Saraste, M. (1984) *FEBS Lett. 166*, 367-372.
Simpkin, D., Palmer, G., Devlin, F. J., McKenna, M. C., Jensen, G. M., & Stephen, P. J. (1989) *Biochemistry 28*, 8033-8039.
Thöny-Meyer, L., Stax, D., & Hennecke, H. (1989) *Cell 57*, 683-697.
Vandeyar, M. A., Weiner, M. P., Hutton, C. J., & Batt, C. A. (1988) *Gene 65*, 129-133.
Verbist, J., Lang, F., Gabellini, N., & Oesterhelt, D. (1989) *Mol. Gen. Genet. 219*, 445-452.
Widger, W. R., Cramer, W. A., Herrmann, R. G., & Trebst, A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 674-678.
Yu, L., & Yu, C. A. (1984) *Biochem. Biophys. Res. Commun. 123*, 1234-1239.
Yun, C.-H., Beci, R., Crofts, A. R., Kaplan, S., & Gennis, R. B. (1990) *Eur. J. Biochem. 194*, 399-411.
Yun, C.-H., Van Doren, S. R., Crofts, A. R., & Gennis, R. B. (1991) *J. Biol. Chem.* (in press).

Evidence for Essential Histidine and Cysteine Residues in Calcium/Calmodulin-Sensitive Cyclic Nucleotide Phosphodiesterase†

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ABSTRACT: Ca/calmodulin-sensitive cyclic nucleotide phosphodiesterase (CaM-PDE) is an important enzyme regulating cGMP levels and relaxation of vascular smooth muscle. This modification study was conducted mostly with bovine brain CaM-PDE to identify essential functional groups involved in catalysis. The effect of pH on V_{max}/K_m indicates two essential residues with pK_a values of 6.4 and 8.2. Diethyl pyrocarbonate (DEP), a histidine-modifying agent, inhibits CaM-PDE with a second-order rate constant of 130 M^{-1} min^{-1} at pH 7.0 and 30 °C. Activity is restored by NH_2OH. The pH dependence of inactivation reveals that the essential residue modified by DEP has an apparent pK_a of 6.5. The difference spectrum of the intact and DEP-treated enzyme shows a maximum between 230 and 240 nm, suggesting formation of carbethoxy derivatives of histidine. The enzyme is also inactivated by *N*-ethylmaleimide (NEM) and 5,5′-dithiobis-(2-nitrobenzoic acid), both sulfhydryl-modifying agents, with the latter effect reversed by dithiothreitol, which suggests inactivation resulting from modification of cysteine residue(s). Partial inactivation of the enzyme by DEP or NEM results in an apparent decrease in the V_{max} without a change in the K_m or the extent of CaM stimulation. The rate of inactivation by DEP is greater in the presence than in the absence of Ca/CaM. A substrate analogue, Br-cGMP, and the competitive inhibitor 3-isobutyl-1-methylxanthine partially protect the enzyme against inactivation by DEP or NEM, suggesting that the modification of histidine and cysteine residues occurs at or near the active site. DEP also inactivated porcine brain CaM-PDE. Free carboxyl-, amino- or hydroxyl-modifying agents such as N^2-(bromoacetyl)-cGMP, 4-nitro-2,1,3-benzoxadiazole chloride, and 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine had no effect on the enzyme activity. The present study indicates for the first time the presence of catalytically essential histidine and cysteine residues at the active site of CaM-PDE.

Guanosine cyclic 3′,5′-phosphate (cGMP)[1] plays an essential role in the modulation of vascular smooth muscle tone [for reviews, see Murad et al. (1985) and Ignarro and Kadowitz (1985)]. Although there are five known families of cyclic nucleotide phosphodiesterase (PDE) (Beavo, 1988), CaM-PDE is one of the most important enzymes for the regulation of vascular cGMP levels and vasorelaxation (Lorenz & Wells, 1983; Hagiwara et al., 1984; Lugnier et al., 1986; Ahn et al., 1989; Weishaar et al., 1990).

CaM-PDE preferentially hydrolyzes cGMP in the vascular tissues and is detected in most tissues including vascular smooth muscle cells (Lorenz & Wells, 1983; Hagiwara et al., 1984; Lugnier et al., 1986; Ahn et al., 1991; Beavo & Reifsnyder, 1990; Nicholson et al., 1991). CaM-PDE isozymes exhibit a very high overall homology with the homologous (catalytic) domain being nearly identical and the regulatory (CaM binding) domain heterologous (Novack et al., 1990).

Despite the importance of CaM-PDE, little is known about its active site and catalytic mechanism. On the basis of the

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[1] Abbreviations: cGMP, guanosine cyclic 3′,5′-phosphate; CaM, calmodulin; PDE, cyclic nucleotide phosphodiesterase; CaM-PDE, Ca/CaM-sensitive PDE; DEP or DEPC, diethyl pyrocarbonate; NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); *p*CMB, *p*-(chloromercuri)benzoic acid; DTT, dithiothreitol; Br-cGMP, 8-bromoguanosine cyclic 3′,5′-phosphate; MIX, 3-isobutyl-1-methylxanthine; DMSO, dimethyl sulfoxide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; BSA, bovine serum albumin; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; BICINE, *N*,*N*-bis(2-hydroxyethyl)glycine; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); MOPS, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PDP-CaM, [3-(2-pyridyldithio)propionyl]calmodulin; *p*CMS, *p*-(chloromercuri)benzenesulfonic acid.

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published partial sequence data (Charbonneau et al., 1986; Li et al., 1990), an earlier report (Epstein et al., 1982), and our preliminary pH study, we chose to focus on histidine and cysteine. Although several modification studies on PDE's have been reported (Epstein et al., 1982; Billingsley et al., 1984; Kincaid, 1984; Loten & Redshaw-Loten, 1986), they provided no evidence for an essential role of any functional group. In order to understand the mechanism and aid in designing mechanism-based inhibitors, we have examined the role of histidine and cysteine using chemical modifying agents. CaM-PDE from bovine or porcine brain rather than aorta was used because of the ease of preparation of adequate amounts of apparently homogeneous enzyme. This study provides evidence for the existence of essential histidine and cysteine residues at the active site of CaM-PDE. Part of this study was presented in abstract from (Ahn et al., 1990).

