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One-Electron Reduction of D-Amino Acid Oxidase. Kinetics of Conversion from the Red Semiquinone to the Blue Semiquinone†

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ABSTRACT: The reduction of D-amino acid oxidase (DAAO) by hydrated electrons (e_{aq}^-) has been studied in the absence and presence of benzoate by pulse radiolysis. The e_{aq}^- did not reduce the flavin moiety in DAAO and reacted with the amino acid residues in the protein. In the presence of benzoate, e_{aq}^- first reacted with benzoate to yield benzoate anion radical. Subsequently, the benzoate anion radical transferred an electron to the complex of DAAO–benzoate to form the red semiquinone of the enzyme with a second-order rate constant of 1.2×10^9 M^{-1} s^{-1} at pH 8.3. After the first phase of the reduction, conversion of the red semiquinone to the blue semiquinone was observed in the presence of high concentration of benzoate. This process obeyed first-order kinetics, and the rate increased with an increase of the concentration of benzoate. In addition, the rate was found to be identical with that of the formation of the complex between benzoate and the red semiquinone of DAAO as measured by a stopped-flow method. This suggests that bound benzoate dissociates after the reduction of the benzoate–DAAO complex by benzoate anion radical and that free benzoate subsequently recombines with the red semiquinone of the enzyme to form the blue semiquinone.

It is well-known that a one-electron reduction of flavoproteins produces a stable free radical of the flavin molecule, which is produced by partial dithionite titration or photochemical processes in the presence of EDTA[1] (Massey & Palmer, 1966). These are protonated (the blue semiquinone) or unprotonated (the red semiquinone) in the physiological pH range. The pK value associated with these flavin radical species free in solution is 8.5 (Ehrenberg et al., 1967; Land & Swallow, 1969; Meisel & Neta, 1975). Most flavoproteins, however, form only blue or red semiquinones of the radical species independently of the external pH (Massey & Palmer, 1966; Massey et al., 1969) except glucose oxidase (Stankovich et al., 1978). This suggests that the particular protein to which the flavin is bound stabilizes either the blue or red semiquinone (Massey & Hemmerich, 1980). For example, the semiquinone of flavodoxin is the blue semiquinone even at a pH value above 10 (Mayhew & Massey, 1969; Mayhew, 1971; Edonondson & Tollin, 1971). Burnett et al. (1974) proposed that the blue semiquinone of flavodoxin is stabilized by a hydrogen bond between the N-5 H of the flavin radical and the carbonyl oxygen of glycine from X-ray analysis of the oxidized and semiquinone forms of flavodoxin. On the other hand, there are other interesting examples in which the ionization state of a flavin radical is influenced by the presence of a ligand (Massey & Palmer, 1966; Yasuda et al., 1967; Mizzer & Thorp, 1981; Yagi et al., 1972). For example, the semiquinone form of DAAO is the red semiquinone in the physiological pH range, whereas it converted into the blue semiquinone upon formation of the benzoate–enzyme complex (Yagi et al., 1972). Here, benzoate combines with DAAO at the substrate site (Yagi, 1962; Massey & Ganther, 1965). From this point of view, kinetic behavior of the semiquinone of flavin is expected to give interesting information about the environment of the flavin prosthetic group of these flavoproteins.

On the other hand, some of the advantages of the pulse radiolysis technique for determining the spectral and kinetic behavior of one-electron-reduction products of flavin have been demonstrated (Land & Swallow, 1969; Meisel & Neta, 1975; Faraggi et al., 1975). Recently this technique has been employed in the studies of flavodoxin (Faraggi & Klapper, 1979) and ferredoxin-NADP reductase (Maskiewicz & Bielski, 1982).

The present paper describes the reduction of DAAO by e_{aq}^- in the presence and absence of benzoate by the use of pulse radiolysis. We focus our attention on the semiquinone form of DAAO.

