A comparison of Zn(II) and Co(II) in the kinetics of inactivation of aminoacylase by 1,10-phenanthroline and reconstitution of the apoenzyme

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The kinetics of reconstitution of apoacylase with either Zn(II) or Co(II) and the inactivation of the Co(II) reconstituted enzyme by 1,10-phenanthroline (OP) has been studied by following the substrate reaction continuously in presence of the metal ion or OP respectively. Although the native Zn(II)-containing and the Co(II)-reconstituted enzymes have closely similar Michaelis constants and maximal velocities, the kinetics for both the inactivation by OP and the reconstitution of the apoenzyme with the metal ions differs considerably. For Co(II), both the inactivation by OP and the reconstitution show simple kinetics, but for Zn(II), the inhibition by OP is a multi-phasic process [Wang, Wu, Wang, Zhou and Tsou (1992) Biochem. J. **281**, 285–290], and the kinetics of reconstitution is also much more complicated. Both the native and the Co(II)-reconstituted enzymes are inhibited by excess of Zn(II), but not by Co(II). The inhibition by Zn(II) in excess and the reconstitution of the apoenzyme with Zn(II) are co-operative processes. The inhibition by Zn and its effect on the fluorescence emission of 1-anilinonaphthalene-8-sulphonic acid bound to the native enzyme indicate multiple Zn(II)-binding sites.

INTRODUCTION

The importance of Zn(II) in the catalytic processes of a number of different enzymes is well established (Vallee and Williams, 1968; Vallee and Galdes, 1984; Vallee, 1988). Removal of Zn(II) from the enzymes by metal chelators leads invariably to inactivation, and the enzymes can usually be re-activated by restoration of the metal (Vallee and Williams, 1968; Kidani and Hirose, 1977; Billo, 1979). As the d-shell electrons are filled for Zn(II), it is completely without absorption, e.s.r. or other signals for spectroscopic studies of the role of the metal ion in the catalytic process; paramagnetic metals have been used as probes for such studies (Latt and Vallee, 1971; Van Wart and Vallee, 1978; Vallee and Galdes, 1984; Kuo and Makinen, 1985; Heese et al., 1990). Co(II) is sufficiently similar to Zn so that it can replace Zn in most of the Zn enzymes with nearly full catalytic activity, and consequently it has been extensively used as a spectroscopic probe for studies on the structure of the active sites of Zn enzymes and the role of the metal ion during catalysis (Vallee and Wacker, 1970; Vallee and Holmquist, 1980; Geoghegan et al., 1983). However, although for enzymes such as carboxypeptidase A, the Co(II)-substituted enzyme is fully active and the structure sufficiently similar to that of the native enzyme (Rees et al., 1981; Vallee and Galdes, 1984; Zhang et al., 1992), it does show some differences in relative rate of hydrolysis towards peptide and ester substrates as compared with the native, Zn(II)-containing enzyme (Auld and Vallee, 1987). In addition, whether the Co(II)-reconstituted enzymes are sufficiently similar to the native enzymes in other catalytic properties, such that the knowledge acquired by the use of Co(II) can be applied to the native enzyme, remains to be demonstrated.

The kinetics of either inactivation or re-activation during metal removal and restoration has been relatively little explored, as these are usually very rapid reactions. Wolz and Zwilling (1989) studied the kinetics of inactivation of *Astacus* (crayfish) proteinase by Zn removal by monitoring the substrate reaction (Tian and Tsou, 1982; Tsou, 1988) in the presence of 1,10-phenanthroline (OP) and presented evidence that the inhibition is of the non-competitive type and that the binding of two OP molecules during metal removal is co-operative. Aminoacylase (EC 3.5.1.14) is a Zn-enzyme catalysing the hydrolysis of acyl-L-amino acids (Kordel and Schneider, 1976, 1977). Zn(II) can be replaced by a number of bivalent metals and the Co(II)-substituted enzyme has been studied by Gilles et al. (1984) and Loffler et al., (1986). In a previous paper the kinetics of the inactivation of aminoacylase by OP has been examined by following the substrate reaction in the presence of OP and a mechanism involving a rapid reversible OP-binding step followed by removal of the metal, leading to the inactivation proposed (Wang et al., 1992).

In the present investigation it is shown that, although the Co(II)-reconstituted aminoacylase is as fully active as the Zn(II) enzyme, the two enzymes behave considerably differently in respect of their kinetics of inactivation by OP and reconstitution of the apoenzyme. Moreover, when present in excess, Zn not only inhibits both the native and the Co(II)-reconstituted enzymes, but also affects the enzyme-bound fluorescence probe 1-anilinonaphthalene-8-sulphonic acid (ANS), whereas Co(II) does not.

