The specificity of yeast low- K_m cyclic AMP phosphodiesterase towards free bivalent metal ions and the diastereoisomers of cyclic adenosine phosphorothioate

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The relative activity of a zinc-containing cyclic AMP phosphodiesterase towards the (S_p)- compared with the (R_p)-diastereoisomer of cyclic adenosine phosphorothioate varied with the identity of the free bivalent metal ion from more than 35 to 0.074 along the series $Mg^{2+} > Mn^{2+} > Co^{2+} > Zn^{2+} > Cd^{2+}$, showing that this ion, and not the tightly bound zinc, bonds to the phosphorothioate moiety of the substrate.

Most cyclic nucleotide phosphodiesterases need a free bivalent metal ion. Mg^{2+} has usually been used *in vitro*, but other metals, e.g. Mn^{2+} , Co^{2+} and Zn^{2+} , are often also active (e.g. Donnelly, 1977). Cyclic adenosine phosphorothioate (cyclic APT) is a substrate for several phosphodiesterases (Eckstein *et al.*, 1974; Jarvest *et al.*, 1982) and activates some cyclic AMP-dependent protein kinases (Rothermel *et al.*, 1983). Jarvest *et al.* (1982) showed that bovine heart cyclic nucleotide phosphodiesterase greatly prefers the (S_p)-diastereoisomer of cyclic APT, but that a yeast high- K_m phosphodiesterase, which contains two zinc atoms per subunit and does not need free bivalent metal ions, hydrolyses (S_p)- and (R_p)-isomers about equally.

A low- K_m cyclic AMP phosphodiesterase from yeast both needs free bivalent metal ions and contains tightly bound zinc (Suoranta & Londesborough, 1984). At least two mammalian phosphodiesterases also contain tightly bound metal (Londesborough, 1985*a,b*). It was interesting to know whether the specificity of the yeast low- K_m enzyme for (S_p)- and (R_p)-cyclic APT is determined by the tightly bound metal or the free bivalent metal ion. Here we show that: (1) the free bivalent metal ion can be Mg^{2+} , Mn^{2+} , Zn^{2+} , Co^{2+} or Cd^{2+} ; (2) with Mg^{2+} the specificity towards cyclic APT diastereoisomers is high and the same as for the bovine heart enzyme; but (3) the relative activity towards the (R_p)-diastereoisomer increases along the series $Mg^{2+} < Mn^{2+} < Co^{2+} < Zn^{2+} < Cd^{2+}$ in

the same order as increasing ability of the metal to bond to sulphur.

Materials and methods

(S_p)- and (R_p)-diastereoisomers of cyclic APT were kindly given by Dr. J. Baraniak, Polish Academy of Sciences, Lodz, Poland. Chelex 100 was from Bio-Rad (Richmond, CA, U.S.A.) and bivalent metal salts were analytical grade from E. Merck A.G., Darmstadt, Germany. Low- K_m cyclic AMP phosphodiesterase of specific activity 1.2 units/mg was '70% nicked' (70% of the polypeptide chains were cleaved; see Suoranta & Londesborough, 1984) and stored at 170 munits/ml in 10 mM-potassium phosphate (pH 7.5)/0.15 M-NaCl/1 mM- $MgCl_2$ /20 μ M-zinc acetate/20 μ M-EDTA/40% (v/v) glycerol. A unit hydrolyses 1 μ mol of cyclic AMP/min at 1 μ M-cyclic AMP, 30°C, pH 7.9. Other chemicals were from the sources previously described (Suoranta & Londesborough, 1984).

