Evidence for the presence of essential histidine and cysteine residues in platelet cGMP-inhibited phosphodiesterase

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cAMP is a major regulator of platelet function. cGMP-inhibited phosphodiesterase (cGI-PDE) is the predominant platelet enzyme hydrolysing cAMP. The pH–rate profile plot for this enzyme yields pK_a values of 6.5 and 9.0, consistent with histidine and cysteine residues respectively. Diethyl pyrocarbonate (DEP) inactivates cGI-PDE in a time- and concentration-dependent manner, and this effect was rapidly reversed by hydroxylamine. It was estimated that 2 mol of histidine residues per mol of enzyme were responsible for the loss of catalytic activity, as deduced from the correlation of the difference spectrum at 240 nm of the DEP-modified cGI-PDE with the enzymic activity. *N*-Ethylmaleimide (NEM) and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) inactivate cGI-PDE in a time- and concentration-dependent manner,

suggesting the selective modification of a cysteine residue. AMP protects the enzyme against DEP, NEM and DTNB, suggesting the presence of histidine and cysteine residues at the active site of cGI-PDE. [14 C]DEP incorporation in the presence of AMP or cGMP indicates the protection of two histidine residues by each nucleotide. These residues are different for each agent, since the combination of AMP and cGMP protects four histidine residues. [3 H]NEM incorporation showed that 1 mol of cysteine per mol of cGI-PDE was protected by AMP, but not by cGMP. We conclude that cGI-PDE possesses two essential histidine residues for activity, two additional histidines for cGMP inhibition, and one cysteine residue at the active site.

INTRODUCTION

cAMP, a ubiquitous second messenger, is involved in signal transduction and other intracellular processes related to diverse cellular functions. The intracellular level of cAMP is regulated by its synthesis by adenylate cyclase and its hydrolysis by cyclic nucleotide phosphodiesterases (PDEs). Regulation of PDE activity comprises an important mechanism for the control of cyclic nucleotide concentrations that in turn mediate cellular responses to biological stimuli. In human platelets, an increase in intracellular cAMP is associated with the inhibition of all platelet responses to agonists, including shape change, aggregation, adhesion and release of granule contents [1–3].

Based on substrate specificity, kinetic characteristics, physical properties and mode of regulation, PDEs have been classified into families [4–6], now numbering eight [7]. Some of them respond allosterically to cGMP; others are regulated by interaction with other proteins (e.g. calmodulin in calcium/calmodulin-dependent PDE). The hydrolysis of cAMP by the cGMP-stimulated PDE is increased by micromolar concentrations of cGMP, whereas the activity of cGMP-inhibited PDE (cGI-PDE) is decreased by micromolar concentrations of cGMP.

The amino acid sequences of each PDE family share moderate sequence identity within a region of approx. 270 amino acids in the C-terminal portion. This congruence of the primary sequences, as well as the fact that enzymes truncated at the N-terminal regions express full catalytic activity, suggest that the C-terminal region contains the catalytic domain of these enzymes [5,6,8–11]. In this domain, several histidine and glutamate residues, as well as a single cysteine residue, are invariant in all mammalian PDEs. The conserved histidine and glutamate residues have been suggested to play a critical role in ligating a required bivalent metal cation (Zn^{2+} , Mg^{2+} , Mn^{2+} or Co^{2+})

[12,13], as in known metallohydrolases such as thermolysin, carboxypeptidase and alkaline phosphatase [14].

Human platelets have three different forms of cyclic nucleotide PDE activity [15]. The first is relatively specific for cGMP (cGMP-binding cGMP-specific PDE); the second is a cGMP-stimulated PDE that hydrolyses cAMP and cGMP at nearly equal rates, but with a relatively high K_m (35–50 μ M); and the third form is cGI-PDE, the predominant enzyme hydrolysing intracellular cAMP in platelets [15] with a low K_m (0.2 μ M).

In order to understand the catalytic mechanism of cGI-PDE, we have performed chemical modification studies on the active site of the enzyme isolated from human platelets. Previous studies from our laboratory suggested the presence of a cysteine residue and a tyrosine residue at or near the active site of cAMP-specific PDE [16]. Other studies have also demonstrated the presence of a cysteine residue and a histidine residue at the active site of calcium/calmodulin-dependent PDE [17]. The kinetic and incorporation studies with group-specific chemical modification reagents described here also suggest the presence of catalytically essential histidine and cysteine residues for cAMP binding or catalysis, as well as essential histidines involved in cGMP inhibition, in human platelet cGI-PDE.

