Purification, characterization and analysis of rolipram inhibition of a human type-IV_A cyclic AMP-specific phosphodiesterase expressed in yeast

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Analyses were done on a human type-IV cyclic AMP (cAMP) phosphodiesterase (hPDE-IV_{A-h6.1}) expressed in an engineered strain of *Saccharomyces cerevisiae*. This strain (YMS6) expressed soluble PDE activity, together with an insoluble activity which was not released by re-homogenization, treatment with high-ionic-strength solutions or with the detergent Triton X-100. Pellet and soluble PDE activities were typical of type-IV PDE. They were cAMP-specific, insensitive to the addition of either cGMP (1 μ M) or Ca²⁺/calmodulin, and inhibited by rolipram. Thermostability studies showed both activities to decay as single exponentials, indicating the presence of homogeneous PDE protein species in each fraction. Pellet PDE activity was more thermostable than the soluble enzyme. Mg²⁺ and Mn²⁺ dose-dependently increased PDE activity and reversed the inactivating

effect of EDTA. h6.1 was engineered to express a C-terminal five-histidine motif (h6.1_{his5}). This allowed purification of the PDE to apparent homogeneity in a simple two-step process involving a rolipram affinity column and a Ni²⁺-chelate column. A single monomeric protein of subunit molecular mass $\sim$ 73 kDa and native molecular mass $\sim$ 74 kDa resulted after a $\sim$ 53 000-fold purification. This exhibited a K_m for cAMP of 8 μ M, a true V_{max} of 0.8 μ mol of cAMP hydrolysed/min per mg of PDE protein, a k_{cat} of 3702 s⁻¹, and a value of the specificity constant k_{cat}/K_m of 4.6×10^8 M⁻¹ s⁻¹, the last implying a diffusion controlled reaction. Rolipram (K_i 0.4 soluble; 0.7 μ M pellet) and 3-isobutyl-1-methylxanthine (K_i 15 soluble; 19 μ M pellet) served as simple competitive inhibitors for both soluble and pellet forms of h6.1, respectively.

INTRODUCTION

The second messenger cyclic AMP (cAMP) is inactivated in cells through the action of cAMP phosphodiesterases (PDE), which effect its hydrolysis to 5'-AMP (Beavo, 1990). This activity is represented by a multigene family of enzymes having distinct characteristics (Beavo, 1990; Conti and Swinnen, 1990; Conti et al., 1991; Davis et al., 1990). The type-IV PDE sub-family consists of a group of enzymes which specifically hydrolyse cAMP in a fashion which is insensitive to the addition of low concentrations of cyclic GMP (cGMP) and of Ca²⁺/calmodulin and whose members are specifically inhibited by the compound rolipram (Beavo, 1990). The first of the type-IV PDEs to have been purified to apparent homogeneity and characterized were the peripheral-plasma-membrane PDE (rPPM-PDE) from rat hepatocytes (Marchmont et al., 1981) and a cytosolic PDE (dS-PDE) from dog kidney (Thompson et al., 1979). These enzymes proved to be extremely difficult to purify, due not only to their paucity but also to their marked susceptibility to proteolysis and inherent lability; such factors have inhibited the characterization of all but a few type-IV PDEs. cDNAs for various type-IV PDEs have been isolated (Davis et al., 1990; Conti and Swinnen, 1990; Conti et al., 1991). This was done initially for the *Drosophila dunc* enzyme (Chen et al., 1986). This cDNA was used as a probe to clone a series of type-IV isoforms from rat (Davis, 1990; Conti et al., 1990, 1991) and from human cDNA libraries (Livi et al., 1990; Mchale et al., 1991; Bolger et al., 1993; Sullivan et al., 1994). To date, however, no detailed

characterization of type-IV PDEs of human origin has been done, and no such species has been purified to apparent homogeneity.

We have described (Sullivan et al., 1994) the molecular cloning of a type-IV PDE, called hPDE-IV_{A-h6.1} (h6.1). This PDE is highly related to a type-IV_A species reported previously by others (hPDE-IV_{A-Livi}; Livi et al., 1990), but differs with respect to five residues located within, or adjacent to, the putative catalytic domain (Sullivan et al., 1994). These changes are Y³¹⁷/D, R⁵²³/M, R⁵²⁷/P, E⁵⁸⁹/A and N⁵⁹⁸/S. The sequence of hPDE-IV_{A-h6.1} would appear, however, to reflect the native situation, with that of hPDE-IV_{A-Livi} (Livi et al., 1990) reflecting an aberrant form (see for discussion Sullivan et al., 1994); it is also possible that both these enzymes may reflect N-terminally truncated species (see Bolger, 1994). Here we detail the generation of an engineered version of this enzyme (h6.1_{his5}) which expresses five histidyl residues at its C-terminus. Using this, we have achieved the first purification to apparent homogeneity of a type-IV PDE encoded by a human gene, the determination of its turnover number, selectivity constant and requirement for Mg²⁺/Mn²⁺, and have shown that both rolipram and 3-isobutyl-1-methylxanthine (IBMX) serve as simple competitive inhibitors of its action.

