Evidence that the peripheral cyclic AMP phosphodiesterase of rat liver plasma membranes is a metalloenzyme

John LONDESBOROUGH

Research Laboratories of the Finnish State Alcohol Company, Alko Ltd., P.O. Box 350,
SF-00101 Helsinki 10, Finland

(Received 18 June 1984/Accepted 18 September 1984)

Cyclic nucleotide phosphodiesterase activity in salt extracts of rat liver plasma membranes was progressively inactivated by treatment with the metal chelators 8-hydroxyquinoline and *o*-phenanthroline, but not the non-chelating *m*-phenanthroline isomer. Activity at 20 μ M-cyclic AMP was lost more slowly than activity at 0.4 μ M-cyclic AMP. The activity of treated preparations was partially restored by incubation with Zn^{2+} or Mn^{2+} ions (in the presence of 1 mM- MgCl_2) but not with Ca^{2+} , Cd^{2+} , Co^{2+} , Cu^{2+} or Fe^{2+} ions, nor by MgCl_2 alone. The results suggest the presence in the membrane extracts of a cyclic AMP phosphodiesterase containing tightly bound metal, possibly Zn or Mn, that affects the enzyme's affinity for cyclic AMP.

Nearly all cyclic nucleotide phosphodiesterases (EC 3.1.4.17) have an absolute requirement for a free bivalent-metal ion such as Mg^{2+} or Mn^{2+} . One of the few exceptions is a high- K_m enzyme from yeast, which has been shown to contain essential Zn (Londesborough, 1978; Londesborough & Suoranta, 1983). This at first suggested that the free bivalent-metal ions required by the other enzymes carried out the functions fulfilled in the yeast enzyme by tightly bound Zn. However, a low- K_m Mg^{2+} -requiring cyclic AMP phosphodiesterase from yeast also contains Zn, removal of which decreases the enzyme's affinity for cyclic AMP (Suoranta & Londesborough, 1984). The question arose whether the presence of tightly bound Zn is a peculiarity of the yeast enzymes or a common feature of cyclic nucleotide phosphodiesterases. The peripheral cyclic AMP phosphodiesterase of rat liver plasma membranes studied by Houslay and colleagues (Marchmont & Houslay, 1980; Marchmont *et al.*, 1981) was chosen for an initial examination of this question because it has been well characterized by these workers and is thought to be the only cyclic nucleotide phosphodiesterase in salt extracts of these membranes. Furthermore, like the low- K_m yeast enzyme (Suoranta & Londesborough, 1984), it is monomeric and exhibits non-hyperbolic kinetics (the deviation is marked for the liver enzyme, with a Hill coefficient of 0.6; Marchmont *et al.*, 1981).

Materials and methods

Materials

Bivalent metal salts were 'pro analysis grade' from E. Merck A.G., Darmstadt, Germany. *o*-(1,10-)Phenanthroline, *m*-(1,7-)phenanthroline and other chemicals were from the sources previously described (Londesborough & Suoranta, 1983).

Preparation of plasma-membrane extracts

Plasma membranes were prepared from livers (each 11–16 g) of male Long-Evans rats by the method by Pilkis *et al.* (1974), except that the sucrose-gradient centrifugation was done in a Kontron TST 28.38 swing-out rotor (75 min at 28000 rev./min) with 7 ml of 42.5% (w/v) sucrose and 10 ml of 48.2% (w/v) sucrose layered over 19 ml of the sample. The membranes collected at the 48.2%/42.5%-sucrose interface were washed with 1 mM- NaHCO_3 and usually stored overnight at -70°C . Salt extracts of the membranes were made by the method of Marchmont *et al.* (1981). Thawed membranes were adjusted to final concentrations of 20 mM-Tris/HCl buffer, pH 7.4, 0.4 M-NaCl, 20 mM-2-mercaptoethanol and about 4 mg of membrane protein/ml, kept at 0°C for 1–3 h, and then centrifuged at 300000g for 30 min. The supernatants ('salt extracts') were usually stored at

