Characterization of the Covalent Enzyme Intermediates Formed during Pyruvate Phosphate Dikinase Catalysis[†,‡]

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ABSTRACT: The intermediacy of a pyrophosphorylenzyme (E-PP) and phosphorylenzyme (E-P) in the *Clostridium symbiosum* pyruvate phosphate dikinase catalyzed interconversion of adenosine 5′-triphosphate (ATP), orthophosphate (P_i), and pyruvate with adenosine 5′-monophosphate (AMP), inorganic pyrophosphate (PP_i), and phosphoenolpyruvate (PEP) was examined using transient kinetic techniques. Single-turnover experiments with [γ-^{32}P]ATP or [^{14}C]ATP and PPDK were carried out in the presence and absence of P_i to test for pyrophosphorylenzyme and AMP formation, respectively. Formation of the E-PP·AMP complex was found to be followed by P_i binding and the formation of the E-P·AMP·PP_i complex. The level of pyrophosphorylenzyme accumulated during a single turnover was found to be dependent on the divalent metal cofactor used ($Mn^{2+} > Co^{2+} > Mg^{2+}$). Single-turnover experiments with [^{32}P]PEP and PPDK were carried out in the presence and absence of PP_i and pyruvate to test for phosphorylenzyme formation in the reverse, ATP-forming direction of the reaction. Phosphorylenzyme formed from the reaction of the E·PEP complex was converted in the presence of AMP and PP_i to free enzyme at a rate exceeding the steady-state turnover rate. The reaction sequence for pyrvate phosphate dikinase was determined to be

$$E + ATP \rightleftharpoons E\cdot ATP \rightleftharpoons E\text{-}PP\cdot AMP \overset{P_i}{\rightleftharpoons} E\text{-}PP\cdot AMP\cdot P_i \rightleftharpoons$$

$$E\text{-}P\cdot AMP\cdot PP_i \underset{PP_i}{\rightleftharpoons} E\text{-}P\cdot AMP \underset{AMP}{\rightleftharpoons} E\text{-}P \overset{pyruvate}{\rightleftharpoons} E\text{-}P\cdot pyruvate \rightleftharpoons E\cdot PEP \rightleftharpoons E + PEP$$

^{31}P NMR analysis of the phosphorylenzyme in the native (–4.0 ppm) and denatured form (–3.9 ppm) revealed a 3-*N*-phosphohistidine residue. Complexation of Mg^{2+} resulted in a 0.3 ppm upfield shift of the phosphorus resonance from native phosphorylenzyme while Mn^{2+} complexation lead to extensive line broadening, indicative of metal cofactor binding in close vicinity to the phosphoryl group. Complexation of the phosphorylenzyme with Mg^{2+} and oxalate led to extensive line broadening of the ^{31}P NMR signal, signifying immobilization of the phosphoryl group through coordination to the Mg·oxalate chelate complex.

During the 1970s Wood and his co-workers provided compelling evidence that the enzyme pyruvate phosphate dikinase (PPDK) isolated from the bacteria *Propionibacterium shermanii* and *Clostridium symbiosum* (formerly known as *Bacteroides symbiosus*) catalyzes the interconversion of ATP, P_i, and pyruvate with AMP, PP_i, and PEP via a novel multiple phosphoryl transfer mechanism (Scheme I) (Wood et al., 1977).[1]

The unique feature of the PPDK mechanism is that an active site histidine residue abstracts the β- and γ-phosphoryl groups from ATP by displacement of AMP from the β-phosphorus and delivers the two phosphoryl groups sequentially to orthophosphate and pyruvate, bound at separate and independent reaction sites, by a process which has become known as the "swinging arm mechanism". The pyrophosphorylenzyme (E-PP) and phosphorylenzyme (E-P) are the key chemical intermediates of this remarkable example of covalent catalysis. A confusing picture of PPDK catalysis emerged from our own studies of the *C. symbiosum* enzyme almost a decade later. Where Wood and co-workers had observed (Milner & Wood, 1976; Milner et al., 1978) parallel initial velocity plots for all substrate pairs (diagnostic of the tri-uni-uni kinetic mechanism of Scheme I), we found (Wang et al., 1988) intersecting initial velocity plots for ATP/P_i and AMP/PP_i substrate pairs and parallel patterns for AMP/PEP and PP_i/PEP pairs (diagnostic of the bi-bi-uni-uni kinetic mechanism of Scheme II). Wood and co-workers had also reported observing [^{14}C]AMP $\rightleftharpoons$ ATP, [^{32}P]P_i $\rightleftharpoons$ PP_i and [^{14}C]pyruvate $\rightleftharpoons$ PEP exchange in the absence of cosubstrates (diagnostic of the tri-uni-uni mechanism of Scheme I), yet we found that the P_i/PP_i pair was required for [^{14}C]AMP $\rightleftharpoons$ ATP exchange and the AMP/ATP pair was required for the [^{32}P]P_i $\rightleftharpoons$ PP_i exchange while the [^{14}C]pyrvate $\rightleftharpoons$ PEP exchange reaction took place independent of cosubstrates (diagnostic of the bi-bi-uni-uni kinetic mechanism of Scheme II). Finally, Wood and co-workers had reported that radi-