MATERIALS AND METHODS

Materials

Diethyl pyrocarbonate (DEP), *N*-ethylmaleimide (NEM), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), hydroxylamine-dithiothreitol, cAMP, DEAE-Sephadex-A25, Caps, Pipes, Mes, Hepes, Tris/HCl and most other chemicals were obtained from Sigma. [3 H]cAMP (28.4 μ Ci/mmol) was purchased from DuPont/New England Nuclear, and [3 H]AMP (57 μ Ci/mmol) was from ICN. Boronate Affi-Gel 601 resin used in the separation

Abbreviations used: PDE, phosphodiesterase; cGI-PDE, cGMP-inhibited PDE; DEP, diethyl pyrocarbonate; NEM, *N*-ethylmaleimide; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

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of [^3H]AMP from cAMP was purchased from Bio-Rad. Outdated human platelets were kindly supplied by the American Red Cross (Penn-Jersey).

Enzymic activity assay

PDE activity assays were performed in 100 μl reaction volumes consisting of 100 mM Tris buffer, pH 7.5, 0.5 mg/ml BSA and 5 mM MgCl_2 . The reaction was initiated by adding 25 μl of 4 μM [^3H]cAMP (20000 c.p.m.), incubated at 25 °C and terminated by adding 200 μl of 0.25 M zinc sulphate followed by 200 μl of 0.25 M barium hydroxide [18]. The mixture was then centrifuged and radioactivity in 200 μl of the supernatant was counted in 3 ml of Ecolite scintillation fluid (ICN) to determine [^3H]cAMP remaining.

A second method used to assay the PDE activity consisted of boiling the above incubation mixture for 2 min, then diluting it to 1 ml and applying it to the boronate affinity column (Bio-Rad). The resin binds the vicinal diol [^3H]AMP (product of hydrolysis), but not [^3H]cAMP. After washing the column with 50 mM Hepes, pH 8.5, buffer to remove unbound excess reagents, the [^3H]AMP was eluted with 0.2 M acetic acid and the radioactivity determined as in the precipitation assay. When this assay was used in the pH-rate profile experiments, the reaction mixtures were adjusted to pH 8.5 after boiling. The activity was calculated based on identically treated control samples, and all assays were conducted in the linear range of the reaction whereby less than 30 % of the initial substrate was hydrolysed.

Isolation of platelet cGI-PDE

The enzyme was isolated by a modification of the method of Grant and Colman [8]. Outdated platelets were centrifuged at 7000 g for 8 min and the pellet was removed with a spatula to avoid the red blood cells at the bottom. It was then resuspended in 100 mM Tris/acetate buffer containing 0.15 M NaCl, pH 6.0. The resuspended platelets were then centrifuged and recovered as above. The washed platelets were centrifuged again and the pellet was stored at -70 °C in 100 mM Tris/acetate buffer, pH 6.5, containing 10 mM MgCl_2 and the following proteinase inhibitors: 20 mM benzamidine, 5 mM ϵ -amino-n-caproic acid, 2 mM EGTA, 5 μM tosyl-lysylchloromethane (TLCK) and 0.01 % soybean trypsin inhibitor. All purification procedures were carried out at 4 °C. The platelets (from 200 donors; approx. 10^{11} platelets/donor) were resuspended in 300 ml of buffer, and sonicated for 3×5 min at 70 % power output using a macro tip cell disruptor (model W-225R; Ultrasonic Inc.). The lysate was centrifuged at 45000 g for 1 h and the resulting supernatant was loaded, in batches, onto 140 ml of DEAE-Sepharose CL-6B (Pharmacia). After 2 h the resin was washed, using a filter funnel, with 50 mM Tris/acetate buffer, pH 6.5, containing 10 mM MgCl_2 . The loaded resin was then packed into a 3 cm $\times$ 25 cm column. The PDE activity was recovered by applying a 1 litre linear salt gradient of 0–1.0 M sodium acetate (flow rate = 1.0 ml/min). The fractions were assayed for cAMP hydrolysis and, in order to distinguish the fractions containing cGMP-stimulated PDE from those containing cGI-PDE, the cAMP hydrolysis assay was performed in the presence or absence of 10 μM cGMP. All fractions containing PDE activity inhibited by cGMP were combined, and loaded (flow rate 0.4 ml/min) on to a Blue Dextran affinity column (2.5 cm $\times$ 18 cm) as previously described by Grant and Colman [8]. The progress of purification was monitored by protein quantification by the Coomassie Blue method, the enzyme activity assay and SDS/PAGE (9 % gels) [19]. The purified enzyme was similar to that previously described [8] and contained a mixture of the native enzyme (110 kDa)

