The Zinc-containing High $K_m$ Cyclic Nucleotide Phosphodiesterase of Bakers’ Yeast*

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The high $K_m$ cyclic nucleotide phosphodiesterase of Saccharomyces cerevisiae was purified by an improved procedure. Its amino acid composition is reported. Its pl is 5.85 ± 0.1. Sedimentation equilibrium analysis of the native enzyme gave $M_w = 88,000 ± 6,000$, whereas gel electrophoresis in the presence of dodecyl sulfate gave a molecular weight of 43,000, indicating that the enzyme is a dimer. Preparations of 94 ± 4% purity contained about 2.4 atoms of zinc/43,000 daltons. Inactivation of the enzyme by 8-hydroxyquinoline was accompanied by loss of this zinc, and the enzyme is a dimer. Preparations of 94 ± 4% purity contained about 2.4 atoms of zinc/43,000 daltons. Inactivation of HIS-phosphodiesterase by 8-mercaptopropionic acid, and 4,7-phenanthrolines and by thiols and KCN, but not by NaN₃. These inhibitors probably act by binding to, but not chelating, enzyme-bound zinc.

Highly purified but still inhomogenous preparations of the soluble high $K_m$ cyclic nucleotide phosphodiesterase (HK-phosphodiesterase) from bakers’ yeast contain tightly bound zinc (1) and are active in the absence of added divalent metal ions (2, 3). We report here that homogenous preparations contain about 2 atoms of tightly bound zinc per monomer (43,000 daltons). Inactivation of HK-phosphodiesterase by 8-hydroxyquinoline is accompanied by loss of this zinc, and the enzyme can be reactivated by dialysis against zinc acetate. Gel electrophoretic and ultracentrifugal analyses indicate that HK-phosphodiesterase is a dimer of molecular weight 86,000, which is at variance with an earlier report (2) that it is a monomer of molecular weight about 65,000.

EXPERIMENTAL PROCEDURES*

Metal (1,7) and para (4,7)-phenanthroline were from ICN Pharmaceuticals, Plainview, NY, U.S.A. Ethanol 1,10-phenanthroline was from Tiska Ao, Buchs, Switzerland, and 8-hydroxyquinoline from E. Merck AG, Darmstadt, Germany. 8-Hydroxyquinoline was dissolved in 1 M HCl and 30% EtOH/1 M HCl or 2% mercaptoethanol/0.1 M monochloroacetic acid to give a final concentration of 10 mM and was stored at −20°C.

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‡The abbreviations used are: HK-phosphodiesterase, the high $K_m$ cyclic nucleotide phosphodiesterase (EC 3.1.4.17) from bakers’ yeast; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid.

§"Experimental Procedures" and Table I are presented in mini-print as prepared by the authors. Mini-print is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 82M-2270, cite the authors, and include a check of money order for $2.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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was chosen for illustration because it is the one used for the purification summarized in Table 2.8-ml fractions were analyzed for zinc (0).

The purification of HK-phosphodiesterase was assayed at 30°C by four methods. (a) The standard assay for HK-phosphodiesterase, using spectrophotometric assay with adenosine 5'-triphosphate and 4-methylumbelliferone as substrate, was used for the detection of enzyme activity. (b) A modification (5) of the snake venom assay (6) was used to check the purity of the enzyme fraction that might interfere with coupling enzymes. (c) Enzyme activity in the presence of 100 mM DTT was determined by a reaction mixture containing 50 mM Pipes/KOH pH 7.0, 1 mM EDTA, 0.5 mM bovine serum albumin, 75 mM KCl and 0.5% of dodecyl mercaptoethanol.

Metal determinations—Elk (13.9 mg), cadmium (12.6 mg) and iron (246.3 mg) were determined in native enzyme samples at the wavelengths indicated with a Varian model 70 atomic absorption spectrophotometer equipped with an AAS 510 system. Standards were prepared in the identical buffer solutions used in preparation of the enzyme. For zinc determinations, 10 ml samples of enzyme solutions were used. The samples were evaporated to dryness on the surface, dried for 24 h at 110°C, charred for 25 min at 500°C, and atomized in a furnace, dried for 25 s at 110°C, charred for 25 min at 500°C, and atomised in a furnace, dried for 25 s at 110°C.