Materials and Methods

DEP, [^{14}C]DEP (9.4 mCi/mmol), NEM, DTNB, NH_2OH, DTT, Br-cGMP, MIX, cGMP, L-histidine, *Crotalus atrox* (snake venom), and most other chemicals, all of reagent grade, were obtained from Sigma Chemical Co. [^{3}H]cGMP (5.7 Ci/mmol) was purchased from DuPont NEN. Liquid scintillation cocktails, Ready Safe and Ready Protein, were from Beckman. DEAE-Sephacel and CaM-Affigel 15 were obtained from Millipore and Bio-Rad, respectively. DEP solution (0.1 M) was freshly prepared in absolute ethanol. DTNB and NEM solutions (30 mM) were prepared in water. Br-cGMP and cGMP solutions (10 mM) were prepared in 10% DMSO. MIX solution (10 mM) was prepared in 100% DMSO. [^{3}H]cGMP was purified by anion-exchange chromatography as previously described (Ahn et al., 1989). NH_2OH solution (2 M) was prepared by dissolving in water and adjusted to pH 7.0 with NaOH.

Bovine brain CaM-PDE was prepared as described previously (Ahn et al., 1991). This enzyme preparation migrated as a doublet with molecular weights of 60K and 63K on SDS-PAGE, suggesting the presence of both 60K and 63K CaM-PDE isozymes. This enzyme (0.52 mg/mL) had a specific activity of 10 μmol mg^{-1} min^{-1} when assayed with 5 μM cGMP under our assay conditions and was used in most modification experiments. A homogeneous 60K isozyme had a specific activity of about 400 μmol mg^{-1} min^{-1} when assayed with 100 μM cAMP in the presence of supermaximal concentrations of Ca/CaM (Sharma & Wang, 1988), and was used in spectroscopic and [^{14}C]DEP incorporation experiments. A porcine brain CaM-PDE with a specific activity of 300–400 μmol mg^{-1} min^{-1} in the presence of a saturating concentration of Ca/CaM (Keravis et al., 1987) was used in some experiments.

CaM-PDE Assay. The activity of the DEAE-Sephacel-adsorbed bovine brain CaM-PDE was measured by a modified version of the method previously reported (Ahn et al., 1989). Briefly, the final reaction mixture (0.2 mL) contained, unless otherwise indicated, 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.5 μM CaM, 0.5 mM $CaCl_2$, 1 μM cGMP, [^{3}H]cGMP (about 80 000 cpm), and 10–20 μL of DEAE-Sephacel-adsorbed CaM-PDE. After 10–15 min of incubation at 30 °C, the reaction mixture was boiled for 1 min and incubated for 10 min with 100 μg of Crotalus atrox to liberate guanosine. After addition of AG1-X2 resin chloride form [0.8 mL of a 1:2 (v/v) mixture of resin and water], the reaction mixture was vortex-mixed and centrifuged at 2000*g* for 10 min. The resulting supernatant fraction (0.4 mL) was transferred to counting vials with 3.5 mL of Ready Safe cocktail for the counting of radioactivity.

Inactivation with DEP, NEM, or DTNB. Bovine brain CaM-PDE (1.3 μg/mL) was incubated at 30 °C with an agent or vehicle in 50 mM Tris-HCl (pH 7.0 for DEP experiments; pH 7.5 for NEM or DTNB experiments) or 25 mM sodium/potassium phosphate buffer (pH 7.0 for DEP experiments) containing 5 mM $MgCl_2$, 0.5 μM CaM, 0.5 mM $CaCl_2$, and 0.2 mg/mL bovine serum albumin (BSA). The reaction was initiated by addition of DEP, NEM, or DTNB. The vehicle for DEP (1–5% ethanol) had no effect on enzyme activity or stability. After incubation for various periods of time, aliquots (100 μL) were removed and mixed with DEAE-Sephacel suspension (100 μL of a 1:1 mixture of preswollen DEAE-Sephacel and water) in 0.9 mL of 50 mM Tris-HCl, pH 7.0, containing 20 mM L-histidine (DEP experiments) or 10 mM DTT (NEM experiments). The mixture was incubated at room temperature for 10 min and then centrifuged at 10000*g* for 2 min. The supernatant solution was removed by aspiration. The enzyme adsorbed on the DEAE-Sephacel in the pellet was washed 3 times with cold 50 mM Tris-HCl buffer (pH 7.0) in a similar manner. The final washed Sephacel-adsorbed enzyme was resuspended with 50 mM Tris-HCl (pH 7.5) to the original volume of the aliquot and was used for determination of enzyme activity. The final recovery of CaM-PDE adsorbed on the Sephacel was 70–80% with an intraexperimental variation of less than 5% of the mean value. In reactivation experiments, DEP- and DTNB-modified CaM-PDE's were incubated with NH_2OH and DTT, respectively. The concentrations of NH_2OH and DTT used did not interfere with the assay. Porcine brain CaM-PDE modified by DEP was diluted 200 000 times with Tris-HCl buffer (pH 7.0) rather than adsorbed onto DEAE-Sephacel and assayed for activity.

Spectral Measurements. The diluted homogeneous 60K CaM-PDE isozyme solution (0.4 mL containing 0.15 mg/mL in 25 mM phosphate buffer, pH 7.0, and 5 mM $MgCl_2$) was mixed with DEP (0.1–0.3 mM) or vehicle (ethanol) in the cuvettes. The difference spectrum between the DEP- and vehicle-added samples was monitored at intervals after DEP addition and later NH_2OH addition using a Perkin-Elmer Lambda 3 UV/Vis spectrophotometer.