MATERIALS AND METHODS

Materials

Aminoacylase and *N*-chloroacetyl-L-leucine (CAL) were Sigma products; $CoSO_4,7H_2O$ (99.999% pure) and $ZnSO_4,6H_2O$ (99.99% pure) were from Alfa. Sephadex G-25 was from Pharmacia, the protein assay reagent was a Bio-Rad product and ANS was from BDH. Tris was a local guaranteed reagent, nitrilotriacetic acid (NTA) was an analytical reagent from the Chinese Medical Co. (Beijing, China), and all other reagents were local products of analytical grade.

Preparation of apo- and reconstituted acylase

Apoenzyme

The enzyme was dissolved in 0.1 M Tris/maleate buffer, pH 6.0,

Abbreviations used: ANS, 1-anilinonaphthalene-8-sulphonic acid; CAL, *N*-chloroacetyl-L-leucine; NTA, nitrilotriacetic acid; OP, 1,10-phenanthroline.

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containing 10 mM EDTA. It was left for 48 h at 4 °C and then passed through a Sephadex G-25 column pre-equilibrated with Tris/HCl buffer, pH 7.9, containing 10 mM NTA and washed with the same buffer to remove completely the EDTA–Zn complex and excess free EDTA. The apoenzyme thus prepared was completely inactive.

Co(II)-reconstituted acylase

The apoenzyme was prepared as above, except that the buffer used for the elusion contained instead 5 mM NTA. To the apoenzyme thus prepared was added $CoSO_4$ to a final concentration of 6 mM, and the mixture was then passed through Sephadex G-25 to remove NTA-Co and excess NTA. The Co(II)-reconstituted acylase was completely active with a k_{+2} ($k_{cat.}$; see Scheme 1 below) = 108 s^{-1}.

The determination of protein concentration and enzyme activity

Protein concentration was measured as described by Bradford (1976), with Bio-Rad Protein Assay Reagent. Enzyme activity was monitored at 30 °C by absorbance changes due to hydrolysis of CAL at 240 nm in a Shimadzu UV-250 spectrophotometer. The difference molar absorption coefficient for the hydrolysis of CAL, $\Delta\epsilon_{240}$ (= 132 $M^{-1}\cdot cm^{-1}$), was determined by monitoring its hydrolysis to completion in the presence of excess enzyme.

Kinetics of reconstitution with Zn(II) and Co(II)

The kinetics of the re-activation by reconstitution of apoacylase with the metal ions was monitored continuously by measuring the activity regenerated from the apoenzyme in a reaction mixture containing both the substrate and the metal ion. With excess Zn(II) or Co(II) the re-activation rate was too high to be conveniently monitored. In order to keep a sufficiently low, and at the same time a constant, level of the metal ions, a metal buffer containing NTA was employed. The reaction mixture contained 0.1 M Tris/HCl buffer, pH 7.9, 1 mM NTA, the indicated concentrations of Zn(II) or Co(II) and the substrate. The concentration of free metal ion ($[Me]_{free}$) can be calculated thus:

$$[Me]_{free} = K_{x'}\{[Me]_T/([NTA]_T - [Me]_T)\}$$

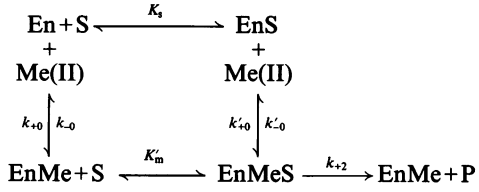
where $[Me]_T$ and $[NTA]_T$ are total concentrations of Me(II) and NTA respectively, and $K_{x'}$ is the dissociation constant of the NTA-Me(II) complex at pH 7.9 and has values of 2.3 and 1.7 nM for the Zn(II) and Co(II) complexes respectively, calculated for pH 7.9 from the dissociation constant of the NTA–Me(II) complex, which has values of $10^{-10.45}$ and $10^{-10.61}$ respectively (Schwarzenbach and Freitag, 1951).

Inhibition of the Co(II) reconstituted enzyme by OP

The kinetics of OP inhibition of the Co(II)-reconstituted enzyme were observed essentially as described previously (Wang et al., 1992) for the native enzyme.