Zinc-containing Cyclic Nucleotide Phosphodiesterase

RESULTS

Purification and Specific Activity of HK-phosphodiesterase—The purification summarized in Table I is typical and was chosen for illustration because it is the one used for the zinc analyses shown in Fig. 1. About 15% of the total enzyme activity in the 34,000 mg supernatant fails to bind to hydroxyapatite under the conditions used in step 2 with buffer volumes decreased in step 3 of the purification procedure and, therefore, greater enzyme concentrations in the eluate (see below). After the final hydroxyapatite step, the enzyme solutions containing 10 μM EDTA could be stored for 2 months at 0°C with no loss of activity. Higher concentrations of EDTA (e.g., 0.1 mM) caused a slow loss of activity (about 10% per month) from purified enzyme. However, EDTA and mercaptoethanol seemed to be necessary in the early stages of the purification, especially fractions with (NH₄)₂SO₄, presumably to protect the enzyme from heavy metal contaminants, to which it is sensitive (2).

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of enzyme eluted from the final hydroxyapatite column showed a band accounting for 94 ± 4% of the material stainable with Coomassie blue (Fig. 2). Isoelectric focusing between pH 3 and 8 on polyacrylamide gels showed a single activity peak between pH 5.75 and 5.95 (3 separate experiments), which accounted for 65–70% of the applied activity and coincided with the only visible band after staining with bromphenol blue (pH 7.7).

Not enough pure enzyme was available to determine its dry weight. Protein concentrations were estimated instead by the methods of Murphy and Kies (11) and Scopes (12), which agreed within 5% and indicated specific activities of 170 ± 25 units/mg for the (dialyzed) peak enzyme fraction in Fig. 1. The range of uncertainty here reflects loss of enzyme activity during storage and dialysis before the protein determinations.

The lower specific activity recorded in Table I is mainly due to underestimation of the blank absorption when calculating the net A₂₈₀. Thus, enzyme prepared by using the protamine and octyl-Sepharose modifications (see under "Experimental Procedures") was already over 90% pure after the DEAE-Sephadex column, and the final hydroxyapatite column could then be run as a concentration step, with a very steep phosphate gradient, so that enzyme eluted at up to 48 units/ml. Under these circumstances, reliable A₂₈₀ values could be measured, and the protein concentrations (of dialyzed enzyme) based on an assumed Z₇₅₇₅₆ng/ml of 1.0 agreed within 5% with those found by the methods of Murphy and Kies (11) and Scopes (12), and lead to estimates for the specific activity close to 170 units/mg of protein. This value has been used in subsequent calculations.

Molecular Weight and Subunit Composition—The Ferguson (16) plot for purified enzyme in the presence of sodium dodecyl sulfate was linear (Fig. 3), and molecular weight values of 43,000 and about 48,000, respectively, were obtained (Fig. 4) by the method of Banker and Cotman (17) from its slope (retardation coefficient, Kₐ) and intercept (log free mobility, M₀). The possibility that less than 1% is lost in the two phosphate washes. The modification using protamine saves hydroxyapatite and facilitates handling material from more than 2 kg of yeast. The modification using octyl-sepharose permits steeper salt gradients on the final hydroxyapatite column and, therefore, greater enzyme concentrations in the eluate (see below). After the final hydroxyapatite step, the enzyme solutions containing 10 μM EDTA could be stored for 2 months at 0°C with no loss of activity. Higher concentrations of EDTA (e.g., 0.1 mM) caused a slow loss of activity (about 10% per month) from purified enzyme. However, EDTA and mercaptoethanol seemed to be necessary in the early stages of the purification, especially fractions with (NH₄)₂SO₄, presumably to protect the enzyme from heavy metal contaminants, to which it is sensitive (2).
Zinc-containing Cyclic Nucleotide Phosphodiesterase