The hydrolysis of cyclic AMP was assayed at 30°C by a modification (Londesborough, 1976) of the snake-venom assay (Thompson & Appleman, 1971). $MgCl_2$ (2 mM) was always added with the snake venom. Buffers for these enzyme assay mixtures or enzyme dilution (50 M-Hepes/KOH, pH 7.9, containing respectively 2.5 or 10 mg of bovine serum albumin/ml) were freed of bivalent-metal contaminants by passage through a pre-equilibrated column of Chelex 100. Stock enzyme was diluted about 2000-fold with buffer containing the same concentration of the same bivalent metal ion as the reaction mixture, and reactions were

Abbreviation used: cyclic APT, cyclic adenosine phosphorothioate.

started by adding 10 μl of diluted enzyme to 200 μl of reaction mixtures. Reaction mixtures for the hydrolysis of cyclic APT contained Chelex 100-treated 50 mM-Hepes/KOH, pH 7.9, but only 0.22 mg of albumin/ml (to keep the A_{265} low) and higher enzyme concentrations (which introduced 3 μM - Mg^{2+} and 67 nM- Zn^{2+}). Reactions were stopped by addition of EDTA (3.5 mM final concn.) and the adenosine phosphorothioate product was immediately determined (Chassin & Harris, 1972) from the change in A_{265} on addition of adenylic acid deaminase (70 munits/ml) by assuming $\epsilon = 7800 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

Results

When reaction mixtures treated with Chelex 100 were used, the blank rate measured in the absence of bivalent metal ions decreased with the cyclic AMP concentration, and at 0.25 μM -cyclic AMP was $6 \pm 1\%$ of the rate at 1 mM- Mg^{2+} . Rates at 0.25 μM -cyclic AMP were not significantly increased by 10 or 50 μM - Cu^{2+} , - Ca^{2+} , - Fe^{2+} or - Ni^{2+} , but were increased (Fig. 1) by the following bivalent metal ions (metal concentration for half-maximal stimulation in parentheses): Mg^{2+} (50 μM), Mn^{2+} (10 μM), Zn^{2+} (50 μM), Cd^{2+} (100 μM) and Co^{2+} (60 μM).

Hofstee (1959) plots at various concentrations of Mg^{2+} , Zn^{2+} and Mn^{2+} were approximately linear between 35 nM- and 1 μM -cyclic AMP (results not shown). Median estimates of the apparent K_m and V_{max} values calculated by the direct linear plot (Eisenthal & Cornish-Bowden, 1974) are shown in Table 1. Blank rates with no added metal ions were subtracted from the observed rates, and were generally less than 20%. When the concentrations of Zn^{2+} and cyclic AMP were both low, however, the blank rate correction increased to 40%, and the K_m and V_{max} estimates of 25 μM - Zn^{2+} would be 40% smaller and 10% higher, respectively, if no correction were made.

Rates of enzyme-catalysed hydrolysis of 44 μM -(S_p)- and (R_p)-cyclic APT in the presence of 100 μM concentrations of different bivalent metal ions are shown in Table 2, together with data for cyclic AMP obtained by the same spectrophotometric method. The ratio of the rates of hydrolysis of the diastereoisomers, $[v(S_p)/v(R_p)]$, depended on the free metal ion, and decreased from more than 35 to 0.074 along the series $\text{Mg}^{2+} > \text{Mn}^{2+} > \text{Co}^{2+} > \text{Zn}^{2+} > \text{Cd}^{2+}$. The same order of metals was found when apparent maximum velocities were used. However, this enzyme shows small deviations from Michaelis-Menten kinetics at high cyclic

Fig. 1. Influence of the type and concentration of free bivalent metal ion on hydrolysis of 0.25 μM -cyclic AMP. Rates with MgCl_2 ($\circ$), MnCl_2 ($\bullet$), zinc acetate ($\triangle$), CoSO_4 ($\square$) and CdCl_2 ($\blacksquare$) are shown as percentages of that at 1 mM- MgCl_2 .

Table 1. Influence of the type and concentration of free bivalent metal ion on kinetic parameters for cyclic AMP hydrolysis. Median values of the apparent K_m (μM) for cyclic AMP and V_{max} ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{l}^{-1}$, normalized to an enzyme concentration of 1 $\text{mg} \cdot \text{l}^{-1}$) were obtained from data between 35 nM- and 1 μM -cyclic AMP. Results of single experiments or means $\pm$ ranges of duplicates are given.

