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Kinetics and Mechanism of Inhibition of *Escherichia coli* Alkaline Phosphatase by Permanganate Ion†

R. A. Thomas and Jack F. Kirsch*

ABSTRACT: The interaction between *Escherichia coli* alkaline phosphatase (EC 3.1.3.1) and permanganate ion has been investigated. The kinetics of the inactivation of this enzyme by permanganate ion at pH 9.0 and 25 °C are described by a two-step mechanism in which the rapid reversible formation of a noncovalent enzyme–permanganate complex with a dissociation constant of 87 μM is followed by a first-order transformation of the complex to a second form with a first-order rate constant of 1.5 min^{-1}. The dissociation rate constant from the final inactive complex is 0.007 min^{-1}, giving an overall dissociation constant of the inactive enzyme–permanganate complex of (0.007/1.5)87 μM = 0.4 μM. The inactivation is inhibited by inorganic phosphate ion, suggesting that the association with permanganate ion most likely occurs at the active site. The effect of phosphate concentration on the inhibition of permanganate inactivation suggests a mechanism where permanganate can inactivate both the free enzyme and enzyme which has only one of the two active sites occupied by phosphate with the latter process occurring at ~40% of the rate of the former. The tetrameric enzyme, formed by dimer association at pH >7 and Zn(II) concentration >10 μM, was inactivated by permanganate with the same rate constant as the dimer. Inactivation by permanganate was significantly more rapid in preparations with low Mg(II) content. Permanganate ion does not act as an oxidant in this process since total enzyme activity is recoverable by simple dilution or by extensive dialysis of the inactivated enzyme. Thiols enhance the rate of reactivation of the permanganate-inactivated enzyme. The effect of thiol concentration on the reactivation rates suggests a mechanism where thiol rapidly reduces enzyme-bound permanganate, thus bypassing the slower dissociation step of permanganate from the inactive permanganate–enzyme complex. The slowness of the overall reaction and the two-step nature of the process suggest the possibility of a reversible covalent bond formed between permanganate ion and some residue at the active site of the enzyme—such as a manganate ester or pentacovalent adduct with the active-site serine residue.

Escherichia coli alkaline phosphatase (EC 3.1.3.1) is a zinc metalloenzyme which catalyzes the hydrolysis of phosphate monoesters. Inorganic phosphate, the product of the hydrolysis, is both a potent inhibitor and, as evidenced by ^{18}O studies (Schwartz, 1963; Applebury et al., 1970), a pseudosubstrate. Permanganate ion was examined as a possible chromophoric analogue of phosphate for transient kinetic studies with this enzyme, since Benisek (1971) had pointed out that MnO_4^- acted as a structural analogue of phosphate with aspartate transcarbamylase. Ohlsson & Wilson (1974) showed that $KMnO_4$ slowly and apparently irreversibly inactivates alkaline phosphatase in a reaction which is suppressed by phosphate, suggesting the possibility that MnO_4^- reacts at the active site. They also noted that this reaction could be reversed by the addition of thiols but were unable to distinguish between a mechanism of inactivation by oxidation vs. a stable complex formation. We report here a detailed study of the kinetics and equilibria of the interaction of $KMnO_4$ with alkaline phosphatase, including quantitative analyses of the phosphate protection and the thiol-mediated reactivation mechanism.

Materials and Methods

Enzyme Purification and Assay. Alkaline phosphatase was prepared from *E. coli* strain C90F1 by using the method of Schlesinger & Olsen (1970). The purified enzyme was stored frozen at a concentration of 5 mg/mL. The specific activity and response to MnO_4^- were unaffected by freezing. Before use, the thawed enzyme was dialyzed against 0.1 M $NaHCO_3$–Na_2CO_3, 1 mM $MgCl_2$, and 0.1 μM $ZnCl_2$ at pH 9.0 and 20 °C. The purified enzyme had a specific activity of 30 μmol of product released per min per mg of protein when assayed with 1 mM *p*-nitrophenyl phosphate in 1 M NaCl and 0.1 M Tris-HCl, pH 8.0 at 25 °C (Halford, 1972). The molar absorbancy of *p*-nitrophenolate was taken to be 16 700 at this pH (Halford, 1971). Product release was followed by using a Unicam SP 800A spectrophotometer equipped with a Texas Instruments recorder.

Protein concentration was determined at 280 nm by using $E_{1cm}^{0.1\%} = 0.72$ as determined by Malamy & Horecker (1964) for the crystalline enzyme. A molecular weight of 86 000 was used for molar concentrations (Applebury & Coleman, 1969).

Reagents. $KMnO_4$ was obtained from Matheson Coleman and Bell, mercaptoethanol was from Aldrich, and *p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate) and Tris base (Trizma) were purchased from Sigma Chemical Co. All other chemicals were reagent grade products of Mallinckrodt.

All $KMnO_4$ studies were done in 0.1 M $NaHCO_3$–Na_2CO_3, 1 mM $MgCl_2$, and 0.1 μM $ZnCl_2$ at pH 9.0, hereafter referred to as 0.1 M bicarbonate pH 9 buffer. Water used for the buffer was distilled from alkaline permanganate, and buffer solutions were filtered through fine-porosity sintered glass before use. It was found that the organic amine buffers commonly used at pH 9 would reduce MnO_4^-.

† From the Department of Biochemistry, University of California, Berkeley, California 94720. *Received January 18, 1980; revised manuscript received June 30, 1980.* This investigation was supported by a grant from the National Science Foundation.

