Measurement of the Zinc Dissociation Constants of Alkaline Phosphatase from *Escherichia coli* by Equilibration with Zinc Ion Buffers*

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ABSTRACT: The dissociation constants for the two zinc ions in alkaline phosphatase from *Escherichia coli* were determined by a new method, the measurement of enzymatic activity after equilibration of the enzyme with different known concentrations of Zn^{2+} maintained by zinc ion buffers. At 25° and pH 8.5 in 1 M NaCl the constants are $K_1 = 10^{-7.66}$ and $K_2 = 10^{-10.22}$ for the stepwise dissociation of the first and the second zinc ions, respectively. The enzymatic activity of the one-zinc form of the enzyme is 0.12 compared to the normal two-zinc form.

The dissociation constant for the 1:1 complex between Zn^{2+} and triply ionized nitrilotriacetic acid was found to be $10^{-9.18}$ in 1 M NaCl at 25°.

Alkaline phosphatase from *Escherichia coli* is a metalloenzyme which consists of two identical polypeptide subunits (Rothman and Byrne, 1963), and which contains two zinc atoms per molecule of enzyme (Plocke *et al.*, 1962). Zinc chelating agents inactivate the enzyme (Garen and Levinthal, 1960; Plocke *et al.*, 1962). With 1,10-phenanthroline this inactivation is due to removal of zinc and activity can be restored by adding Zn^{2+} ions (Plocke and Vallee, 1962).

In the studies reported here this enzyme was equilibrated with zinc ion buffers containing known fixed concentrations of Zn^{2+}, and the activity measured relative to the activity of enzyme which had not been equilibrated with a zinc ion buffer. From the fractional activity of the enzyme as a function of Zn^{2+} concentration, equilibration constants were calculated for the stepwise dissociation of the two zinc ions from the enzyme, and the relative activity of the one-zinc form of the enzyme was estimated.

Materials and Methods

Reagents. Enzyme was Worthington Biochemical Corp. BAP-C (lot no. 6146) prepared from *E. coli* strain C-4 grown in a fortified dextrose medium, 5 mg/ml suspension in 65% saturated ammonium sulfate. The activity given by Worthington is 32 units/mg measured by the procedure of Garen and Levinthal (1960) where 1 unit = 1 μmole of *p*-nitrophenyl phosphate hydrolyzed/min at 25° under defined conditions. Under our different assay conditions the activity is 17 units/mg. Substrate was Sigma 104 *p*-nitrophenyl phosphate (*p*-NPP)[1] disodium salt, tetrahydrate. Eastman no. 5417 nitrilotriacetic acid (NTA) was used as received. Titration with NaOH gives an assay of 100.0 ± 0.3%. $ZnSO_4$ was Merck no. 75213 granular zinc sulfate (ACS grade), carefully calcined and weighed as anhydrous salt. Tris was Fisher T-395 THAM primary standard. Tris–HCl was Sigma Trizma–HCl, anhydrous, reagent grade. Standard buffer was Fisher no. 11-505-230, pH 7.00 ± 0.02 at 25°. All other reagents were reagent grade or ACS grade except the ethanolamine which was Eastman no. 1597.

Solutions. Tris buffer was 0.1 M Tris–Tris·HCl (3:2 mole/mole) in 1 M NaCl; NTA was 0.0124 M in 0.1192 M (3:2) Tris buffer and 1 M NaCl (the NTA was converted to the disodium salt by adding the calculated volume of carbonate-free NaOH solution); zinc stock was 0.062 M $ZnSO_4$ in 1 M NaCl; H_2SO_4 was 2.2 M; ethanolamine was 3.5 M, 5% neutralized with H_2SO_4.

Procedure. A fresh stock solution of enzyme was prepared for each run by adding 50 μl of enzyme suspension to 0.4 ml of Tris buffer. To equilibrate the enzyme with zinc ion buffer, 20 μl of enzyme stock solution was added to 0.5 ml of NTA solution. After 3 min, during which time inhibition became virtually complete, 100 μl of a Zn^{2+} solution (prepared from zinc stock solution and 1 M NaCl) was added and the mixture allowed to equilibrate for 30–120 min (see Table I). During this equilibration period the enzyme activity increased to a steady value. Enzyme activity was measured by adding 0.5 ml of a substrate solution (2 mg/ml of *p*-NPP in Tris buffer) followed after a predetermined hydrolysis period by 1 ml of H_2SO_4 to stop the reaction. A 1-ml aliquot was transferred to a 10-ml volumetric flask containing 4 ml of water, then made

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[1] Abbreviations used: NTA, nitrilotriacetic acid; *p*-NPP, *p*-nitrophenyl phosphate.