Kinetic Measurements. The kinetic constants of the partially DEP- or NEM-inactivated and native enzyme were determined from Eadie–Hofstee plots of cGMP hydrolysis data. The DEP-modified and intact enzymes were prepared by incubating CaM-PDE with 3 mM DEP or vehicle at 30 °C for 12 min in 50 mM Tris-HCl (pH 7.0) containing 5 mM $MgCl_2$, 0.5 μM CaM, 0.5 mM $CaCl_2$, and 0.2 mg/mL BSA and adsorbing the enzyme onto DEAE-Sephacel as described above. The NEM-modified enzyme was similarly prepared by incubating CaM-PDE (1.2 μg/mL) with 5 mM NEM for 10 min at pH 7.5. The Sephacel-adsorbed enzyme activity was assayed as above using varying concentrations of cGMP. In the pH–activity study, CaM-PDE (13 ng) was incubated with increasing concentrations of cGMP at 30 °C for 5 min in 200 μL of 50 mM buffer at various pHs (see below) containing $MgCl_2$, CaM, and $CaCl_2$ as above. V_{max} and K_m were determined by Lineweaver–Burk plots of the cGMP hydrolytic activity of CaM-PDE at various concentrations (0.3, 0.6, 1, 1.5, 3, and 8 μM) of cGMP at each pH. The buffers used were (pH in parentheses) MES (6, 6.5), HEPES (7, 7.5, 8), BICINE (8.5, 9, 9.5), Borax–NaOH (10, 10.5), and CAPS (11). CaM-PDE was found to be stable at pHs ranging from 6 to 10. For the study of the pH dependence of enzyme inactivation by DEP, $t_{1/2}$ (the time required for 50% inactivation of enzyme) was determined from a time-dependent

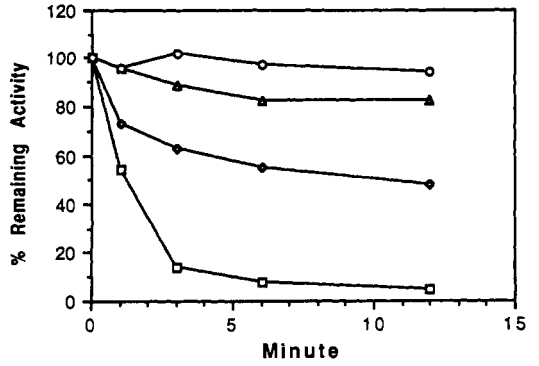

FIGURE 1: Inactivation of bovine brain CaM-PDE by DEPC. CaM-PDE was incubated with increasing concentrations of DEPC at 30 °C in 50 mM Tris-HCl (pH 7.0) modification medium containing 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.5 μM CaM, and 0.2 mg/mL BSA. After this incubation, the enzyme was adsorbed onto DEAE-Sephacel, washed, and assayed to determine the remaining activity as described under Materials and Methods. Control activity was 30–40 pmol of cGMP hydrolyzed min^{-1} (0.2 mL)$^{-1}$. The DEPC concentrations were none (○), 1 (△), 3 (◇), and 5 mM (□).

inactivation at various pHs. The apparent rate constant (K_{app}) for inactivation was calculated from the relationship $K_{app} = 0.693/t_{1/2}$. The buffers used were MES (5.7, 6.1), PIPES (6.5, 6.9), and MOPS (7.2, 7.5).

Incorporation of the [^{14}C]Carbethoxy Group into the 60K CaM-PDE Isozyme. The 60K isozyme (26 μg/mL) was incubated with 3 mM [^{3}H]DEP at 30 °C for varying durations in 25 mM sodium/potassium phosphate (pH 7.0) containing 5 mM $MgCl_2$, 0.5 μM CaM, and 0.5 mM $CaCl_2$. At the end of the incubation, a 40-μL aliquot was removed and mixed with DEAE-Sephacel in 50 mM Tris-HCl (pH 7.0) containing 20 mM L-histidine and 1 mM EGTA. The DEAE-Sephacel containing adsorbed enzyme was resuspended with buffer after washing 3 times as described above. The Sephacel suspension was transferred to a vial containing 3.5 mL of Ready Protein to count radioactivity.

RESULTS

Dependence of Catalysis on pH. The nature of ionizable groups affecting enzyme activity can be determined by examining the pH dependence of the kinetic parameters V_{max}, K_m, or V_{max}/K_m (Dixon, 1953; Cleland, 1982). The plots of V_{max}/K_m vs pH show a bell-shape curve with a maximum at pH 7–8 and reveal the presence of two groups with approximate pK_a values of 6.4 and 8.2 in bovine brain CaM-PDE.

Inactivation of Bovine Brain CaM-PDE by DEP. The incubation of CaM-PDE with DEP results in a time- and concentration-dependent loss of enzyme activity (Figure 1). Deviation from pseudo-first-order kinetics is apparent because of DEP hydrolysis as observed by others (Larrouquere, 1964; Melchior & Fahrney, 1970; Berger, 1975; Bateman & Hersh, 1987). An almost complete inactivation is observed after 12 min with 5 mM DEP. Control, incubated with vehicle (ethanol), shows no loss of activity under the same conditions. A second-order rate constant for inactivation is calculated from initial rate measurements to be 130 M^{-1} min^{-1} at pH 7.0 and 30 °C in 50 mM Tris-HCl.

To identify the amino acid residues modified in the enzyme by DEP, the following series of experiments were carried out.

(*a*) *Reactivation of the Inactive Enzyme with NH_2OH.* NH_2OH has been shown to remove the carbethoxy group from modified histidine and tyrosine residues, but it does not affect the more stable carbethoxysulfhydryl and *N*-carbethoxylysyl bonds (Miles, 1977). Treatment with 1 M NH_2OH for 20