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Scheme I

$$E + MnO_4^- \underset{fast}{\overset{K_s}{\rightleftharpoons}} E{\cdot}MnO_4^- \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} E{*}MnO_4^-$$

Recent studies have indicated that Mg(II) must be included in order to obtain maximal enzymatic activity and stability of the enzyme (Anderson et al., 1975; Bosron et al., 1975, 1977). To prevent loss of catalytically important Zn(II) (Cohen & Wilson, 1966), we added 0.1 μM Zn(II) to all solutions except where otherwise noted. Final working solutions of $KMnO_4$ were prepared by at least a 100-fold dilution of a concentrated stock solution immediately before use.

Glassware. All vessels used with the dilute $KMnO_4$ solutions were soaked in a concentrated H_2SO_4–HNO_3 (1:1) bath, rinsed with water distilled from alkaline permanganate, and dried in a dust free environment. These precautions were found necessary to oxidize trace contaminants which reduce some of the MnO_4^-, giving rise to variable results.

Incubation of Alkaline Phosphatase with $KMnO_4$. Typically, to a small volume of $KMnO_4$ in 0.1 M bicarbonate pH 9 buffer was added an equal volume of alkaline phosphatase solution to give a final concentration of 1 μM enzyme and from 10 to 40 μM $KMnO_4$. It was found necessary to dialyze exhaustively the 2 μM enzyme stock solution against 0.1 M bicarbonate pH 9 buffer before exposure to permanganate in order to obtain reproducible results. While incubating at 25 °C, suitable aliquots were withdrawn periodically and diluted at least 100-fold into 1 mM *p*-nitrophenyl phosphate buffered by 0.1 M bicarbonate pH 9 buffer for the activity assay. The dilution prevented further inactivation by $KMnO_4$, and no reactivation was observed in the short time period required for the assay.

Data Analysis. Values of the determined parameters and associated standard errors were determined by nonlinear regression analysis using program NLIN of the University of California Computer Center.

Results

The data and discussion which follow relate to the rate and equilibrium constants shown in Scheme I for the interaction of alkaline phosphatase with MnO_4^-, where $E{\cdot}MnO_4^-$ is a Michaelis complex formed in a rapid equilibrium process from the free enzyme (denoted by E) and MnO_4^- with dissociation constant, K_s. The complex undergoes a slow transformation to give a second form, $E{*}MnO_4^-$, with rate constant k_1. The latter process is also reversible with the rate constant k_{-1}.

Rate of Inactivation of Alkaline Phosphatase by MnO_4^-. Figure 1 shows the dependence of the rate of inactivation of alkaline phosphatase upon $[MnO_4^-]$. The rate constant was calculated from the initially rectilinear portion of the progress curve. It was found necessary to perform these experiments with the same enzyme sample on the same day in order to obtain internally consistent results. The variability in inactivation rate constants often differed by as much as 50% between batches of enzyme. It should be noted that Scheme I does not account for the observation that the lines of the progress curve when extended back to the time of mixing do not intercept the axis at 100% initial activity. This appears to be the result of a very rapid initial loss of up to 25% of the initial activity on mixing enzyme and MnO_4^-. This is followed by a first-order decay of the remaining enzyme activity, since $[MnO_4^-]$ initially is at least in a 10-fold molar excess over enzyme, to give pseudo-first-order kinetics. Further, the basic Scheme I does not account for the curvature seen at higher $[MnO_4^-]$ toward the end of the inactivation process (Figure 1, 30 and 40 μM MnO_4^-). These results are considered further under Discussion.

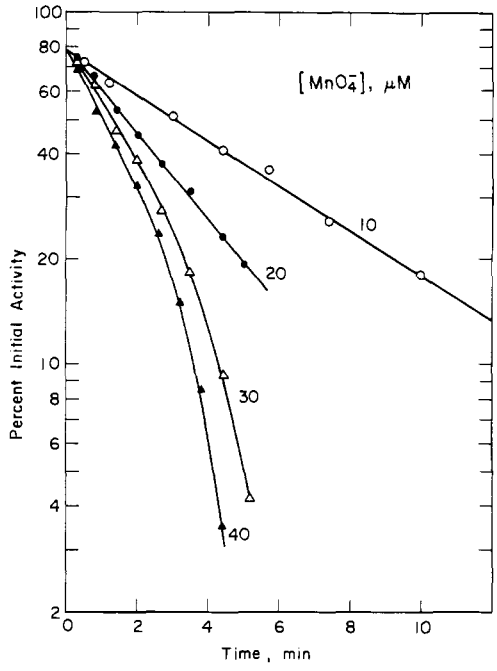


FIGURE 1: The kinetics of the MnO_4^--induced inactivation of alkaline phosphatase. To a 2.0 μM solution of enzyme in 0.1 M bicarbonate pH 9 buffer (see Materials and Methods) was added an equal volume of $KMnO_4$ to give the desired final incubation mixture of 1.0 μM enzyme and the indicated concentration of permanganate. Incubation was done at 25 °C. 25-μL aliquots were diluted 100-fold into a cuvette containing 1.0 mM *p*-nitrophenyl phosphate buffered by 0.1 M bicarbonate pH 9 buffer for assay of remaining activity.