TABLE I: Activity of Alkaline Phosphatase Equilibrated with Zinc Ion Buffers.[a]

Run No.	Relative $[Zn^{2+}] = [Zn^{2+}]/K_X'$	pZn	Equilibration Periods (min)	Av Fractional Activity (F)
55	0.00251	11.37	60, 80, 100, 120	0.0075[b]
55	0.00334	11.25	60, 80, 100, 120	0.011[b]
55	0.00502	11.07	60, 80, 100, 120	0.016[b]
54	0.00502	11.07	50, 70, 90	0.016
51	0.0101	10.77	30, 50, 70, 90	0.026
52	0.0204	10.46	30, 50, 70, 90	0.049
53	0.0526	10.05	30, 50, 70, 90	0.077
54	0.111	9.72	30, 50, 70, 90	0.096
51	0.250	9.37	30, 50, 70, 90	0.116
52	0.500	9.07	30, 50, 70, 90	0.16
50	1.00	8.77	30, 50, 70, 90	0.22
53	2.00	8.47	30, 50, 70, 90	0.25
54	3.00	8.29	30, 50, 70, 90	0.27
53	5.00	8.07	30, 50, 70, 90	0.36
51	9.00	7.82	30, 50, 70, 90	0.41
52	19.0	7.49	50, 90	0.52
50	*c*	4.0	30, 50, 70	1.17[d]

[a] Conditions: 25°, pH 8.5, 1 M NaCl, 0.1 M Tris–Tris·HCl buffer, 0.01 M NTA, and various concentrations of $ZnSO_4$. Values of F are ±6%. [b] Hydrolysis period (9 min) for equilibrated enzyme. [c] Enough $ZnSO_4$ added to saturate NTA and provide 10^{-4} M zinc ion in excess. [d] Hydrolysis period 3 min for equilibrated enzyme.

basic with 4 ml of a solution of ethanolamine (5% neutralized to lessen atmospheric oxidation of the *p*-nitrophenate ion at high pH), mixed, and brought to volume with water. The amount of *p*-nitrophenol liberated was determined by measuring the absorbance at 400 mμ using a Hitachi P-E 139 spectrophotometer with 10-mm cuvets. The reference for full enzyme activity in the absence of zinc ion buffer was 20 μl of enzyme stock solution added to 0.6 ml of Tris buffer, incubated, and assayed as above. The substrate blank was 0.5 ml of substrate solution added to 0.6 ml of Tris buffer and assayed as above. For the inhibition control 20 μl of enzyme stock solution was added to 0.5 ml of NTA and the enzyme activity measured immediately after the 3-min inhibition period without adding Zn^{2+}. Before taking the 1-ml aliquot, 0.1 ml of water was added to replace the 100 μl of Zn^{2+} solution which was omitted. The hydrolysis period was always 3 min for the reference. It was 5 min for the equilibrated enzyme, the inhibition control, and the substrate blank in all runs except run 55 where it was 9 min for these solutions, and the pZn 4.0 solutions in run 50 where the hydrolysis period was 3 min for the equilibrated enzyme but 5 min for the blank and control (see Table I). The fractional activity of equilibrated enzyme is the ratio of the rate of increase in absorbance of the solution of the enzyme equilibrated with zinc ion buffer to the rate of increase in absorbance of the reference enzyme solution. No error was introduced by allowing hydrolysis to proceed for different periods of time in these two solutions, because preliminary experiments showed the extent of enzymatic hydrolysis of the substrate is strictly proportional to the hydrolysis time under our experimental conditions.

All solutions and reactions were in a thermostat at 25.0°. The micropipets were treated on the outside with a water-repellent silicone film (Desicote, Beckman Instruments, Inc.) to reduce loss of liquid.