Determination of rate constants of reconstitution

The reconstitution reaction is as follows:



Scheme 1

From Scheme 1 it can be shown that the concentration of the product formed is given by (Tsou, 1988):

$$P = v'\left(t - \frac{1}{A[Y]+B}\{1 - e^{-(A[Y]+B)t}\}\right)$$

where P is the concentration of the product formed at time t, v' is the final rate of the substrate reaction at a fixed level of Y. If $R = A[Y]+B$, then the above equation can be written as:

$$P = v'\left[t - \frac{1}{R}(1 - e^{-Rt})\right] \quad (1)$$

Where:

$$v' = \frac{k_{+2}[E][S]}{K_m+[S]}\cdot\frac{A[Y]}{A[Y]+B} = V\cdot\frac{A[Y]}{A[Y]+B} \quad (2)$$

V is the maximal activated rate when Y is in large excess. A and B are the apparent forward and backward rate constants respectively, and:

$$A = \frac{k_{+0}K_S + k'_{+0}[S]}{K_S+[S]} \quad (3)$$

$$B = \frac{k_{-0}K'_m + k'_{-0}[S]}{K'_m+[S]} \quad (4)$$

By curve-fitting of the experimental data with equation (1), R and v' can be obtained; A and B can then be obtained by a plot of R against [Y].

From eqns. (3) and (4), when $k_{+0} = 0$, a plot of $1/A$ against $1/[S]$ gives a straight line; when $k'_{+0} = 0$, a plot of $1/A$ against [S] gives a straight line; when $k_{+0} = k'_{+0}$, A is independent of [S]; when $k_{-0} = 0$, a plot of $1/B$ against $1/[S]$ is a straight line; when $k'_{-0} = 0$, a plot of $1/B$ against [S] is a straight line, and when $k_{-0} = k'_{-0}$, B is independent of [S]. From the slopes and intercepts, k_{+0}, k_{-0}, K_s and K'_m can all be obtained.

From eqn. (2), the plots of $1/v'$ against $1/[Y]$ and $1/V$ against $1/[S]$ are both straight lines, and from the intercepts at the ordinate and abscissa respectively, k_{+2} and K'_m can be obtained.

All the curve-fitting for analysis of the kinetic data was carried out with the computer program Sigma Plot 4.0.

Inhibition of native and Co(II)-reconstituted acylase by excess Zn(II)

The inhibition of native and Co(II)-reconstituted enzymes was studied by monitoring the substrate reaction continuously in the presence of excess Zn(II). The reaction mixture contained 0.1 M Tris/HCl buffer, pH 7.9, and the indicated concentrations of Zn(II) and substrate.

Fluorescence measurements

ANS fluorescence was measured in a Perkin–Elmer MPF-66 spectrofluorimeter at 30 °C with an exciting wavelength of 375 nm.

RESULTS

Kinetic properties of native and Co(II)-reconstituted aminoacylases

The Co(II)-reconstituted enzyme shows kinetic properties similar to those of the native enzyme. With CAL as substrate it has an activity (k_{+2} = 108 s^{-1}) slightly higher than that for the native enzyme (k_{+2} = 98 s^{-1}) and the same K_m (8.3 mM) as that obtained by a double-reciprocal plot for the two enzymes. With *N*-acetyl-L-leucine as the substrate, Loffler et al. (1986) reported k_{+2} values

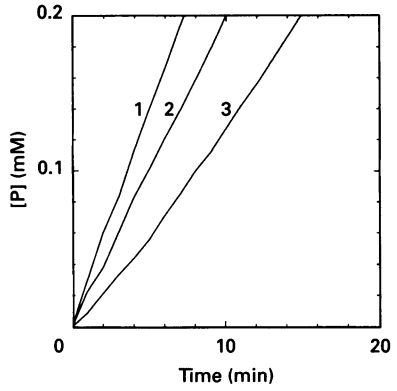


Figure 1 Course of substrate hydrolysis of the Co(II)-reconstituted enzyme in the presence of different concentrations of OP

The enzyme (5 μl of a 2.79 μM solution) was added to a 1 ml reaction mixture containing 0.1 M Tris/HCl buffer, pH 7.9, 1.67 mM substrate and OP concentrations of 0, 20, and 40 μM for curves 1–3. The absorbance decrease at 240 nm was monitored at 30 °C.

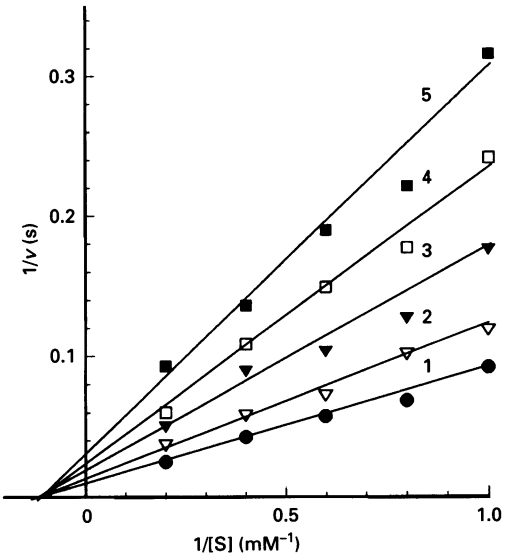


Figure 2 Double-reciprocal plots of the inhibition of the Co(II)-reconstituted enzyme by OP

Reaction conditions were as for Figure 1, except that the initial enzyme concentration was 33.4 μM; OP concentrations were, for lines 1–5, 0, 10, 20, 30 and 40 μM respectively.

for the native and the Co(II)-reconstituted enzymes as 5400 and 20400 s^{-1}, and K_m values of 34.8 and 20.4 mM, respectively.