–70°C. The yield of plasma-membrane protein (determined by the biuret reaction; Gornall *et al.*, 1949) was 0.6mg/g of fresh liver, and their cyclic AMP phosphodiesterase activity at 0.4 μ M-cyclic AMP was close to 40 μ units/mg of protein. This agrees well with the results obtained by Marchmont *et al.* (1981); however, only 40–60% of this activity could be solubilized by the high-salt treatment, which is lower than reported by those authors (85%). Like their material, my salt extracts showed departures from Michaelis–Menten kinetics, with apparent K_m values (limiting slopes of v -versus- $v/[S]$ plots between 50 μ M- and 0.4 μ M-cyclic AMP) of 5 μ M and 0.7 μ M (cf. 15 μ M and 0.9 μ M reported by Marchmont & Houslay, 1980).

Cyclic AMP phosphodiesterase assay

Cyclic AMP phosphodiesterase was measured at 30°C by a modification (Londesborough, 1976) of the snake-venom assay method of Thompson & Appleman (1971). Assay mixtures (100 μ l) contained 40 mM-Tris/HCl buffer, pH 7.4, 5 mM-MgCl₂, 20 mM-2-mercaptoethanol, 2 mg of bovine albumin/ml and, unless otherwise stated, 0.4 μ M-cyclic [³H]AMP (about 20000 c.p.m.). Under these conditions, 1 unit of phosphodiesterase hydrolyses 1 μ mol of cyclic AMP/min. The albumin had no effect on the activity of enzyme bound to the membranes, but caused an 80% increase in the activity of the salt extracts. Under the conditions used, less than 10% of adenosine binds to the Dowex resin. When [³H]AMP (0.1–10 μ M) was incubated with plasma membranes and then treated with the snake venom and Dowex resin, a constant, high (86%), recovery of radioactivity was obtained, showing the absence of enzymes (Rutten *et al.*, 1973) that metabolize AMP and adenosine to products poorly recovered from the Dowex resin. Assays at 0.4 μ M-cyclic AMP were unaffected by the presence of up to 40 μ M-zinc acetate.

Treatment with phenanthrolines and 8-hydroxyquinoline

Portions of salt extracts were mixed at 20°C (in the dark for phenanthrolines) with bovine albumin and 5 mM solutions of phenanthroline or 8-hydroxyquinoline in 50 mM-Pipes/KOH buffer, pH 7.0 [containing 5% (v/v) ethanol for the phenanthrolines] to give final concentrations of 1 mg of albumin/ml, 15 mM-Tris/HCl, 10 mM-Pipes/KOH, 300 mM-NaCl, 15 mM-2-mercaptoethanol, 0–1% ethanol and usually 1 mM reagent, pH 7.2. Controls lacked only phenanthroline and 8-hydroxyquinoline. Portions (5 μ l at 0.4 μ M-cyclic AMP and 10 μ l at 20 μ M-cyclic AMP) were added to phosphodiesterase assay mixtures at appropriate times.

Re-activation of *o*-phenanthroline- and 8-hydroxyquinoline-treated enzyme

Treated enzyme was dialysed at 5°C for about 18 h against at least 300 vol. of 20 mM-Tris/HCl buffer, pH 7.4, containing 100 mM-NaCl, 1 mM-MgCl₂, 7 mM-2-mercaptoethanol, 50 μ M-EDTA and any indicated bivalent-metal salt. The recovered enzyme solution was weighed and assayed, and corrections were applied for volume changes. Enzyme dialysed in the absence of bivalent-metal salts (except MgCl₂) was tested for re-activation by incubating 100 μ l portions with 10 μ l of the appropriate metal salt solution at 0°C.

