Resistance to inactivation by EGTA of the enzyme–substrate and enzyme–phosphate complexes of alkaline phosphatase

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Bovine intestinal mucosal alkaline phosphatase is inactivated by the chelating agent EGTA. Several concentrations of the enzyme were incubated with EGTA and a range of concentrations of the substrate *p*-nitrophenyl phosphate to determine the substrate concentration as a function of time. As predicted by a recently developed theory [Duggleby (1986) *J. Theor. Biol.* **123**, 67–80], catalysis ceases before all substrate is exhausted. An analysis of these final substrate concentrations according to the theory revealed that, whereas the free enzyme is unstable, the effect of EGTA is counteracted when either the substrate or product (phosphate) is bound. Comparison of the results with those obtained by direct stability measurements and steady-state kinetic experiments gave a qualitatively and quantitatively consistent body of evidence in support of this interpretation.

INTRODUCTION

The alkaline phosphatases from both bacterial and mammalian sources are very-well-studied zinc metallo-enzymes (Fernley, 1971; Reid & Wilson, 1971; Coleman & Gettins, 1983). These enzymes usually contain several zinc atoms per subunit that are tightly bound and are not lost during enzyme purification, although they can be removed by treatment with chelating agents. The resulting apoenzyme is inactive, suggesting that at least one of the zinc atoms forms part of the active site. It is well accepted that inorganic phosphate and, presumably, the phosphate group of the substrate bind directly, or in close proximity, to the metal atom (Wyckoff *et al.*, 1983).

As might be expected from this close association between the substrate and the zinc, the presence of substrate has been reported (Harkness, 1968) to protect against inactivation by the chelating agent EDTA, although it is not clear whether this protection is partial or complete. Substrate protection was studied by Ackermann & Ahlers (1976), who proposed a two-stage 'induced-fit' model in which binding of one substrate molecule per enzyme dimer gives partial protection against EDTA whereas binding of a second substrate molecule affords complete protection. It should be noted that the interpretation of the effects of EDTA is difficult because it causes an immediate inhibition as well as a time-dependent inactivation, leading to bizarre kinetics (Conyers *et al.*, 1967).

A recently developed theory (Duggleby, 1986) provides an approach to the quantification of enzyme instability. The analysis is based upon measurements of progress curves of substrate utilization and yields the inactivation rate constant of an enzyme together with those of the enzyme–substrate and enzyme–product complexes. We have applied this theory to the inactivation by zinc chelation of bovine intestinal mucosal alkaline phosphatase. However, to avoid the kinetic complexities that arise with EDTA, we have used EGTA, which is non-inhibitory at 10 mM, a concentration that is sufficient to cause complete inactivation (half-life 3.4 min) within half an hour.

The results obtained suggest a model in which only the free enzyme is susceptible to inactivation by EGTA whereas the complexes of the enzyme with either its substrate (*p*-nitrophenyl phosphate) or its product (phosphate) are resistant to this chelating agent. Quantitative analysis indicates complete agreement between this model and data obtained from progress-curve experiments, direct enzyme-inactivation studies and steady-state kinetic measurements.

MATERIALS AND METHODS

Alkaline phosphatase (bovine intestinal mucosa, Sigma type VII) was diluted to approx. 40 mg/l in 0.15 M-sodium carbonate buffer, pH 10.0, before use. This diluted enzyme solution was stable for several days when stored at 4 °C. All preincubation and reaction mixtures described below were prepared in this carbonate buffer and all experiments were conducted at 30 °C.

Stability experiments

The EGTA-induced inactivation of alkaline phosphatase was examined by adding a small volume (usually 20 µl) of the diluted enzyme to 3 ml of a solution containing 10 mM-EGTA. In some instances this solution contained up to 14 mM-trisodium phosphate as well. After preincubation for various times, samples (usually 100 µl) were added to 3 ml of a reaction mixture containing 2 mM-*p*-nitrophenyl phosphate. Activity was determined by recording the change in absorbance at 410 nm due to formation of the *p*-nitrophenolate ion over the first 2–3 min, with a Perkin–Elmer Lambda 3 spectrophotometer.

Steady-state kinetic experiments

The diluted enzyme solution was diluted a further 150-fold with carbonate buffer, and 100 µl samples were added to 3 ml reaction mixtures containing between 0.4 mM- and 10 mM-*p*-nitrophenyl phosphate and between 0 and 20 mM-trisodium phosphate. Activity was determined as described above.