$[\text{M}^{2+}]$ (μM)	Mg^{2+}		Zn^{2+}		Mn^{2+}	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
0					0.39	0.63
10	0.19 $\pm$ 0.01	0.33 $\pm$ 0.01				
25			0.47 $\pm$ 0.06	0.27 $\pm$ 0.02	0.34	1.07
50			0.61 $\pm$ 0.03	0.98 $\pm$ 0.04		
100	0.17 $\pm$ 0.03	0.82 $\pm$ 0.21	0.62 $\pm$ 0.04	1.75 $\pm$ 0.06	0.33	1.31
1000	0.17	1.18				

Table 2. Effect of bivalent metal ion type on the specificity towards (*S_p*)- and (*R_p*)-diastereoisomers of cyclic APT. Reaction rates ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{l}^{-1}$, normalized to an enzyme concentration of $1 \text{ mg} \cdot \text{l}^{-1}$) are means $\pm$ S.D. of four or six estimates at $44 \mu\text{M}$ -substrate and $100 \mu\text{M}$ -free bivalent metal ion. Apparent maximum velocities are shown in parentheses, and were obtained by extrapolation of data at 44 and 17 and/or $110 \mu\text{M}$ -substrate, Michaelis-Menten kinetics being assumed.

Metal ion	Reaction rate or apparent maximum velocity with:			
	Cyclic AMP	(<i>S_p</i>)-Cyclic APT	(<i>R_p</i>)-Cyclic APT	$v_{(S_p)}/v_{(R_p)}$
Mg ²⁺	1.7 $\pm$ 0.3	0.14 $\pm$ 0.003 (0.19)	<0.004	> 35
Mn ²⁺	3.0 $\pm$ 0.3	1.8 $\pm$ 0.4 (2.0)	0.80 $\pm$ 0.05 (1.7)	2.3 (1.2)
Co ²⁺	2.4 $\pm$ 0.1	2.1 $\pm$ 0.5 (2.6)	3.0 $\pm$ 0.2 (3.6)	0.70 (0.72)
Zn ²⁺	1.7 $\pm$ 0.4	0.10 $\pm$ 0.01 (0.28)	0.17 $\pm$ 0.06 (0.76)	0.59 (0.37)
Cd ²⁺	5.4 $\pm$ 0.6	0.23 $\pm$ 0.02 (0.36)	3.1 $\pm$ 0.4 (4.8)	0.074 (0.075)

AMP concentrations with Mg²⁺ (Suoranta & Londesborough, 1984), so the absolute significance of these apparent maximum velocities is uncertain.

The diastereoisomers of cyclic APT were tested as inhibitors of the hydrolysis of $0.25 \mu\text{M}$ -cyclic AMP. In the presence of 1 mM-MgCl_2 , the (*S_p*)-form was a much stronger inhibitor [K_i values: (*S_p*), $6 \mu\text{M}$; (*R_p*), $> 80 \mu\text{M}$], whereas at 0.1 mM-ZnCl_2 there was little difference [K_i values: (*S_p*), $35 \mu\text{M}$; (*R_p*), $20 \mu\text{M}$].

Discussion

The rate of hydrolysis of $0.25 \mu\text{M}$ -cyclic AMP by yeast low- K_m cyclic AMP phosphodiesterase in the absence of added bivalent metal ions was increased more than 15-fold by 1 mM-Mg^{2+} , and Mn²⁺, Co²⁺, Zn²⁺ and Cd²⁺ could also satisfy this bivalent-metal-ion requirement, whereas Cu²⁺, Ca²⁺, Fe²⁺ and Ni²⁺ could not. At a physiological cyclic AMP concentration ($0.25 \mu\text{M}$) the rates at optimal concentrations of Mg²⁺, Mn²⁺ and Zn²⁺ were nearly equal, and those with Cd²⁺ and Co²⁺ only a little lower (Fig. 1). Zn²⁺ and Co²⁺ inhibited above their optimal concentrations, whereas Mn²⁺ had the lowest concentration ($10 \mu\text{M}$) for half-maximal activation. These factors may affect the activity *in vivo*.