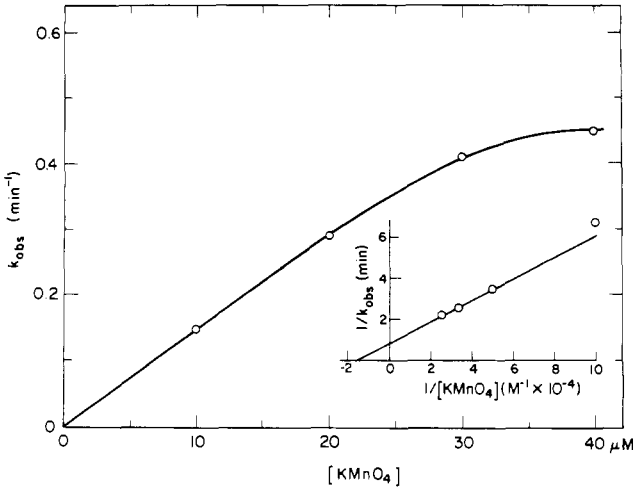


FIGURE 2: The observed first-order rate constants for the experiment of Figure 1 plotted as a function of permanganate concentration. (Inset) Double-reciprocal plot of the data. The solid lines are drawn for a dissociation constant K_s = 87 μM and limiting rate constant for inactivation of 1.5 min^{-1}.

Evidence for an intermediate $E{\cdot}MnO_4^-$ complex is presented in Figure 2, where a maximal rate constant for inactivation is approached at high MnO_4^- concentration. The dissociation constant for permanganate and the maximal inactivation rate constant were calculated from the least-squares fit to eq 1.

$$k_{obsd} = \frac{k_1[MnO_4^-]}{K_s + [MnO_4^-]} \quad (1)$$

Equation 1 is readily derived from Scheme I with the restriction $k_{-1} \ll k_1$. Under the experimental conditions employed here, no reactivation was observed. The apparent

Table I: Effect of Endogenous Phosphate on the Rate Constant for Inactivation of Alkaline Phosphatase by Permanganate Ion

enzyme sample[a]	k_{obsd} (min^{-1})
native	0.19
phosphate free[b]	0.18
reconstituted[c]	0.16

[a] The experiments were performed as described under Materials and Methods. 1 μM alkaline phosphatase was inactivated by 10 μM $KMnO_4$. [b] The protocol outlined by Bloch & Schlesinger (1973) was followed to remove endogenous phosphate. [c] 1.25 equiv of Na_2HPO_4 was added to a second sample of the phosphate-freed enzyme.

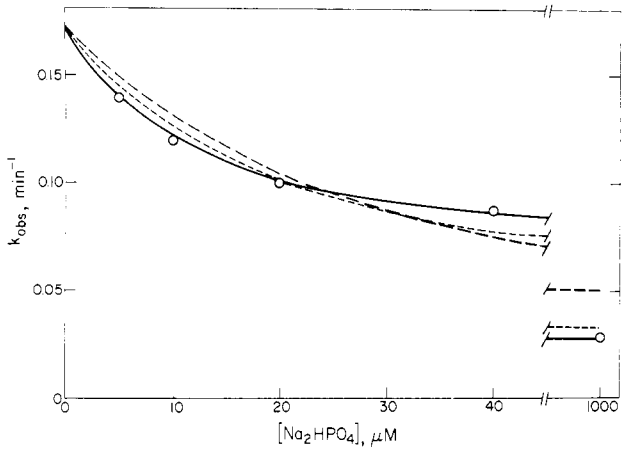


FIGURE 3: Inhibition of the MnO_4^--mediated inactivation of alkaline phosphatase by inorganic phosphate. Incubations were performed as described under Materials and Methods by using 1 μM enzyme, 10 μM $KMnO_4$, and the indicated concentrations of Na_2HPO_4. The experimental data are depicted by the open circles. The theoretical curves represent the least-squares fits to Schemes II (heavy dashes), III (light dashes), and IV (solid line).

Scheme II

$$E + P_i \overset{K_{1p}}{\rightleftharpoons} E{\cdot}P_i$$

$$E + MnO_4^- \xrightarrow{k_1/K_s} E{*}MnO_4^- \text{ (inactive)}$$

dissociation constant K_s is 87 ± 33 μM, and the maximal rate constant for inactivation k_1 is 1.5 ± 0.5 min^{-1}.

Effect of Inorganic Phosphate. Bloch & Schlesinger (1973) have found that alkaline phosphatase purified from *E. coli* contains up to two tightly bound inorganic orthophosphate ions per enzyme molecule. The data in Table I indicate no significant difference in the inactivation rate between the native enzyme, the phosphate-freed (purged) enzyme, and a sample of purged enzyme reconstituted with inorganic phosphate. Excess inorganic phosphate decreases the rate constant for the reaction of $KMnO_4$ with alkaline phosphatase as shown in Figure 3. Three mechanisms to account for the data were evaluated. The first (Scheme II) assumes that only free enzyme is inactivated by MnO_4^-; i.e., binding of inorganic phosphate to form the $E{\cdot}P_i$ complex excludes MnO_4^- and subsequent formation of the inactive complex. The observed rate constants for the inactivation by MnO_4^- as a function of the concentrations of phosphate and MnO_4^- are given by eq 2 (Appendix I). The value of k_1/K_s = 1.5 min^{-1}/87 μM =

$$k_{obsd} = \frac{(k_1/K_s)[MnO_4^-]}{1 + [P_i]/K_{1p}} \quad (2)$$

1.73 × 10^4 M^{-1} min^{-1} was fixed from the results of the data in Figures 1 and 2. The theoretical curve for this scheme is represented by the heavy dashes in Figure 3.