Scheme I: Pyruvate Phosphate Dikinase Catalyzed Reaction Sequence According to Wood et al. (1977)

$$E + ATP \rightleftharpoons E\cdot ATP \rightleftharpoons E\text{-}PP\cdot AMP \underset{AMP}{\rightleftharpoons} E\text{-}PP \overset{P_i}{\rightleftharpoons}$$

$$E\text{-}PP\cdot P_i \rightleftharpoons E\text{-}P\cdot PP_i \underset{PP_i}{\rightleftharpoons} E\text{-}P \overset{pyruvate}{\rightleftharpoons} E\text{-}P\cdot pyruvate \rightleftharpoons$$

$$E\cdot PEP \rightleftharpoons E + PEP$$

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‡ This paper is dedicated to the late Harland G. Wood.

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[1] Abbreviations: PPDK, pyruvate phosphate dikinase; E-PP, pyrophosphorylenzyme; E-P, phosphorylenzyme; ATP, adenosine 5′-triphosphate; AMP, adenosine 5′-monophosphate; PEP, phosphoenolpyruvate; PP_i, inorganic pyrophosphate; P_i, inorganic phosphate; NADH, dihydronicotinamide dinucleotide; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EGTA, ethylene glycol bis(*β*-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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olabeled pyrophosphorylenzyme could be formed in near quantitative yield by incubating PPDK (in the presence of Mg^{2+} and NH_4^+ cofactors) with PEP and [^{32}P]PP_i, with [γ-^{32}P]ATP, or with [β-^{32}P]ATP, but we were unable, despite repeated attempts, to obtain radiolabeled enzyme in yields exceeding a few percent by any of these methods. Further support for the bi-bi-uni-uni mechanism came from positional isotope exchange studies with [α,β-^{18}O,β,β-$^{18}O_2$]ATP, which showed that PPDK catalyzed the transfer of ^{18}O from the α,β-bridge position to the α-nonbridge position in the presence but not in the absence of P_i (Wang et al., 1988), and from the kinetic studies of the plant PPDK reported by Hatch and co-workers (Andrews & Hatch, 1969; Jenkins & Hatch, 1985).

Scheme II: Bi-Bi-Uni-Uni Kinetic Mechanism of Pyruvate Phosphate Dikinase According to Wang et al. (1988)

$$E + ATP \rightleftharpoons E\cdot ATP \overset{P_i}{\rightleftharpoons} E\cdot ATP\cdot P_i \rightleftharpoons E\text{-}P\cdot AMP\cdot PP_i \underset{PP_i}{\rightleftharpoons}$$

$$E\text{-}P\cdot AMP \underset{AMP}{\rightleftharpoons} E\text{-}P \overset{pyruvate}{\rightleftharpoons} E\text{-}P\cdot pyruvate \rightleftharpoons$$

$$E\cdot PEP \rightleftharpoons E + PEP$$

The apparent discrepancy between our results and those reported by Wood and co-workers coupled with the feasibility of an alternate chemical pathway involving the intermediacy of E·ADP·PP_i rather than E-PP·AMP led us to investigate the intermediates of PPDK catalysis using transient kinetic and ^{31}P NMR techniques.[2] In this paper, we present data which demonstrate that the pyrophosphorylenzyme is formed by the reaction of the enzyme with ATP in the absence of phosphate, that the position of the internal equilibrium (E·ATP $\rightleftharpoons$ E-PP·AMP) varies greatly with the divalent metal ion used as cofactor, and that the E-PP·AMP complex is a competent intermediate in the forward reaction. Data are also presented which show that the E-P·pyruvate complex generated from reaction of PPDK with PEP is a competent intermediate in the reverse, ATP-forming reaction and that the phosphorylenzyme contains a 3-phosphohistidine moiety, which becomes immobilized upon complexation with Mg^{2+} and oxalate.