Inhibition of the native and the Co(II)-reconstituted enzymes by OP

It has been shown in a previous report that the inhibition of native acylase by OP is multiphasic. The initial rapid formation of a reversible enzyme–OP complex is followed by a relatively slow reversible conformational change step leading to an irreversible removal of the metal from the enzyme (Wang et al., 1992). However, the kinetics of inhibition of the Co(II)-reconstituted enzyme by OP is considerably different.

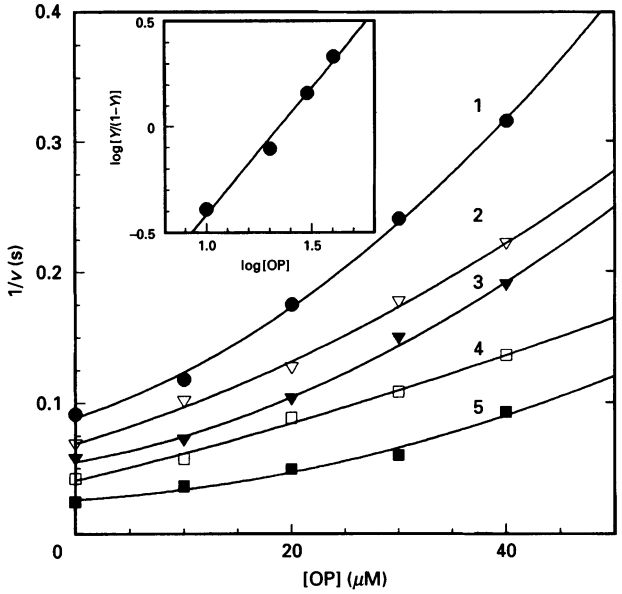


Figure 3 1/*v*-versus-[OP] plots at different substrate concentrations

Data are taken from Figure 2. The substrate concentrations were 1, 1.25, 1.67, 2.5 and 5 mM for curves 1–5 respectively. The inset show a Hill plot of the results.

Instead of a multiphasic process, by addition of the Co(II)-reconstituted enzyme to the reaction mixture containing different concentrations of OP, a series of straight lines were obtained for the substrate reactions (Figure 1) with decreasing slopes as the concentration of OP increases. It appears that the rate of inhibition is so fast that it is completed within the time of mixing. Double-reciprocal plots of $1/v$ against $1/[S]$ at different OP concentrations give straight lines with the same K_m values (Figure 2), suggesting that the inhibition is of the reversible non-competitive type.

However, the inhibition is different from the simple non-competitive type in that Dixon plots of $1/v$ against [OP] gives, instead of a series of straight lines at different substrate concentrations, a series of curves bending upward, suggesting co-operative binding of the inhibitor (Figure 3). A Hill plot gives a straight line with a Hill coefficient of 1.2, as shown in the inset to Figure 3.

Reconstitution of Zn(II) with the apoenzyme

The re-activation rate for the apoenzyme with excess Zn(II) was too fast to be conveniently studied. In order to keep a sufficiently low and, at the same time, a constant, level of the free metal ion, a metal buffer containing NTA was employed. The course of the substrate reaction during the reconstitution of the apoenzyme with Zn(II) shows a gradual increase in activity until a constant rate is reached (Figure 4). Both the rate of increase in activity and the final activity of the re-activated enzyme increase with Zn(II) until, at higher free Zn(II) concentrations, both approach constant levels. Curve-fitting of experimental points with eqn. (1) gives satisfactory results, and the parameters R and v' can be obtained. However, plots of R against Zn(II) at different substrate concentrations give, instead of a series of straight lines as in the case of Co(II) reconstitution (see below), a group of upward bending curves, suggesting co-operative binding of Zn(II) during reconstitution (Figure 5).