Materials and Methods

Hog kidney D-amino acid oxidase (DAAO) was purified by the procedure of Kubo et al. (1960) with the modifications of Massey et al. (1961) and Curti et al. (1973). The enzyme was prepared as the benzoate-bound form, and benzoate was freed from the enzyme, prior to each experiment, by passage of the enzyme, after reduction with excess D-alanine, through a column of Sephadex G-25 (Yagi & Ozawa, 1962a,b). Enzyme concentration was determined on the basis of FAD bound to the enzyme, using a molar extinction coefficient of 11.3 mM^{-1} cm^{-1} at 455 nm, and is expressed as subunit moles per liter. All other reagents were obtained commercially as the analytical grade.

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[1] Abbreviations: DAAO, D-amino acid oxidase; FAD, flavin adenine dinucleotide; e_{aq}^-, hydrated electron; EDTA, ethylenediaminetetraacetic acid.

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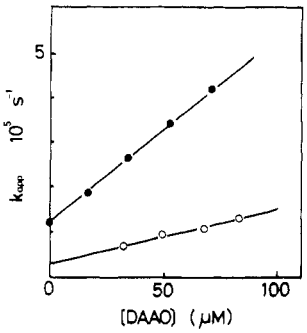


FIGURE 1: Concentration dependence of the pseudo-first-order rate constants for reaction of e_{aq}^- (●) and the benzoate anion radical (○) with DAAO. Phosphate buffer (pH 8.3) 5 mM; *tert*-butyl alcohol 0.1 M.

A sample of DAAO for pulse radiolysis is prepared as follows. Argon gas was passed over the surface of solution of DAAO containing 5 mM phosphate buffer (pH 5–8) or 10 mM borate buffer (pH 8–9) and 0.1 M *tert*-butyl alcohol for scavenging the OH· with stirring. The effect of *tert*-butyl alcohol (0.1 M) on the optical absorption spectrum and catalytic activity of DAAO was not seen. The bottle containing approximately 100 mL of sample solution was connected to the flow cell with a 1- or 1.5-cm light path, placed on the front of the accelerator. Fresh solution was used for each irradiation. Pulse radiolysis experiments were performed with the electron linear accelerator of the Institute of Scientific and Industrial Research, Osaka University (Kobayashi & Hayashi, 1981). The pulse width and energy were 8 ns and 20 MeV, respectively. Photolysis by the analyzing light was minimized by means of an optical shutter and selected filters.

The stopped-flow apparatus used was a Union Giken Model RA-401 spectrometer. A semiquinone form of DAAO was prepared as follows. The enzyme solution in the presence of 25 mM EDTA was placed in the main chamber of a Thunberg-type cuvette. Then, the content of the cuvette was carefully deaerated by repeated cycles of degassing and flushing with nitrogen gas. The cuvette was placed in an ice–water bath and illuminated for 5 min with a 1-kW tungsten lamp. The red semiquinone of the enzyme thus prepared was introduced into one of the reservoirs of the flow system. This enzyme was rapidly mixed at 20 °C in the stopped-flow apparatus with an equal volume of various concentrations of sodium benzoate. The sodium benzoate solutions containing 0.1 M phosphate buffer (pH 5–8) or 0.1 M borate buffer (pH 8–9) were also prepared by careful equilibration with nitrogen gas, before mixing.

Results

When deaerated solutions containing DAAO were subjected to a single pulse, the transient absorption due to e_{aq}^- is produced. The decay of the e_{aq}^- followed at 600 nm was found to obey pseudo-first-order kinetics. In Figure 1, such pseudo-first-order rate constants are plotted against DAAO concentration at pH 8.3. In the same figure, the reaction of benzoate anion radical with DAAO is also shown, which is described below. The rate increases with increase of the concentration of DAAO. This suggests that the decay of e_{aq}^- is due to the reaction of DAAO with e_{aq}^-. From the slope of Figure 1, the second-order rate constant of the reaction e_{aq}^- with DAAO is calculated to be 4×10^9 M^{-1} s^{-1}. The intercept value, the rate of the decay of e_{aq}^- in the absence of DAAO, is due to its reaction with the matrix solution. However, no decrease in the absence at 450 nm due to reduction of the FAD of DAAO could be detected, even 30 s after pulse. A transient