(< 10 %) and a proteolytic product (61 kDa) (> 90 %). Both components had the same specific activity, and both were recognized by a polyspecific affinity-purified antibody to cGI-PDE on Western blotting. The molarity was based on a molecular mass of 61 kDa, and no correction was applied for the < 10 % that presents as the 110 kDa form.

pH-rate profile

The pH-rate profiles using saturating cAMP concentrations (2 μM) were determined over the pH range 5.0–10.5. The following buffers were utilized at 50 mM concentration for the pH ranges indicated: Mes, pH 5.0–6.0; Pipes, pH 6.5–7.0; Hepes, pH 7.5–8.5; Caps, pH 9.0–10.5. The activity assay for the pH-rate profile was performed as described above, with slight modifications as follows. The enzyme was added and, after boiling to stop the reaction, the mixture was diluted with 5 ml of 150 mM Hepes, pH 8.5, buffer containing 100 mM NaCl and then treated as described above.

Inactivation of cGI-PDE with DEP, NEM or DTNB

cGI-PDE (50–60 $\mu\text{g/ml}$) was incubated at 25 °C with a modifying agent or vehicle in 50 mM Tris/HCl, pH 7.0, containing 5 mM MgCl_2 and 0.2 mg/ml BSA. The reaction was initiated by addition of DEP, NEM, DTNB or vehicle. Aliquots were withdrawn periodically and diluted 100-fold to determine the residual activity, as described above. Protection against inactivation was performed by first incubating the enzyme with AMP for 5 min before adding the reagent. Grant et al. [20] showed that AMP was more potent than cAMP in protecting cGI-PDE against modification by cAMP affinity reagents.

Spectral measurements

The difference spectrum in the UV region of DEP-modified cGI-PDE compared with the native enzyme was determined using a Cary 230 double-beam spectrophotometer at 25 °C. The enzyme solution (0.2 ml, containing 1 μM cGI-PDE dissolved in 50 mM Tris/HCl, pH 7.0, 10 mM MgCl_2) was pipetted into two quartz cuvettes. At various time intervals the difference spectrum between the DEP- and vehicle-added samples was monitored, and an aliquot of the reaction mixture was diluted 100-fold with 50 mM Tris/HCl, pH 7.5, 1 mg/ml BSA and 5 mM MgCl_2 to measure the enzyme activity. The number of modified histidine residues was calculated using the molar absorption coefficient of $3200 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for *N*-carbethoxyhistidine [21].

Incorporation of [^{14}C]DEP and [^3H]NEM into cGI-PDE

The enzyme (30–60 μg) was preincubated in the absence or presence of AMP (30 mM) or cGMP (30 mM), or both, for 15 min in 50 mM Tris/HCl, pH 7.0, containing 5 mM MgCl_2 . [^{14}C]DEP (3 mM; 10^6 c.p.m./ μmol) was added and, after 10 min at 25 °C, the [^{14}C]DEP incorporation was stopped by addition of 50 mM L-histidine, followed by extensive dialysis against 50 mM Tris/HCl, pH 7.0, containing 5 mM MgCl_2 . [^3H]NEM (5 mM; 2×10^6 c.p.m./ μmol) was added to enzyme solutions previously incubated in the absence or presence of AMP (50 mM) and/or cGMP (50 mM) for 15 min, under the conditions described above. In the case of the combined protection experiments, both nucleotides were preincubated simultaneously with the enzyme at 25 °C for 15 min. [^3H]NEM incorporation was stopped by addition of 50 mM L-cysteine and dialysis. [^{14}C]cGI-PDE or [^3H]cGI-PDE was transferred to a vial containing Ecolite scintillation fluid (ICN) for radioactivity measurements. Aliquots

were withdrawn to assay the amount of protein by the Coomassie Blue method.