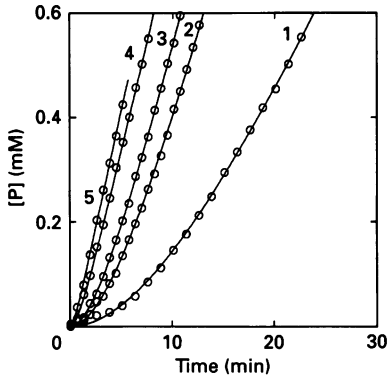


Figure 4 Course of substrate hydrolysis during reconstitution of Zn(II) to the apoenzyme

Final concentrations were: enzyme, 70 nM; NTA, 50 μM; substrate, 4 mM; Zn(II) for curves 1–5, 9.1, 16.7, 23.1, 28.6 and 33.3 μM respectively; the calculated free Zn(II) concentrations were 0.51, 1.15, 1.98, 3.07 and 4.58 nM respectively.

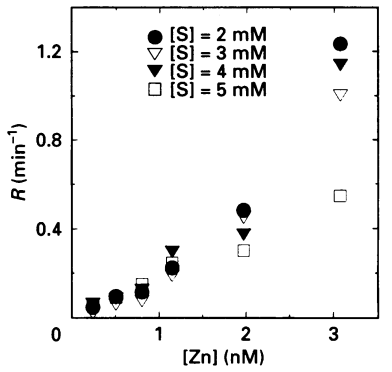


Figure 5 Plot of *R* against [Zn] for Zn(II) reconstitution

Values of *R* were obtained from data obtained for substrate hydrolysis during reconstitution by curve-fitting. Experimental conditions were as for Figure 4, with substrate concentrations as indicated.

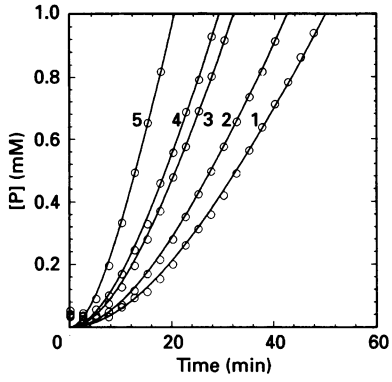


Figure 6 Course of substrate hydrolysis during reconstitution of Co(II) to the apoenzyme

Final concentrations were: enzyme, 70 nM, NTA, 1 mM; substrate, 4 mM; Co(II), for curves 1–5, 0.48, 0.57, 0.67, 0.77 and 0.86 mM respectively; the calculated free Co(II) concentrations were 1.56, 2.31, 3.54, 5.6 and 10.8 nM respectively.

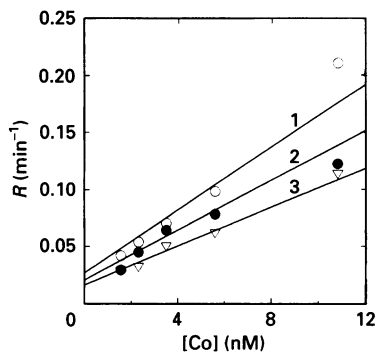


Figure 7 Plot of *R* against [Co] for Co(II) reconstitution

Values of *R* were obtained from data obtained for substrate hydrolysis during reconstitution by curve-fitting. Experimental conditions were as for Figure 6, with substrate concentrations of 1, 3 and 5 mM respectively for lines 1, 2 and 3.

Reconstitution kinetics of the apoenzyme with Co(II)

The course of the re-activation of the apoenzyme by Co(II) has also been monitored by measuring the substrate reaction (Figure 6) in the presence of NTA to ensure a low, but constant, level of free Co(II). Comparison of Figures 4 and 6 shows that the courses of re-activation are closely similar, except that much higher Co(II) concentrations are required as compared with Zn(II), and, at the higher free-metal-ion-concentration range, neither the rate of re-activation nor the final enzyme activity approaches a constant value. However, unlike the re-activation by Zn(II), plots of R against Co(II) at different substrate concentrations give a series of straight lines, as shown in Figure 7, from which the apparent forward and reverse rate constants, A and B, can be obtained.

The plots of $1/A$ against [S] and $1/B$ against [S] both give straight lines, as shown in Figures 8(a) and 8(b), indicating that $k_{+0} \gg k'_{+0}$ and $k_{-0} \gg k'_{-0}$. From the slopes and intercepts, k'_{+0}, k'_{-0}, K_s and K'_m can all be obtained, and these are given in Table 1. From eqn. (2) the plots of $1/v'$ against 1/[Y], and $1/V$ against 1/[S] are both straight lines as shown in Figures 8(c) and 8(d). From the intercepts at the ordinate and abscissa respectively of Figure 8(d), k_{+2} and K'_m can be obtained, as is also given in Table 1. It is to be noted that the dissociation constants for substrate binding are similar for the apo-, the native and the Co(II)-reconstituted enzymes. However, the rate constant for the binding of Co(II) with the enzyme–substrate complex (ES) is much lower than that for its binding with the enzyme (E). It is suggested that Co(II) is not essential for the binding of the substrate, but the binding of substrate greatly hinders the binding of Co(II).