Discussion and Results

The dissociation constants for the two zinc atoms in alkaline phosphatase were found by equilibrating the enzyme with known zinc ion buffers and measuring the fractional enzymatic activity in the buffer. As far as we know this method has not previously been used to determine the dissociation constants for metal ions in metalloenzymes. It has the advantages over equilibrium dialysis, the usual method, of not requiring an exacting technique, of avoiding corrections for complexes between the metal ion in solution and anions or even other proteins in the medium, and especially of making it unnecessary to reduce trace metal contamination to extremely low levels in all the materials used. In addition it permitted us to study the enzyme over a range of four orders of magnitude of zinc concentration, including concentrations far too low to be obtained by other methods, and to measure directly the enzymatic activity of the one-zinc form of the enzyme.

Metal ion buffers are completely analogous to hydrogen ion buffers. They consist of a metal ion complex in solution with the free complexing or chelating agent. (Similarly a hydrogen ion buffer may be thought of as a hydrogen ion complex, the weak Brønsted acid, in solution with free complexing agent, the conjugate Brønsted base.) They may be conveniently prepared by adding a solution of a salt of the metal to a solution containing a stoichiometric excess of a complexing agent. Consider the metal ion, M, and the complexing agent, X, which form the 1:1 complex, MX (charges omitted), that dissociates reversibly $MX \rightleftharpoons M + X$. In an M–X metal ion buffer, the concentration of the metal ion is $M = K_X[MX]/[X]$ where K_X is the dissociation constant of the complex. If the total amount of M used to prepare the buffer is high compared to all other sources of M, the concentration, [M], will be fixed and controlled by the buffer. Furthermore, even if the metal ion forms complexes with other substances present in the solution, the concentration of uncomplexed metal ion, [M], can still be computed from the expression above as though these other complexing agents were not present, if these accidental complexes are much less stable or if the amounts of the accidental complexing agents are small compared to the amount of metal ion which was added. This remains true even though most of the trace of M not present as MX may be present as other complexes. If the

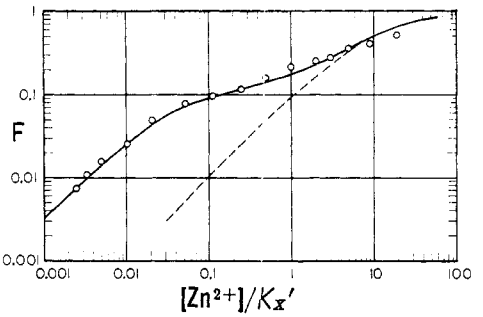

FIGURE 1: Activity of unfractionated enzyme equilibrated with zinc ion buffers. ——— Computed curve for $K_1 = 10^{-7.66}$, $K_2 = 10^{-10.22}$, $\sigma = 0.117$. - - - - Computed curve for $K_1 = 10^{-7.66}$, $K_2 = 0$, $\sigma = 0$. Each data point is an average of several determinations (see Table I).

complexing agent X forms stable complexes with a number of heavy metal ions, the concentration of trace impurities of these ions will be reduced to extremely low levels without disturbing the metal ion buffer. Assume that the concentration of X is 10^{-2} M, and the dissociation constant of the 1:1 complex of X with a multiply-charged cation is 10^{-8} or smaller. (These assumptions approximate the actual conditions of our experiments.) If the heavy metal, Y, with an atomic weight of 50 is present as an impurity of 5 ppm (10^{-7} M), then the concentration of free uncomplexed Y will be $(10^{-8} \times 10^{-7})/10^{-2} = 10^{-13}$ M or less, while the amount of X used up by this process, 10^{-7} M, will be completely insignificant compared to 10^{-2} M.

The alkaline phosphatase was equilibrated with zinc ion buffers using the chelating agent nitrilotriacetic acid, $N(CH_2COOH)_3$ (to be abbreviated NTA or H_3nta). This forms only one important zinc complex, $Znnta^-$ (Bjerrum *et al.*, 1957), which is so stable that with a stoichiometric excess of NTA the concentration of $Znnta^-$ is essentially equal to the total concentration of zinc. Consequently once K_X', the concentration dissociation constant of this complex under the experimental conditions, is known, the concentration of uncomplexed zinc may be calculated from