Results

Cyclic AMP phosphodiesterase activity in salt extracts of liver plasma membranes was progressively inactivated by the metal-chelating *o*-phenanthroline, but not by the non-chelating *meta* isomer (Fig. 1*a*). At 1 mM-*o*-phenanthroline and 20°C the decrease in activity measured at 0.4 μ M-cyclic AMP stopped after 2 h, and a residual 23% of the activity persisted up to at least 6 h, whereas activity measured at 20 μ M-cyclic AMP decreased more slowly, although it also fell to 23% after 6 h (Fig. 1*b*). The metal chelator 8-hydroxyquinoline also caused a progressive inactivation (Fig. 1*c*). 8-Hydroxyquinoline did not cause instantaneous inhibition of the phosphodiesterase activity at the concentration (47 μ M) introduced to the assay mixtures with the enzyme sample. However, the data at 0.4 μ M-cyclic AMP in Fig. 1 (and Figs. 2 and 3) have been corrected for instantaneous inhibitions of 9% and 35% respectively, caused by the 47 μ M-*o*- and -*m*-phenanthroline carried over to the assays with the enzyme samples. *p*-Phenanthroline was a slightly stronger instantaneous inhibitor than *m*-phenanthroline (result not shown). Instantaneous inhibition by this concentration of *o*-phenanthroline was negligible at 20 μ M-cyclic AMP.

The activity lost during treatment with *o*-phenanthroline or 8-hydroxyquinoline was not recovered during dialysis to remove the reagent, but could be partially restored by certain bivalent-metal ions. In some experiments the enzyme was dialysed for about 18 h in the presence and in the absence of these metal ions (Figs. 1*a* and 1*c*), and in others the metal ions were added only after the dialysis (Figs. 1*b* and 2; 1 mM-MgCl₂ was always present in the dialysis buffer and seemed to improve the stability of the enzyme, especially after treatment with *o*-phenanthroline, but did not by itself cause any re-activation). At 0°C and 45 μ M-EDTA, more than 90% of the final re-activations caused by 450 μ M- and 90 μ M-zinc acetate

Fig. 1. *Progressive inactivation of cyclic AMP phosphodiesterase by o-phenanthroline and 8-hydroxyquinoline* Plasma-membrane salt extracts were incubated at 20°C in the absence (□) or in the presence of 1 mM-*m*-phenanthroline (△), *o*-phenanthroline (● and ○) or 8-hydroxyquinoline (▲), and then dialysed (broken lines) and treated with bivalent-metal ions as described in the Materials and methods section. (a) Phosphodiesterase activity at 0.4 μM-cyclic AMP is shown as μunits/ml of incubation mixture, and the enzyme was dialysed in the presence or in the absence of 90 μM-zinc acetate. (b) The activities at 0.4 μM- (●) and 20 μM- (○) cyclic AMP are shown as percentages of the values before addition of *o*-phenanthroline. After 2 h treatment, the enzyme was dialysed in the absence of bivalent-metal ions (except Mg²⁺), and the dialysed enzyme was assayed before and 4 h after addition of 90 μM of the indicated metal ions. (c) Enzyme treated with 8-hydroxyquinoline was dialysed in the presence and in the absence of 90 μM-zinc acetate.

were obtained within 1 min and 30 min respectively, whereas 18 μM-zinc acetate (which is nearly all complexed by 45 μM-EDTA even at 1 mM-MgCl₂) caused only a small slow re-activation (Fig. 2). MnCl₂ was nearly as effective as zinc acetate (Fig. 1b). Ca²⁺, Co²⁺, Fe²⁺ (Fig. 1b), Cd²⁺ (Fig. 2) and

Fig. 2. *Time-dependence of re-activation of o-phenanthroline-inactivated cyclic AMP phosphodiesterase by Zn²⁺* Plasma-membrane salt extracts were treated with 1 mM-*o*-phenanthroline (○) and dialysed (broken line) without bivalent-metal ions. Dialysed enzyme was then incubated at 0°C without further treatment (△) or after addition of 450 μM-zinc acetate (▲), 90 μM-zinc acetate (●), 18 μM-zinc acetate (■), 90 μM-NiSO₄ (□) or 90 μM-CdSO₄ (▽). Experimental details are described in the Materials and methods section.