RESULTS

Dependence of catalysis on pH

The nature of ionizable groups affecting enzyme activity can be determined by examining the pH-dependence of $V_{\max}$, K_m or $V_{\max}/K_m$ values [22]. A plot of the $V_{\max}$ versus pH for the human platelet cGI-PDE showed parabolic dependence, with a maximum between pH 7.0 and 8.5 (Figure 1). The pH-rate profile indicates two essential residues with pK_a values of 6.5 and 9.0. These pK_a values are consistent with histidine ($pK_a = 6-7$) and cysteine ($pK_a = 8-9$), but other amino acids could give similar values depending on the local environment.

Figure 1 Effect of pH on cGI-PDE catalysis

cGI-PDE was incubated with $2 \mu\text{M}$ cAMP at 25°C for 30 min in 50 mM buffer at various pH values containing 5 mM MgCl_2 and 0.5 mg/ml BSA. The buffers used were Mes (pH 4.5–5.5), Pipes (pH 6.0–7.0), Hepes (pH 7.5–8.5) and Caps (pH 9.0–10.5). Units of $V_{\max}$ are pmol of cAMP hydrolysed/min per ml.

Figure 2 Inactivation of cGI-PDE by DEP

cGI-PDE was incubated with increasing concentrations of DEP [1 (●), 3 (■) and 6 (▲) mM] at 25°C in 50 mM Tris/HCl (pH 7.0) containing 5 mM MgCl_2 and 0.5 mg/ml BSA. A control was performed in the presence of vehicle (○). After incubation, the enzyme was diluted 100-fold with the same buffer, and assayed to determine the remaining activity. Data are expressed as means $\pm$ S.E.M. of three independent experiments, each performed in duplicate.

Table 1 Kinetic parameters

Kinetic studies of native enzyme, DEP-modified (30% of native activity) and NEM-modified (30% of native activity) cGI-PDE were analysed by both non-linear regression and a Lineweaver–Burk plot. The enzyme shows classical Michaelis–Menten kinetics with excellent agreement of the two analytical methods and no evidence of departure from linearity up to $3.0 \mu\text{M}$ cAMP.

Enzyme	K_m (μM)	$V_{\max}$ (nmol/min per mg)
Native cGI-PDE	0.24 ± 0.03	855 ± 23
DEP-modified cGI-PDE	0.40 ± 0.05	298 ± 32
NEM-modified cGI-PDE	0.24 ± 0.03	236 ± 17

Effect of DEP on cGI-PDE

Loss of cGI-PDE activity on exposure to DEP was time- and concentration-dependent (Figure 2). DEP at 6 mM almost completely inactivated the enzyme in 10 min. The kinetic properties of the DEP-modified cGI-PDE were determined with the inactivated (70%) enzyme. Both the native and DEP-modified enzymes showed linear Michaelis–Menten kinetics. The apparent $V_{\max}$ was markedly reduced from 855 ± 23 nmol/min per mg for the control enzyme to 298 ± 32 nmol/min per mg for the DEP-modified enzyme. However, the K_m values for cAMP hydrolysis was similar: $0.235 \pm 0.03 \mu\text{M}$ for the native enzyme and $0.40 \pm 0.05 \mu\text{M}$ for the modified enzyme (Table 1). Although DEP has been reported to react preferentially with histidine residues, it can modify other unprotonated nucleophiles such as thiol, amine and hydroxy groups [23]. However, the DEP reaction with histidine (to give *N*-carbethoxyhistidine) can be rapidly reversed by hydroxylamine. The reaction of DEP with other residues either is irreversible (lysine, arginine) or is only slowly reversed (tyrosine) [23–25]. Hydroxylamine re-activated cGI-PDE inactivated by DEP (Figure 3): treatment of the DEP-inactivated enzyme with 0.5 M hydroxylamine restored 50% of the activity in 4 min.

Number of histidine residues modified by DEP

The reaction between DEP and histidine and tyrosine residues on the enzyme can be followed by spectral changes [23]. An increase in absorbance around 240 nm is expected for histidine modification by DEP due to *N*-carbethoxylation of histidine residues, whereas a decrease in absorbance at 280 nm is caused by *O*-carbethoxylation of tyrosine residues. Figure 4 shows the difference spectra during the inactivation of cGI-PDE by DEP at pH 7.0, taken at different time intervals. The absorbance between 230 and 250 nm markedly increased over time, while that at 280 nm did not change. The spectral analysis supports the modification of histidine residues, without tyrosine modification, under these conditions.