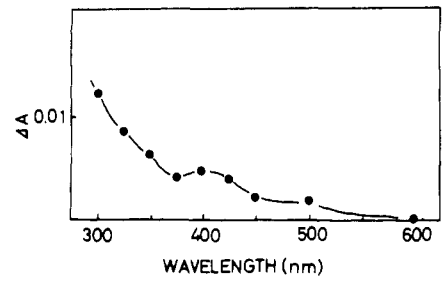


FIGURE 2: Kinetic difference spectrum from reaction of e_{aq}^- with DAAO at pH 8.3. The spectrum was taken at 8 μs after pulse radiolysis. Phosphate buffer (pH 8.3) 5 mM; *tert*-butyl alcohol 0.1 M; DAAO 51 μM.

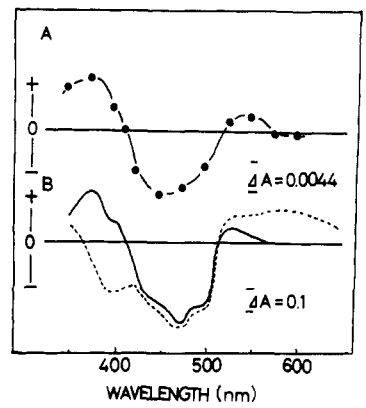


FIGURE 3: (A) Kinetic difference spectrum of pulse radiolysis of DAAO. The spectrum was taken at 500 μs after pulse radiolysis. Absorption increases in the upward direction. Phosphate buffer (pH 8.3) 5 mM; *tert*-butyl alcohol 0.1 M; DAAO, 5 μM; sodium benzoate 25 mM. (B) Difference spectra of the red semiquinone (—) and the blue semiquinone (- - -) of DAAO minus the benzoate–DAAO (oxidized) complex. DAAO 51 μM; phosphate buffer (pH 8.3) 5 mM; sodium benzoate 0.1 M.

species with absorption in the visible region was detected. This transient absorption spectrum at 8 μs after the pulse is shown in Figure 2. The spectrum, which has a peak near 400 nm and below 350 nm, is considered to be due to the radical ions of the amino acid residues in the protein (Adams, 1972).

The reaction of DAAO with e_{aq}^- was performed in the presence of equimolar benzoate. Under this condition essentially all benzoate is bound to DAAO in the oxidized state, since the dissociation constant in the benzoate–enzyme is 1.5×10^{-6} M (Quay & Massey, 1977). The e_{aq}^- did not reduce FAD in the benzoate–DAAO complex but reacted with protein moiety of the enzyme, in resemblance to the reaction of e_{aq}^- with DAAO in the absence of benzoate. On the other hand, in the presence of excess benzoate, a transient spectrum with an observed maximum at 450 nm was observed within 1 μs after the pulse. This spectrum is identical with those obtained upon pulse radiolysis of benzoate free in aqueous solution (Sangster, 1966). In this system, e_{aq}^- is converted to the benzoate anion radical nearly quantitatively. The benzoate anion radical reacted with FAD of DAAO. Figure 3A shows the difference spectrum obtained at 500 μs after pulse radiolysis. For comparison, the corresponding difference spectra of the semiquinone form of DAAO minus the benzoate–DAAO (oxidized) complex are shown in Figure 3B. These spectra were obtained from separate experiments and were normalized against the spectrum of oxidized DAAO. The red semiquinone was formed by irradiation of the fully oxidized enzyme anaerobically in the presence of 25 mM EDTA. The blue semiquinone was formed by the addition of 0.1 M sodium benzoate to the red semiquinone anaerobically. The kinetic