Inhibition of the native and Co(II)-reconstituted enzyme by excess Zn

Although Zn is essential for the activity of acylase, an excess of Zn(II) produces inhibition for both the native and the Co(II)-reconstituted enzymes. In addition to its effect on the initial rate, the inhibition is also a time-dependent process, approaching a final constant inhibited rate in about 60 min, as shown in Figure 9, for the inhibition of the native enzyme at different substrate concentrations. Similar results were obtained for the Co(II)-reconstituted enzyme. The enzyme was found to be stable at this

Table 1 Kinetic constants for the re-activation of aminoacylase with Co(II)

k_{+0}, k_{-0}, K_s, K_m, K'_m, and k_{+2} are the binding and dissociation rate constants of Co(II), dissociation constant of substrate with the apoenzyme, and Michaelis constants for the native and Co(II) enzymes and rate constant of substrate reaction of Co(II)-reconstituted enzyme respectively.

k_{+0} ($M^{-1} \cdot s^{-1}$)	k_{-0} (s^{-1})	K_s (mM)	K'_m (mM) 1/B—[S]	K'_m (mM) 1/V—[S]	K_m (mM)	k_{+2} (s^{-1})
2.4×10^5	5.4×10^{-4}	6.9	5.34	9.13	9.1	70

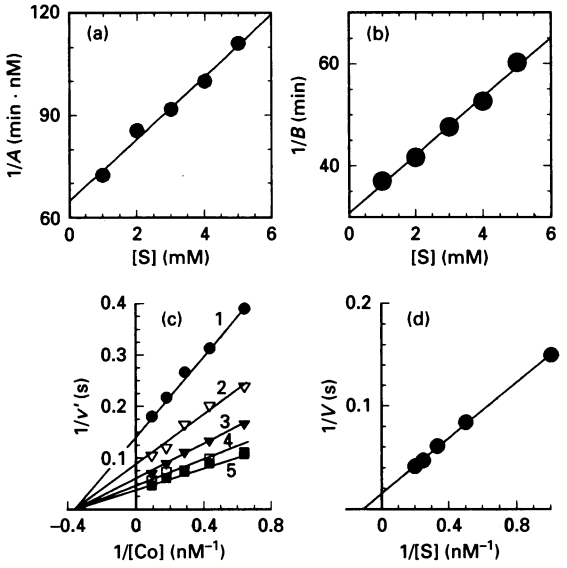


Figure 8 Double-reciprocal plots for the determination of kinetic constants for the reconstitution of Co(II) with the apoenzyme

(**a**) $1/A$ against [S] plot; A values were obtained from a plot of R against [Co(II)]. Experimental conditions were as for Figure 6, with substrate concentrations as indicated. (**b**) $1/B$ against [S] plot; B values were obtained from a plot of R against [Co(II)]. Experimental conditions were as for Figure 6, with substrate concentrations as indicated. (**c**) $1/v'$ against 1/[Co(II)] plots; v' values were obtained from data obtained for substrate hydrolysis during reconstitution by curve-fitting. Experimental conditions were as for Figure 6, with substrate concentrations of 1, 2, 3, 4, and 5 mM for lines 1–5 respectively. (**d**) The $1/V$ against 1/[S] plot; data are from (**c**).

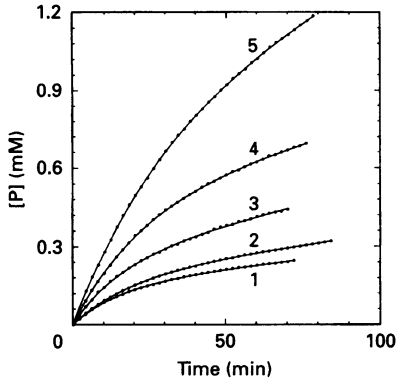


Figure 9 Effect of substrate concentration on the inhibition of excess Zn(II) on native acylase

The course of substrate hydrolysis was monitored continuously at 240 nm. Final concentrations of enzyme and Zn(II) were 49 nM and 200 μM respectively, and substrate concentrations were, for curves 1–5, 1, 1.25, 1.67, 2.5 and 5 mM respectively. Points are experimental ones, and curves are results of curve-fitting by the program Sigma plot 4.0.