Figure 5 shows the relationship between the remaining activity and the number of carbethoxyhistidine residues, related to loss of activity, at different concentrations of DEP (0.2–2 mM). The number of modified histidine residues was linearly related up to 70% inactivation of the enzyme. Extrapolation to 0% residual activity showed that 2.4 mol of histidine per mol of cGI-PDE was modified at that point. After 70% inactivation of cGI-PDE, the number of histidine residues modified was not correlated with the inactivation of the enzyme, and probably represents non-specific loss of activity with the modification of multiple histidines. These results indicate that approx. 2.0 mol of histidine

Figure 3 Reversal of DEP-induced inactivation of cGI-PDE by hydroxylamine

cGI-PDE was incubated with 4 mM DEP at 25 °C in 50 mM Tris/HCl, pH 7.0, containing 5 mM $MgCl_2$ and 0.5 mg/ml BSA (■). After 6 min hydroxylamine was added to the enzyme at different concentrations: 125 (△), 250 (□) and 500 (○) mM. After incubation, the enzyme was diluted 100-fold with the same buffer, and assayed to determine the remaining activity. Data are expressed as means $\pm$ S.E.M. of three independent experiments, each performed in duplicate.

per mol of 61 kDa enzyme are responsible for most of the cGI-PDE activity.

Effects of DTNB and NEM on cGI-PDE

DTNB, a thiol-modifying agent [26,27], inactivates cGI-PDE in a time- and concentration-dependent manner (Figure 6). The residual cGI-PDE activity was less than 20% after 20 min of

Figure 4 Spectral changes accompanying the inactivation of cGI-PDE by DEP

The enzyme solution (20 μ g in 0.2 ml of 50 mM Tris/HCl, pH 7.0, containing 10 mM $MgCl_2$) was placed in two cuvettes. DEP (2 mM) or ethanol (vehicle) was added to the cuvette, and the difference spectrum was monitored after 0.5, 5, 15 and 30 min. At each time point an increase in absorbance at 240 nm was seen. These tracings are representative of four similar experiments.

Figure 5 Relationship between cGI-PDE activity and the amount of modified histidine residues responsible for the decrease in activity

cGI-PDE (20 μ g in 0.2 ml of 50 mM Tris/HCl, pH 7.0, containing 10 mM $MgCl_2$) was incubated with different concentrations of DEP (0.2–2 mM). The number of modified histidines responsible for the decrease in activity was calculated from molar absorption coefficient of *N*-carboxyhistidine at 240 nm ($3200 \text{ M}^{-1} \cdot \text{cm}^{-1}$). Aliquots were removed, diluted 100-fold in 50 mM Tris/HCl, pH 7.5, containing 5 mM $MgCl_2$ and 0.5 mg/ml BSA, and then assayed to determine the activity. The solid line is fitted by non-linear regression including all of the values. The broken line is fitted by linear regression including only the values at 30% of initial activity or higher (see the text).

DTNB (15 mM) incubation. Also, the hydrolysis of cAMP mediated by native cGI-PDE increased by 60% when the enzyme was treated with 5 mM dithiothreitol, suggesting that cysteine residues play a role during catalysis. NEM, another thiol-modifying reagent [28], also inactivated cGI-PDE. When 50% of the activity had been lost, 0.7–1.0 mol of [3 H]NEM was incorporated per mol of enzyme. Incorporation of another 7–9 mol of [3 H]NEM resulted in only an additional 20% loss of activity. Although the apparent $V_{\max}$ was decreased from the control value of $855 \pm 23 \text{ nmol/min per mg}$ to $236 \pm 17 \text{ nmol/min per mg}$ for the NEM-modified (70% inactivated) enzyme, the K_m of the NEM-modified cGI-PDE was $0.236 \pm 0.025 \mu\text{M}$ for cAMP hydrolysis, essentially the same as that of the native enzyme

Figure 6 Inactivation of cGI-PDE by DTNB

cGI-PDE was incubated with increasing concentrations of DTNB [1 (●), 5 (■) and 15 (▲) mM] at 25 °C in 50 mM Tris/HCl (pH 7.0) containing 5 mM $MgCl_2$ and 0.5 mg/ml BSA. A control was performed in the presence of vehicle (○). After incubation, the enzyme was diluted 100-fold with the same buffer, and assayed to determine the activity. Data are expressed as means $\pm$ S.E.M. of three independent experiments, each performed in duplicate.