MATERIALS AND METHODS

Materials

Tris, benzamidine hydrochloride, phenylmethanesulphonyl fluoride, aprotinin, pepstatin A, antipain, EDTA, cGMP, cAMP,

Abbreviations used: cAMP, cyclic AMP; cGMP, cyclic GMP; PDE, cAMP phosphodiesterase activity; hPDE-IV, human type-IV PDE; hPDE-IV_A, human type-IV PDE splice variant family 'A'; h6.1, a human type-IV PDE reported by Sullivan et al. (1994) and referred to as hPDE-IV_{A-h6.1} (HPDE4A1D; proposed descriptor); h6.1_{his5}, h6.1 engineered to express five histidine residues at its C-terminus; hPDE-IV_{A-Livi}, a human type-IV PDE reported by Livi et al. (1990) (HPDE4A1A; proposed new Gene Bank descriptor); dS-PDE, soluble type-IV PDE from dog kidney (Thompson et al., 1979); rPPM-PDE, a peripheral-plasma-membrane type-IV PDE from rat hepatocytes (Marchmont et al., 1981); IBMX, 3-isobutyl-1-methylxanthine; rolipram, 4-[3-(cyclopentoxyl)-4-methoxyphenyl]-2-pyrrolidone; Ro-20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidine; ORF, open reading frame; 5-FOA, 5-fluoro-orotic acid; GPD, glyceraldehyde-3-phosphate dehydrogenase.

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Table 1 Yeast strains

This lists the strain designations referred to in the text and details their genotype.

Strain	Genotype	Reference or source
YMS4	MAT α leu2 ura3 his4 lys2 pde1::LYS2 pde2::LEU2 pep ₄ ::URA3	Sullivan et al. (1994)
YMS5	Derivative of YMS4 selected for loss of functional URA3 by growth on 5-FOA plates	Sullivan et al. (1994)
YMS5X	MAT α leu2 ura3 his4 lys2 pde1::LYS2 pde2::LEU2 pep ₄ ::ura3 Δ AS	This work
YMS6	MAT α leu2 ura3 his4 lys2 pde1::LYS2 pde2::LEU2 pep ₄ ::ura3::YlpMS1-6.1	Sullivan et al. (1994)
YMS8	MAT α leu2 ura3 his4 lys2 pde1::LYS2 pde2::LEU2 pep ₄ ::ura3 Δ AS::YlpMS1-6.1(HIS)	This work

Dowex 1X8-400 (Cl⁻ form, 200–400 mesh), IBMX, snake venom (*Ophiophagus hannah*), bovine brain calmodulin, low-melting-point agarose and Triton X-100 were from Sigma Chemical Co. (Poole, Dorset, U.K.). [³H]cAMP and [³H]cGMP were from Amersham International (Amersham, Bucks., U.K.). Leupeptin was from Peptide Research Foundation (Scientific Marketing Associates, London, U.K.). Dithiothreitol and triethanolamine/HCl were from Boehringer (Lewes, Sussex, U.K.). AH-Sepharose 4B resin was from Pharmacia U.K. Bradford reagent was from Bio-Rad (U.K.). ProBond Nickel resin was from Invitrogen. Restriction enzymes were from New England Biolabs and Boehringer Mannheim. T4 DNA ligase was from Gibco BRL. T4 DNA polymerase was from Boehringer Mannheim. *Taq* polymerase and dNTPs were from Perkin-Elmer. T7 sequencing kits and 5-fluoro-orotic acid (5-FOA) were from Pharmacia. Magic Minipreps were from Promega. Dimethyl sulphoxide, MgCl₂ and all other chemicals, which were of A.R. grade, were from Fisons (FSA Laboratory Supplies, Loughborough, Leics., U.K.).

Strains of *Saccharomyces cerevisiae*

The strains of *S. cerevisiae* used in this study are described in Table 1. All yeast strains were constructed by standard genetic manipulations and were grown at 30 °C in rich (YPD) medium, on complete (SC) medium lacking nutrients, or on YPD medium containing uracil and 5-FOA as specified (Sikorski and Boeke, 1991; Sullivan et al., 1994).

Plasmids, DNA manipulations, and sequencing

The plasmid (pME69 Δ AS) used to disrupt the *ura3* gene in the *pep4*::URA3 locus was constructed as follows. pME69, containing the *ura3* gene on a *Hind*III fragment flanked by DNA from the *pep4* gene locus, as used to disrupt the *PEP4* gene previously (Sullivan et al., 1994), was digested to completion with *Apa*I and *Stu*I sites, which are both present in the URA3 gene and are unique in the plasmid. The digested plasmid was incubated with T4 DNA polymerase and all four dNTPs, then self-ligated to create pME69 Δ AS.

A plasmid [YIpMS1-6.1(HIS)₅] was constructed to allow for expression of a His₅-tagged form of h6.1. Initially a 3' histidine cassette containing five histidine codons and a stop codon was created by annealing complementary oligonucleotides, 3'HIS-A (5'-GATCCGATATCACCGGTCATCACCAACCACCATGAG-3') and 3'HIS-B (5'-TCGACTCAATGGTGTATGGTGTGACCGGTGATATCGG-3') and then ligating with *Bam*HI/*Sal*I-digested p19MCS9 to create pMCS9-3'(HIS)₅. The small insert in this plasmid was sequenced to ensure that the cloned oligonucleotide sequence was as designed. The plasmid pMCS9 is a derivative of pUC19 with different restriction sites in the