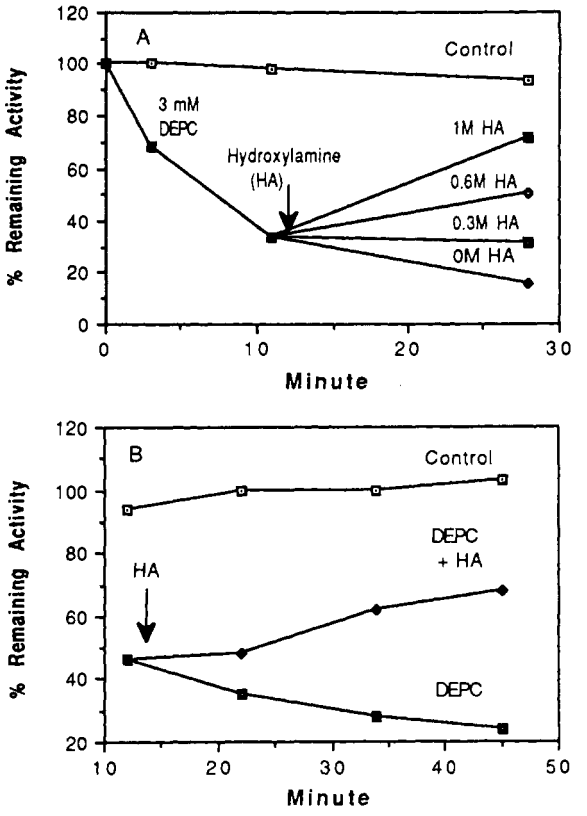

FIGURE 2: Reversal of DEPC-induced inactivation of CaM-PDE by NH_2OH. HA was the abbreviation for NH_2OH. (A) CaM-PDE (1.3 μg/mL) was incubated with 3 mM DEP for 12 min in 50 mM Tris-HCl (pH 7.0) modification medium. NH_2OH was then added to the enzyme to varying concentrations (0, 0.3, 0.6, and 1 M). (B) CaM-PDE was incubated with 3 mM DEPC for 13 min and then incubated with or without NH_2OH (0.6 M) for an additional 32 min.

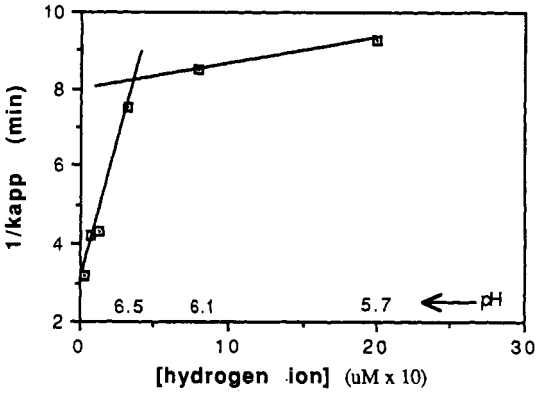

FIGURE 3: pH dependence of inactivation of CaM-PDE by DEP. CaM-PDE was incubated at 30 °C for varying times (0, 2.5, 5, 10, and 20 min) with 5 mM DEP or vehicle (5% ethanol) in 50 mM buffer at various pHs. At the end of incubation, enzyme was adsorbed on Sephacel and assayed for the remaining activity.

min almost fully restores the activity of the inactivated enzyme in a concentration- and time-dependent manner (Figure 2). This finding indicates that the inactivation of the enzyme is not due to the modification of cysteine or lysine residues.

(*b*) *Effect of pH.* Since DEP is known to react with the unprotonated imidazole group but not with the protonated group (Holbrook & Ingram, 1973), the effect of pH on inactivation was investigated. The pseudo-first-order rate constant for inactivation was determined at different pH values and plotted against pH. The inactivation of the enzyme by DEP is pH-dependent and was slower at acidic pHs and faster at alkaline pHs as shown in Figure 3. The apparent pK_a of the reactive group is about 6.5, a value within the range ex-

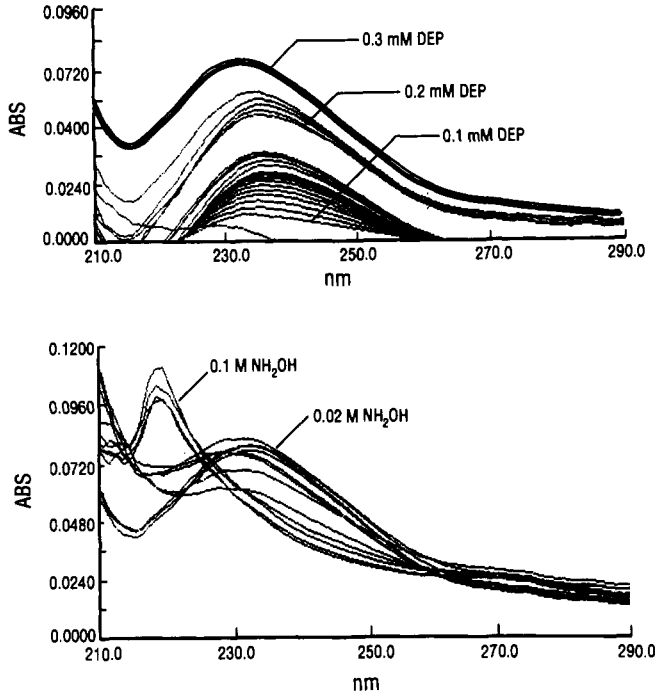

FIGURE 4: Ultraviolet difference spectra after inactivation and reactivation of 60K CaM-PDE isozyme. (Top) Effect of addition of DEP. The enzyme solution (60 μg in 0.4 mL of 25 mM sodium/potassium phosphate, pH 7.0) was placed in two cuvettes. DEP or ethanol (vehicle) was addeed to the cuvette, and the difference spectrum was monitored at 1.5-min intervals (for the first 10 tracings) and later 5-min intervals until no further increase. (Bottom) Effect of addition of NH_2OH. NH_2OH was then added to both cells, and the difference spectrum was monitored at 3-min intervals.

pected for a histidine residue. This result is consistent with the proposal that the inactivation is due to the modification of histidine.

(c) *Spectral Changes Accompanying Inactivation and Reactivation.* Figure 4 illustrates the changes in the absorption spectrum of CaM-PDE during carbethoxylation at pH 7.0. The absorption between 230 and 240 nm markedly increases in a time- and concentration-dependent manner while that at 278 nm does not change. The increase near 240 nm is indicative of histidine modification (Ovadi et al., 1967; Muhlrad et al., 1969; Melchior & Fahrney, 1970). The O-acetylation or O-alkylation of tyrosine residues is known to decrease the absorption at 278 nm (Shaltiel & Patchornik, 1963; Simpson et al., 1963; Burstein et al., 1974). The NH_2OH treatment (0.01–0.1 M) results in a time-dependent decrease in the absorption between 230 and 240 nm (Figure 4). Similar opposite changes by DEP and NH_2OH treatment are observed for L-histidine (Ahn, unpublished observation). The number of histidine residues modified by DEP in CaM-PDE is calculated to be approximately 10 per each molecule of enzyme using a molar extinction coefficient of 3000 M^{-1} cm^{-1} (Melchior & Fahrney, 1970).