Cu²⁺ (results not shown) ions did not restore activity measured at 0.4 μM-cyclic AMP. Ni²⁺ ions sometimes caused a small re-activation (up to 1.3-fold), but usually had little or no effect (Figs. 1b and 2). However, after short treatments with *o*-phenanthroline the ratio of activities at 0.4 μM- and 20 μM-cyclic AMP ($v_{20}/v_{0.4}$) increased from 4 ± 0.5 (the value with untreated enzyme) to about 14, and Ni²⁺ ions could decrease this ratio. In the experiment of Fig. 1(b), $v_{20}/v_{0.4}$ increased from 4.2 before treatment with *o*-phenanthroline to 13.6 after 2 h treatment, and was 6.7 for the dialysed enzyme; after addition of Zn²⁺, Ni²⁺ or Ca²⁺ ions to the dialysed enzyme, $v_{20}/v_{0.4}$ values of 5.3, 4.5 and 6.9 respectively were observed, although only the Zn²⁺ ions caused an increase in the activity at 0.4 μM-cyclic AMP.

The results in Figs. 1 and 2 clearly show that phosphodiesterase activity in the salt extracts is inactivated by metal chelators and specifically re-activated by certain bivalent-metal ions in a time-dependent reaction, implying that a phosphodiesterase using tightly bound metal is present. According to Houslay and colleagues (Marchmont & Houslay, 1980; Marchmont *et al.*, 1981), the phosphodiesterase activity at different cyclic AMP concentrations of salt extracts of plasma membranes decays at 45°C as a single exponent with a half-life of 6.1 min, and is therefore due to a single enzyme. If my salt extracts also contain only one phosphodiesterase, then the more rapid loss of activity at 0.4 μM- than at 20 μM-cyclic AMP shown in Fig. 1(b) would imply that loss of tightly bound metal decreases the enzyme's affinity for cyclic AMP. All the experiments with metal chelators

were done with salt extracts that had been frozen at least once, and Fig. 3 shows that, indeed, the activities at 20 μM -, 0.4 μM - and 0.1 μM -cyclic AMP of frozen material decayed as a single exponent with a half-life of 2.5 min. Furthermore, heat treatment did not alter the rate of inactivation by *o*-phenanthroline: the initial activity and that left (36%) after 4 min at 45°C both decreased by $49 \pm 2.5\%$ in 2 h and by $59 \pm 2\%$ in 6 h (Fig. 3, inset). These results suggest that only one phosphodiesterase was present, and hence that *o*-phenanthroline treatment must decrease the affinity.

It is not clear why these frozen extracts have a shorter half-life at 45°C than reported by Marchmont & Houslay (1980). My fresh extracts exhibited biphasic decay curves, about 65% of the activity decaying with a half-life of about 0.9 min and the remainder with a half-life of 5.2 min (Fig. 3), which

Fig. 3. Thermal inactivation of cyclic AMP phosphodiesterase activity in salt extracts

Salt extracts were heated for 1–20 min at 45°C and then transferred to ice/water and assayed. The activities at 0.4 μM -cyclic AMP of fresh (●), once-frozen (■) and twice-frozen (▼) samples of the same extract are expressed as percentages of the activity of the unheated fresh enzyme. For the twice-frozen sample, activities at 20 μM - (△) and 0.1 μM - (▽) cyclic AMP are also shown at 0 and 4.25 min. The inset shows the effect of treating the unheated (●) and 4 min-heated (○) once-frozen sample with 1 mM *o*-phenanthroline. Also shown, in the main Figure, is the thermal-decay curve at 45°C of a once-frozen salt extract of membranes prepared in the presence of 50 μM -EDTA and 50 μM -zinc acetate (○): the activity at 0.4 μM -cyclic AMP is here expressed as a percentage of the unheated value, which was 86% of the value before freezing. For the biphasic plots (○ and ●), the late linear phases are extrapolated backwards (broken lines) to estimate the extent of the initial rapid reaction.

is reasonably close to the value of 6.1 min reported by Marchmont & Houslay (1980). Apparently, storage at -70°C destroyed the labile fraction and decreased the half-life of the stable fraction. The yields of plasma-membrane protein and of phosphodiesterase activity at 0.4 μM -cyclic AMP in plasma membranes and their salt extracts were doubled by including 50 μM -zinc acetate/50 μM -EDTA in all buffers. Such salt extracts lost less of their phosphodiesterase activity (14%) during freezing and thawing than did normal extracts (50%), and after thawing still exhibited a component (60% of the total activity) with a half-life at 45°C of 5.2 min (Fig. 3).