Two frozen and thawed samples of purified HK-phosphodiesterase were analyzed in the ultracentrifuge after extensive dialysis against 50 mM Na phosphate, pH 8.0, containing 50 mM KCl, 0.5 mM Mg acetate, and 10 µM EDTA. Sedimentation equilibrium runs at 5 °C on the first sample (initial concentration, about 0.25 mg/ml) by the meniscus depletion method gave linear plots of log fringe displacement against radius squared (not shown) with Mr of 82,000 (after 14 h at 22,000 rpm) and 89,000 (after 27 h at 18,000 rpm). A similar run on the second sample (0.3 mg/ml) gave Mr of 94,000. After a further two months storage at 5 °C a value for Mr of 57,000 was obtained at 20 °C, and in a still later analysis at 20 °C high molecular weight aggregates appeared but material near the meniscus had Mr of 42,000. A sedimentation coefficient of 3.80 × 10⁻¹³ s⁻¹ was obtained at 4 °C with freshly thawed material, equivalent to s₂₀,₅₀ = 5.95 × 10⁻¹³ s⁻¹. Combined with the gel electrophoresis results, these data suggest that HK-phosphodiesterase is a roughly spherical dimer with a molecular weight of 86,000 and that at any rate aged samples have a tendency to dissociate at 20 °C.

Amino Acid Composition—Table II shows the amino acid composition of a sample of enzyme of specific activity 160 units/mg of protein assuming E₅₆₅₀₋₅₀ = 1.0. The amount of methionine found (not more than 0.11 nmol in the 4.3 µg of hydrolysate applied to the column) was close to the sensitivity limit.

Metal Content—Analysis of fresh eluate from the second hydroxyapatite column showed a main peak of zinc coincident with the enzyme activity peak (Fig. 1). The zinc contents of the buffers used to develop this column were 6 ng/ml at 50 mM phosphate and 23 ng/ml at 200 mM phosphate. After correction for this amount of zinc contamination, the excess zinc in fractions 27 through 31 varied between 19 and 24 ng/unit of enzyme, and the total excess zinc in these fractions divided by their total enzyme activity was 21.5 ng/unit. The hydroxyapatite column appears to bind some zinc from the buffer because the elution pattern after the enzyme peak contained less zinc (about 10 ng/ml at 220 mM phosphate) than did the input buffer. The error in the calculated zinc content of the enzyme due to possible overestimation of contaminating zinc is less than 10%.

In earlier work (1), the excess zinc contents of partially purified preparations of HK-phosphodiesterase with specific activities between 20 and 61 units/mg were also found to be between 20 and 24 ng/unit, whilst 16-h dialysis against 10 mM EDTA changed the excess zinc content to 29 ng/unit of remaining activity or 16 ng/unit of original activity (45% of the activity was lost during the dialysis). Although the enzyme has a pI of 5.85 (see above), the zinc is probably not nonspecifically bound, because this long dialysis against 10 mM EDTA caused a smaller loss of zinc than of activity.

Table II

| Amino acid composition of HK-phosphodiesterase |
|-----------------------------------------------|
| Results from two 24-h and one 72-h hydrolysates are shown as residues/43,000 g to the nearest integer. |

| Amino Acid | Residue/43,000 g |
|------------|-----------------|
| Asx        | 39              |
| Thr        | 20              |
| Ser        | 28              |
| Glx        | 47              |
| Pro        | 21              |
| Gly        | 23              |
| Ala        | 13              |
| Val        | 21              |
| Ile        | 26              |

| Amino Acid | Residue/43,000 g |
|------------|-----------------|
| Leu        | 44              |
| Tyr        | 13              |
| Phe        | 15              |
| Lys        | 25              |
| His        | 13              |
| Arg        | 16              |
| Met        | 1 (?)⁺         |
| Cys        | ND⁺            |

⁺ See text.
⁺ ND Not determined.

Fig. 2. Polyacrylamide gel electrophoresis of purified enzyme in sodium dodecyl sulfate. About 2 µg of protein from the peak enzyme fraction shown in Fig. 1 was loaded onto a 7.7% T polyacrylamide gel in 0.1% sodium dodecyl sulfate. After electrophoresis and staining, the gel was scanned at 570 nm. The pyronin G standard proteins (0, 68,000), ovalbumin (43,000), lactate dehydrogenase (36,000), and myoglobin (17,200) is shown.

Fig. 3. Ferguson (16) plots for standard proteins and HK-phosphodiesterase. Logarithms of the mobilities relative to that of pyronin G (Rg) are plotted against the sum (T) of the acrylamide and bisacrylamide concentrations for standard proteins (C, from top to bottom: myoglobin, lactate dehydrogenase, ovalbumin, and bovine serum albumin) and HK-phosphodiesterase (○).