$$[Zn^{2+}] = Zn_0/([NTA]_0 - [Zn]_0)K_X'$$

where $[Zn]_0$ is the stoichiometric concentration of zinc and $[NTA]_0$ is the stoichiometric concentration of NTA. For these studies we measured K_X' under our experimental conditions (see the Appendix), because it could not be computed with sufficient accuracy from published values in dilute solutions of electrolytes. Because these measurements were made in 1 M $NaNO_3$ solutions where the unchelated Zn^{2+} does not complex with the anions of the supporting electrolyte, this expression gives the concentration of free uncomplexed Zn^{2+} ions, and can be used to compute this concentration even in the 1 M NaCl zinc ion buffers in which the enzyme was studied. Therefore, although much of the trace of unchelated zinc in these buffers exists as chloro complexes, the observed dissociation constants for the enzyme do not have to be corrected for complexing between zinc and chloride ions in solution. Unfortunately NTA binds Zn^{2+} slightly too strongly to be ideal for these studies. Although we could reduce the Zn^{2+} concentration as low as we wished, we could not raise it high enough to study the range above 50% fractional activity of alkaline phosphatase.

The experimental conditions for equilibration of enzyme with the zinc ion buffer—25°, pH 8.50, 1 M NaCl, 0.1 M Tris buffer, and 0.01 M NTA–zinc ion buffer—were chosen to keep the salt concentration high compared to the buffers so that moderate changes in the buffers would not influence any salt effect and to keep the Tris buffer concentration high compared to the NTA concentration so that displacement of hydrogen ion from the NTA (added as the disodium salt) by zinc ion would not change the pH significantly. To reach equilibrium between the enzyme and the zinc ion buffer within 0.5 hr, 0.01 M NTA was necessary.

Equilibrium was not approached from both directions because the net rate of removal of zinc from the enzyme is strongly slowed by even small amounts of zinc in the zinc ion buffer. Instead, equilibrium was always approached from below by first removing the zinc from the enzyme with NTA, then adding zinc sulfate solution to the incubation mixture to produce the desired zinc ion buffer, and finally, after an equilibration period, adding the substrate and measuring the activity. Equilibrium with the zinc ion buffer was indicated by constant fractional activity (within experimental scatter) for four solutions which had been equilibrated for different lengths of time. To minimize irreversible changes in the enzyme both the inhibition period and the equilibration period were kept as short as possible. This procedure was checked with a few preliminary experiments in which equilibrium was approached from both directions. In three studies at pZn 9.72 ($[Zn^{2+}]/K_X' = 0.11$), after 45 min the fractional activity of enzyme placed directly into the zinc ion buffer was constant and the same as the activity of enzyme which had been first completely inhibited with NTA. At pZn 9.37 ($[Zn^{2+}]/K_X' = 0.25$), the activity of enzyme placed directly into zinc ion buffer was still decreasing after 120 min, but the activity at different times was consistent with a first-order decay to the fractional activity of enzyme which had been first inhibited with NTA and then equilibrated with zinc ion buffer. In contrast to the time required to reach equilibrium from above, enzyme was completely inhibited by NTA within 3 min under comparable conditions. [In these studies we noticed that the substrate, *p*-nitrophenyl phosphate, protects alkaline phosphatase from further attack by NTA. This agrees with the observation that the competitive inhibitor orthophosphate protects this enzyme against inactivation by EDTA (Garen and Levinthal, 1960).]

Equilibrium constants were computed for the simplest scheme consistent with the observed relation between fractional enzyme activity and zinc ion concentration (see Figure 1), namely, the stepwise removal of the two zinc ions from the enzyme. The concentration dissocia-

tion constants, K_1 and K_2, for this stepwise dissociation by the reactions

$$E{\cdot}Zn_2 \rightleftharpoons E{\cdot}Zn + Zn^{2+}$$

and

$$E{\cdot}Zn \rightleftharpoons E + Zn^{2+}$$

(charges omitted from enzyme) are defined as

$$K_1 = [E{\cdot}Zn][Zn^{2+}]/[E{\cdot}Zn_2] \quad (1)$$

and

$$K_2 = [E][Zn^{2+}]/[E{\cdot}Zn] \quad (2)$$

The fractional activity of the enzyme in equilibrium with a zinc ion buffer is given by

$$F = \frac{[Zn^{2+}]^2/K_1 + \sigma[Zn^{2+}]}{[Zn^{2+}]^2/K_1 + [Zn^{2+}] + K_2} \quad (3)$$

where F is the fractional activity, K_1 and K_2 are the concentration dissociation constants for the first and second zinc ions as defined by eq 1 and 2 above, and σ is the relative activity of the one-zinc form of the enzyme compared to the two-zinc form. In accordance with our experimental results and those of others, the zinc-free form is taken to be inactive. We found it convenient to make all measurements and calculations relative to K_X', the dissociation constant of the Zn^{2+}–NTA chelate. In these terms eq 3 becomes

$$F = \frac{([Zn^{2+}]/K_X')^2(K_X'/K_1) + \sigma([Zn^{2+}]/K_X')}{([Zn^{2+}]/K_X')^2(K_X'/K_1) + ([Zn^{2+}]/K_X') + (K_2/K_X')} \quad (4)$$