multicloning region. Plasmid pMCS9-3'(HIS)₅ consists of 5'-*Hind*III, *Bam*HI, *Eco*RV, *Age*I (underlined in 3'HIS-A), five histidine codons (double-underlined), stop codon, *Sal*I site-3'. The 3'-end of the open reading frame (ORF) of 6.1 was mutated by amplification using YIpMS1-6.1 as template DNA in a PCR with the primers p13 and 3'Age. The 5' primer p13 (5'-ATGTGTGACAAGCACACT-3') binds upstream of a unique *Bg*II site in the 6.1 cDNA. The 3' primer 3'Age (5'-AGCTgtcgacGGA-ACCGGTAGGGTCTCCACC-3') primes at the extreme 3'-end of the 6.1 ORF. The PCR results in the loss of the stop codon of 6.1 and insertion of an *Age*I site (upper case underlined) and a *Sal*I site (lower case underlined) at the extreme 3'-end in the sequence of the 3'Age primer given above. The PCR fragment was purified and digested with *Bg*II and *Sal*I to release a 665 bp fragment that was ligated into *Bam*HI/*Sal*I-digested p19MCS6 to create the plasmid pMCS6-Age. The plasmid p19MCS6 is a derivative of pUC19 with different restriction sites in the multicloning region. The insert of pMCS6-Age was sequenced to ensure that no mutations had occurred. This plasmid was then digested with *Hind*III and *Eag*I; the *Hind*III site lies in the vector next to the *Bam*HI/*Bg*II fusion, whereas the *Eag*I site lies within the insert, 128 bp upstream of the engineered *Age*I site. The large *Hind*III-*Eag*I fragment was ligated with the 1.6 kb *Hind*III-*Eag*I fragment from YIpMS1-6.1 containing the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter and the 5'-end of the ORF of 6.1. The insert of the resulting plasmid, pMCS6-GPD6.1Age, consisted of the GPD promoter fused to the entire ORF of 6.1, with the stop codon being replaced by the last three bases of the *Age*I site. The large *Hind*III/*Age*I insert from pMCS6-GPD6.1Age, consisting of the GPD promoter and the ORF of 6.1, was ligated to the large *Hind*III/*Age*I fragment of pMCS9-3'(HIS)₅ (see above) in order to create the plasmid pMCS9-GPD6.1(HIS)₅. This contained the ORF of 6.1, one additional Gly codon, five His codons and a stop codon, and was flanked by unique *Bam*HI and *Sal*I sites at the 5'- and 3'-ends respectively. The *Bam*HI-*Sal*I fragment was ligated into *Sal*I/*Bam*HI-digested YIpMS1 to create YIpMS1-6.1(HIS)₅.

PCRs (Saiki et al., 1988) were performed on plasmid DNA (1 ng) in 50 mM KCl/10 mM Tris/HCl (pH 8.3)/2 mM MgCl₂ containing 200 μ M each of the four deoxyribonucleotides, 0.1–0.5 μ M of each oligonucleotide primer, 5% formamide (Sarkar et al., 1990) and 2 units of *Taq* polymerase. After 20 cycles (each 30 s at 94 °C, 1 min at 45 °C, 1 min at 72 °C) and then incubation at 72 °C for 5 min, the reaction products were analysed by agarose-gel electrophoresis and DNA fragments were purified (Weislander, 1979).

Sequencing was performed by the dideoxynucleotide chain-termination method (Sanger et al., 1977; Biggin et al., 1983; Chen and Seeburg, 1985) using T7 sequencing kits. The Bio-Rad gene pulser was used for transformation of yeast cells by

electroporation (Becker and Guarente, 1991). Verification of correct single integration and gene-replacement events was carried out by Southern analysis. *Escherichia coli* NM522 and K803 were used for plasmid transformation and propagation as previously described (Maniatis et al., 1982; Nishimura et al., 1990).

Yeast cell culture and extraction

Cells grown at 30 °C in yeast-extract/peptone/dextrose medium were harvested some 20 h after inoculation with a starter culture (10 ml per 500 ml). Harvesting was achieved by centrifugation at 1400 *g*_{av.} for 15 min at 4 °C. Cell pellets (25 ml) were suspended by addition of 5 ml of 80 mM triethanolamine/HCl/5 mM MgCl₂ buffer (adjusted to pH 7.4 with KOH) containing a mixture of protease inhibitors at a final concentration of 40 µg/ml phenylmethanesulphonyl fluoride, 156 µg/ml benzamidine and 1 µg/ml each of aprotinin, leupeptin, pepstatin A and antipain. The protease inhibitors were initially dissolved as a 1000 × stock in 100 % dimethyl sulphoxide before addition to the buffer. The cells in the pellet were lysed by four passes through a French Pressure Cell Press (SLM Instruments Inc.; 40 ml cell) at 6624 kPa (960 lb/in²). The resultant lysate was centrifuged at 210000 *g*_{av.} for 45 min. Samples of both pellet and supernatant were assayed for PDE activity immediately and residual material was stored at -80 °C for later analysis.

PDE assay

PDE activity was assayed by a modification of the two-step procedure of Thompson and Appleman (1971) and Rutten et al. (1973) as described previously by Marchmont and Houslay (1980). All assays were conducted at 30 °C, and in all experiments a freshly prepared slurry of Dowex/water/ethanol (1:1:1, by vol.) was used for determination of activities. Initial rates were taken from linear time courses of activity. For the determination of kinetic parameters, the PDE assays were conducted with [cAMP] over the range 0.1–100 µM. Double-reciprocal plot data were analysed by computer fitting to the hyperbolic form of the Michaelis–Menten equation by using an iterative least-squares procedure in order to obtain estimates of *K*_m and associated errors (Ultrafit, Marquardt algorithm; Biosoft, Cambridge, U.K.). Dose–effect inhibitor analyses were performed with 3 µM cAMP as substrate over a range of inhibitor concentrations as shown in the appropriate Figures. PDE inhibitors were dissolved in 100 % dimethyl sulphoxide as a 10 mM stock and diluted in 20 mM Tris/HCl/10 mM MgCl₂ buffer at final pH 7.4 to provide a range of concentrations for use in the assay. The residual levels of dimethyl sulphoxide were shown not to affect PDE activity over the ranges used in this study.

Thermal denaturation

Pellet and soluble fractions were prepared as described above. Each sample was pipetted into pre-warmed 20 mM Tris/HCl/10 mM MgCl₂, final pH 7.4, contained in a glass bijoux which had been placed on a stirrer in a water bath at the indicated temperature. At the time points shown on the appropriate Figure a 100 µl sample was removed and placed in an Eppendorf Microfuge tube, which had been kept on ice. PDE activities were then measured with 1 µM cAMP as substrate.