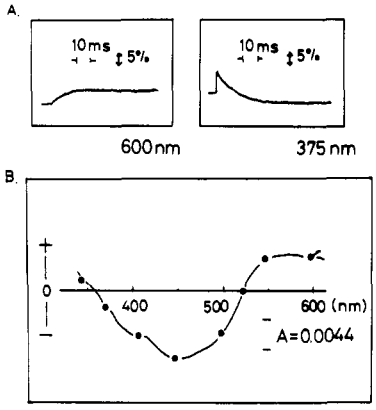


FIGURE 4: (A) Oscilloscope traces of the transmittance change after pulse radiolysis of DAAO in the presence of benzoate. Phosphate buffer (pH 8.3) 5 mM; *tert*-butyl alcohol 0.1 M; DAAO 5 μM; sodium benzoate 25 mM. (B) Kinetic difference spectra of pulse radiolysis of DAAO. The spectrum was taken at 30 ms after pulse radiolysis.

difference spectrum, which has an absorption minimum at 450 nm and maximum at 375 nm, is similar to that of the red semiquinone of DAAO minus the benzoate–DAAO (oxidized) complex, as shown in Figure 3B. Therefore, it is concluded that the benzoate anion radical transfers an electron to the benzoate–DAAO complex to form the red semiquinone of this enzyme.

For determination of the rate constant of reduction of DAAO by the benzoate anion radical, the decay of the benzoate anion radical was monitored at 409 nm, the isosbestic point between the benzoate–DAAO complex and the red semiquinone form. This absorption change at 409 nm obeys pseudo-first-order kinetics, since 2.3 μM benzoate anion radical, using a molar extinction coefficient of 9 mM^{-1} cm^{-1} at 450 nm, is generated in a solution containing ~30–100 μM DAAO. This reaction is expressed by

$$E\cdot FAD + Bz^{-}\cdot \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot FAD^{-}\cdot + Bz \qquad (1)$$

Figure 1 shows the dependence of the apparent first-order rate constant on the concentration of DAAO. From the slope and the intercept of Figure 1, the second-order rate constant for the reaction of the benzoate anion radical with DAAO (k_1) and its backward reaction (k_{-1}) is estimated to be 1.2×10^9 M^{-1} s^{-1} and 3×10^4 s^{-1}, respectively.

In the presence of excess benzoate, following the spectral changes due to the reduction of FAD group, other spectral changes took place in the time range of milliseconds. Typical oscilloscope traces at 600 and 375 nm are shown in Figure 4A. Figure 4A shows the kinetic difference spectrum at 30 ms after the pulse. The spectrum has a broad absorption from 400 to 450 nm and around 600 nm characteristic of the blue semiquinone (Figure 4B) and is similar to that of the blue semiquinone of DAAO minus the benzoate–DAAO (oxidized) complex in Figure 3B. Therefore, it is concluded that this slow process is due to the conversion from the red semiquinone to the blue semiquinone of this enzyme. This process obeys the first-order kinetics, and its apparent rate constant is found to increase with the increase of concentration of benzoate.

For examination of whether the formation of the blue semiquinone observed after pulse radiolysis is due to the process of binding of benzoate to the red semiquinone of DAAO, kinetics of the binding of benzoate to the red semiquinone form of DAAO were studied under pseudo-first-order condition by means of stopped-flow apparatus; the results are compared with those obtained by pulse radiolysis. The binding of benzoate to the enzyme was followed by the conversion from the

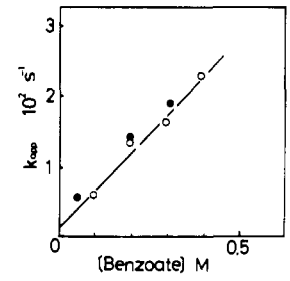


FIGURE 5: Correlation between benzoate concentration and the first-order rate constant in the formation of blue semiquinone of DAAO. (○) Pulse radiolysis method; (●) stopped-flow method.