Progress-curve experiments

Reaction mixtures (3 ml) containing 10 mM-EGTA and between 2 mM- and 10 mM-*p*-nitrophenyl phosphate were prepared, and then the reaction was started by addition of between 10 and 80 μ l of diluted enzyme. At 15–20 timed intervals, the absorbance at 479 nm was measured. This wavelength was chosen so as to avoid excessively large absorbances.

Data analysis

Absorbances were converted into concentrations by reference to solutions of *p*-nitrophenyl phosphate that had been fully hydrolysed by incubation for several hours with alkaline phosphatase. Data were further analysed by fitting the appropriate equation by non-linear regression, with the use of the DNRP53 computer program (Duggleby, 1984). First-order decay curves were fitted to the data from the stability experiments, and the equation for competitive inhibition was fitted to the results from the steady-state kinetic experiments. Progress curves were analysed (Duggleby, 1985) to calculate the residual substrate concentration at infinite time, and this information was then utilized as described by Duggleby (1986) to determine various enzyme inactivation rate constants. This latter procedure is documented more fully in the Results section.

RESULTS

EGTA is a chelating agent that, at a concentration of 10 mM, does not inhibit bovine intestinal mucosal alkaline phosphatase (results not shown), but does cause a first-order inactivation (Fig. 1). When the enzyme is incubated with the substrate *p*-nitrophenyl phosphate in the presence of EGTA, this inactivation prevents the complete hydrolysis of the substrate. The extent of substrate utilization depends upon the quantity of enzyme added (Fig. 2).

According to the theory developed by Duggleby (1986), the amount of substrate that remains unchanged at infinite time ($[A]_f$) is described by eqn. (1):

$$\frac{[E]_0}{[A]_0 - [A]_f} = \frac{K_a}{V_m} \left[\left(j_1 + \frac{j_3[A]_0}{K_p} \right) \left(\frac{\ln([A]_0/[A]_f)}{[A]_0 - [A]_f} \right) + \frac{j_2'}{K_a} - \frac{j_3}{K_p} \right] \quad (1)$$

In this equation, $[E]_0$ and $[A]_0$ are the initial concentrations of enzyme and of substrate, V_m is the maximum velocity, K_a is the Michaelis constant and K_p is the inhibition constant for the product (phosphate). The inactivation rate constant for the free enzyme is represented by j_1 and that for the enzyme-product complex is j_3 . The symbol j_2' is an apparent inactivation rate constant for the enzyme-substrate complex. This equation is the basis of the *J* plot (Duggleby, 1986), in which $[E]_0/([A]_0 - [A]_f)$ is plotted against $\ln([A]_0/[A]_f)/([A]_0 - [A]_f)$. The amount of unchanged substrate at infinite time was determined at several concentrations of enzyme and of substrate, and the data were used to construct a *J* plot as shown in Fig. 3.

Two significant features emerge from the *J* plot. First, all points lie near to a common straight line, irrespective of the initial concentration of substrate. This indicates that the enzyme-product complex is resistant to the inactivating effect of EGTA. Incubation of the enzyme with EGTA in the presence of phosphate (Fig. 1)

Fig. 1. Inactivation of alkaline phosphatase by EGTA

Experiments were performed as described in the Materials and methods section by incubating the enzyme at a concentration of 0.07 mg/l ($\square$), 0.28 mg/l ($\circ$ and $\bullet$) or 1.13 mg/l ($\blacksquare$) with 10 mM-EGTA. In one experiment ($\circ$) 10.5 mM-trisodium phosphate was also present. Samples (approx. 28 ng of enzyme) were removed at intervals and assayed for residual activity, which is plotted as a percentage of the initial activity. The lines drawn are best fits obtained by non-linear regression and are characterized by inactivation rate constants of 0.2091 min^{-1} (—) or 0.0391 min^{-1} (---).

Fig. 2. Progress curves for the hydrolysis of *p*-nitrophenyl phosphate by alkaline phosphatase in the presence of EGTA

Various amounts of alkaline phosphatase ($[E]_0$) as indicated were added to solutions containing 8 mM-*p*-nitrophenyl phosphate and 10 mM-EGTA, and the absorbance at 479 nm was measure at 15–20 timed intervals. From the absorbance measurements, the concentration of unchanged substrate was calculated and plotted against the time of measurement. The lines represent smooth curves drawn through the experimental points, which have been omitted for the sake of clarity.