Purification of PDE activity from YMS8 yeast cells

Aminoalkyl-Sepharose 4B resin was substituted with the rolipram analogue AAL 115, which had been modified at the carboxyl end

with 4-(3-carboxypropyloxy-4-methoxyphenyl)-2-pyrrolidine (Marivet, 1986), as described by Fougier et al. (1986). This was then used to bind h6.1_{his5} as described by Fougier et al. (1986). For a typical small-scale preparation a 50 % resin/water slurry (400 µl) was first equilibrated for 1 h in 20 ml of TMD buffer (50 mM Tris/HCl, pH 7.5, 5 mM MgCl₂ and 0.1 mM dithiothreitol). The resin was sedimented by centrifugation at 4000 *g*_{av.} and the supernatant removed. A soluble high-speed supernatant fraction from the disrupted YMS8 yeast cells (10 ml; as detailed above) was added to the resin and left, with mixing, at 30 °C for 1 h, over which time the PDE activity bound to the resin. In practice, the amount of affinity resin used was that found to be sufficient to bind all of the PDE activity. Non-specifically bound proteins were removed by washing the resin with TMD buffer containing 500 mM KCl (30 °C; 1 h). This was done by sedimenting the resin, as above, and resuspending it in 10 ml of the KCl/TMD buffer. PDE activity was then specifically eluted with TMD buffer containing 2 mM cAMP as described by Fougier et al. (1986), i.e. for 1 h at 30 °C. The eluate was then mixed, for 1 h at 4 °C, with a Nickel ProBond Metal affinity-chromatography resin (400 µg of a 50 % resin/20 % ethanol slurry) which had previously been equilibrated with TMD buffer. The resin, with adsorbed PDE, was washed by first sedimenting the resin and then resuspending it in 10 ml of TMD buffer containing 500 mM KCl at 4 °C (1 h) in order to remove any non-specifically adsorbed proteins. This was repeated at least three times or until no 280 nm-absorbing material was eluted. PDE activity was then eluted from the resin (1 h at 4 °C) with a minimal volume (1 ml) of TMD buffer containing 50 mM EDTA. After recovering the supernatant (1 ml), 250 µl of 1 M MgCl₂ was added and the sample was stored frozen, in batches, until required.

Protein determination

Protein was routinely measured by the method of Bradford (1976), with BSA as a standard. The assessment of protein concentration in the final purified PDE preparation was determined by subjecting samples to SDS/PAGE on a 10 % gel, followed by silver staining. Standard samples of BSA (0.005–0.2 µg) were run on separate lanes on the same gel. The gel was then scanned while wet in a Shimadzu CS-9000 dual-wavelength flying-spot scanner at a wavelength of 575 nm. A linear relationship was found between absorbance and applied protein over the range examined. This allowed us to construct a calibration curve from which the protein content of the applied PDE fraction was determined relative to BSA as a standard.

Molecular-mass determination by SDS/PAGE

Purified PDE samples were freeze-dried and resuspended in one-tenth of their original volume. Samples (50 µl) were boiled in an equal volume of sample buffer (125 mM Tris/HCl, final pH 6.8, 25 % glycerol, 4 % SDS and 5 % 2-mercaptoethanol) for 5 min. The samples were subjected to SDS/PAGE at 60 mA through a 10 % gel as described by Laemmli (1970). Further samples containing prestained molecular-mass markers (200, 97.4, 68, 43, 29, 18.4 and 14.3 kDa) were treated in the same way. After electrophoresis, the gel was washed in aq. 50 % (v/v) methanol for 18–24 h before silver staining. The stain was prepared by adding solution A (0.8 g of AgNO₃ in 4 ml of water) dropwise with stirring to solution B (21 ml of 0.36 % NaOH, 1.4 ml of conc. NH₄OH) and then diluting with water to 100 ml. The gel was stained by shaking in the stain for 15 min at room temperature. It was washed in water in a similar manner (3 × 5 min)

and then shaken in developer (500 ml of water, 5 ml of 1% citric acid, 500 μ l of formaldehyde) until protein bands were revealed.

Molecular-mass determination by gel filtration

A Sephadex G-150 column (45 cm $\times$ 1 cm) was poured and equilibrated, at 4 °C, with 20 mM Tris/HCl buffer (final pH 7.4) containing 5 mM MgCl₂. The void volume (V_0) of the column was determined by monitoring the elution of Blue Dextran (2000 kDa) at 280 nm. The elution volumes (V_e) of the various protein standards were determined in a similar manner. These were β -amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.4 kDa). Fractions (0.5 ml) were collected from the column, which was run at 36 ml/h at 4 °C. A linear standard curve was constructed of log molecular mass against V_e/V_0 . Samples (0.75 ml) containing PDE activity were applied to the column and eluted similarly in order to determine the V_e of the PDE activity, and hence the native molecular size.

Figure 1 SDS/PAGE and gel filtration of purified h6.1_{his5}

(a) A typical silver-stained SDS/PAGE gel of two different preparations of purified h6.1_{his5}. (b) Elution profiles of PDE activity found in the soluble fraction of YMS6, representing h6.1, and also that for the activity purified from YMS8, namely h6.1_{his5}. These data are typical of experiments done at least three times using different cultures.