Fig. 4. Apparent molecular weight of HK-phosphodiesterase in 0.1% sodium dodecyl sulfate. The retardation coefficients (Kₑ) and free relative mobilities (Mₑ) found from Fig. 3 are plotted against the molecular weight. Arrows show the values for HK-phosphodiesterase.
Analyses were also made for cadmium (because the sensitivity and analytic wavelengths for atomic absorption spectroscopy of cadmium and zinc are similar) and iron (because, like zinc, it is a nearly ubiquitous contaminant, which might become nonspecifically bound). No excess cadmium was present (Fig. 1). A sample of enzyme (11 units/ml and 90 units/mg) dialyzed overnight against 10 mM K phosphate/90 mM NaCl/50 μM EDTA, pH 7.8, contained 19.2 ng of zinc/unit and 2.9 ng of iron/unit. This iron may have been picked up from the buffer, which contained about 18 ng of iron/ml, but less than 2 ng of zinc/ml.

These results show that HK-phosphodiesterase contains tightly bound zinc that cannot be accounted for by nonspecific binding. For a specific activity of the pure enzyme of about 170 units/mg of protein, the zinc content of pure fresh enzyme (21.5 ng/unit) corresponds to about 2.4 atoms of zinc/43,000-dalton subunit.

**Inactivation by 8-Hydroxyquinoline**—HK-phosphodiesterase was inactivated by 8-hydroxyquinoline in a temperature- and concentration-dependent reaction that was first order with respect to time, although the pseudo-first order rate constant decreased when the initial concentration of HK-phosphodiesterase was increased (Fig. 5a). At an initial enzyme concentration of 280 milliunit/ml, the pseudo-first order rate constant was proportional to the 8-hydroxyquinoline concentration, with an apparent second order rate constant of 6.0 M⁻¹ min⁻¹ at 30 °C (Fig. 5b). Addition of 1 mg of bovine albumin/ml caused almost complete protection (not shown). The amounts of 8-hydroxyquinoline transferred to the assay in these experiments (not more than 50 μM) did not disturb the assay system and caused less than 5% competitive inhibition of HK-phosphodiesterase (see below).

Inactivation was accompanied by loss of zinc from the enzyme. In one experiment, a sample containing 30.5 ng of excess zinc and 1.30 units of HK-phosphodiesterase/ml (i.e., 23.5 ng of excess zinc/unit) was incubated with 2.5 mM 8-hydroxyquinoline for 7 h and then dialyzed for 15 h against 10 mM K phosphate, pH 8.0, containing 50 mM NaCl and 10 mM EDTA (this buffer contained 6 ng of zinc/ml). The sample lost 88% of its enzyme activity and 81% of its excess zinc. Spectrophotometric measurements at 225 and 215 nm showed that the HK-phosphodiesterase protein (about 10 μg/ml) was recovered through the dialysis, so that loss of zinc was not due to precipitation or adsorption of inactivated enzyme. A control sample, treated identically except that 8-hydroxyquinoline was omitted, lost 20% of its enzyme activity and 36% of its excess zinc, so that the final excess zinc content was 19 ng/unit.

HK-phosphodiesterase that had been inactivated by 8-hydroxyquinoline could be partially reactivated by dialysis against small concentrations of zinc acetate. Mercaptoethanol increased the rate and extent of reactivation and also stabilized untreated enzyme during the dialysis (Fig. 6, a and c). Co²⁺ ions consistently caused a definite but smaller reactivation than Zn²⁺ (e.g., Fig. 6c), but Mn²⁺, Cu²⁺, and Cd²⁺ caused no significant recovery of activity (Fig. 6, b and c). Results with Ni²⁺ were variable, but the reactivation was always less.

![Fig. 5. Inactivation by 8-hydroxyquinoline in 0.1 M K phosphate, pH 7.5. HK-phosphodiesterase was incubated with and without 8-hydroxyquinoline, and its activity (milliunit/ml incubation mixture) was measured by transferring 20-μl samples to 1.0-ml standard assay mixtures. a, HK-phosphodiesterase at 550, 230, or 120 milliunits/ml (see log activity intercepts) incubated with 2.5 mM 8-hydroxyquinoline at 30 °C (O) or 0 °C ([]). b, HK-phosphodiesterase at 280 milliunits/ml incubated with zero (Δ), 0.63 mM (□), 1.25 mM (△), or 2.5 mM (O) 8-hydroxyquinoline at 30 °C. Inset, pseudo-first order rate constants from b plotted against the 8-hydroxyquinoline concentration (80Q).](http://www.jbc.org/)