Inactivation of Enzyme with NEM or DTNB. NEM inactivates CaM-PDE in a time- and concentration-dependent manner (Figure 5A,B). DTNB also inactivates the enzyme (Figure 5B). While the inactivation induced by NEM is irreversible, the DTNB-induced inactivation is reversed by treatment with DTT. The DTNB (2 mM)-treated enzyme activity was 12 ± 0.5 and 21.5 ± 0.5 pmol min^{-1} $(0.2\ mL)^{-1}$, respectively, after a 20-min incubation with or without 20 mM DTT. The control activity was 22.5 ± 0.5 pmol min^{-1} $(0.2\ mL)^{-1}$. This result indicates a 90% reversal of activity by DTT. In another study, a 70% reversal was observed.

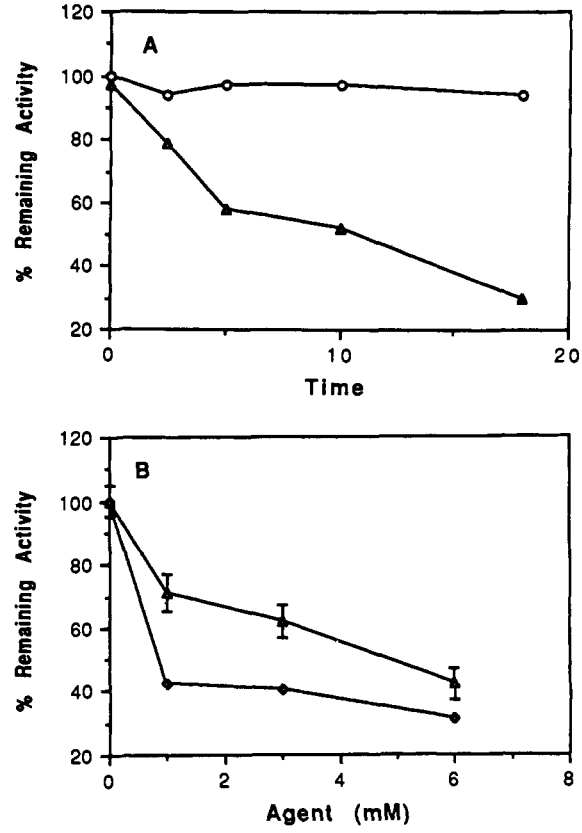

FIGURE 5: Inactivation of CaM-PDE by NEM or DTNB. (A) Time course of activity of control (○) or NEM-incubated CaM-PDE (△). CaM-PDE was incubated with 3 mM NEM in 50 mM Tris-HCl (pH 7.5) medium. (B) CaM-PDE was incubated for 5 min with varying concentrations of NEM (△) in 50 mM Tris-HCl (pH 7.5) medium or with DTNB (◇) in 25 mM sodium/potassium phosphate (pH 7.5) medium.

Protection of Enzyme from DEP-, NEM-, or DTNB-Induced Inactivation. A substrate analogue, Br-cGMP, and the competitive inhibitor MIX protect the enzyme from inactivation by DEP in a concentration-dependent manner (Table I). Br-cGMP also protects the enzyme from inactivation by varying concentrations of DEP (1–6 mM) with the extent of protection inversely related to DEP concentration (Table I). Likewise, Br-cGMP and MIX protect the enzyme from inactivation by NEM (Table II). Also, Br-cGMP protects the enzyme from inactivation by DTNB (Table II). In contrast with the above agents, CaM, an allosteric activator of CaM-PDE in the presence of $CaCl_2$, accelerates inactivation of the enzyme by DEP (Figure 6).

Effect of Modification on Kinetic Parameters. The effect of DEP or NEM modification on the kinetic properties of the enzyme was determined with partially inactivated enzyme (about 50% and 21% inactivated by DEP and NEM, respectively). The K_m of the DEP-modified enzyme is essentially identical with that of the control enzyme (Table III). However, a large change in the apparent V_{max} is observed between the control and modified enzyme (Table III). In addition, the control and DEP-modified enzyme preparations exhibit the same degree of stimulation (about 2-fold) by Ca/CaM (Table III). Similarly, modification with NEM leads to a decrease in V_{max} without any change in K_m (Table III). Also, the NEM-modified enzyme was stimulated by Ca/CaM to the same extent as the control enzyme (Table III).

Number of Histidine Residues Modified by DEP. The number of histidine residues that react with DEP was estimated from the incorporation of [^{14}C] from [^{14}C]DEP into

Table I: Protection of CaM-PDE from DEP-Induced Inactivation[a]

addition (mM)	activity (pmol min^{-1} tube^{-1})	% residual activity	% protection
experiment 1			
none	30 ± 0.2	100	
MIX (1)	31 ± 1.0	103	
DEP (3)	13 ± 0.3	43	
DEP (3) + MIX (1)	17 ± 0.8	57	24[b]
experiment 2			
none	34 ± 1.4	100	
Br-cGMP	34 ± 1.9	100	
DEP (1)	28 ± 1.2	82	
DEP (1) + Br-cGMP (1)	33 ± 0.4	97	83[b]
DEP (3)	17 ± 0.8	50	
DEP (3) + Br-cGMP (1)	26 ± 1.5	76	52[b]
DEP (6)	8.8 ± 0.3	26	
DEP (6) + Br-cGMP (1)	16 ± 0.7	47	28[b]
experiment 3			
none	25 ± 0.4	100	
DEP (3)	19 ± 0.3	76	
DEP (3) + Br-cGMP (0.01)	21 ± 0.7	83	29
DEP (3) + Br-cGMP (0.1)	23 ± 0.2	93	71[b]
DEP (3) + Br-cGMP (1)	23 ± 0.4	94	75[b]
DEP (3) + MIX (0.003)	21 ± 0.2	87	46[b]
DEP (3) + MIX (0.03)	22 ± 0.1	91	63[b]
DEP (3) + MIX (0.3)	22 ± 0.4	92	67[b]