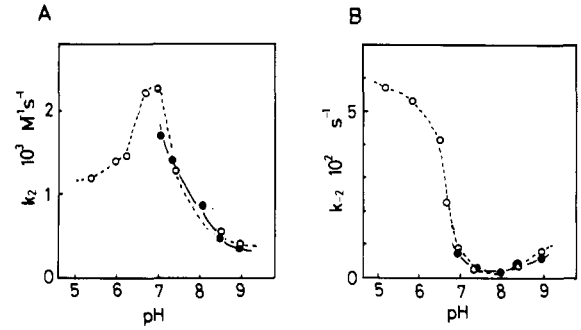


FIGURE 6: (A) pH dependence of the rate constants of the complex formation of benzoate with the red semiquinone of DAAO. (B) pH dependence of the rate constants of the dissociation of benzoate. (●) Stopped-flow method; (○) pulse radiolysis method.

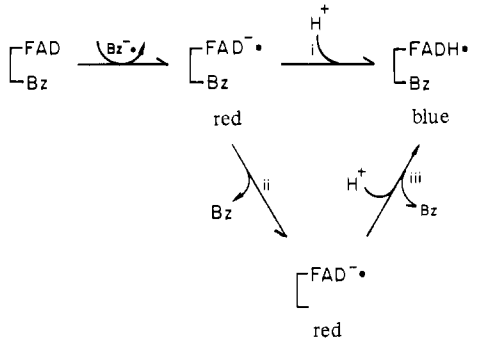
red semiquinone to the blue semiquinone. The pseudo-first-order rate constants are plotted against the benzoate concentration obtained by the pulse radiolysis and the stopped-flow methods, as shown in Figure 5. The rate constants obtained by the two methods are identical. From the slope and intercept in Figure 5, the rate constants for the complex formation of benzoate (k_{on}) and the dissociation of benzoate (k_{off}) can be estimated. Figure 6 shows the pH–rate constant curves for the conversion from the red semiquinone to the blue semiquinone obtained by pulse radiolysis and stopped-flow methods. The formation of the blue semiquinone below pH 7.0 was complete in less than 3 ms, the dead time of the stopped-flow apparatus. The rate constants obtained by the two methods agree with each other at each pH value examined.

Discussion

The nonspecific reductant e_{aq}^{-}, which is very reactive toward flavin (Land & Swallow, 1969), was not able to reduce the flavin moiety of DAAO in the absence of benzoate. A similar result was obtained in the case of glucose oxidase. In contrast to these flavoprotein oxidases, the flavin in electron-transferring flavoprotein, flavodoxin (Faraggi & Klapper, 1979), can be reduced easily by e_{aq}^{-}. This difference can be explained by the fact that in the flavodoxin case the isoalloxazine ring is found at the periphery of the molecule where the dimethylbenzene end is accessible to solvent, as has been suggested by X-ray analysis (Burnett et al., 1974). The reduction by e_{aq}^{-} is assigned mainly to a direct reaction proceeding via the exposed edge of the flavin. The flavin moiety in flavoprotein oxidase is not directly exposed to the solvent and is masked by the protein moiety. In addition, the nonreducible FAD of DAAO by e_{aq}^{-} suggests that other acceptors, which e_{aq}^{-} reduces easily, may be located near the active site of DAAO. These amino acid residues then act as "electron sinks" and do not transfer to the flavin. It was proposed that tyrosyl (Kotaki et al., 1968), cysteinyl (Tu & McCormick, 1973), and histidyl (Walsh et al., 1973) residues are located near the active site.