RESULTS

Disruption of URA3

The strain YMS5 is a derivative of YMS4, which had been selected for loss of functional *ura3* gene product by counter-selection on media containing 5-FOA (Sullivan et al., 1994). We found, however, that when YMS5 was used as the host for transformations, then many transformants were spontaneous revertants lacking plasmid sequences. To improve the recovery rate for true transformants, we carried out one-step disruption of

Figure 2 Thermal denaturation of h6.1 in soluble and pellet forms

Experiments were carried out as detailed in the Materials and methods section. Data shown are typical of experiments done at least three times. Lines are fitted by least-squares analyses and half-lives given in Table 2. Inactivation was done (a) at 45 °C and (b) at 50 °C for soluble (●) and pellet (□) enzyme.

Table 2 Parameters of h6.1 activity in soluble and pellet form

Soluble and particulate/pellet fractions of h6.1 were obtained from YMS6 cells. For the determination of K_m values, the hydrolysis of cAMP was determined over a range of cAMP concentrations (0.01–100 μ M). Double-reciprocal plots of initial rates against [cAMP] were linear, indicative of simple saturation kinetics. Substrate-utilization data were fitted with high correlation (> 0.99), by using an iterative method, to the hyperbolic Michaelis equation $v_{obs} = (V_{max} [cAMP]) / (K_m + [cAMP])$ in order to determine K_m values. In thermostability studies, both soluble and pellet extracts from YMS6 were incubated for various periods of time (see Figure 2) before removal of material to ice as a prelude to performing an assay to determine the residual PDE activity in these fractions. Semi-log plots were made of log of percentage of PDE activity remaining against time, from which the half-life of decay ($t_{0.5}$) was determined. Dose-dependent changes in activity with Mg²⁺ and Mn²⁺ and EDTA were done in the presence of 3 μ M cAMP as substrate by using procedures described in the legend to Figure 6. K_i values for rolipram and IBMX were determined from both slope and Dixon (1953) replots of data generated as shown in Figures 3 and 5. K_i values for Ro-20-1724 and for cilostimide were calculated by using the dose-response data generated in Figure 4 on the basis that these inhibitors were competitive in action. Re-activation is for EDTA inactivated enzyme. Experiments were done with *n* separate enzyme preparations, with assays done in triplicate in each case. Values are means $\pm$ S.D.; n.d., not determined.

Parameter	Soluble form	Pellet form
K_m for cyclic AMP (μ M)	4.1 $\pm$ 1.7 (<i>n</i> = 6)	5.2 $\pm$ 1.5 (<i>n</i> = 6)
K_i for rolipram (μ M)	0.4 $\pm$ 0.1 (<i>n</i> = 6)	0.7 $\pm$ 0.3 (<i>n</i> = 6)
K_i for IBMX (μ M)	15 $\pm$ 3 (<i>n</i> = 3)	19 $\pm$ 3 (<i>n</i> = 3)
K_i for Ro-20-1724 (μ M)	n.d.	7 $\pm$ 1 (<i>n</i> = 3)
K_i for cilostimide (μ M)	n.d.	129 $\pm$ 21 (<i>n</i> = 3)
$t_{0.5}$ at 45 °C	10 $\pm$ 2 (<i>n</i> = 3)	88 $\pm$ 9 (<i>n</i> = 3)
$t_{0.5}$ at 50 °C	2 $\pm$ 0.3 (<i>n</i> = 3)	27 $\pm$ 3 (<i>n</i> = 3)
EC ₅₀ for Mg ²⁺ (mM)	n.d.	1.8 $\pm$ 0.5 (<i>n</i> = 3)
EC ₅₀ for Mn ²⁺ (mM)	n.d.	1.5 $\pm$ 0.4 (<i>n</i> = 3)
EC ₅₀ for Mg ²⁺ (mM) (re-activation)	n.d.	2.4 $\pm$ 0.5 (<i>n</i> = 3)
IC ₅₀ for EDTA (mM)	n.d.	0.08 $\pm$ 0.03 (<i>n</i> = 3)

Figure 3 Kinetics of rolipram inhibition

Soluble (a, b, c) and pellet (d, e) forms of h6.1 were used in these typical experiments done at least three times. Shown are double-reciprocal plots of cAMP hydrolysis against [cyclic AMP] (a, d), Dixon (1953) replots of the reciprocal of cAMP hydrolysis against [rolipram] for the soluble enzyme (b), and replots of the slopes of the double-reciprocal plots (a) and (d) against [rolipram] (c, e respectively). Linear Dixon (1953) replots were also obtained for the pellet enzyme (results not shown). In (f) is a plot of PDE activity versus [PDE] in the absence (●) and presence (□) of 4 μM rolipram with assays done at 1 μM cAMPs. The line with protein given here for soluble crude extract of 6.1 were drawn by least squares analysis with correlation coefficient (r) > 0.99. Similar data were obtained for both the pellet and soluble forms of h6.1 and h6.1_{his5}. Velocity measurements are in arbitrary units.

the *ura3* gene in YMS4 to create YMS5X (Rothstein, 1983). Plasmid pME69 Δ AS had 62 bp deleted within the ORF of the *ura3* gene, rendering it non-functional. Digestion of the plasmid with *Hind*III releases the defective *ura3* gene, which was purified and introduced into YMS4 by electroporation (Becker and Guarente, 1991). After electroporation, the cells were washed twice with YPD media and then grown overnight without selection before being plated on to 5-FOA-containing plates. Southern analysis of ten transformants growing on the 5-FOA plates revealed that in all ten colonies the *ura3* gene within the disrupted *pep4* locus carried the 62bp deletion. One colony was chosen for further use, and was named YMS5X.