[a] Each value (activity) was the mean ± SEM of three determinations. Enzyme (1.3 μg/mL) was preincubated with a protecting agent (Br-cGMP or MIX) for 5 min before DEP was added. Enzyme was incubated for 6 min (experiment 1) or 3 min (experiments 2 and 3) with DEP in 50 mM Tris-HCl (pH 7.0) modification medium. After the incubation for modification, the enzyme was adsorbed onto Sephacel, washed free of agents (DEP, Br-cGMP, or MIX), and assayed for activity as described under Materials and Methods. [b] $P < 0.05$ vs DEP alone (Student's t test).

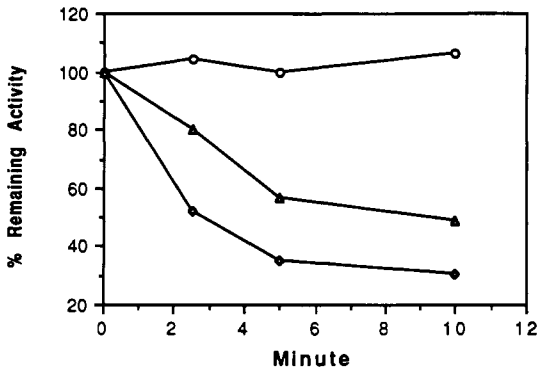

FIGURE 6: Effect of Ca/CaM on inactivation of CaM-PDE by DEP. CaM-PDE was incubated with 5 mM DEP in the presence (◇) or absence (△) of 0.5 mM Ca/0.5 μM CaM or without DEP (○) in the presence of Ca/CaM in 50 mM Tris-HCl (pH 7.0) containing 5 mM $MgCl_2$ and 0.2 mg/mL BSA.

the enzyme. ^{14}C incorporation increased with time, reaching a plateau after 15-min incubation with 3 mM DEP. A maximum of 10–11 mol of [^{14}C]carbethoxy groups is incorporated per mole of 60K CaM-PDE isozyme (data not shown).

Inactivation of Porcine Brain CaM-PDE by DEP. DEP (0.4 mM) inactivated this CaM-PDE 63% after incubation of the enzyme at 30 °C for 15 min. Free carboxyl-, amino-, or hydroxyl-modifying agents such as N^2-(bromoacetyl)-cGMP, 4-nitro-2,1,3-benzoxadiazole chloride, or 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine failed to inactivate the enzyme under the same conditions (data not shown).

Discussion

The present study demonstrates for the first time that CaM-PDE contains essential histidine and cysteine residues.

Table II: Protection of CaM-PDE from NEM- or DTNB-Induced Inactivation[a]

addition (mM)	activity (pmol min^{-1} tube^{-1})	% residual activity	% protection
experiment 1			
none	21.6 ± 0.4	100	
Br-cGMP	18.5 ± 1.1	86	
MIX (1)	18.7 ± 0.9	87	
NEM (2)	12.2 ± 0.2	56	
NEM (2) + Br-cGMP (2)	14.7 ± 1.5	68	60[b]
NEM (2) + MIX (1)	15.6 ± 1.3	72	67[b]
experiment 2			
none	15.3 ± 0.6	100	
NEM (1)	3.4 ± 0.4	22	
NEM (1) + Br-cGMP (2)	8.9 ± 1.4	58	46[b]
NEM (3)	0.67 ± 0.4	4.4	
NEM (3) + Br-cGMP (2)	4.6 ± 1.4	30	27[b]
NEM (6)	0.84 ± 0.19	5.5	
NEM (6) + Br-cGMP (2)	7.2 ± 1.7	47	44[b]
experiment 3			
none	16.5 ± 0.7	100	
NEM (5)	11.6 ± 0.2	70	
NEM (5) + Br-cGMP (0.1)	12.8 ± 0.3	78	24[b]
NEM (5) + Br-cGMP (1)	14.7 ± 0.3	89	63[b]
NEM (5) + MIX (0.003)	14.7 ± 0.3	89	63[b]
NEM (5) + MIX (0.03)	15.8 ± 0.3	96	86[b]
experiment 4			
none	19.6 ± 0.01	100	
DTNB (1)	8.1 ± 0.06	41	
DTNB (1) + Br-cGMP (2)	9.9 ± 0.26	51	16[b]

[a] Each value (activity) was the mean ± SEM of three determinations. After 5-min preincubation with a protecting agent, enzyme was incubated for 5–10 min with a modifying agent in Tris-HCl (experiments 1–3) or 25 mM sodium/potassium phosphate (experiment 4) modification medium (pH 7.5). [b] $p < 0.05$ vs NEM or DTNB alone (Student's t test).

Table III: Properties of Control and DEP- or NEM-Incubated CaM-PDE[a]

enzyme	activity (pmol min^{-1} tube^{-1}) EGTA	Ca/CaM	n-fold stimulation	K_m (μM)	V_{max} (pmol/min)
experiment 1					
control	8.8	20	2.3	5.4	134
DEP-incubated	4.8	9.6	2.0	5.3	60
experiment 2					
control	14.6	26.2	1.8	6.8	198
NEM-incubated	10.1	20.8	2.1	6.3	148

[a] Each value (activity) was an average of two determinations. Data shown are representative of two separate experiments. Enzyme was partially inactivated by incubating with 3 mM DEP or with 5 mM NEM as described under Materials and Methods. K_m and V_{max} values were obtained from Eadie–Hofstee plots of cGMP hydrolysis by intact and modified CaM-PDE. The enzyme activity was assayed with varying concentrations (0.5, 1, 1.5, 3, and 8 μM) of cGMP in 50 mM Tris-HCl (pH 7.5) containing 5 mM $MgCl_2$, 0.5 μM CaM, and 0.5 mM $CaCl_2$.