![Fig. 6. Reactivation by zinc of HK-phosphodiesterase inactivated by 8-hydroxyquinoline. HK-phosphodiesterase (about 0.7 units/ml) was incubated at 30 °C in 0.1 M K phosphate/10 mM EDTA, pH 8.0, in the presence (O) or absence (□) of 2.5 mM 8-hydroxyquinoline. After the indicated times (about 1.5 h in a, b, and c), the incubations were stopped by transfer to ice and dilution with an equal volume of the same buffer containing 2 mg of bovine albumin/ml, and 500-μl samples were dialyzed at 5 °C against 300-m1 lots of 0.1 M K phosphate, pH 8.0, containing: a, 1 mM MgCl₂ plus 20 μM EDTA (□), 20 μM Zn acetate (△), 20 μM Zn acetate/20 μM EDTA (△), or 20 μM Zn acetate/20 μM EDTA/2.8 mM mercaptoethanol (□); b, 20 μM EDTA/2.8 mM mercaptoethanol plus no addition (□), 20 μM MnCl₂ (△), 20 μM NiSO₄ (□), 20 μM Zn acetate, or 30 μM Zn acetate (□); c, 20 μM EDTA/2.8 mM mercaptoethanol plus no addition (□), 30 μM CuCl₂ (△), 30 μM Cd acetate (□), 30 μM Co(NO₃)₂ (□), 30 μM MnCl₂/10 mM EDTA (□), 30 μM Zn acetate with the mercaptoethanol omitted (□), 30 μM Zn acetate (△), or 80 μM Zn acetate (△) and (□); and d, 20 μM EDTA/2.8 mM mercaptoethanol/60 μM Zn acetate. In d, the control incubation (□) was stopped after 128 min and the incubations with 8-hydroxyquinoline (□) after 25 (△), 60 (△), 95 (□), and 144 (□) min. After about 24-h dialysis against three (□) or two (b, □, and d) lots of buffer, the samples were stored in glass tubes at 5 °C. Correcations for changes of volume during dialysis were made using the change in albumin concentration determined from A₂₈₀ and A₂₅₄ measurements. HK-phosphodiesterase activity was measured with the adenylate deaminase assay (see under “Experimental Procedures”).](http://www.jbc.org/)
than with Zn$^{2+}$ and usually less than with Co$^{2+}$ (e.g. Fig. 6b). The proportion of initial activity that could be recovered decreased with progressive inactivation by 8-hydroxyquinoline, so that after 18% inactivation about 93% of the original activity was recoverable, but after 75% inactivation only 50% was recoverable (Fig. 6d). The loss of recoverable activity during 8-hydroxyquinoline treatment was also close to first order, with a rate constant about half (51% at 30 °C and 55% at 35 °C) that for the original loss of activity under the corresponding conditions (Fig. 7).

**Instantaneous Inhibition by Metal Chelators and Similar Compounds**—Although it did not cause a time-dependent inactivation of the enzyme (1), 1,10-phenanthroline behaved as a simple competitive inhibitor when added to reaction mixtures ($K_i = 0.6$ mM; Fig. 8). The nonchelating isomers, 1,7- and 1,4-phenanthroline, were also inhibitors (Table III). Attempts to prevent the inhibition by 1,10-phenanthroline by addition of Zn, Ni, or Co salts are complicated by the strong inhibition exerted by the metals themselves (2). Maximum protection by NiSO$_4$ was reached when the concentration of NiSO$_4$ was about a third of that of the 1,10-phenanthroline (Table III).

In ordinary reaction mixtures (snake venom assay), 8-hydroxyquinoline caused mixed inhibition ($K_i = 1.1$ mM; but the increases in 1/ V were nonlinear and poorly reproducible), probably because irreversible inactivation of the enzyme was significant during the time required to measure the reaction rate. The effect on $V$ was almost completely suppressed by addition of 1 mg of albumin/ml, leaving a predominantly competitive inhibition ($K_i = 1.1$ mM, Fig. 8).