Integration of YIpMS1-6.1(HIS)₆

Plasmid YIpMS1-6.1(HIS)₆ was linearized with *Eco*RV as it cuts the plasmid once within the URA3 gene. DNA was purified and

introduced into YMS5X cells by electroporation, whereupon the cells were plated on media lacking uracil. Transformants were picked and grown in YPD media overnight before being tested for heat-shock resistance/sensitivity (see Sullivan et al., 1994). More than 90% of transformants tested were resistant to heat shock. This demonstrated that the histidine-tagged h6.1 human PDE was able to hydrolyse cAMP in intact yeast. Southern analysis of transformants exhibiting heat-shock resistance was carried out in order to determine the site of integration and whether single or multiple integration events had occurred. One transformant (YMS8) that carried a single copy of YIpMS1-6.1(HIS)₆ integrated at the *pep4* locus was used for all subsequent analysis of the expressed histidine-tagged h6.1.

Expressed activity

The disrupted cell homogenates from YMS5 failed to achieve the

hydrolysis of cAMP. In marked contrast with this, strain YMS6, with a single copy of the h6.1 cDNA engineered for expression and integrated into the genome, showed a profound ability to hydrolyse cAMP (5–9 pmol of cyclic AMP hydrolysed/min per mg of protein at 30 °C; $n = 25$, range). This activity (1 μ M cAMP substrate) was not stimulated by addition of Ca^{2+} (100 μ M)/calmodulin (20 ng/ml), nor was it affected by the presence of cGMP (10 μ M). No hydrolysis of cGMP (< 1 % activity seen with cAMP) was observed. Thus the PDE activity expressed in YMS6 was typical of a type-IV PDE.

The yeast extract was subjected to centrifugation at 210000 g_{av} for 45 min. In the supernatant fraction we observed that some 22–48 % of the activity occurred as a soluble species, with the rest of the activity found in the pellet (range; $n = 25$). Subjecting the pellet to further extraction led to the release of < 9 % of the pelleted activity. Pellet PDE activity was not rendered soluble by treatment with EGTA (1 mM), high concentrations of NaCl (200 mM–2 M) or with the detergent Triton X-100 (0.1–3 %). This pellet activity is thus not an adsorbed species, nor a peripheral protein nor an integral membrane protein.

PDE activity in the strain YMS8, which contained a single copy of the h6.1 cDNA tagged to express the enzyme with five histidine residues at its C-terminus (h6.1_{his5}), was also cAMP-specific. It was similarly typical of a type-IV PDE (see above), with an activity of 2–6 pmol of cAMP hydrolysed/min per mg of protein seen in soluble extracts (range; $n = 16$). Activity was found in both the soluble fraction of the yeast homogenate (25–33 %) as well as in the pellet fraction (range; $n = 16$).

Purification of h6.1_{his5}

This was performed as described in the Materials and methods section by applying a two-stage affinity-column procedure to a soluble extract (210000 g /45 min supernatant) from the strain YMS8. The first stage involved the use of a rolipram affinity column, which effected a purification of ~ 160 -fold with a 20–25 % yield. The second stage utilized a Ni^{2+} column, which effected an overall purification of ~ 53000 -fold with an overall yield of 5–10 %. The PDE produced was apparently homogeneous by virtue of it giving a single protein band which co-migrated with activity on non-denaturing gels (results not shown) and a single silver-stained band of 73 ± 2 kDa on SDS/PAGE (Figure 1a).

Molecular size

Gel filtration (Figure 1b) of the homogeneous enzyme purified from YMS8 indicated a native molecular mass of 74 ± 3 kDa. Similar results were found for the single peak of soluble PDE activity occurring in the crude soluble fraction from YMS8 (76 ± 2 kDa) and for the single peak of activity found by using the crude soluble fraction from YMS6 (73 ± 3 kDa). h6.1 thus appears to be a monomeric protein, and the addition of five histidine residues at its C-terminus does not appear to alter this. That single peaks of PDE activity were observed in analyses done on the crude soluble extracts is consistent with their being a single homogeneous population of soluble h6.1 expressed. This is consistent with the thermostability studies (see below).

Thermostability of yeast-expressed h6.1 PDE

The thermostability of h6.1 PDE activity (YMS6) found in both the soluble and pellet fractions was assessed. In each instance, activities decayed as a single exponential, yielding linear semi-log plots of residual activity against time for studies done at both

Figure 4 Dose-dependent inhibition by rolipram, Ro-20-1724, IBMX and cilostimide

Shown for h6.1 in these typical experiments, done at least three times, are the comparative dose-dependent inhibitory effects of rolipram, Ro-20-1724, IBMX and cilostimide performed at 3 μ M cAMP.

45 °C and 50 °C (Figures 2a and 2b). The activity in the pellet, however, was considerably more thermostable than that in the soluble fraction (Figures 2a and 2b; Table 2). That these activities each decayed as a single exponential was indicative of a single homogeneous PDE protein population occurring in each fraction.

Kinetics of cAMP hydrolysis by h6.1 PDE preparations

Lineweaver–Burk plots for the hydrolysis of cAMP by the homogeneous preparation of h6.1_{his5}, as well as for the soluble and pellet h6.1, activities were linear over a wide range of cAMP concentrations, 0.01–100 μ M (results not shown, but see Figures 3a and 3d). These were indicative of simple saturation kinetics. Analysis of these data yielded similar K_m values in the low- μ M range for all the enzymes (Table 2).

To date, however, all V_{max} values calculated for PDE enzymes expressed by human cDNAs have been arbitrary values, as these enzymes had not been purified to apparent homogeneity. Here, however, we were able to determine kinetic constants for the homogeneous enzyme preparation afforded by h6.1_{his5}. This yielded values for the K_m for cAMP of 8 ± 1 μ M and a true V_{max} of 0.8 ± 0.1 μ mol of cAMP hydrolysed/min per mg of h6.1_{his5} protein. From these, and knowing the molecular mass of the pure protein, we could calculate that the turnover number, k_{cat} , was 3702 s^{-1} . The efficiency of the enzyme in performing the hydrolysis of cAMP could be assessed by determination of the specificity constant, k_{cat}/K_m , for which we obtained a value of 4.6×10^8 $M^{-1} \cdot s^{-1}$ (mean $\pm$ S.D. for $n = 3$ different preparations).