EXPERIMENTAL PROCEDURES

General. PPDK (*C. symbiosum*) was purified from *Escherichia coli* JM101 cells transformed with the PPDK-encoding recombinant plasmid pACYC184D12 (Pacolyko, 1990) by using the purification procedure described in Wang et al. (1988).

Rapid Quench Experiments. Rapid quench experiments were performed at 25 °C using a rapid quench instrument from KinTek Instruments (3-syringe model) equipped with a thermostatically controlled circulator. A typical experiment was carried out by mixing a 40-μL solution of 50 mM K^+Hepes (pH 7.0) containing enzyme and monovalent and divalent metal cofactors with an equal volume of substrates in 50 mM K^+Hepes (pH 7.0). The reactions were quenched after a specified period of time with 164 μL of 0.6 M HCl. Immediately afterwards, 100 μL of CCl_4 was added to the quenched sample followed by vigorous vortexting to precipitate the protein. The mixture was centrifuged, and the supernatant and protein pellet were separated. The protein pellet was dried using a Kimwipe and the dissolved in 10 N H_2SO_4 at 100 °C. The solution was then added to scintillation fluid for counting.

Preparation of the Phosphorylenzyme (E-P) and ^{31}P NMR Analysis. A typical reaction contained 100 μM PPDK (based on 90-kDa subunit weight), 200 μM PEP, 5 mM $MgCl_2$, 10 mM NH_4Cl, 400 μM NADH, and 0.5 unit of lactate dehydrogenase in 5.6 mL of 50 mM K^+Hepes (pH 7.0) at 25 °C. The reaction was monitored at 340 nm until completion at which point 100 μM NADH had undergone oxidation. The reaction mixture was chromatographed on a 1 × 30 cm Sephadex G-75 column at 4 °C using 50 mM K^+Hepes (pH 7.0) as eluant. The fractions containing PPDK were combined and concentrated to 0.3 mL with an Amicon ultrafiltration cell. ^{31}P NMR samples (0.6 mL) contained 30% D_2O, 1 mM EGTA, 50 mM K^+Hepes (pH 7.0) and approximately 1 mM phosphorylated enzyme in the presence or absence of 5 M urea. Data collection was made at 27 °C over an 8-h period. ^{31}P NMR spectra were recorded on a Bruker AM-400 multinuclear spectrometer operating at a frequency of 162 MHz, a Bruker WP-200 multinuclear spectrometer operating at a frequency of 84 MHz, or a Bruker AMX-500 multinuclear spectrometer operating at 203 MHz. Typical acquisition parameters used were 60 ppm sweep width, 0.50 acquisition time, 0.5 s relaxation delay, and 30 000 scans.

[^{14}C]AMP $\rightleftharpoons$ ATP Isotope Exchange Experiments. All reactions (0.5 mL) contained 0.25 mM ATP, 0.05 mM ATP, 0.25 mM PP_i, 1 mM P_i, 20 mM NH_4Cl, 150 mM imidazole hydrochloride (pH 7.0), and 2 mM $MgCl_2$, $CoCl_2$, or $MnCl_2$. After incubation of the reaction mixture with a catalytic amount of PPDK at 25 °C for 30 min, 0.1 μmol of [^{14}C]AMP (sp act. = 590 mCi/mmol) was added. Fifty-microliter aliquots were removed at varying conversions and quenched with 4 μL of 2 M HCl. Next, 5 μL of 20 mM solutions of AMP and ATP was added to the quench solutions along with 3 μL of 50 mM EGTA. Ten-microliter aliquots of the resulting mixtures were spotted on PEI–cellulose TLC plates which were developed with 0.25 M triethylamine bicarbonate (pH 9.0). The areas on the plates containing ATP and AMP were scraped off and assayed for radioactivity using liquid scintillation techniques. The velocity of each exchange reaction was calculated by using

$$V_{ex} = \frac{-[AMP][ATP]}{[AMP] + [ATP]} \times \frac{2.3}{t}(\log (1 - f)) \qquad (1)$$

where f = % exchanged at time t (min)/% exchanged at equilibrium.