| Inhibitor                  | % Inhibition |
|----------------------------|--------------|
| 1,10-Phenanthroline: 2.5 or 5.0 mM | 58 or 77    |
| 1,7-Phenanthroline: 2.5 or 5.0 mM | 75 or 80    |
| 1,4-Phenanthroline: 2.5 or 5.0 mM | 63 or 75    |
| 3 mM 1,0-phenanthroline    | 90           |
| +0.75 mM NiSO$_4$          | 56           |
| +1.5 mM NiSO$_4$           | 37           |
| +3.0 mM NiSO$_4$           | 80           |
| 1.5 mM 1,10-phenanthroline | 42           |
| +0.25 mM NiSO$_4$          | 37           |
| +0.5 mM NiSO$_4$           | 19           |
| +1.0 mM NiSO$_4$           | 61           |
| +1.5 mM NiSO$_4$           | 73           |
| NiSO$_4$: 0.5 or 3.0 mM    | 85 or 96     |
| KCN: 12, 24, or 48 mM      | 14, 36, or 80|
| NaN$_3$: 24 or 48 mM       | 5 ± 5 or 5 ± 5|

**Table III**

**Instantaneous inhibition by metal chelators and similar compounds**

The concentration of cAMP was 0.5 mM. The buffer was 225 mM Tris/HCl, pH 7.8, for experiments with the phenanthrolines and 100 mM Tris/HCl, pH 7.8, containing 0–50 mM KCl (to maintain a constant ionic strength of about 0.1) for experiments with KCN and NaN$_3$. Rates of reaction were measured with the snake venom assay in the first three experiments and by isolating remaining $^3$H-CAMP by thin layer chromatography in the others.
seemed to increase during the course of the reaction; results in Table III are from average rates during the first 5 min after addition of enzyme. By contrast, NaN₃ did not inhibit significantly at concentrations up to 48 mM.

Mercaptoethanol ($K_F = 0.43$ mm; $K_{RS} = 1.6$ mm) and mercaptoethylamine ($K_F = 0.71$ mm; $K_{RS} = 4.1$ mm) were both mixed inhibitors (Fig. 9).

**DISCUSSION**

The amount of HK-phosphodiesterase in bakers’ yeast is small and a roughly 50,000-fold purification is necessary, so that preparations must start from relatively large amounts of yeast. Fujimoto et al. (2) overcame this problem by drying the yeast and allowing it to autolyze in the presence of toluene at 37 °C. The present method of purification avoids this potentially damaging step, is shorter, and gives a 2-fold higher yield. However, the final specific activity obtained by Fujimoto et al. (2) (285 units/mg at 1 mM cAMP, 37 °C; equivalent (4) to 170 units/mg at 0.5 mM cAMP, 30 °C) and the general properties of their preparation, such as $K_m$ value, divalent metal ion independence, and inhibition by thiol, are similar to those of our preparation.

Although we did not use protease inhibitors, it is unlikely that our procedure causes major changes in the structure of the enzyme, because the pH dependence of $V_{max}$ and $K_m$ of the EDTA-insensitive cAMP phosphodiesterase activity of permeabilized yeast (18) agrees well with that of enzyme purified by the method of Table I (4). However, proteolytic modification is always a possibility, especially with yeast enzymes (19), and can occur without obvious change in catalytic properties (e.g. Ref. 20). Two, so far unexplained, observations caution that some modification of the enzyme may occur during its purification. First, the activity of the 34,000 × g supernatants increases by about 25% if they are stored at 30 °C in the presence or absence of the protease inhibitors, phenylmethylsulfonyl fluoride or pepstatin A.² Second, all enzyme fractions through step 4 exhibit Hofstee plots at pH 7.8 in the presence of 2 mM EDTA that bend slightly upward below about 70 μM cAMP (19). This behavior disappears after the first hydroxyapatite column, but attempts to demonstrate an effector molecule or an EDTA-insensitive enzyme with a low $K_m$ have so far been unsuccessful. Most of the Mg²⁺-dependent low $K_m$ cAMP phosphodiesterase that is found partly in the cytosol of bakers’ yeast (21) is removed during step 3, and the remainder elutes from the first hydroxyapatite column before application of the (NH₄)₂SO₄ gradient.