Action of selective inhibitors

Assaying at a concentration of cAMP which approximates to that of the K_m for cAMP, we found that h6.1 activity was potently inhibited by low concentrations of the selective type-IV PDE inhibitors rolipram (Table 2; Figures 3 and 4) and Ro-20-1724 (Table 2; Fig. 4). It was also inhibited by the non-selective PDE inhibitor IBMX and, at much higher concentrations, by the type-

Figure 5 Kinetics of IBMX inhibition

Shown is the inhibitory effect of IBMX on h6.1 in these typical experiments done at least three times. In (a) are double-reciprocal plots of cAMP hydrolysis against [cAMP], and in (b) are replots of their slopes against [IBMX]. Dixon (1953) replots of the reciprocal of cAMP hydrolysis against [IBMX] were linear in all instances (results not shown). Velocity measurements are in arbitrary units.

III selective inhibitor cilostimide (Table 2; Figure 4). This profile is typical for a type-IV PDE activity (Houslay and Kilgour, 1990; Conti and Swinnen, 1990; Conti et al., 1991).

Kinetics of inhibition of rolipram and IBMX

Both rolipram (Figure 3) and IBMX (Figure 5) acted as simple competitive inhibitors of h6.1 activity expressed in both the soluble and the pellet fractions of YMS6. This was evident from the fact that double-reciprocal plots of PDE activity against cAMP concentrations remained linear over a wide range of substrate and inhibitor concentrations, and that both the slope and Dixon (1953) replots of these data (Figures 3 and 5) were similarly linear.

The relationship of PDE activity to protein concentration was also linear for the soluble, pellet and homogeneous enzyme preparations of h6.1, and remained linear even in the presence of half-maximal inhibitory concentrations of rolipram; typical data

Figure 6 Effect of bivalent cations and EDTA

Shown are experiments typical of ones done at least three times for the dose-dependent (a) activation of h6.1 by Mg²⁺ and Mn²⁺ (M^{2+}) and in (b) the inhibition caused by EDTA. In (a) is also shown the re-activation of EDTA-treated h6.1 by Mg²⁺.

for the soluble enzyme are shown in Figure 3(f). This indicates that rolipram is not serving as a tight-binding inhibitor of h6.1.

Action of EDTA and the bivalent cations Mg²⁺ and Mn²⁺

Addition of either Mg²⁺ or Mn²⁺ to PDE assays of h6.1 led to a dose-dependent increase in activity (Figure 6a). In contrast, addition of the chelating agent EDTA either to assays or directly to h6.1 caused the dose-dependent abolition of PDE activity (Figure 6b). However, in both instances, the inhibitory effect of EDTA could be simply reversed by addition of either Mg²⁺ or Mn²⁺ to h6.1 (Figure 6a).

DISCUSSION

h6.1 is clearly a type-IV PDE in that it specifically hydrolyses cAMP, is unaffected by Ca²⁺/calmodulin and by low concentrations of cGMP, and is potentially inhibited by the selective inhibitors rolipram and Ro-20-1724 (Table 2). It also shares sequence similarity with the various type-IV PDEs cloned from rat, in particular RD1 (the rat brain rolipram-sensitive form), which would indicate that it can be classified as a PDE-IV_A species (Sullivan et al., 1994).

In order to purify h6.1 to apparent homogeneity the cDNA was engineered so that the expressed protein would exhibit a

Zn²⁺-binding motif in the form of five histidyl residues at its C-terminus (h6.1_{hiss}). In such a form it might be expected to bind to a Ni²⁺-chelate column. However, as yeast contains various Zn²⁺-binding proteins, we first allowed h6.1_{hiss} to bind to a rolipram affinity column. It was then specifically eluted with cAMP and immediately bound to the chelate column. This strategy had the advantage of allowing for the easy removal of cAMP, being effected through washing the enzyme absorbed to the chelate column. Elution of a homogeneous preparation of h6.1_{hiss} from the chelate column was then performed with EDTA. However, re-activation of the eluted PDE required treatment with Mg²⁺ (see below).

Using this apparently homogeneous enzyme preparation, we were able to determine a true $V_{\max}$ value (Table 2) as well as calculating a turnover number, k_{cat} ($\sim 3702 \text{ s}^{-1}$). For comparison, from the published $V_{\max}$ data for two type-IV PDEs purified from native sources (Thompson et al., 1979; Marchmont et al., 1981) we can calculate a k_{cat} of 342 s^{-1} for dS-PDE and k_{cat} values of 28392 s^{-1} and 3869 s^{-1} for rPPM-PDE (representing the limiting conditions seen for the apparent 'low' and 'high'-affinity components). The specificity constant k_{cat}/K_m for h6.1_{hiss} was calculated as $4.6 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$. This suggests that h6.1_{hiss} works near maximum efficiency and the reaction is essentially diffusion controlled. This also appears to be true for rPPM-PDE ($\sim 6.6 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$) and also for dS-PDE ($1.6 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$). The similarity of these specificity constants would support the contention made in each case that such enzymes were purified to apparent homogeneity.