Alkaline phosphatase at a concentration of 18 μg/ml was equilibrated with zinc ion buffers with pZn ranging from 7.49 to 11.37. The average fractional activity for the several equilibration times is listed in Table I. The best values for the parameters (K_1/K_X'), (K_2/K_X'), and σ in eq 4 were found by trial and error. These are: $(K_1/K_X') = 13$, $(K_2/K_X') = 0.036$, and $\sigma = 0.12$. Taking K_X' as $10^{-8.77}$ (see the Appendix) gives $K_1 = 10^{-7.66}$ and $K_2 = 10^{-10.22}$. The solid curve in Figure 1 was computed from these values. Data points from Table I are shown for comparison. Logarithmic scales are used to accommodate the large range of values. It is hard to estimate the precision of the parameters because of the complicated way in which they appear in eq 4. From the variation of the fit of the calculated curve with different trial values, we estimate pK_1 and pK_2 to be precise within 0.1 or 0.15 pK unit, and σ to be precise within 0.01 unit. The ratio σ/K_2, which is the quantity least affected by adjustment of the parameters, is probably precise to within 6% relative error.

The precision of the average fractional activity, F, in Table I is $\pm 6\%$ relative error with no significant variation with pZn. This is roughly 10 times the variation observed in dummy runs in which enzyme and substrate were replaced by a *p*-nitrophenol solution. We have no explanation for this. To eliminate the effect of any slow drift in the properties of the enzyme, the zinc ion buffers were used in random order.

We checked our interpretation of the data by considering various simpler equilibrium schemes which do not require both enzymatic activity of the one-zinc form of the enzyme and stepwise dissociation of the two zinc ions from the enzyme. None of these simpler possibilities could be made to fit the data. The consequences of these various simpler schemes are best seen by making the corresponding simplifying modification in eq 4. If both σ and K_2 are assumed to be 0 (*i.e.*, if the one-zinc form is assumed to be inactive and if the second dissociation may be neglected), the expected fractional activity will follow the broken curve shown in Figure 1. Changing the data point through which this one-parameter curve passes cannot improve the agreement with data because it merely displaces the curve horizontally without changing the shape. If K_2 is assumed to be greater than 0 (*i.e.*, if the second dissociation is considered) with σ still assumed to be 0, the agreement is even poorer, because the expected fractional activity is less for all values of $[Zn^{2+}]$. The poorest correspondence is obtained in the limit of so large a value of K_2 that the equilibrium process becomes the one-step removal of both zinc ions. (In this case it does not matter whether the one-zinc form has activity or not.) If σ is 0, K_2 must be negative to fit the data, which is meaningless. Even for σ greater than 0, K_2 must also be greater than 0 to fit the data, because for K_2 equal to 0 the expected fractional activity will asymptotically approach the horizontal line, $F = \sigma$, at low zinc concentration, and not drop more rapidly as our data do below a relative zinc concentration of 0.1. From this analysis above it is clear that the data can be explained by an equilibrium scheme with stepwise dissociation of the zinc ions and enzymatic activity of the one-zinc form, but cannot be explained by any simpler scheme. The behavior of the enzyme at very low zinc ion concentrations is also consistent with our proposed scheme. In this region, where $([Zn^{2+}]/K_X')^2$ becomes small compared to $\sigma([Zn^{2+}]/K_X')$, and both $([Zn^{2+}]/K_X')^2$ and $[Zn^{2+}]/K_X'$ become small compared to (K_2/K_X'), eq 4 reduces to $F = \sigma([Zn^{2+}]/K_X')(K_X'/K_2)$, or logarithmically to $\log F = \log([Zn^{2+}]/K_X') + \log(\sigma K_X'/K_2)$, and the log–log graph of the fractional activity against concentration of zinc ion should approach a straight line of unit slope as it actually does (Figure 1).