Native enzyme exhibited a molecular weight of between 82,000 and 94,000 in the ultracentrifuge, and the enzyme-dodecyl sulfate complex behaved as a typical polypeptide of molecular weight 43,000 during gel electrophoresis. The agreement of the molecular weight estimates from the slope (43,000) and intercept (about 48,000) of Fig. 3 excludes the presence of more than a small amount of carbohydrate (ovalbumin, with 3% carbohydrate, also behaves “ideally”) or unusual structural arrangements. Thus, HK-phosphodiesterase appears to be a dimer of probably identical subunits, with an overall molecular weight of 86,000. The sedimentation coefficient ($s_{20, w} = 5.95 \times 10^{-13}$) is typical for a roughly spherical globular protein of this size. Fujimoto et al. (2) obtained a molecular weight of 65,000 for their preparation by gel electrophoresis in the presence of sodium dodecyl sulfate. We have been unable to repeat this observation (see under "Results").

The anomalous retardation of HK-phosphodiesterase during gel filtration through Sephadex G-100 at 5 °C (apparent molecular weights of about 65,000 have been reported (2, 3)) is probably due to some interaction between the enzyme and the gel matrix, although reversible dissociation of the enzyme cannot be excluded completely.

Few data are available concerning the amino acid compositions of cyclic nucleotide phosphodiesterases. The relative proportions of different classes of amino acids found in HK-phosphodiesterase (Table II) broadly resemble those for the monomeric low $K_m$ cAMP phosphodiesterase from dog kidney (22) which has a molecular weight (48,000-61,000) possibly close to that of the HK-phosphodiesterase subunit. Both enzymes contain 23.1 ± 0.4% of acidic residues (Asp and Glu), 14.8 ± 0.5% of basic residues (Lys, His, and Arg), 34.5 ± 0.8% aliphatic residues (Gly, Ala, Val, Ile, and Leu), 12.5 ± 0.3% Thr + Ser, and only small amounts of Met (3 residues/61,000 daltons for the kidney enzyme). Neither enzyme resembles the calmodulin-dependent enzyme from bovine brain (23) so closely in these respects, and in particular, the brain enzyme is relatively rich in Met (44 residues/116,000 daltons).

Pure fresh enzyme contained 21.5 ng of zinc/unit (Fig. 1), corresponding to 4.8 atoms of zinc/86,000-dalton dimer, assuming a specific activity of 170 units/mg of protein. This zinc cannot be removed by dialysis against 10 mM EDTA, without a proportionately greater loss of activity (1). Probably each monomer contains two tightly bound zinc atoms and smaller amounts of nonspecifically bound metals. Inactivation by 8-hydroxyquinoline was accompanied by loss of zinc, and the inactivated enzyme could be partially reactivated by dialysis against low concentrations of zinc and cobalt salts. Reactivation by zinc was facilitated by mercaptoethanol; very likely zinc in a hydrophobic zinc-mercaptoethanol complex can approach its binding site and be incorporated into the enzyme more easily than hydrated zinc ions. If reactivation by cobalt is due to formation of an active cobalt enzyme, which has not yet been proved, then the properties of this artificial enzyme should illuminate the role of zinc in the native enzyme. The observations of Jarvest et al. (24) suggest this role may be catalytic. Thus, HK-phosphodiesterase, but not the Mg²⁺-dependent high $K_m$ enzyme from beef heart, hydrolyzes both the (S)- and (R)-diastereoisomers of adenosine cyclic 3’,5’-phosphorothioate, and the authors argue (24) that the Zn⁺⁺ of the beef heart enzyme for the (S)-diastereoisomer reflects the inability of Mg²⁺ to coordinate to sulfur, whilst, since zinc can form both M(II)-O and M(II)-S complexes, the yeast enzyme can accept either configuration of the phosphorothioate. It is not yet known whether metals such as nickel, manganese, cadmium, and copper, which cause little or no reactivation, can be incorporated into the enzyme.

Several features of the kinetics of inactivation by 8-hydroxyquinoline are unusual and require further investigation. 1) The loss of activity is not strictly first order; the inactivation seems to slow after loss of about 75% of the activity (Figs. 5 and 7). Furthermore, the pseudo-first order rate constant decreased when the initial enzyme concentration was increased (Fig. 5) at enzyme concentrations (<3.2 pg/ml) far smaller than those (1 mg/ml) at which albumin causes partial protection. Possibly some dissociation of HK-phosphodiesterase to monomers is occurring under the conditions of the experiment (small enzyme concentration, 30 °C). 2) Although the instantaneous competitive inhibition of enzyme by 8-hydroxyquinoline indicated a dissociation constant for the enzyme-8-hydroxyquinoline complex of 1.1 mM (Fig. 8) in the presence of enzyme and absence of albumin, the rate of inactivation did not show saturation kinetics but was proportional to the concentration of 8-hydroxyquinoline up to at least 2.5 mM (Fig. 5). This may indicate that different zinc atoms are involved in the two processes or even that zinc is not directly involved in the competitive inhibition by 8-hydroxyquinoline.