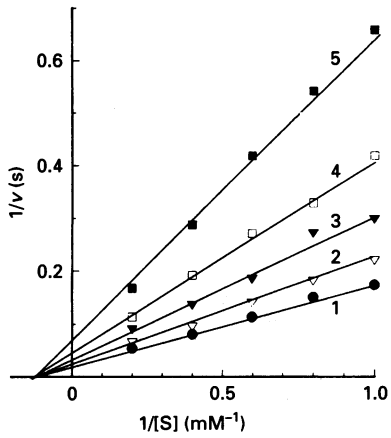


Figure 10 Double-reciprocal plot of $1/v$ against 1/[S] for the inhibition of the native enzyme by excess Zn(II)

The course of substrate hydrolysis was monitored continuously, with a final enzyme concentration of 49 nM. Zn(II) concentrations were, for lines 1–5, 0, 100, 200, 300 and 400 μM respectively.

temperature for the time period examined. Curve-fitting with eqn. (6) gives both A and B for the reaction:

$$[P] = \frac{v}{A[Y]+B}\left\{Bt + \frac{A[Y]}{A[Y]+B}[1-e^{-(A[Y]+B)t}]\right\} \quad (6)$$

Both the initial and final rates of reaction decrease with increasing concentrations of Zn(II), and decreasing concentrations of substrate, for both the native and the Co(II)-reconstituted enzymes. Plots of the reciprocal of the initial rates, $1/v$, against 1/[S] at different Zn(II) concentrations give a series of straight lines meeting at the abscissa for both the native and the Co(II)-reconstituted enzymes. The results for the native enzyme are given in Figure 10. The above results show that, as far as the initial rate is concerned, the inhibition by an excess of Zn(II) is similar to reversible non-competitive inhibition; however, as the time course of the inhibition reaction shows, the binding of Zn(II) also involves a slow process leading to a state of the enzyme with a lower level of activity. Neither Zn(II) or substrate concentrations have any appreciable effect on the forward and reverse rates of the slow step of inactivation of both the native and the Co(II)-reconstituted enzymes (results not shown), suggesting that the slow step involves conformational changes of the complex of the enzymes with excess Zn(II). In sharp contrast, Co(II) at comparable concentrations has no appreciable effects on the activity of either the native or the Co(II)-reconstituted enzymes (results not shown).

Like the inhibition of Co(II)-reconstituted enzyme by OP, plots of $1/v$ against Zn(II) give upward-bending curves for both the native (Figure 11), and a Hill plot (Figure 11 inset) gives a

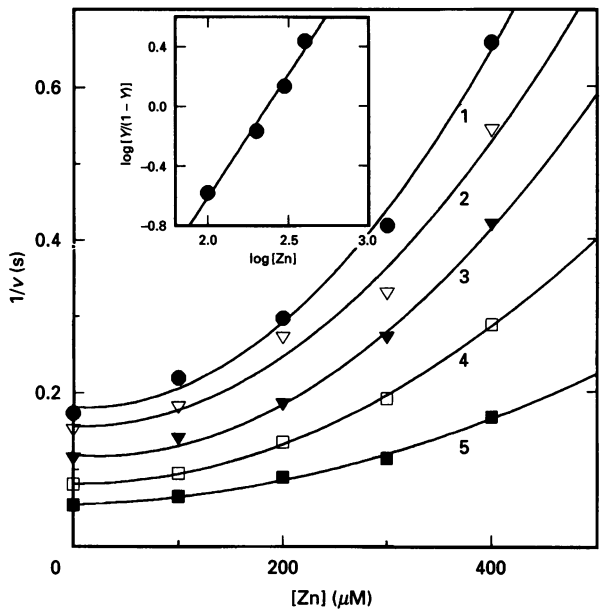


Figure 11 Effect of Zn(II) concentration on the initial velocity of the native acylase

The course of substrate hydrolysis was monitored continuously at 240 nm. The final enzyme concentration was 49 nM, and substrate concentrations were, for curves 1–5, 1, 1.25, 1.67, 2.5 and 5 mM respectively. The inset shows a Hill plot of the results.

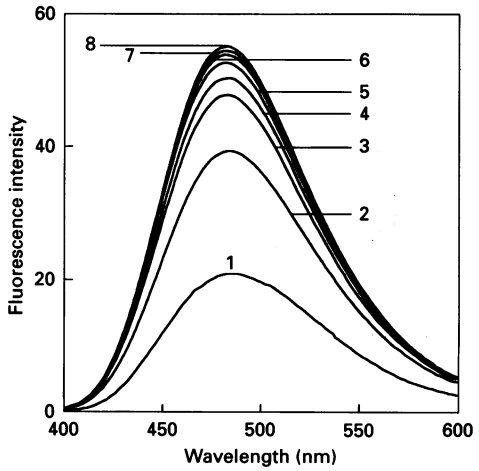


Figure 12 Fluorescence emission spectra of ANS–acylase I complex in the presence of excess Zn(II)

The Figure shows fluorescence spectra of ANS during Zn(II) binding to the native acylase in 0.1 M Tris/HCl buffer, pH 7.9 at 30 °C, at 0, 0.5, 16, 28, 40, 52, 64, and 76 min after mixing for curves 1–8 respectively. The enzyme and zinc concentrations were 0.2 and 240 μM respectively. The excitation wavelength was 375 nm.