The formation of transient absorption centered at 400 nm due to cysteine radical ions (Adams, 1972) was observed in Figure 2. On the other hand, the use of a benzoate anion radical, formed by the reaction of benzoate with e_{aq}^-, results in a rapid reduction of the oxidized flavin in the active site of the enzyme to the corresponding semiquinone state. Since [benzoate]/[benzoate–DAAO] is sufficiently high in the present experiments, the benzoate anion radical is formed preferentially in the bulk of the solution and not on the enzyme active site. Furthermore, in view of the kinetic evidence shown in Figure 1, which shows the second-order reaction between the benzoate anion radical and DAAO, the observed reaction represents an intermolecular electron transfer from the benzoate–DAAO complex, not an intramolecular electron transfer from the benzoate anion radical bound to the enzyme to the FAD. Since the value of the second-order rate constant (1.2×10^9 M^{-1} s^{-1}) is close to that for a diffusion-controlled process, this reaction cannot be attributed to the exchange between benzoate bound to the enzyme and benzoate anion radical in the bulk solution. But it is considered that an electron probably enters DAAO via another site of electron acceptance which is different from the binding site for benzoate on DAAO. Similar rates for the reaction of the benzoate anion radical with cytochrome *c* and metMb have been reported (Simic et al., 1975). In these cases, it was proposed that the interaction between the donors and porphyrin ring plays an important role. In the present case, however, the mechanism for electron transfer from the benzoate anion radical to FAD is not clear at present.

An important finding in the present work is that the red semiquinone is found transiently and is subsequently converted to the blue semiquinone in the presence of a high concentration of benzoate. Two possible pathways for the formation of the blue semiquinone after reduction can be schematized as follows:



The benzoate anion radical transfers an electron to the benzoate–DAAO complex to form a red semiquinone in a nonequilibrium state, and then the red semiquinone is protonated to form the blue semiquinone (path i). Alternatively, a transient red semiquinone is formed, and then the bound benzoate dissociates from the enzyme to yield the stable red semiquinone (path ii). Subsequently, benzoate recombines with the red semiquinone to form the blue semiquinone. Here, we demonstrate that the bound benzoate dissociates from the enzyme in the red semiquinone after reduction of the benzoate–DAAO complex. This is verified by the fact that rate constants of the formation of the blue semiquinone obtained by the use of pulse radiolysis are identical with those of binding of benzoate with the red semiquinone (path iii) obtained by the stopped-flow method. Therefore, path i, i.e., the protonation of the red semiquinone of DAAO, can be excluded, and the latter mechanism via path ii is likely. This suggests that the dissociation of benzoate from the enzyme is faster than the protonation of the red semiquinone of DAAO ($k_i \ll k_{ii}$). If reaction i is simply explained by the protonation of the red semiquinone, the rate should be very fast. This slow rate indicates that N-5 of the flavin radical protonates with a conformational change in the protein. In this scheme, however, we were unable to observe spectrophotometrically the red semiquinone–benzoate complex and path ii, in which the bound benzoate dissociates after reduction. Another possible explanation, other than the mechanisms discussed above, is considered as follows. After reduction of the benzoate–DAAO complex, free benzoate binds to the benzoate-binding site of the semiquinoid enzyme, which is different from that of the oxidized enzyme. In this case, the process of the dissociation of bound benzoate from the enzyme after reduction can be ruled out. If so, 2 mol of benzoate is bound in this enzyme. This possibility, however, can be excluded by the evidence that benzoate competes with a substrate, D-alanine, in combining with the semiquinoid enzyme (Yagi et al., 1972).

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Registry No. DAAO, 9000-88-8; benzoic acid, 65-85-0.

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Catalytic Specificity of Yeast Inorganic Pyrophosphatase for Magnesium Ion as Cofactor. An Analysis of Divalent Metal Ion and Solvent Isotope Effects on Enzyme Function†