Although DEP can react with functional groups of histidine, lysine, tyrosine, cysteine, serine, and arginine as well as the α- and ε-amino groups of amino acids (Larrouquere, 1964; Muhlrad et al., 1969; Melchior & Fahrney, 1970), the usual group modified in neutral or slightly acidic media is the histidine imidazole (Miles, 1977). However, because no chemical agent has absolute specificity, selective modification must be demonstrated to attribute inactivation to the modification of particular residues. The specificity of the DEP reaction for histidine residues was supported by the following. (a) There was an increase in absorption between 230 and 240 nm without any change in absorption near 278 nm after addition of DEP

and a decrease in absorption after addition of NH_2OH. The increase near 240 nm is indicative of histidine modification (carbethoxylation) (Melchior & Fahrney, 1970; Muhlrad et al., 1969) whereas the decrease at 278 nm is associated with O-acetylation or O-alkylation of tyrosine residues (Burstein et al., 1974). (b) Rapid reversal of inactivation of NH_2OH was observed. The reaction of DEP with other residues either is irreversible (lysin, arginine) or is only slowly reversed (tyrosine) (Miles, 1977). (c) The reaction rate (for inactivation) was dependent on a residue with a pK_a of 6.5, and the reactive form was unprotonated, in agreement with earlier studies on other proteins (Holbrook & Ingram, 1973; Dickenson & Dickinson, 1975). (d) There was an essentially identical maximal number of DEP-modified histidine residues derived by both spectroscopic and [^{14}C]DEP incorporation methods, indicating only histidine residues are modified by DEP. (e) A relatively high rate constant of inactivation was found. Inactivation of protein by modification of imidazole groups with DEP has generally resulted in second-order rate constants greater than 100 M^{-1} min^{-1}, as compared with the rates of modification of other residues, which are at least 10 times lower (Holbrook & Ingram, 1973; Wells, 1973).

The selective modification of cysteine residues is indicated by (a) inactivation of CaM-PDE by sulfhydryl-modifying agents, NEM and DTNB, (b) reversal of DTNB-inactivated enzyme by DTT, and (c) a rapid inhibition by $CuCl_2$ (Ahn, unpublished observation) which is known to inactivate readily cysteine-containing enzymes. DTNB reacts with sulfhydryl groups in proteins, forming a 2-nitro-5-mercaptobenzoyl derivative of cysteine (Lundblad & Noyes, 1984), which is readily reversed by the addition of a reducing agent such as DTT (Lundblad & Noyes, 1984; Tian et al., 1985).

Substrates or competitive inhibitors are likely to protect essential residues at the active site from modification. The protection of CaM-PDE from DEP or NEM by the substrate analogue Br-cGMP or the inhibitor MIX, which binds to the active site of PDE's, suggests that the essential histidine or cysteine residue is located at or near the catalytic site of CaM-PDE. Acceleration of DEP-induced inactivation of the enzyme in the presence of Ca/CaM provides additional support for an active-site location of the essential histidine residue. Ca/CaM is reported to increase not only V_{max} but also the affinity of CaM-PDE for substrate (Sharma & Wang, 1988), thus implying the facilitation of substrate interaction with the active site.

Many histidine residues are modified by DEP. However, the results showing a decrease in the apparent V_{max} without a change in the K_m for cGMP or the extent of Ca/CaM stimulation following partial CaM-PDE inactivation by DEP or NEM are consistent with a single essential histidine and a single essential cysteine residues. The above kinetic results are expected when any enzyme with a single essential residue is modified, and they reflect the presence of a mixed population of completely active and completely inactive enzyme molecules (Bazaes et al., 1980).

As shown in this study, the pH dependence of V_{max}/K_m reveals two essential ionizing groups with pK_a's of 6.4 and 8.2, similar to those of histidine and cysteine. Since cGMP has no functionality that is ionizable in the pH range studied, these pK_a's apparently reflect functional groups on the enzyme. Additionally, similar pK_a values are obtained even when V_{max} is plotted against pH (data not shown). Since V_{max} is quite independent of the ionized state of the substrate or substrate affinity for enzyme (Dixon, 1953), those two groups appear to belong to the enzyme and to be involved in catalysis rather than binding of substrate to the enzyme. On the basis of the above modification data, the group with a pK_a of 6.5 may represent a histidine residue and the group with a pK_a of 8.2 a cysteine residue.

The involvement of sulfhydryl groups and carboxyl groups in allosteric activation of PDE's has been suggested (Billingley et al., 1984; Kincaid, 1984; Loten & Redshaw-Loten, 1986). Low concentrations of *p*CMB and *p*CMS reliably activated the insulin-sensitive particulate low-K_m cAMP-PDE from adipose tissue and liver (Loten & Redshaw-Loten, 1986). Enzymatic carboxymethylation of brain CaM-stimulated cAMP-PDE resulted in decreased (20%) responsiveness to Ca/CaM without any effect on the basal activity (Billingley et al., 1984). CaM-PDE was activated by the affinity label PDP-CaM but not by its thiolated form, and this was reversed by DTT (Kincaid, 1984). The residues modified appeared to reside in the regulatory site since modifying agents mimicked allosteric activators and have no effect on the basal activity (Billingley et al., 1984; Kincaid, 1984; Loten & Redshaw-Loten, 1986). By contrast, this study indicates that a cysteine residue in the active site is essential for the catalytic activity of CaM-PDE.

The present data and inactivation by DEP of cGMP-PDE (Ahn, unpublished data) together with the reported sequence data (Charbonneau et al., 1986; Li et al., 1990) showing conserved histidine residues in the catalytic domain of diverse cyclic nucleotide PDE's raise the possibility that a histidine imidazole group is involved in the catalytic mechanism of most cyclic nucleotide PDE's. Unlike the imidazole group, the cysteine thiol group does not appear to be universally involved in the catalysis of various PDE's because of a minimal inactivation of some PDE's by sulfhydryl-modifying agents (Loten & Redshaw-Loten, 1986; Makino et al., 1980) and the lack of conserved cysteine residues (Charbonneau et al., 1986).