Scheme III

$$E + P_i \overset{K_{1p}}{\rightleftharpoons} E{\cdot}P_i$$

$$E + MnO_4^- \xrightarrow{k_1/K_s} E{*}MnO_4^-$$

$$E{\cdot}P_i + MnO_4^- \xrightarrow{k_{1p}/K_{sp}} E{\cdot}P_i{*}MnO_4^-$$

Scheme IV

$$E + P_i \overset{K_{1p}}{\rightleftharpoons} E{\cdot}P_i$$

$$E{\cdot}P_i + P_i \overset{K_{2p}}{\rightleftharpoons} E{\cdot}(P_i)_2$$

$$E + MnO_4^- \xrightarrow{k_1/K_s} E{*}MnO_4^-$$

$$E{\cdot}P_i + MnO_4^- \xrightarrow{k_{1p}/K_{sp}} E{\cdot}P_i{*}MnO_4^-$$

In an attempt to find a model which would better fit the data (especially for higher phosphate concentrations) the model shown in Scheme III where MnO_4^- also inactivates phosphate-bound enzyme, but perhaps at a rate constant different from that for the free enzyme, was evaluated.

Equation 3 for the rate of inactivation (see Appendix I) applies to Scheme III. The minimizing values of the ad-

$$k_{obsd} = \left(\frac{(k_1/K_s)K_{1p} + (k_{1p}/K_{sp})[P_i]}{K_{1p} + [P_i]} \right)[MnO_4^-] \quad (3)$$

justable parameters are K_{1p} = 20 ± 3 μM and k_{1p}/K_{sp} = (2.9 ± 0.7) × 10^3 M^{-1} min^{-1} (given that k_1/K_s = 1.73 × 10^4 M^{-1} min^{-1}). These parameters for Scheme III (light dashes, Figure 3) give a much improved fit to the experimental data, especially at 1 mM phosphate.

Alkaline phosphatase can reversibly bind a second phosphate ion under certain conditions (Block & Bickar, 1978; Otvos et al., 1979). Scheme IV takes this factor into account. Scheme IV differs from Scheme III in that some of the enzyme may exist in the $E{\cdot}(P_i)_2$ form, which is assumed not to react with MnO_4^- at an appreciable rate. The derivation of eq 4 describing the kinetics for Scheme IV is given in Appendix I.

$$k_{obsd} = \left(\frac{(k_1/K_s) + (k_{1p}/K_{sp})[P_i]/K_{1p}}{1 + [P_i]/K_{1p} + [P_i]^2/K_{1p}K_{2p}} \right)[MnO_4^-] \quad (4)$$

The best estimates for the parameters were found to be k_{1p}/K_{sp} = (6.8 ± 0.6) × 10^3 M^{-1} min^{-1}, K_{1p} = 10 ± 1.5 μM, and K_{2p} = 0.67 ± 0.10 mM. The calculated curve for these values gives an excellent fit to the experimental data throughout the phosphate concentration range employed (solid line, Figure 3).

The significance of including the parameter K_{2p} in the above model was evaluated by considering the extent to which the residual sum of squares is reduced when this parameter is included. The difference in the residual sum of squares obtained from Schemes III and IV is compared with an estimate of the variance of the errors by an $F(1,3)$ test in order to test the hypothesis $H_0/K_{2p} = \infty$ (Draper & Smith, 1966). Formally the test is for $H_0/(1/K_{2p}) = 0$. The calculated value of F_c is 26.2, and the hypothesis $H_0/(1/K_{2p}) = 0$ can be rejected at the 97.5% confidence level since $F(1,3,0.975) = 17.4$. Thus it appears statistically significant to include the binding of a second phosphate ion in the model.

Effect of Zn(II) and Mg(II). Reynolds & Schlesinger (1969) have demonstrated the self-association of alkaline phosphatase dimers to form a tetramer at pH >7 and Zn(II) concentrations >10 μM. The rate of inactivation by permanganate is uninfluenced by Zn(II) concentration over the

Table II: Effect of Zn(II) and Mg(II) on the Rate Constant for Inactivation of Alkaline Phosphatase by Permanganate Ion

enzyme sample	k_{obsd} (min^{-1})
dimer [0.1 μM Zn(II)][a]	0.31
tetramer [100 μM Zn(II)][a]	0.33
dialyzed (no $MgCl_2$)[b]	0.48
dialyzed (1 mM $MgCl_2$)[b]	0.20

[a] The inactivation was performed as described under Materials and Methods. 1 μM alkaline phosphatase was inactivated by 10 μM $KMnO_4$ in the presence of the indicated Zn(II) concentration. [b] A different enzyme preparation from that used in the Zn(II) experiments was employed.

range where the protein is expected to form tetramers (Table II).

Recent studies have clarified the role of the Mg(II) ion in stabilizing and in modulating the enzymatic activity of alkaline phosphatase (Anderson et al., 1975; Bosron et al., 1975, 1977). We found that when an enzyme sample which had been exhaustively dialyzed at 25 °C against 0.1 M bicarbonate pH 9 buffer without 1 mM $MgCl_2$ was assayed and compared to a sample to which 1 mM $MgCl_2$ was included, the former not only had a lower specific activity (12 compared to 18 units/mg) but also was inactivated by MnO_4^- almost 2.5 times more rapidly, as shown in Table II. These results further demonstrate that Mg(II) is protecting the enzyme against MnO_4^- inactivation.