Our dissociation constants, as is usual in studies of enzymes, are concentration constants, rather than true, thermodynamic constants and, therefore, may be different under different equilibrium conditions. The size of the dissociation constants, especially K_2, suggests that the zinc is attached by at least two groups as the binding is much stronger than the observed binding between zinc ions and unidentate ligands. The constants tell little about the bonding groups, because the zinc complexes of a large number of polydentate ligands, especially those with amino groups, or carboxylic acid groups, or both, have dissociation constants in the observed range (Bjerrum *et al.*, 1957.)

It is clear from the observed fractional enzymatic

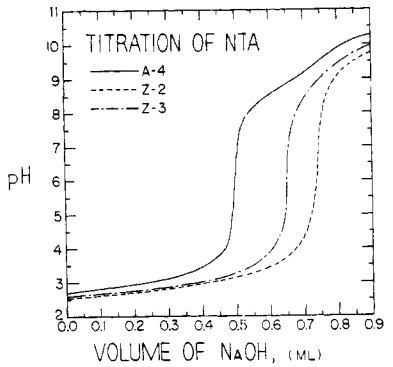

FIGURE 2: pH titration of nitrilotriacetic acid with and without added $ZnSO_4$. A-4 1 M $NaNO_3$, 0.001 M NTA; Z-2 1 M $NaNO_3$, 0.001 M NTA, 0.001 M $ZnSO_4$; Z-3 1 M $NaNO_3$, 0.001 M NTA, 0.0006 M $ZnSO_4$.

activity at low Zn^{2+} concentrations (*e.g.*, relative zinc ion concentration 0.2 or less) that NTA inhibits alkaline phosphatase by removing the zinc rather than forming a complex with the enzyme. If it had formed a complex with the enzyme, the addition of small amounts of Zn^{2+} compared to the amount of NTA present would not have restored partial activity, because the amount of free NTA used up as the Zn^{2+}–NTA complex would have been too small to cause the NTA–enzyme complex to dissociate significantly. Previous studies have shown that complexing agents can remove zinc from *E. coli* alkaline phosphatase (Plocke and Vallee, 1962), but have not demonstrated that the inhibition is due to the removal of zinc. This is a point of some interest for two reasons: because the use of metal ion buffers depends upon the removal of metal from the metalloenzyme by the complexing agent, and because it is possible that in some cases (see, *e.g.*, Plocke *et al.*, 1962) the metal complexing agent inhibits by forming a complex with the enzyme rather than by removing the metal ion.

To test whether the enzyme activity could be completely restored by adding zinc ions to the inhibited enzyme, 102% of the amount of zinc sulfate needed to saturate the NTA was added in one run, producing a concentration of 10^{-4} M excess zinc ion (much of it probably as chloro complexes) after the addition of substrate. The fractional activity was 1.17, showing not only complete restoration of activity but enhancement of the activity by low concentrations of zinc ion.

Appendix. The Dissociation Constant of the Zn^{2+}–NTA Chelate

Following the method used to find the constants for the Fe^{2+}–NTA and Mn^{2+}–NTA chelates (Schwarzenbach and Freitag, 1951), the dissociation constant for zinc chelate was found from pH titrations of nitrilotriacetic acid solutions in the presence and absence of Zn^{2+}. (Three procedures are discussed and illustrated by Schwarzenbach and Freitag. We used the first one rather than the one used by the authors to study the Zn^{2+}–NTA chelate.) This method depends on the apparent increase in K_{a_3}, the ionization constant of the 3rd hydrogen of nitrilotriacetic acid, by the displacement equilibrium