The role of histidine and cysteine residues in the reaction mechanism of CaM-PDE remains to be elucidated. Ribonuclease, a phosphodiesterase, is known to utilize two histidine residues in catalysis, each serving as an acid and a base (Wharton & Eisenthal, 1981). Histidine and cysteine have been found to play an essential role in catalysis of other enzymes such as papain, alcohol dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase (Wharton & Eisenthal, 1981). The modification and pH–activity data suggest that histidine and cysteine residues may serve as a base and an acid, respectively, in CaM-PDE catalysis.

In summary, the present study indicates the presence of essential histidine and cysteine residues at the active site of CaM-PDE and an acid–base catalytic mechanism of CaM-PDE.

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Registry No. cGMP, 7665-99-8; PDE, 9040-59-9; His, 71-00-1; Cys, 52-90-4; diethyl pyrocarbonate, 1609-47-8.

References

Ahn, H. S., Crim, W., Romano, M., Sybertz, E., & Pitts, B. (1989) *Biochem. Pharmacol. 38*, 3331–3339.

Ahn, H. S., Foster, M., Cable, M., Foster, C., & Sybertz, E. (1990) *Pharmacologist 32*, 170.

Ahn, H. S., Foster, M., Cable, M., Pitts, B., & Sybertz, E. (1991) *Adv. Exp. Med. Biol.* (in press).

Bateman, R. C., Jr., & Hersh, L. B. (1987) *Biochemistry 26*, 4237–4242.
Bazaes, S., Beytia, E., Jabalquinto, A. M., Solis de Ovando, F., & Eyzaguirre, J. (1980) *Biochemistry 19*, 2305–2310.
Beavo, J. A. (1988) *Adv. Second Messenger Phosphoprotein Res. 22*, 1–38.
Beavo, J. A., & Reifsnyder, D. H. (1990) *Trends Pharmacol. Sci. 11*, 150–155.
Berger, S. L. (1975) *Anal. Biochem. 67*, 428–437.
Billingsley, M., Kuhn, D., Velletri, P. A., Kincaid, R., & Lovenberg, W. (1984) *J. Biol. Chem. 259*, 6630–6635.
Burstein, Y., Walsh, K. A., & Neurath, J. (1974) *Biochemistry 13*, 205–210.
Charbonneau, H., Beier, N., Walsh, K. A., & Beavo, J. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 9308–9312.
Cleland, W. W. (1982) *Methods Enzymol. 87*, 390–405.
Dickenson, C. J., & Dickinson, F. M. (1975) *Eur. J. Biochem. 52*, 595–603.
Dixon, M. (1953) *Biochem. J. 55*, 161–170.
Epstein, P. M., Strada, S. J., Sarada, K., & Thompson, W. J. (1982) *Arch. Biochem. Biophys. 218*, 119–133.
Hagiwara, M., Endo, T., & Hidaka, H. (1984) *Biochem. Pharmacol. 33*, 453–457.
Holbrook, J. J., & Ingram, V. A. (1973) *Biochem. J. 131*, 729–738.
Ignarro, L. J., & Kadowitz, P. J. (1985) *Annu. Rev. Pharmacol. Toxicol. 25*, 171–191.
Keravis, T. M., & Wells, J. N. (1987) *J. Cyclic Nucleotide Protein Phosphorylation Res. 11*, 365–372.
Kincaid, R. L. (1984) *Biochemistry 23*, 1143–1147.
Larrouquere, J. (1964) *Bull. Sco. Chim. Fr.* 1543.
Li, T., Volpp, K., & Applebury, M. L. (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87, 293–297.
Lorenz, K. L., & Wells, J. N. (1983) *Mol. Pharmacol. 23*, 424–430.
Loten, E. G., & Redshaw-Loten, J. C. (1986) *Int. J. Biochem. 18*, 847–851.
Lugnier, C., Shoeffter, P., LeBec, A., Strouthou, E., & Stoclet, J. C. (1986) *Biochem. Pharmacol. 35*, 1743–1751.
Lundblad, R. J., & Noyes, C. M. (1984) *Chemical Reagents for Protein Modification*, Vol. I, pp 62–88, CRC Press, Inc., Boca Raton, FL.
Makino, H., De Buschiazzo, P. M., Pointer, R. H., Jordan, J. E., & Kono, T. (1980) *J. Biol. Chem. 255*, 7845–7849.
Melchior, W. B., & Fahrney, D. (1970) *Biochemistry 9*, 252–258.
Miles, E. W. (1977) *Methods Enzymol. 47*, 431–442.
Muhlrad, H., Hegyi, G., & Horanyi, M. (1969) *Biochim. Biophys. Acta 181*, 184–190.
Murad, F., Rapoport, R. M., & Fiscus, R. (1985) *J. Cardiovasc. Pharmacol. 7 (Suppl. 3)*, S111–S118.
Nicholson, C. D., Challis, R. A. J., & Shahid, M. (1991) *Trends Pharmacol. Sci. 12*, 19–27.
Novack, J. P., Charbonneau, H., Blumenthal, D. K., Walsh, K. A., & Beavo, J. A. (1990) *Adv. Exp. Med. Biol. 255*, 387–395.
Ovadi, J., Libor, S., & Elodi, P. (1967) *Acta Biochim. Biophys. 2*, 455–458.
Shaltiel, S., & Patchornik, A. (1963) *Isr. J. Chem. 1*, 187.
Sharma, R. K., & Wang, J. H. (1988) *Methods Enzymol. 159*, 582–594.
Simpson, R. T., Riordan, J. F., & Vallee, B. L. (1963) *Biochemistry 2*, 616–622.
Tian, W. X., Hsu, R. Y., & Wang, Y. S. (1985) *J. Biol. Chem. 260*, 11375–11387.
Weishaar, R. E., Kobylarz-Singer, J. T., & Panek, R. (1990) *Hypertension 15*, 528–540.
Wells, M. A. (1973) *Biochemistry 12*, 1086–1093.
Wharton, C. W., & Eisenthal, R. (1981) *Molecular Enzymology*, pp 220–274, John Wiley & Sons, New York.