Reversibility of the Permanganate-Induced Inactivation. Preliminary experiments showed some slow recovery upon dilution of inactive E*MnO_4 complex. Complete recovery is obtained on exhaustive dialysis. An enzyme solution (14 μM) which had been completely inactivated by 4 mol equiv of MnO_4^- (56 μM) was placed in a dialysis bag, which had been previously treated with MnO_4^- in order to oxidize reducing contaminants, and exhaustively dialyzed against 0.1 M bicarbonate pH 9 buffer. Protein and activity assays performed on the dialyzed sample and another dialyzed sample of the enzyme which had not been treated with MnO_4^- showed complete restoration of the original specific activity of the enzyme. This result shows conclusively that the reactivation process is not a reduction of MnO_4^--oxidized enzyme but represents the slow dissociation of a very tight complex between the enzyme and MnO_4^-. This conclusion is further supported by the observation that the rate for reactivation of MnO_4^--inhibited enzyme is enhanced by the addition of a quantity of neutral protein presumably acting as a scavenging agent for the nascent released MnO_4^-. In a typical experiment it was found that 5 μg/mL chymotrypsinogen enhanced the rate of reactivation of a 1 μg/mL solution of permanganate-inactivated alkaline phosphatase some three- to fivefold.

While our work was in progress, Ohlsson & Wilson (1974) reported the observation of strong inhibition of *E. coli* alkaline phosphatase by MnO_4^- and the complete restoration of enzymatic activity by added low concentrations of mild reducing agents, but they were unable to distinguish between a mechanism in which the reducing agents reactivated oxidized enzyme or simply trapped MnO_4^- released from an enzyme–MnO_4^- complex. The fact that reactivation is achieved by simple dilution unambiguously excludes the former proposal.

Dissociation Rate Constant. Solutions of 1 μM alkaline phosphatase were inactivated completely by treatment with 10 μM $KMnO_4$ for 30 min. Samples were diluted 100-fold into buffer and assayed for recovery of activity as a function of time. The reappearance of activity was observed to be first order with k_{obsd} = 0.007 min^{-1}. This is the value of k_{-1} in Scheme I.

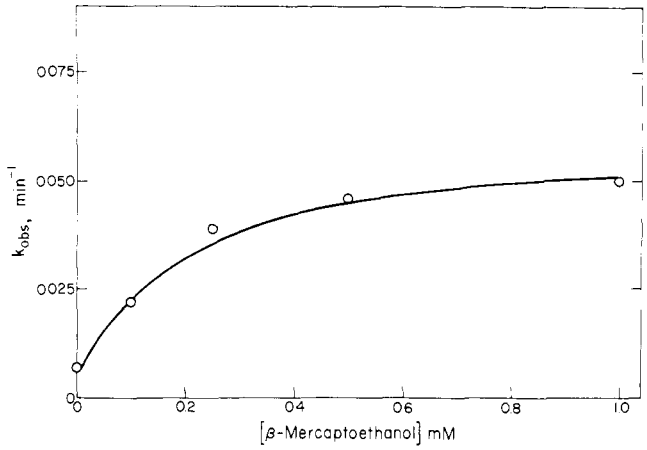
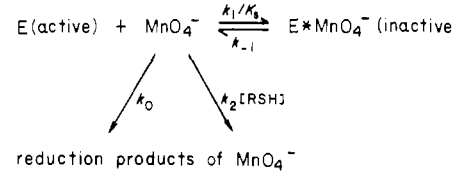


FIGURE 4: The thiol-mediated reactivation of the enzyme–permanganate complex. A solution of 1 μM alkaline phosphatase was inactivated by incubation with 10 μM $KMnO_4$ until all activity was lost, after which 25-μL aliquots of the mixture were diluted 100-fold into β-mercaptoethanol solutions at the indicated concentrations buffered by 0.1 M bicarbonate pH 9 and the rate of recovery of active enzyme was monitored. The values for k_{obsd} were obtained from the observed first-order plots of log ($E_T - E$) vs. time. E_T is the enzyme activity at infinite time but is achieved in practice within 24 h after dilution into the mercaptan. This value was found to agree well with a similarly diluted enzyme sample which had not been treated with MnO_4^-. E is the assayed activity at time t. The curve is theoretical for eq 2 with the constants given in Table III.

Scheme V



Effects of Mercaptans. The recovery of activity from permanganate-treated alkaline phosphatase is facilitated by mercaptans such as β-mercaptoethanol or dithiothreitol. Figure 4 shows the effect of added mercaptoethanol on the observed rate constant for reactivation of the permanganate-inhibited enzyme.

Two mechanisms for the enhancement of the rate of reactivation by thiols were evaluated. It was assumed for the first that added thiol reagents (RSH) react exclusively with free MnO_4^- after its dissociation from the inactive E*MnO_4^- complex, i.e., where k_2 is the second-order rate constant for the reduction of MnO_4^- by added thiol and k_0 is the endogenous reduction rate constant in the absence of thiol (Scheme V). Other constants are as defined in Scheme I.