Fig. 3. *J* plot for inactivation of alkaline phosphatase by EGTA

From a series of curves such as those shown in Fig. 2, the final substrate concentration ($[A]_t$) was determined and the functions shown on the axes were calculated. The various symbols correspond to different initial substrate concentrations ($[A]_0$): 10 mM (●), 8 mM (○), 6 mM (■), 4 mM (□) and 2 mM (not shown).

demonstrated this protective action of phosphate. The half-life was determined over a range of phosphate concentrations and found to conform to a linear relationship (Fig. 4), as would be expected if the enzyme-phosphate complex is not inactivated by EGTA.

The second feature to note from the *J* plot is that the common straight line that describes the data passes through the origin. This indicates that the enzyme-substrate complex, like the enzyme-product complex, is resistant to inactivation by EGTA. Thus only the free enzyme is affected by EGTA, and in these circumstances the data will be described by eqn. (2):

$$[A]_t = [A]_0 / \exp([E]_0 V_m / j_1 K_a) \quad (2)$$

A plot of $[A]_t$ versus $[A]_0$ (Duggleby, 1986) should consist of a family of straight lines through the origin with a slope that depends on the quantity of enzyme added. This expectation was confirmed (Fig. 5a); the slope of each of the lines was calculated and $\ln(1/\text{slope})$ plotted against the enzyme concentration (Fig. 5b). Again in accord with the theory, this secondary plot yielded a straight line through the origin.

The results presented so far indicate qualitative agreement between the data and a model for alkaline phosphatase in which the free enzyme is inactivated in the presence of EGTA whereas the enzyme-substrate complex and the enzyme-product complex are resistant to the effect of this chelating agent. We now consider whether there is quantitative agreement.

Fig. 4. Stabilization of alkaline phosphatase by phosphate

From a series of experiments such as those shown in Fig. 1, but over a range of concentrations of trisodium phosphate, the half-life of EGTA-induced inactivation was determined. The line drawn corresponds to a situation in which the enzyme-phosphate complex is stable to EGTA and has a dissociation constant of 2.249 mM.

The observation that all points in the *J* plot (Fig. 3) appear to fall on a common straight line is the principal basis for the conclusion that the enzyme-product complex is stable. In order to test the validity of this conclusion, eqn. (1) was fitted to the data, with $[A]_t$ being treated as the fitted variable, by obtaining a numerical solution for the equation. This analysis yielded a value for j_3 of $0.0035 \pm 0.0022 \text{ min}^{-1}$, less than 2% of the estimate of j_1 ($0.1838 \pm 0.0060 \text{ min}^{-1}$), and not significantly different from zero. Thus it is reasonable to conclude that the enzyme-product complex is stable. From the same analysis, the best fit value of j_2' was found to be negative ($-0.0047 \pm 0.0023 \text{ min}^{-1}$); since a negative inactivation rate constant has no physical meaning, we conclude that the enzyme-substrate complex is stable.

It has been shown in Fig. 4 that there is a linear dependence of the half-life for enzyme inactivation on the phosphate concentration. The concentration of phosphate that doubles the half-life should correspond to the dissociation constant for this product, which itself should equal the kinetically determined inhibition constant. The latter value was found to be $2.100 \pm 0.182 \text{ mM}$ (results not shown), in good agreement with the dissociation constant from Fig. 4 of $2.249 \pm 0.057 \text{ mM}$.

The slope of the line in Fig. 5(b) ($1.662 \pm 0.021 \text{ litre} \cdot \text{mg}^{-1}$) should equal $V_m / j_1 K_a$, and from the known value of V_m / K_a (results not shown) of $0.326 \pm 0.011 \text{ litre} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ the inactivation rate constant for the free enzyme was calculated to be $0.1960 \pm 0.0068 \text{ min}^{-1}$. This is in excellent agreement with the directly determined value from Fig. 1 of $0.2091 \pm 0.0040 \text{ min}^{-1}$. An alternative estimate of the value of this rate constant can be obtained from the *J* plot (Fig. 3), which has an expected slope of $j_1 K_a / V_m$. The line drawn in Fig. 3 corresponds to an inactivation rate constant for the free enzyme of 0.1998 min^{-1} .

Thus results from three entirely different types of experiment, namely steady-state kinetics, inactivation studies and progress-curve end points, yield a quali-

Fig. 5. $[A]_t$ plot for inactivation of alkaline phosphatase by EGTA

Experiments were as described in Fig. 3. In (a) the final substrate concentration ($[A]_t$) is plotted against the initial substrate concentration ($[A]_0$) at enzyme concentrations of 0.14 mg/l (○), 0.28 mg/l (●), 0.56 mg/l (□), 0.85 mg/l (■) and 1.13 mg/l (△). Each line is a best fit to the data of a straight line passing through the origin. From these lines $\ln(1/\text{slope})$ was calculated and is plotted in (b) against the enzyme concentration. The line, which has a slope of $1.662 \text{ litre} \cdot \text{mg}^{-1}$, is the best fit to the data of a straight line through the origin.

tatively and quantitatively consistent model in which EGTA inactivates free alkaline phosphatase with a half-life of approx. 3.4 min but is without effect on the enzyme-substrate and enzyme-product complexes.