Determination of the Turnover Number for Mg^{2+}/NH_4^+ and Co^{2+}/NH_4^+ Activated PPDK. The turnover number was determined for both the Mg^{2+}/NH_4^+ and Co^{2+}/NH_4^+ activated PPDK enzyme systems. PPDK was assayed at 25 °C under maximum velocity conditions in the ATP-forming direction using the spectrophotometric assay described by Wang et al. (1988). The reaction solutions contained (in a total volume of 1 mL) 150 mM imidazole hydrochloride (pH 7.0), 20 mM NH_4Cl, 5 mM $MgCl_2$ or $CoCl_2$, 1 mM PP_i, 0.4 mM AMP, 0.5 mM PEP, and a catalytic amount of PPDK. PPDK was assayed in the PEP forming direction under maximum velocity conditions at 25 °C by following the conversion of [^{14}C]ATP to [^{14}C]AMP. The reaction solutions contained (in a total volume of 500 μL) 150 mM imidazole hydrochloride (pH 7.0), 10 mM NH_4Cl, 5 mM $MgCl_2$ or 1 mM $CoCl_2$, 10 mM P_i, 0.5 mM ATP (spiked with [^{14}C]ATP, sp act. = 560 mCi/mmol), 1 mM pyruvate, and a catalytic amount of PPDK. Yeast inorganic pyrophosphatase was included to drive the reaction to completion. Samples were analyzed for [^{14}C]ATP and [^{14}C]AMP as described for the AMP $\leftrightarrow$ ATP exchange reactions. The turnover numbers for

[2] A preliminary report of this work is found in Carroll et al. (1989).

ATP formation were determined to be 16 s^{-1} with the Mg^{2+}/NH_4^+ activated PPDK and 3 s^{-1} with the Co^{2+}/NH_4^+ activated PPDK and for PEP formation, 5 s^{-1} with the Mg^{2+}/NH_4^+ activated PPDK and 2 s^{-1} with the Co^{2+}/NH_4^+ activated PPDK. The specific activity of the PPDK used to measure the single-turnover data presented in Figure 2 as well as the ^{31}P NMR data was approximately 1.5–2.5-fold higher (i.e., ~20 units/mg vs 8–12 units/mg).

Hydrolysis of the Pyrophosphorylenzyme. The E-PP used for the hydrolysis studies was prepared by reaction of 40 μM PPDK, 10 μM [γ-^{32}P]ATP, 10 mM NH_4Cl, and 5 mM $MnCl_2$ in 50 mM K^+Hepes (pH 7.0) at 7 °C. The reaction was started by the addition of the [γ-^{32}P]ATP. Aliquots (86 μL) were removed at specific times and added to 165 μL of 0.6 M HCl and 100 μL of CCl_4 with vortexing to precipitate the enzyme. For the hydrolysis carried out in 0.4 M HCl, 330 μL of 0.6 M HCl was added to the 172-μL reaction mixture immediately following the addition of [γ-^{32}P]ATP. Aliquots (130 μL) were removed at specific times and added to 100 μL of CCl_4 with vortexing to precipitate the enzyme. The enzyme precipitate was pelleted by centrifugation and analyzed as described above.

RESULTS AND DISCUSSION

Formation of the E-PP·AMP Complex. Previous attempts in our laboratory to generate radiolabeled PPDK by reaction with [γ-^{32}P]ATP (or [β-^{32}P]ATP) in buffered solution containing $MgCl_2$ and NH_4Cl in yields exceeding a few percent failed (Wang et al., 1988). This failure coupled with the observed requirement of P_i/PP_i for PPDK catalyzed [^{14}C]-AMP $\rightleftharpoons$ ATP exchange and $\alpha,\beta \rightarrow \alpha$ positional isotope exchange in [α,β-^{18}O,β,β-$^{18}O_2$]ATP suggested that P_i binding to the enzyme–ATP complex may be required for pyrophosphorylenzyme formation. To test for pyrophosphorylenzyme formation from the reaction of PPDK and ATP in the presence and absence of P_i, we turned to transient kinetic techniques.