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ABSTRACT: This work extends our functional studies of yeast inorganic pyrophosphatase, previously performed in the presence of Mg^{2+} in H_2O [Springs, B., Welsh, K. M., & Cooperman, B. S. (1981) *Biochemistry 20*, 6384–6391], to studies in the presence of Zn^{2+}, Mn^{2+}, or Co^{2+} in H_2O and of Mg^{2+} in D_2O. Measurements of equilibrium formation of enzyme-bound pyrophosphate as a function of added inorganic phosphate and of the rates of enzyme catalysis of inorganic pyrophosphate hydrolysis and H_2O–inorganic phosphate oxygen exchange are used to calculate microscopic rate constants for (a) pyrophosphate hydrolysis and formation on the enzyme surface and (b) release of inorganic phosphate from enzyme. These rate constants allow the following conclusions to be drawn: (1) The solvent isotope effect on overall enzyme-catalyzed pyrophosphate hydrolysis that we previously measured [Konsowitz, L., & Cooperman, B. S. (1976) *J. Am. Chem. Soc. 98*, 1993–1995] derived primarily from an effect on the rate of inorganic phosphate release from enzyme. Only a modest effect is found on the rate of pyrophosphate hydrolysis on the enzyme surface. (2) The lower effectiveness, compared with Mg^{2+}, of Zn^{2+}, Mn^{2+}, and Co^{2+} as cofactors for inorganic pyrophosphate hydrolysis is due mainly or entirely to the slower rates of phosphate release from enzyme in the presence of each of these ions. Put another way, the specificity of inorganic pyrophosphatase for Mg^{2+} as a cofactor derives in large measure from the rapid rates of phosphate release achievable in its presence. Similar considerations might explain the specificity of other phosphoryl enzymes for Mg^{2+} as cofactor. In a second series of experiments, solvent isotope effects on overall enzyme-catalyzed PP_i hydrolysis are determined in the presence of Zn^{2+}, Co^{2+}, and Mn^{2+} and compared with that found in the presence of Mg^{2+}. The magnitudes of the effect are found to decrease in the order $Mn^{2+} > Zn^{2+} > Co^{2+} > Mg^{2+}$. This result supports conclusions 1 and 2 reached above, since the rate of inorganic phosphate release is uniquely rate determining in the presence of Zn^{2+} or Mn^{2+} and only partly rate determining in the presence of Co^{2+} or Mg^{2+}, and, as already mentioned, inorganic phosphate release displays a higher solvent isotope effect than does pyrophosphate hydrolysis on the enzyme surface.

Yeast inorganic pyrophosphatase (EC 3.6.1.1) (PPase),[1] a dimeric enzyme composed of identical subunits, has long been known to require divalent metal ions for activity [Kunitz, 1952; for a recent review of the properties of this enzyme, see Cooperman (1982)]. With PP_i as a substrate, the relative activity conferred by divalent metal ions falls in the order $Mg^{2+} > Zn^{2+} > Co^{2+}$, $Mn^{2+} \gg Cd^{2+}$ (Butler & Sperow, 1977; Janson et al., 1979; Hackney, 1980; O. A. Moe, Jr., S. Pham, B. Selinsky, and T. Dang, unpublished experiments; Welsh et al., 1983). PPase catalysis of PP_i hydrolysis in the presence of Mg^{2+} displays a solvent isotope effect of just under 2 (Konsowitz & Cooperman, 1976), measured at the pH and pD maxima.

In recent work (Springs et al., 1981), we presented a unified scheme for PPase action and demonstrated the following.

(1) Equation 1, in which three Mg^{2+} are bound per PPase subunit in the presence of P_i or PP_i, quantitatively accounts

$$MgPP_i + Mg_2E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} Mg_2EMgPP_i \underset{k_4}{\overset{k_3}{\rightleftharpoons}} MgE(MgP_i)_2 \underset{k_6}{\overset{k_5}{\rightleftharpoons}} MgEMgP_i + MgP_i \underset{k_8}{\overset{k_7}{\rightleftharpoons}} Mg_2E + P_i \quad (1)$$

for PPase catalysis of PP_i hydrolysis, of H_2O–P_i oxygen exchange, and of P_i–PP_i exchange at Mg^{2+} concentrations below 10 mM. At higher Mg^{2+} concentrations, it is also necessary to consider eq 2, in which four Mg^{2+} are bound per subunit in the presence of P_i or PP_i. In these equations, we are con-

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[1] Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonate; PPase, yeast inorganic pyrophosphatase; P_i, inorganic phosphate; PP_i, inorganic pyrophosphate; Tris, tris(hydroxymethyl)aminomethane; the subscript T indicates the total stoichiometric concentration of a species added to a solution.

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