The rate of reactivation of MnO_4^--inactivated alkaline phosphatase is given by eq 5 for this mechanism (Appendix II) where E_T = [E] + [E*MnO_4^-] and $k_0' = k_0 + k_2$[RSH].

$$\frac{d[E]}{dt} = \frac{k_{-1}k_0'(E_T - [E])}{(k_1/K_s)[E] + k_0'} \quad (5)$$

Equation 5 can be integrated by separation of variables to give a solution which can be reduced to a first-order rate expression (see Appendix II):

$$\ln\left(\frac{E_T}{E_T - [E]}\right) = k_{obsd}t \quad (6)$$

where

$$k_{obsd} = \frac{k_{-1}(k_0 + k_2[RSH])}{(k_1/K_s)E_T + k_0 + k_2[RSH]} \quad (7)$$

Scheme VI

$$E + MnO_4^- \underset{k_{-1}}{\longleftarrow} E{*}MnO_4^- \overset{RSH}{\underset{K_t}{\rightleftharpoons}} E{*}MnO_4^- \cdot RSH \xrightarrow{k_2'}$$

$$E + \text{reduced } MnO_4^- + \text{oxidized RSH}$$

Scheme VII

$$E + MnO_4^- \overset{\text{fast}}{\underset{K_s}{\rightleftharpoons}} E{\cdot}MnO_4^- \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} E{*}MnO_4^- \overset{[RSH]}{\underset{K_t}{\rightleftharpoons}}$$

$$E{*}MnO_4^- \cdot RSH \xrightarrow{k_2'} E + MnO_2 + RSO_3^-$$

It is clear from eq 7 that the limiting value of k_{obsd} at [RSH] $\rightarrow \infty$ is k_{-1}, and no effect of added thiol on the rate of reactivation should be observed under conditions where the protein-permanganate complex is sufficiently dilute such that the forward reaction in Scheme V (k_1/K_s) is insignificant. Since mercaptoethanol clearly accelerated the rate of reactivation, Scheme V was discarded in favor of Scheme VI.

In this scheme the active enzyme E can be recovered either by dissociation of the inactive $E{*}MnO_4^-$ complex with rate constant k_{-1} or, in the presence of added thiol, a ternary complex, $E{*}MnO_4^-{\cdot}RSH$, formed initially, is reduced in a unimolecular step with the rate constant k_2'. The existence of such a ternary complex is suggested by the saturation of k_{obsd} as [RSH] is increased (Figure 4).

The rate of reactivation of MnO_4^--inactivated alkaline phosphatase for this scheme is given by (see Appendix III)

$$\frac{d[E]}{dt} = k_{obsd}(E_T - [E]) \tag{8}$$

where

$$k_{obsd} = \frac{k_{-1}K_t + k_2'[RSH]}{K_t + [RSH]} \tag{9}$$

which may be integrated to give an expression for an exponential appearance of the active enzyme with the first-order rate constant k_{obsd}. In the absence of added thiol, $k_{obsd} = k_{-1}$, which has a value of 0.007 min^{-1} (see above). K_t and k_2' were determined by nonlinear regression analysis of eq 9 with k_{-1} fixed.

The kinetic and equilibrium constants for the interaction of MnO_4^- with alkaline phosphatase and the reactivation of the MnO_4^--treated enzyme according to the completed scheme (Scheme VII) are summarized in Table III.

Table III: Kinetic and Equilibrium Constants for the Interaction of Permanganate with Alkaline Phosphatase

parameter[a]	value[b]
K_s	87 (33) μM
k_1	1.5 (0.5) min^{-1}
k_{-1}	7 (0.7) $\times 10^{-3}$ min^{-1}
K_{eq}[c]	0.40 (0.02) μM
k_2'	0.060 (0.005) min^{-1}
K_t	200 (60) μM

[a] The parameters are defined in Scheme VII except as noted. [b] Figures in parentheses represent associated standard errors. [c] $K_{eq} = k_{-1}K_s/k_1$.

Discussion

A still unexplained phenomenon is the occurrence of a biphasic inactivation rate profile (see Figure 1), characterized by a very rapid loss in activity in which up to 25% of the initial activity may be lost at a rate too rapid to measure by the enzyme activity assay. This is followed by a first-order decay of the remaining enzyme activity, since MnO_4^- initially is at least in a 10-fold molar excess over enzyme, to give pseudo-first-order kinetics. No correlation of the initial rapid phase with the usual experimental parameters, varied pH, concentration of enzyme or MnO_4^-, or ionic strength, was found.

The basic Scheme I does not account for the curvature seen at higher [MnO_4^-] near the end of the inactivation (Figure 1, 30 and 40 μM $KMnO_4$). One explanation would be the formation of an $E{\cdot}(MnO_4^-)_2$ complex by binding of a second MnO_4^-, perhaps in a cooperative manner, and that this complex forms the inactive $E{*}(MnO_4^-)_2$ complex even more rapidly than the $E{*}MnO_4^-$ complex is formed. It is also possible that this *second* phase represents a simple oxidation of a critical residue remote from the active site.

Ohlsson & Wilson (1974) find MnO_4^- to be a somewhat more potent inhibitor of *E. coli* alkaline phosphatase (with $K_{eq} < 0.1$ μM) compared with 0.4 μM observed in these experiments. This may be due to the different experimental conditions or to a different source and method of isolation and purification of the phosphatase.

There are a number of oxyanions of similar size and shape to phosphate and permanganate which strongly inhibit *E. coli* alkaline phosphatase and related phosphohydrolases. Enzymatic phosphoryl transfer reactions are thought to proceed through a trigonal-bipyramidal transition state in which the entering and leaving groups occupy apical positions (Benkovic & Schray, 1973). Some of these inhibitory oxyanions are known to form adducts with trigonal-bipyramidal geometry. Thus, it can be argued that perhaps these oxyanions inhibit the enzyme by binding with the geometry which resembles the transition state formed during the hydrolysis of phosphate esters.