² J. Londesborough, unpublished work.
3) Reactivation by zinc was never complete; instead the recoverable activity decreased with time in an apparently first order process with a rate constant close to half that for the original loss of activity (Fig. 7). A possible explanation, which is being further examined, is that dimers in which one protomer has lost essential zinc atom(s) can be reactivated, but that removal of zinc from both protomers is followed by a rapid and irreversible structural change. If the rate constants for removal of zinc from the two protomers are identical and independent of the zinc status of the other protomer, then the following scheme results,

\[
\begin{align*}
A & \xrightarrow{2k} B \\
& \xrightarrow{k} C \\
& \xrightarrow{k'} C' \\
& \xrightarrow{+\text{Zn}^{2+}} A \\
& \xrightarrow{+\text{Zn}^{2+}} B \\
& \xrightarrow{+\text{Zn}^{2+}} C \\
& \xrightarrow{+\text{Zn}^{2+}} C'
\end{align*}
\]

where \(A\) is native dimer, \(B\) and \(C\) are dimers in which, respectively, one or both protomers have lost zinc, and \(C'\) is irreversibly inactivated. Assuming that each \(A\) dimer has twice the enzyme activity of each \(B\) dimer, the activity immediately after 8-hydroxyquinoline treatment is \(E = 2A + B\), and the activity after maximum replacement of zinc is \(E = 2A + 2B + 2C\). For the simplest case, that \(k' \gg k\), the concentration of \(C\) is negligible, so \(E = 2A + 2B\). It can then be shown that \(E = 2A_0 e^{-kt}\) and \(R = 2A - (2e^{-kt} - e^{-kt})\), where \(A_0\) is the initial enzyme concentration. The curved lines in Fig. 7 show \(R\) calculated from this equation and the values of \(k\) determined from the first order decay of \(E\). The agreement between calculated and observed \(R\) is satisfactory only during the first 40% of the decrease in \(R\). At later times, observed \(R\) is greater than predicted, possibly indicating that \(k'\) is small enough for the concentration of \(C\) to become significant during the later stages of treatment with 8-hydroxyquinoline.

In contrast to 8-hydroxyquinoline, 1,10-phenanthroline did not cause progressive inactivation, though it was a competitive inhibitor (\(K_i = 0.6\) mm; Fig. 8). Liver alcohol dehydrogenase also is progressively inactivated by 8-hydroxyquinoline but not by 1,10-phenanthroline (25). The relatively large inhibition constants for 8-hydroxyquinoline and 1,10-phenanthroline suggest that neither compound can rapidly form a bidentate chelate with enzyme-bound zinc. Furthermore, the nonchelating 1,7- and 4,7-phenanthrolines also were effective inhibitors. Nonchelating phenanthrolines also inhibit yeast alcohol dehydrogenase (and a number of other enzymes), and it has been suggested that these and other aromatic nitrogen bases bind hydrophobically to the nucleotide binding site (26). A similar explanation may apply to HK-phosphodiesterase, though there seems no reason to exclude a monodentate interaction with enzyme-bound zinc. Two mammalian cyclic nucleotide phosphodiesterases are also inhibited by 1,10-phenanthroline and nonchelating phenanthrolines (27, 28), but no information is available about their possible metal contents.

Inhibition by KCN, but not NaN\(_3\) (Table III), is consistent with the much smaller stability constants of complexes of Zn\(^{2+}\) with N\(_3\) than with CN\(^-\) (29). All thiols tested inhibit the enzyme. Mercaptoethanol and mercaptoethylamine (Fig. 9) are mixed inhibitors that bind more tightly to free enzyme than to enzyme-substrate complex. Dithiothreitol (2), cysteine, and CoA (3) also inhibit. Thiols probably inhibit by binding to the zinc, rather than by reducing a hypothetical disulfide bond. The possible physiological significance of the inhibition by thiols is not yet clear.

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