Figure 7 Protection by AMP against chemical inactivation of cGI-PDE

Upper panel: protection against inactivation by DEP. cGI-PDE was incubated with 2 mM DEP in the presence (○) or absence (●) of 20 mM AMP in 50 mM Tris/HCl (pH 7.0) containing 5 mM MgCl₂ and 0.5 mg/ml BSA. After this incubation, the enzyme was diluted 100-fold with the same buffer, and assayed for activity. Data are expressed as means ± S.E.M. of three independent experiments, each performed in duplicate. Lower panel: protection against inactivation by DTNB. cGI-PDE was incubated with 10 mM DTNB in the presence (□) or absence (■) of 40 mM AMP in 50 mM Tris/HCl (pH 7.0) containing 5 mM MgCl₂ and 0.5 mg/ml BSA. After this incubation, the enzyme was diluted 100-fold with the same buffer to determine the residual activity. Data are expressed as means ± S.E.M. of three independent experiments, each performed in duplicate.

($0.235 \pm 0.03 \mu\text{M}$) (Table 1). The kinetic parameters of DTNB-modified cGI-PDE were similar to those of the NEM-modified enzyme.

Protection of cGI-PDE from DEP-, NEM- or DTNB-induced inactivation

cGI-PDE is partially protected against DEP inactivation by prior incubation with 20 mM AMP (the product of cAMP hydrolysis mediated by cGI-PDE) (Figure 7, upper panel). AMP also protects the enzyme from inactivation by NEM (results not shown) and by DTNB (Figure 7, lower panel). These results strongly suggest that the inactivation induced by DEP, NEM or DTNB is active-site-directed.

Protection of cGI-PDE by AMP and cGMP from [¹⁴C]DEP incorporation

In order to quantify the number of histidine residues present at the active site of cGI-PDE, incorporation studies using [¹⁴C]DEP were performed in the presence or absence of AMP and cGMP. Approximately 5 mol of [¹⁴C]carboxy groups were incorporated per mol of cGI-PDE following incubation with 3 mM [¹⁴C]DEP for 10 min. However, when the enzyme was pre-incubated with the competitive inhibitor (cGMP, 30 mM) or the product (AMP, 30 mM), two histidine residues were protected against [¹⁴C]DEP modification by each nucleotide (Table 2). In order to ascertain whether these protected histidine residues were the same or different, we performed a separate experiment using a mixture of AMP (30 mM) plus cGMP (30 mM). We found that 4.0 histidine residues (mean of two experiments performed in duplicate) were protected, indicating that each nucleotide protected a different pair of histidines.

Protection of cGI-PDE against [³H]NEM incorporation

The number of cysteine residues that react with NEM was also estimated from the incorporation of [³H]NEM into the enzyme. Approximately 2 mol of cysteine was modified per mol of cGI-PDE when the enzyme was incubated with 5 mM [³H]NEM for 20 min (Table 3). The number of cysteine residues modified by [³H]NEM was the same in the presence or absence of cGMP (50 mM). On the other hand, AMP (50 mM) protected cGI-PDE from inactivation by NEM as well as from [³H]NEM incorporation. In the presence of AMP, 1 mol of cysteine residue per mol of cGI-PDE was protected from modification by [³H]NEM, suggesting that this residue is at or near the active site of this enzyme.

DISCUSSION

PDEs regulate the intracellular concentrations of cAMP by hydrolysing the diester (P–O) bond, thus converting the second messenger into the inactive molecule AMP. The mechanistic details of how this is accomplished are still unclear; however, it

Table 2 Protection by AMP and cGMP against [¹⁴C]DEP incorporation

cGI-PDE (61 kDa form) at 60 μg in 50 mM Tris/HCl, pH 7.0, containing 10 mM MgCl₂, was incubated for 15 min in the absence or presence of AMP or cGMP. [¹⁴C]DEP (2171 ± 330 d.p.m./nmol) was added, and after 10 min the incorporation was stopped by addition of 40 mM L-histidine. The enzyme was dialysed and the radioactivity in the retentate estimated. Results are means ± S.E.M. *n* corresponds to the number of separate experiments. Histidine protected (mol) is the groups modified by [¹⁴C]DEP minus the groups modified in the presence of nucleotide.