$$H\mathit{nta}^{2-} + Zn^{2+} \rightleftharpoons Zn\mathit{nta}^{-} + H^{+}$$

The calculations were greatly simplified because the first two hydrogens are completely ionized, and only the 1:1 complex need be considered (Bjerrum *et al.*, 1957). Seven solutions were titrated: A-1 and A-2 which were 1 M in NaCl and 0.001 M in NTA; A-3 and A-4 which were 1 M in $NaNO_3$ and 0.001 M in NTA; Z-1 and Z-2 which were 1 M in $NaNO_3$, 0.001 M in NTA, and 0.001 M in $ZnSO_4$; and Z-3 which was 1 M in $NaNO_3$, 0.001 M in NTA, and 0.0006 M in $ZnSO_4$, all CO_2 free. The titrant was CO_2-free 0.2008 M NaOH. The base was added with constant stirring from a 1-ml Gilmont micropipet buret, to 50 ml of each solution thermostated at 25.0°. pH was measured with a Corning Model 12 pH meter using a Corning no. 476022 glass electrode and a Corning no. 476002 calomel electrode. The instrument was standardized against pH 7.00 reference buffer before and after each titration. Increments (10 μl) of base were added in the region from the second hydrogen end point to the third hydrogen end point and 25-μl increments were added elsewhere. Figure 2 presents graphs of titrations A-4, Z-2, and Z-3. For clarity data points are omitted. The scatter does not exceed the rated precision of the instrument, ± 0.02 pH unit.

The measured ionization constant for the third hydrogen is best described as a modified Sørensen constant defined by

$$K_{a_3} = 10^{-pH}[\mathit{nta}^{3-}]/[H\mathit{nta}^{2-}] \approx a_{H^+}[\mathit{nta}^{3-}]/[H\mathit{nta}^{2-}]$$

that is nta^{3-} and $H\mathit{nta}^{2-}$ enter the equilibrium expression as concentrations while the H^+ ion enters as an approximate hydrogen ion activity. Solutions A-1 and A-2 give values of 8.70 ± 0.01 and 8.71 ± 0.01, respectively, for pK_{a_3} in 1 M NaCl supporting electrolyte, while solutions A-3 and A-4 give values of 8.65 ± 0.01 and 8.67 ± 0.01, respectively, in 1 M $NaNO_3$. Each value is the average of several values and the uncertainty is the standard deviation. Typical published values for pK_{a_3} at 20° are 9.73 in 0.1 M KCl or KNO_3 and 10.70 at zero ionic strength (Bjerrum *et al.*, 1957). The observed specific salt effect of 0.04 pH unit is satisfactorily small. Measurement of the specific salt effect was important because the enzyme was studied in 1 M NaCl while the stability of the chelate was measured in 1 M $NaNO_3$ to eliminate complications from competing chloro complexes. Sodium perchlorate, which would have been better than $NaNO_3$, could not be used because $KClO_4$ would have precipitated at the electrode salt bridges. Using an average pK_{a_3} of 8.66, pK_X for the dissociation of the $Zn\mathit{nta}$–chelate is computed to be 9.10 ± 0.03, 9.26 ± 0.03, and 9.19 ± 0.07 from solutions Z-1, Z-2, and Z-3, respectively, giving

an average value of 9.18 ± 0.06; as before each value is an average of several values and the uncertainty is the standard deviation. The reported value in 0.1 M KNO_3 at 20° is 10.67 (Bjerrum *et al.*, 1957). K_X is best regarded as the stoichiometric dissociation constant

$$K_X = \frac{[Zn^{2+}][nta^{3-}]}{[Znnta^-]}$$

although ionic activities enter in a complicated fashion, from implicit approximations in the method of computation.

Although in our equilibrium expressions we have written $[nta^{3-}]$ and $[Hnta^{2-}]$ as though these were free and uncomplexed ions, they really represent the total concentration of the free ion plus the various complexes of the ion with sodium ion. Because of the same large excess of sodium ion, the distribution of free ions of NTA and the various sodium complexes of NTA remains constant and the same in both the zinc ion buffers used to study the enzyme and the solutions used to measure the dissociation constant of the zinc chelate. Consequently these equilibrium constants containing the effect of the sodium complexes are the correct ones to use. This competition of sodium ion with both hydrogen ion and zinc ion may be one reason for our values for pK_{a_3} and pK_X being markedly less than literature values for low salt concentration.

At the pH 8.50 of the zinc ion buffers used, the free nitrilotriacetic acid (besides existing partly as various sodium complexes) is a mixture of doubly and triply ionized forms. The required equilibrium constant is not K_X but

$$K_X' = \frac{[Zn^{2+}]([nta^{3-}] + [Hnta^{2-}])}{[Znnta^-]} = K_X\left(1 + \frac{10^{-pH}}{K_{a_3}}\right)$$

Using $pK_{a_3} = 8.70$ for NTA in 1 M NaCl, pK_X' is computed to be 8.77.

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