h6.1 in both soluble and pellet forms (Table 2) in yeast, as well as when expressed in soluble forms in COS-1 cells (Sullivan et al., 1994), showed not only similar K_m values for cAMP hydrolysis but also similar K_i values for inhibition by rolipram. This indicates that, whatever gives rise to the aggregated form of h6.1 in yeast, the catalytic/inhibitor-binding site would appear to be relatively unaffected. Indeed, the only obvious difference between the pellet and soluble forms was that the pellet form was considerably more thermostable (Table 2). Presumably this was because the formation of the immobilized material constrained the conformation of the enzyme such that it resisted thermal denaturation. As with the soluble enzyme, thermal denaturation allowed all ($> 90\%$) of the pellet activity to decay as a single exponential (Figure 2). This is characteristic of a single homogeneous population of enzyme molecules in both instances. Soluble h6.1 in yeast is not then formed from a mixture of partially degraded proteins. This is consistent with the fact that h6.1_{hiss} can be purified to yield a single protein of the size predicted from its sequence and that gel-filtration studies identified a single peak of activity (Figure 1). The soluble form presumably reflects the native state, as h6.1 activity expressed in transfected COS cells is found exclusively in the cytosol (Sullivan et al., 1994).

The bivalent cations Mg²⁺ and Mn²⁺ have been shown by various investigators to be capable of increasing PDE activity (see, e.g., Bergstrand et al., 1978; Grant and Colman, 1984; Lavan et al., 1989). However, although two rolipram-inhibited type-IV PDEs in hepatocytes are clearly activated by these bivalent cations in a dose-dependent fashion (Lavan et al., 1989), a soluble cAMP-specific PDE (type VII) has also been identified in hepatocytes which appears to be insensitive to activation by Mg²⁺ and also inhibition by IBMX (Lavan et al., 1989). Clearly h6.1 is both inhibited by IBMX and activated by Mg²⁺ (Figures 5 and 6; Table 2). Such features are characteristic of the PDE-IV_A family. Interestingly, treatment of h6.1 with EDTA led to the abolition of PDE activity in a fashion which could be reversed by the subsequent addition of either Mg²⁺ or Mn²⁺. This suggests

that there may be a distinct binding site for Mg²⁺ on h6.1, rather than the role of Mg²⁺ being simply to form a complex with cAMP.

There has been allusion to the possibility that the mechanism of inhibition of type-IV PDEs by rolipram may be anomalous, on the basis that log dose-inhibition curves showed a shallow profile (McLaughlin et al., 1993; Bolger et al., 1993) and that inhibition data in double-reciprocal plots of studies done on hPDE-IV_{A-Livi} did not appear to fit simple competitive kinetics (Livi et al., 1990). However, no rigorous analysis of inhibition of a type-IV PDE by either a selective inhibitor such as rolipram or a non-selective inhibitor such as IBMX has been reported before the present study. We show here that both compounds serve as simple competitive inhibitors of h6.1, yielding linear double-reciprocal plots, linear slope replots and linear Dixon (1953) replots (Figures 3 and 5).

In analyses of a human type-IVA PDE, Torphy and co-workers (Livi et al., 1990; Torphy et al., 1992) noted that rolipram did not serve as a simple competitive inhibitor. They observed that the change in slope of double reciprocal plots of rolipram inhibition deviated from that which could be predicted for simple competitive kinetics. It appeared that the value for the slope was lower than that expected. Certainly our formulation of slope replots (data not shown) from data (fig. 5 of Livi et al., 1990; fig. 3 of Torphy et al., 1992) would support this, in that such plots were markedly concave in form. These anomalies would not be expected if the enzyme bound more than one molecule of rolipram as convex slope replots would typify this, with a slope being observed which was bigger than that expected for simple competitive inhibition. The data describing the type-IVA PDE analysed by Torphy and co-workers (Livi et al., 1990; Torphy et al., 1992) appear to indicate that rolipram serves as a partial competitive inhibitor of that enzyme (see Dixon and Webb, 1979). Consistent with such a mechanism, we noted (results not shown) that a secondary replot of the reciprocal of the change in slope against the reciprocal of [rolipram] was linear (see Dixon and Webb, 1979). From such an analysis we could directly calculate a value of $1.8 \mu\text{M}$ for the affinity constant K'_i , which reflects the binding of rolipram to the enzyme-substrate complex of hPDE-IV_{A-Livi}. The application of curve-fitting strategies to this secondary replot then allowed us to determine values for K'_i as $0.24 \mu\text{M}$ and for K_s^{cAMP} as $5 \mu\text{M}$. These observations suggest that the amino acid differences between hPDE-IV_{A-h6.1} and hPDE-IV_{A-Livi} do indeed lead to a functional alteration. This may be because such residues are all located either in or near to the domain of the enzyme which is believed to contain the catalytic site (Chen et al., 1986; Jin et al., 1992). The different inhibitory kinetic mechanisms for the action of rolipram on these two type-IVA PDEs suggests that h6.1 and hPDE-IV_{A-Livi} are conformationally and functionally distinct forms. One explanation for the difference in kinetics between the two closely related PDEs would be that the active site in hPDE-IV_{A-Livi} has been distorted, compared with h6.1, such that it can now accommodate both cAMP and rolipram together. Clearly, confirmation of this will involve independent approaches, such as the structural analysis of these enzymes. However, it is clear from the diverse nature of the range of PDE inhibitors identified (see, e.g., Weishaar et al., 1985), that the active-site area of PDEs is of a considerable size. In some instances it can accommodate molecules with the very different side chains as are seen for specific inhibitors for different classes of PDEs. It is possible that the altered folding occurring in hPDE-IV_{A-Livi} may have so modified the binding pocket that both the inhibitor and cAMP can now bind in tandem.

Although the paucity and lability of PDEs, especially from

human sources, has severely constrained the development of our understanding of members of this important enzyme family, the availability of cloned enzymes which can be efficiently expressed in yeast should help to resolve this.

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