The apparent K_m for cyclic AMP varied with the nature of the free bivalent metal ion, but was insensitive to changes in the concentrations of Mg²⁺, Mn²⁺ and Zn²⁺ that changed the apparent maximum velocity by severalfold (Table 1). This shows that the metal ion·cyclic AMP⁺ complexes (Londesborough & Lukkari, 1979) are not the only substrates (they may not be substrates at all), and suggests, by analogy with Haldane's (1930) argument (see also Cornish-Bowden, 1976), that the kinetically determined Michaelis constants are close to thermodynamic dissociation constants.

Jarvest *et al.* (1982) have shown that a Mg²⁺-dependent cyclic AMP phosphodiesterase from

heart can hydrolyse only the (*S_p*)-diastereoisomer of cyclic APT, whereas the free-bivalent-metal-ion-independent high- K_m enzyme from yeast, which contains two zinc atoms per subunit (Londesborough & Suoranta, 1983), is about equally active with the (*S_p*)- and (*R_p*)-isomers. Jarvest *et al.* (1982) proposed that the metal ion binds to the phosphorothioate moiety of cyclic APT, and that, because Zn can bond equally well to sulphur or oxygen, the high- K_m yeast enzyme can accept either diastereoisomer, whereas the heart enzyme can accept only one [in practice, the (*S_p*)-], because magnesium does not readily bond to sulphur. Our results (Table 2) show that, when the free bivalent metal ion is Mg²⁺, the low- K_m yeast enzyme, although it contains tightly bound zinc (but probably only one atom per monomer; Suoranta & Londesborough, 1984), exhibits a strict specificity for the same (*S_p*)-diastereoisomer as the heart enzyme. However, this specificity can be eliminated [$v_{(S_p)}/v_{(R_p)} \approx 1$ for Mn²⁺, Co²⁺ and Zn²⁺] or reversed [$v_{(S_p)}/v_{(R_p)} = 0.074$ for Cd²⁺] by changing the free bivalent metal ion. This shows that, for the yeast low- K_m enzyme, it is the 'free' bivalent metal ion, rather than the tightly bound zinc, that interacts with the phosphorothioate (or phosphate) moiety of the substrate, and supports the model of Jarvest *et al.* (1982) because the ratio $v_{(S_p)}/v_{(R_p)}$ decreases in the order Mg²⁺ > Mn²⁺ > Co²⁺ > Zn²⁺ > Cd²⁺ expected for decreasing strength of metal-oxygen bonding compared with metal-sulphur bonding. Jaffe & Cohn (1979) found the same order when they compared the preference of hexokinase for A- and B-diastereoisomers of adenosine [β -thio]triphosphate with different metal ions.

The study of the inhibition of cyclic AMP hydrolysis by the diastereoisomers of cyclic APT shows that the interaction between the metal ion and the phosphorothioate moiety contributes at least 6 kJ/mol (i.e., $\Delta G = RT \cdot \ln 13$) to the binding energy of cyclic APT, because although the (*S_p*)-

and (R_p)-diastereoisomers were about equal inhibitors in the presence of Zn^{2+} , (S_p)-cyclic APT was at least 13-fold more effective than (R_p)-cyclic APT in the presence of Mg^{2+} . Quite small changes in the orientation of the cyclic nucleotide in the active site will affect the interaction between this metal ion and the phosphate (or phosphorothioate) moiety to an extent depending on the size of the metal ion and whether its co-ordination geometry is rigid or fairly flexible (as for Zn^{2+}). This factor alone may account for the variation in specificity for cyclic GMP of a heart enzyme in the presence of different bivalent metals (Donnelly, 1977).

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