Discussion

The time-dependent inactivation by metal chelators of the phosphodiesterase activity in liver plasma-membrane salt extracts, the persistence of this inactivation through dialysis and the time-dependent re-activation of dialysed samples by Zn^{2+} ions strongly suggest that the peripheral cyclic AMP phosphodiesterase present in such extracts (Marchmont & Houslay, 1980) requires tightly bound metal ions. Identification of the postulated metals would be an arduous task, because 150 rats provide only about 30 μg of pure enzyme (Marchmont *et al.*, 1981), equivalent to less than 40 ng of metal (assuming 1 atom of metal/52000 Da molecule of enzyme), which is barely adequate for a properly controlled set of Zn analyses, and inadequate for metals with weaker atomic absorption spectra. The present results, however, already indicate that at least one mammalian cyclic AMP phosphodiesterase is probably a metalloenzyme, as are two yeast enzymes (Londesborough & Suoranta, 1983; Suoranta & Londesborough, 1984).

Like the low- K_m yeast enzyme (Suoranta & Londesborough, 1984), phosphodiesterase activity in the salt extracts was progressively inactivated by both 8-hydroxyquinoline and *o*-phenanthroline. The high- K_m yeast enzyme is progressively inactivated by 8-hydroxyquinoline, but not by *o*-phenanthroline, although this latter compound is an instantaneous inhibitor (Londesborough & Suoranta, 1983). The non-chelating *m*-phenanthroline and *p*-phenanthroline are still more powerful instantaneous inhibitors for both the high- K_m yeast enzyme and the present liver enzyme. This is also the case for cyclic nucleotide phosphodiesterase fractions from human lung (Bergstrand & Lundquist, 1978; Bergstrand *et al.*, 1978). The lung enzymes have not been tested for progressive inactivation by metal chelators, but the mutually antagonistic effects of Zn^{2+} and *o*-phenanthroline suggested to Bergstrand & Lundquist (1978) that at

least one of the lung enzymes (fraction I_{BT}) might require Zn²⁺. Another hint that some mammalian phosphodiesterases may contain tightly bound metal is the widely observed modulation of their activity by imidazole, including activation at high cyclic AMP concentration and inhibition at low cyclic AMP concentration of crude heart and brain enzymes (see Chasin & Harris, 1976). Imidazole can replace Zn-bound H₂O in, e.g., liver alcohol dehydrogenase (Evans & Shore, 1980), and the effect of such a replacement on enzyme activity would depend on details of the reaction mechanism.

The heat-inactivation studies (Fig. 3) suggest that at least the frozen salt extracts used for metal-chelator studies contained only one cyclic AMP phosphodiesterase. If so, then the more rapid loss of activity at 0.4 μ M- than at 20 μ M-cyclic AMP caused by *o*-phenanthroline (Fig. 1*b*) means that the affinity of the enzyme is decreased by treatment with *o*-phenanthroline, as has been shown for the low-*K_m* phosphodiesterase of yeast (Suoranta & Londesborough, 1984). However, the discrepancy between my value for the half-life at 45°C (2.5 min) and the Marchmont & Houslay (1980) value (6.1 min) and the different behaviour of fresh and frozen extracts (Fig. 3) remain unexplained. My assay mixtures contained 2 mg of albumin/ml (see the Materials and methods section), which nearly doubled the activity of salt extracts, possibly by stabilizing a heat-labile component. On the other hand, the phosphodiesterase of salt extracts of plasma membranes prepared in a zinc buffer was more stable to freezing and maintained the component with a 5.2 min half-life at 45°C through storage at -70°C (Fig. 3). Thus the availability of zinc during isolation of the phosphodiesterase, and even the bivalent-metal-ion status of the rats, may affect the

enzyme's behaviour. The latter possibility can be tested by dietary studies. If, indeed, cyclic AMP phosphodiesterase activities depend on bivalent-metal ion availability, this might be one mechanism for the multiple effects, including behavioural changes, of zinc deprivation in mammals (see, e.g., Russell *et al.*, 1983).

I thank Sirkka Kanervo for her skilful technical assistance.

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