Van Etten et al. (1975) have reported the early transition metal oxyanions vanadate (VO_4^{3-}), molybdate (MoO_4^{2-}), and tungstate (WO_4^{2-}) to be excellent reversible competitive inhibitors of acid phosphatase. This enzyme is thought to hydrolyze phosphate esters via a covalent *N*-phosphorylhistidine (enzyme) intermediate. The authors propose that the inhibition of acid phosphatase by these oxyanions could be explained by the resemblance of the hydrated or active site chelated transition metal oxide to the trigonal-bipyramidal "transition" state. These authors note that the strong inhibition of *E. coli* alkaline phosphatase by periodate (Ohlsson & Wilson, 1974) might also be due to formation of a periodatozincate chelate as a transition state analogue.

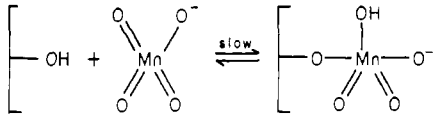
In a recent study Lopez et al. (1976) found both the vanadium ions VO^{2+} and VO_3^- to be potent competitive inhibitors of *E. coli* alkaline phosphatase catalyzed hydrolysis of *p*-nitrophenyl phosphate. The Theorell–Yonetani plots for these ions show mutually exclusive binding with inorganic phosphate, and the authors propose that this enzyme-complexed ion resembles the metastable intermediate formed during the hydrolysis of substrate.

What is the nature of the slow step responsible for loss of enzymatic activity when the phosphatase is incubated with MnO_4^-? One trivial "catch-all" explanation would be that MnO_4^- mediates a conformational change which is much slower than the turnover rate of the enzyme and thus is not part of the usual catalytic cycle. Conformational changes of the free enzyme or enzyme complexed to substrate or inhibitor have been proposed to account for kinetic behavior displayed during the catalytic turnover (e.g., Hull et al., 1976; Hull & Sykes, 1976; Sykes et al., 1974). However, if the inactivation step is due to a conformational change of the enzyme, it must occur with a rate some 3–4 orders of magnitude slower than one which would lie on the catalytic pathway. Estimates of the rate constants for the proposed catalytically important

conformational change have ranged between 20 and somewhat less than 200 s^{-1} at alkaline pH (Aldridge et al., 1964; Fernley & Walker, 1969; Halford et al., 1969; Halford, 1971; Reid & Wilson, 1971; Halford, 1972), whereas the MnO_4^--mediated inactivation rate constant is only 0.025 s^{-1} at pH 9.

The fact that all substrates and most inhibitors of alkaline phosphatase possess one or more negative charges suggests the existence of complementary positively charged site(s) on the enzyme responsible for recognition and binding of the substrate or inhibitor. Daeman & Riordan (1974) reported results which implicate the role of one essential arginine residue per active site in the binding of phosphate. If an arginine residue does function to bind MnO_4^-, one would still have to explain the unusually slow rate of association and dissociation (corresponding to inactivation and recovery rates of enzymatic activity) of this anion with the guanido group.

The relatively slow inactivation of the enzyme suggests that a covalent reaction might be responsible for this slow step. Two likely candidates would be the formation of a pentacovalent adduct between the enzyme and MnO_4^- or of an ester between MnO_4^- and a catalytically important moiety at the active site of the enzyme as depicted in eq 10 and 11 below, respectively, where species I would resemble the trigonal-bi-



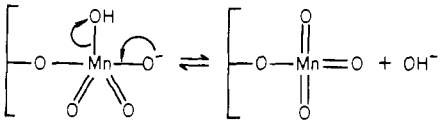
(10)

I

pyramidal transition state proposed for hydrolysis of phosphate esters by alkaline phosphatase (Williams & Naylor, 1971). The reversible formation of such an adduct could account for the inhibition of alkaline phosphatase by MnO_4^-.

Symons (1954) proposed the transient formation of a pentavalent adduct between MnO_4^- and OH^- to account for the base-catalyzed oxygen exchange with MnO_4^-. He envisioned an S_N2 displacement mechanism with a pentacoordinate transition state where the fifth oxygen ligand (from OH^-) is bonded to the one vacant inner d orbital of the manganese. This reaction is difficult because it requires the approach of two negatively charged species in the transition state. This should provide less of a barrier for reaction 10 because of the possibility that the negatively charged permanganate oxygen could be at least partly neutralized by the nearby positively charged arginine.

Disproportionation of I gives a serine–manganese(VII) ester (eq 11).



(11)

I II

Species II is closely analogous to the phosphoryl enzyme which has been established to be the intermediate in the alkaline phosphatase catalyzed hydrolysis of phosphomonoesters, except for the difference in charge. Alkaline phosphatase is known to stabilize substantially the active-site phosphoserine intermediate relative to free phosphoserine (Levine et al., 1969). It is possible that the enzyme sufficiently stabilizes the serine–manganese(VII) ester so that its formation would proceed in preference to MnO_4^--mediated oxidation of the enzyme. This postulate for species II suffers from the lack of available chemical analogy. To our knowledge, no ester of Mn(VII) has yet been prepared, although good evidence exists for esters of lower oxidation states (Stewart, 1965; Lee & Brownridge, 1974; Jaky & Simandi, 1976; Simandi & Jaky, 1976). Nonetheless, it is conceivable that part of the same mechanism by which the enzyme stabilizes the phosphoserine ester by a factor of 10^6 (Levine et al., 1969) may effect the formation of the enzyme-bound Mn(VII) ester.