Reagents	<i>n</i>	¹⁴ C in cGI-PDE (d.p.m./nmol)	Histidines in cGI-PDE (mol/mol)	Histidine protected (mol)
3 mM [¹⁴ C]DEP	5	11 460 ± 2180	5.15 ± 0.65	—
3 mM [¹⁴ C]DEP + 30 mM AMP	3	6517 ± 711	3.03 ± 0.50	2.10
3 mM [¹⁴ C]DEP + 30 mM cGMP	4	6933 ± 491	3.41 ± 0.60	1.75

Table 3 Protection by AMP and cGMP against [³H]NEM incorporation

cGI-PDE (61 kDa form), (at 60 µg) in 50 mM Tris/HCl, pH 7.0, containing 10 mM MgCl₂, was incubated for 15 min in the absence or presence of AMP or cGMP. [³H]NEM (8115 ± 1600 d.p.m./nmol) was added and after 20 min the incorporation was stopped by addition of 50 mM L-cysteine. The enzyme was dialysed and the radioactivity in the retentate estimated. Results are means ± S.E.M. *n* corresponds to number of separate experiments. Cysteine protected (mol) is the groups modified by [³H]NEM minus the group modified in the presence of nucleotide.

Reagents	<i>n</i>	³ H in cGI-PDE (d.p.m./nmol)	Cysteines in cGI-PDE (mol/mol)	Cysteine protected (mol)
5 mM [³ H]NEM	5	19127 ± 2000	2.11 ± 0.5	—
5 mM [³ H]NEM + 50 mM AMP	4	9179 ± 1100	1.14 ± 0.3	0.97
5 mM [³ H]NEM + 50 mM cGMP	5	18110 ± 2050	2.00 ± 0.5	0.10

has been proposed that the process may parallel the mechanism of RNA degradation by ribonuclease A, in which a nucleophilic attack of the P=O centre by an imidazole moiety of histidine ultimately results in diester bond cleavage [29].

The effect of pH on the activity of an enzyme depends on the p*K*_a of the ionizing group on the enzyme and the substrate that participates in the catalytic mechanism. The optimum pH, unlike the *K*_m, is a characteristic of the enzyme rather than of the substrate. The pH sensitivity of the kinetic parameters (*V*_{max}, *K*_m and *V*_{max}/*K*_m) is most often an indication of the ionizable groups at or near the site of enzyme reactivity [22]. The reactions catalysed by human platelet cGI-PDE exhibit parabolic pH-rate profiles. This relationship indicates that at least two ionizable groups are important for enzyme activity, of which one functional group needs to be protonated and the other must be deprotonated during catalysis. A p*K*_a value around 6.5 is consistent with an imidazole group of histidine, whereas a critical role for cysteine is suggested by the higher p*K*_a value of 9.0.

In order to examine the involvement of histidine and cysteine residues in cGI-PDE, we performed chemical modification experiments using the group-selective reagents DEP, NEM and DTNB. DEP reacts preferentially with histidine, but it can also react with other functional groups such as tyrosine, lysine, cysteine, serine and arginine [23,25,26,30]. However, selective modification by DEP can be achieved and the modified amino acids distinguished by the reversibility of the reaction and the spectral characteristics of the adducts formed. Reversal of the enzyme-DEP reaction by hydroxylamine is characteristic of histidine and/or tyrosine modification, since the reaction of DEP with other amino acid residues is irreversible [23]. Distinction between histidine and tyrosine modification can be made by measuring the rate of re-activation with hydroxylamine [23,25]. DEP-modified cGI-PDE was rapidly re-activated after treatment with hydroxylamine (Figure 3). Since the reversal of tyrosine residue modification is usually very slow, the data suggest histidine modification. A reaction of a tyrosyl group can be identified by a characteristic decrease in absorbance at 275–280 nm after reaction with DEP [24]. Tyrosine modification is unlikely here, since there was no decrease in absorbance observed in the difference spectrum for cGI-PDE within this wavelength region. There was, however, a large and progressive increase over time in absorbance at 240 nm, as would be expected for the modification of histidine residues [24,25]. In addition, the increase in absorbance at 240 nm that appeared over time was rapidly reversed by hydroxylamine (results not shown).

