\log v$ against pH. This suggests that the rate of formation of the enzyme-phosphate-inosine complex was influenced by two dissociation processes.

DISCUSSION

Many phosphatases with different substrate specificities and cellular locations have been described to catalyse a phosphotransferase reaction [7–9], while several enzymes that are called phosphotransferases have been demonstrated to hydrolyse phosphoesters [14,15]. In fact, the ability to transfer a phosphate group is shared by different phosphohydrolases and is probably due to a unique mechanism of action involving an enzyme-phosphate intermediate [7,8]. This reaction mechanism has been proposed also for cytosolic 5'-nucleotidase purified from rat liver, on the basis of kinetic studies on the inhibition exerted by added inosine on phosphate formation from IMP [5]. In previous papers we described the kinetic behaviour of the enzyme purified from calf thymus, which supported this hypothesis [4,6]. In fact, by measuring the rate of nucleoside formation from nucleoside monophosphates and the rate of phosphate formation in the presence or in the absence of a nucleoside acceptor, we concluded that inosine and water could compete for the same phosphorylated intermediate. Furthermore, the activatory effect exerted by the phosphate acceptor on the rate of nucleoside formation from a mononucleotide might be caused by the partitioning of a phosphorylated intermediate between a nucleoside acceptor and water, where the rate-limiting step is hydrolysis of the intermediate [10]. The formation of a covalent enzyme-substrate intermediate has been demonstrated for several enzymes catalysing the transfer of phosphate, such as phosphoglucomutase [16] and phosphoglyceromutase [17]. This has also been demonstrated for various hydrolases, such as alkaline phosphatase [9] and low-molecular-mass cytosolic phosphotyrosine protein phosphatase [7], which are also phosphotransferases.

The results of the present study demonstrated that the covalent enzyme-phosphate intermediate was formed by incubating active cytosolic 5'-nucleotidase with [32 P]IMP, and was trapped by rapid mixing with SDS. When cytosolic 5'-nucleotidase was inactivated by low pH or high temperature, it still showed the same electrophoretic and immunochemical behaviour as the native enzyme, but was no longer able to bind radioactive phosphate. Under these experimental conditions, a radioactive band of higher molecular mass (66 kDa) appeared, which,

Figure 7 Effect of pH on the kinetic parameters of cytosolic 5'-nucleotidase

In (A) and (B), $\log V_{\max}$ and pK_m are plotted as a function of pH, with deoxy-GMP as substrate ranging between 0.09 mM and 1.6 mM. In (C) $\log v$ for IMP formation from saturating deoxy-GMP (2 mM) and subsaturating inosine (ranging from 0.2 to 2 mM) is plotted as a function of pH. Solid curves were obtained by computer-assisted analysis by fixing a pK_a for the enzyme-substrate complex at 8 (A), two pK_a values for the free enzyme or substrate at 6.4 and 7.2 (B), and two pK_a values for the enzyme-phosphate complex at 6.4 and 7.8 (C).

however, did not react with specific antibodies. We, therefore, speculate that the treatment of the enzyme preparation at low pH or high temperature is responsible for a possible structural change of a coexisting protein in the final enzyme preparation, which exposes a binding site for IMP. Indeed, a faint silver-stained band with a molecular mass identical to that exhibited by the labelled protein was present in the enzyme preparation after boiling or acid treatment. Although its identity remains obscure, on the basis of the molecular mass and the ability of albumin to bind several compounds, the labelled band might be reasonably ascribed to albumin itself.

Although it is usually very difficult to predict the groups involved in catalysis only from the effect of pH on the kinetic parameters, our results suggest that cysteine and histidine are involved in the enzyme's mechanism of action. The pK_a of 6.4 evaluated from the plot of pK_m against pH might be interpreted as an indication of the involvement of a histidine residue in the formation of both deoxy-GMP-enzyme and enzyme-phosphate-inosine complexes, although it might well be related to the pK_a of the free substrate (deoxy-GMP) dissociation. The pK_a values of 7.2, 7.8 and 8.0 might be indicative of the involvement of cysteine residue(s).

In a previous paper we showed a difference in the pH optima for the hydrolase and phosphotransferase reactions (6.5 and 7.4 respectively) [10]. On the basis of our results, we may speculate that a cysteine residue with a particularly low pK_a (7.2) is present in the free enzyme and that the environment of this residue is significantly affected by the formation of the enzyme-phosphate intermediate, causing the change in the pK_a to closer to 8. This could explain the shift of the dependence of the rate of the phosphotransferase reaction towards more alkaline pH values.

The profiles of the pH-dependence of the photoinactivation of cytosolic 5'-nucleotidase also suggest the involvement of a histidine residue in its action. In the light of our results, since the substrate IMP protects the enzyme from photo-oxidation in the presence of Methylene Blue, it seems reasonable to propose that histidine might be involved in the formation of the covalent enzyme-phosphate intermediate, as in several other phosphate-transferring enzymes [17]. On the other hand, some authors have demonstrated the formation of a thiol-phosphate bond for several protein phosphatases [7,8]. Our results suggest that cysteine might also be involved in enzyme catalysis; however, a direct involvement of a cysteine residue in the bond with phosphate seems unlikely, since the presence of the substrate IMP is unable to protect the enzyme from thiol oxidation.

The use of a specific antiserum against cytosolic 5'-nucleotidase revealed the presence of two bands related to the enzyme on gel electrophoresis, both of which were phosphorylated in the presence of [32 P]IMP. It is highly possible that the occurrence of these two bands is due to proteolysis, as cytosolic 5'-nucleotidases from chicken liver and pig lung have been shown to be very susceptible to proteolysis [18,19], although we observed the bands at very early steps of purification and in the presence of protease inhibitors. However, since recent results indicate the presence in chicken of two types of cDNAs encoding purine 5'-nucleotidase corresponding to two separate genes [20], the existence in the calf thymus of different forms of cytosolic 5'-nucleotidase cannot be ruled out.

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