Appendix I

Equation 4 is derived from Scheme IV as the most general case. Rewriting Scheme IV:

$$E + P_i \overset{K_{1p}}{\rightleftharpoons} E{\cdot}P_i \tag{12}$$

$$E{\cdot}P_i + P_i \overset{K_{2p}}{\rightleftharpoons} E{\cdot}(P_i)_2 \tag{13}$$

$$E + MnO_4^- \xrightarrow{k_1/K_s} E^*MnO_4^- \tag{14}$$

$$E{\cdot}P_i + MnO_4^- \xrightarrow{k_{1p}/K_{sp}} E{\cdot}P_i{}^*MnO_4^- \tag{15}$$

The mass balance expression is

$$E_T = [E] + [E{\cdot}P_i] + [E{\cdot}(P_i)_2] + I \tag{16}$$

where $I = [E^*MnO_4^-] + [E{\cdot}P_i{}^*MnO_4^-]$, the sum of the catalytically inactive forms of the enzyme. The dissociation constants for processes 12 and 13 are

$$[E{\cdot}P_i] = \frac{[E][P_i]}{K_{1p}} \qquad [E{\cdot}(P_i)_2] = \frac{[E][P_i]^2}{K_{1p}K_{2p}} \tag{17}$$

which after substitution into (16) and rearrangement gives

$$[E] = \frac{E_T - I}{1 + [P_i]/K_{1p} + [P_i]^2/(K_{1p}K_{2p})} \tag{18}$$

The rate of appearance of I through reactions 14 and 15 is

$$\frac{dI}{dt} = (k_1/K_s)[E][MnO_4^-] + (k_{1p}/K_{sp})[E{\cdot}P_i][MnO_4^-] \tag{19}$$

which after substitution of the expressions for [E] and [E·P$_i$] and rearrangement (19) becomes

$$\frac{dI}{dt} = k_{obsd}(E_T - I) \tag{20}$$

where

$$k_{obsd} = \left(\frac{(k_1/K_s) + (k_{1p}/K_{sp})[P_i]/K_{1p}}{1 + [P_i]/K_{1p} + [P_i]^2/(K_{1p}K_{2p})} \right)[MnO_4^-] \tag{21}$$

The integration of (20) with the initial conditions $I = 0$ at $t = 0$ gives

$$\ln\left(\frac{E_T}{E_T - I}\right) = k_{obsd}t \tag{22}$$

Substituting (21) into (22) and rearranging gives (23). The

$$\ln [E] = \ln\left(\frac{E_T}{1 + [P_i]/K_{1p} + [P_i]^2/(K_{1p}K_{2p})} \right) - k_{obsd}t \tag{23}$$

first term on the right side of eq 23 is a constant for a fixed [P$_i$], and the second predicts a first-order decay of active enzyme [E] as a function of time.

Equation 3 from Scheme III is obtained by setting $K_{2p} = \infty$ in eq 21. Equation 2 from Scheme II is obtained from eq 21 by setting $k_{1p}/K_{sp} = 0$ and $K_{2p} = \infty$.

Appendix II

Equation 5 is derived from Scheme V with the assumption that $d[MnO_4^-]/dt \ll d[E]/dt$. This is justified at least where

significant thiol is present to trap released MnO_4^-. Separation of variables in eq 5 gives eq 24.

$$\frac{(k_1/K_s)[E] + k_0 + k_2[RSH]}{k_{-1}(k_0 + k_2[RSH])(E_T - [E])}\, d[E] = dt \quad (24)$$

Introducing the variable u for the denominator of eq 24, we obtain

$$\frac{1}{k_{-1}(k_0 + k_2[RSH])}\left[\frac{k_1/K_s}{k_{-1}(k_0 + k_2[RSH])}\, du - [(k_1/K_s)E_T + k_0 + k_2[RSH]]\,\frac{du}{u}\right] = dt \quad (25)$$

which can be integrated directly. The constant of integration is defined by the initial conditions [E] = 0 at t = 0, giving

$$\ln\left(\frac{E_T}{E_T - [E]}\right) = \frac{k_{-1}(k_0 + k_2[RSH])t + k_1[E]/K_s}{(k_1/K_s)E_T + k_0 + k_2[RSH]} \quad (26)$$

Under the experimental conditions employed, the last term in the numerator of eq 26 becomes negligible compared to the other terms after ~10 s leading to eq 7.

Appendix III

The rate of appearance of active enzyme (Scheme VI) is given by

$$\frac{d[E]}{dt} = k_{-1}[E^*MnO_4^-] + k_2'[E^*MnO_4^-\cdot RSH] \quad (27)$$

The mass balance expression is

$$E_T = [E] + [E^*MnO_4^-] + [E^*MnO_4^-\cdot RSH] \quad (28)$$

The dissociation constant of the ternary complex $E^*MnO_4^-\cdot RSH$ is given by eq 29.

$$K_t = \frac{[E^*MnO_4^-][RSH]}{[E^*MnO_4^-\cdot RSH]} \quad (29)$$

Equations 27–29 combine to give eq 30

$$\frac{d[E]}{dt} = \frac{k_{-1}K_t + k_2'[RSH]}{K_t + [RSH]}(E_T - [E]) \quad (30)$$

where

$$k_{obsd} = \frac{k_{-1}K_t + k_2'[RSH]}{K_t + [RSH]} \quad (31)$$

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