Hill coefficient of 1.7. The inhibition of the Co(II)-reconstituted enzyme shows similar results (not shown) and a Hill coefficient of 1.4.

Effect of excess Zn on the fluorescence emission of enzyme-bound ANS

The fluorescence emission of ANS increases markedly when bound to the native Zn-containing aminoacylase, and the maximum blue shifts from 525 to about 500 nm with a difference maximum for the enzyme–ANS complex at 482 nm. With the addition of excess Zn, the emission intensity at 482 nm further increases slowly and reaches a final value nearly three times the original in about 60 min (Figure 12), the same time as that required for the inhibition to reach completion. The addition of Co(II) has no effect on the fluorescence of the enzyme–ANS complex, nor has Zn(II) any effect on the fluorescence of the complex of ANS with BSA.

DISCUSSION

As with a number of Zn enzymes, Co(II) can replace Zn(II) in aminoacylase with nearly full activity, and the Co(II)-reconstituted enzyme has kinetic parameters similar to those of the native enzyme (Loffler et al., 1986; Gilles et al., 1984). However, the two metals show considerable difference in the kinetics of inactivation by metal removal with OP and in the kinetics of reconstitution of the apoenzyme with the respective metals.

It has been shown in a previous paper (Wang et al., 1992) that the removal of Zn from aminoacylase by OP involves a rapid binding of OP at the metal-binding site, followed by a relatively slow step of inactivation with metal removal. However, the reaction of OP with the Co(II)-reconstituted enzymes appears to resemble a simple one-step reversible non-competitive inhibition. This could be the result of a much looser combination of Co(II), as compared with Zn(II), with the enzyme, and the binding of OP with the Co(II)-enzyme leads to rapid removal of the metal and loss of enzyme activity. For the Zn(II)-enzyme, the binding of the metal with the apoenzyme is so tight that a slow conformational change is necessary before the metal can be removed by OP, leading to irreversible inactivation. In the present study, Zn(II) re-activates the apoenzyme at a much lower concentration as compared with Co(II) re-activation.

Although the concentration required for re-activation is more than an order of magnitude lower for Zn(II) than that for Co(II), the courses of re-activation of the apoenzyme with the two metals are apparently similar. However, the kinetics of reconstitution appears to be different for these two metals. Both the initial rate and the final level of activation approach maximal values with the increase in free metal ion concentrations for reconstitution with Zn(II), but not for reconstitution with Co(II). Moreover, the more complicated kinetics of re-activation by Zn(II) suggests co-operative binding of Zn(II) at multiple sites, but the linear function of a plot of R against [Co(II)] suggests binding of Co(II) at a single site, as shown by a comparison of Figure 5 with Figure 7.

The suggestion of Zn binding at multiple sites is further supported by the finding of inhibition of the activity of both the native and Co(II)-reconstituted enzymes by excess Zn(II), whereas Co(II) shows no marked inhibition even at much higher concentrations. Similarly, inhibition by excess Zn(II) has been reported for carboxypeptidase A (Larsen and Auld, 1989); however, the inhibition of carboxypeptidase A by Zn(II) is a simple competitive inhibition, whereas for aminoacylase, Zn not only inhibits the initial velocity non-competitively and co-operatively, but also induces a time-dependent process leading to further inhibition.

The presence of multiple Zn-binding sites has also been shown by a time-dependent increase in fluorescence emission of ANS bound to the native enzyme on the addition of excess Zn. As this is a slow process even in presence of a large excess of Zn, it is not possible to determine either the binding constant or the binding stoichiometry. The increase in ANS fluorescence undoubtedly indicates a conformational change in the enzyme which, with

ANS also in excess, could be either an environmental change at the same ANS-binding site or changes leading to additional ANS binding. This conformational change is closely related to the inhibition process, as both require a similar concentration of Zn and about the same time to approach completion.

The similarity of the kinetics of the substrate reaction for the native and the Co(II)-reconstituted enzymes suggests that, as a probe, Co(II) could reveal important properties of the active site of aminoacylase and other Zn-enzymes. However, the role of Zn(II) in this enzyme is much more complicated than can be represented by Co(II), as shown especially by the binding of Zn(II) at multiple sites, possible conformational changes of the enzyme, as indicated during its removal and reincorporation, as well as time-dependent inhibition when present in excess.

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