DISCUSSION

The inactivation of mammalian alkaline phosphatases by chelating agents is well documented (Ackerman & Ahlers, 1976), and that by EDTA has been examined by several groups (Conyers *et al.*, 1967; Ackermann & Ahlers, 1976; Miggiano *et al.*, 1985). From these studies it is evident that the effects of EDTA are not entirely straightforward in that it causes an immediate inhibition as well as a time-dependent inactivation (Conyers *et al.*, 1967). Although substrate has been reported to protect against inactivation (Harkness, 1968), the kinetics are complicated (Ackermann & Ahlers, 1976). For these reasons we sought a different chelating agent that would not cause any immediate inhibition. Although a low concentration of a very potent zinc chelator seemed to offer a possible answer, this introduces the complication

that allowance must be made for depletion of the free chelator as a result of metal binding. The alternative was to seek a non-inhibitory compound that would inactivate only at concentrations vastly in excess of any bivalent metal ions likely to be present. EGTA at a concentration of 10 mM met these criteria and caused first-order inactivation of bovine intestinal mucosal alkaline phosphatase with a rate constant of 0.209 min^{-1} (Fig. 1). As is the case for the *Escherichia coli* enzyme (Garen & Levinthal, 1960), inclusion of phosphate protects against the effect of chelators. We interpret this as indicating that the enzyme-phosphate complex, an intermediate on the catalytic pathway, has the essential zinc atom locked in. This interpretation was supported by the finding that the protective effect of phosphate was associated with a dissociation constant of 2.25 mM (Fig. 4), a value very similar to the kinetically determined inhibition constant for phosphate of 2.10 mM.

The theory that has been developed recently in this laboratory (Duggleby, 1986) provides a method for the detection and quantification of enzyme inactivation. The principal tool of that theory is the J plot, which is constructed from measurements of the residual substrate concentration at infinite time over a range of initial substrate and enzyme concentrations. For alkaline phosphatase, all points on the J plot were found to fall on a common straight line (Fig. 3), which is exactly what is expected when the enzyme-product complex is stable. Moreover, this straight line passes through the origin, which indicates that the enzyme-substrate complex is stable. The slope of the J plot gave an inactivation rate constant for the free enzyme of 0.200 min^{-1} , in good agreement with the directly determined value of 0.209 min^{-1} . Further confirmation of the stability of the enzyme-substrate complex came from an $[A]_t$ plot (Fig. 5a), which, in complete agreement with the theory, consisted of a series of straight lines through the origin with a slope that depended in a quantitatively predictable manner (Fig. 5b) on the enzyme concentration. Thus it is only in the free enzyme that the zinc atom is sufficiently exposed that it can be removed by EGTA; in the enzyme-substrate and enzyme-product complexes the zinc is not released.

For almost any enzyme it is a straightforward matter to study the stability of the free enzyme, regardless of whether any observed losses of activity result from dissociation of an essential cofactor, chemical modification or denaturation due to extremes of pH, high temperatures, detergents and so on. Similarly, the inactivation of certain enzyme-reactant complexes can be measured directly, as was done in the present study for the enzyme-phosphate complex. However, there will almost always be some intermediates on the catalytic pathway that are inaccessible to direct examination because they will progress rapidly and spontaneously through catalysis. For alkaline phosphatase, or indeed almost every other hydrolase, the enzyme-substrate complex is inaccessible to direct measurements of its inactivation.

The theory developed in this laboratory (Duggleby, 1986) is unique in that it provides a means for studying the inactivation of transient complexes. This unique ability derives from the approach of measuring reaction progress curves; all intermediates on the catalytic pathway are simultaneously present, since the method requires that catalysis is proceeding. The present study,

which has shown that EGTA can only remove zinc from alkaline phosphatase when neither substrate nor product is bound, is the first experimental verification of the theory. It can now be applied with confidence to other enzyme stability problems. Such problems include the identification of functional groups that may become non-reactive (protected) or super-reactive at particular stages of the catalytic cycle, and the characterization of suicide substrates, for which the kinetics represent a special case of the theory of unstable enzymes.

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