The spectral studies showed that several histidine residues in cGI-PDE were modified by DEP; however, the modification of those histidine residues at or near the active site and the loss of activity as a consequence were correlated and modification of two histidine residues was correlated with the loss of 70% of the

catalytic activity. This observation shows that these histidine residues play a critical role in regulating the hydrolytic mechanism in cGI-PDE. Further evidence that DEP modification was within the active site was provided by the protection of cGI-PDE activity in the presence of the cAMP hydrolysis product, AMP. AMP has been shown to effectively protect the active site of this enzyme against inactivation by substrate affinity analogues [20]. The presence of essential histidine residues at the active site of cGI-PDE is also supported by the [¹⁴C]DEP incorporation experiments. Several histidine residues were modified by [¹⁴C]DEP; however, only two residues were protected against ¹⁴C incorporation by AMP or by the competitive inhibitor (cGMP). However, the mixture of AMP and cGMP protected about four histidine residues, indicating that two pairs of histidines were located in different sites.

Dithiothreitol, a disulphide-bond-reducing agent, significantly activated the enzyme, whereas the thiol-modifying reagents NEM and DTNB inactivated the enzyme in a time- and concentration-dependent manner, suggesting modification of a required free thiol moiety, cysteine, in the enzyme. AMP also protected the cGI-PDE activity against NEM and DTNB inactivation, suggesting the presence of essential cysteine residue(s) in this enzyme. The involvement of cysteine residue(s) in the active site of a calcium/calmodulin-dependent PDE has been shown by similar chemical modification [17].

Two cysteine residues in cGI-PDE were modified by [³H]NEM; however, only one residue was protected by AMP. Unlike the [¹⁴C]DEP modification of cGI-PDE, cGMP does not offer protection against [³H]NEM incorporation. It is possible that cGMP, cAMP and AMP interact with different domains of the PDE. A topographical model of the reacting domains [31] suggests that the active site is made up of three regions: two linearly oriented interacting sites (sites 1 and 2) with interference boundaries on both sides (site 3). Site 3 is proposed to serve as protrusions limiting the size of the inhibitor around that area. cAMP, as well as AMP, normally assumes the preferred *anti* conformation and sits perpendicularly to site 1 and site 2. cGMP normally assumes a *syn* conformation which interacts with site 2, but does not interact with site 1. Based on this model, it is possible that two histidines are present in each of the distinct sites for cAMP and cGMP, since both cyclic nucleotides are able to protect the enzyme against [¹⁴C]DEP incorporation. The fact that the combination of both AMP and cGMP protects four histidines indicates that two pairs of histidine are involved. The essential cysteine is probably present at the site occupied only by cAMP, since cGMP cannot offer protection against [³H]NEM incorporation into cGI-PDE, which is again consistent with separate sites for the nucleotides.

The loss of activity associated with the modification of either histidine or cysteine resulted in a decrease of the *V*_{max} similar to

the decrease in activity, without a substantial change in the K_m . Since the inactivation is irreversible, one cannot distinguish kinetically between a mixture of partially inactive enzyme molecules or a mixture of completely active and completely inactivated molecules. The lack of a K_m effect suggests that the histidines are probably not directly involved in substrate binding. However, the data do not allow us to distinguish whether the histidines are involved in catalysis, or metal binding or both. The perfectly conserved histidines in the catalytic domain of all mammalian PDEs may serve as ligands for the required bivalent metal cations [12,13], as in other characterized metallohydrolases [11]. In general, three amino acid residues serve as ligands for binding to bivalent metal cations involved in catalysis, a fourth co-ordinating ligand is usually water. The amino acid frequency for metal co-ordination can be arranged in the order His $\gg$ Glu $>$ Asp $\sim$ Cys [14].

The kinetic studies and spectral data, coupled with incorporation studies, indicate the presence of four essential histidines and one essential cysteine at the active-site domain of cGI-PDE. Furthermore, they indicate that the competitive inhibitor cGMP occupies a site distinct from that of the substrate or product. Since cGMP functions as a competitive inhibitor, it is likely that the nucleotide binding sites are partially overlapping. The localization of each in the primary structure, and the exact role of these residues, are currently under investigation.

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