First, PPDK in buffered solution containing $MgCl_2$ and NH_4Cl was mixed with a limiting amount of [γ-^{32}P]ATP in a rapid quench apparatus. The amount of radiolabeled enzyme formed over the time course of a single turnover was measured by quenching the reaction after the specified aging period (milliseconds) with acid, precipitating the protein from the quench mixture with organic solvent, and assaying the radioactivity contained in the precipitated enzyme using liquid scintillation techniques. The concentration of radiolabeled enzyme formed in the reaction vs reaction time is plotted in Figure 1. The amount of radiolabel transferred from [γ-^{32}P]-ATP to the enzyme plateaued at a maximum of 6%. The control reactions in which [^{14}C]ATP was substituted for [γ-^{32}P]ATP or in which the [γ-^{32}P]ATP was added after, not before, the enzyme was quenched with acid resulted in 1–2% transfer of radiolabel from the ATP to the enzyme pellet.

Next, the level of E-PP·AMP formed by reaction of Mg^{2+}/NH_4^+ activated PPDK with [γ-^{32}P]ATP was measured in the presence of P_i. The results, shown in Figure 1, reflect an increase and then decrease in the level of E-PP·AMP observed during the course of a single turnover of the enzyme. Thus, while the maximum level of E-PP·AMP generated during a single turnover is quite small, its rapid formation and, then in the presence of P_i, rapid decline suggests that it is a competent intermediate in the PPDK reaction.

Alternate reaction conditions were explored for the purpose of enhancing the accumulation of the E-PP·AMP complex during PPDK turnover. Increasing or decreasing the pH of the reaction from 7.0 by 1 pH unit had no significant effect

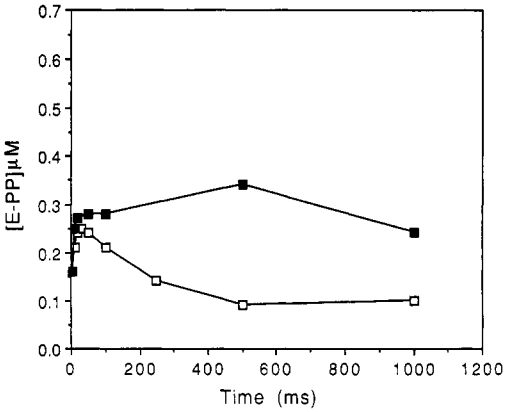

FIGURE 1: Time course profile for the pyrophosphorylenzyme intermediate formed from the active site of Mg^{2+}/NH_4^+ activated PPDK (sp act. = 8 units/mg) at 25 °C. The reaction contained 6.4 μM [γ-^{32}P]ATP, 34 μM PPDK active sites, 1 mM pyruvate, 2.5 mM $MgCl_2$, 10 mM NH_4Cl, 50 mM K^+Hepes (pH 7.0), and (■) 0 mM P_i or (□) 3 mM P_i.

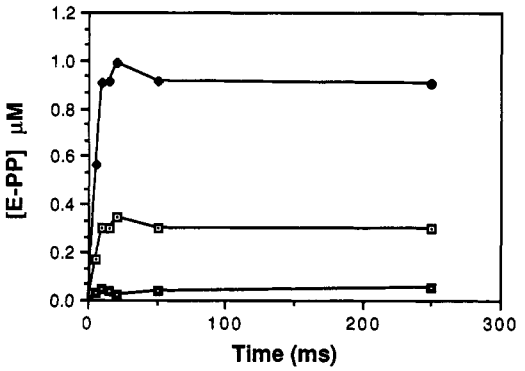

FIGURE 2: Time course profile for the pyrophosphorylenzyme intermediate formed from a single turnover of [γ-^{32}P]ATP in the active site of PPDK (sp act. = 20 units/mg) activated by NH_4^+ and different divalent cations at 25 °C. The reactions contained 1.5 μM [γ-^{32}P]ATP, 40 μM PPDK active sites, 10 mM NH_4Cl, 50 mM K^+Hepes (pH 7.0), and 2.5 mM (□) $MgCl_2$, (⊡) $CoCl_2$ or (◆) $MnCl_2$.

on E-PP·AMP accumulation. Attention was next directed to the divalent metal ion cofactor. While the physiological divalent metal ion cofactor for PPDK is Mg^{2+}, other divalent metal ions (viz., Mn^{2+} and Co^{2+}) have been reported to activate the enzyme (Michaels et al., 1978). In the present study, we found that the velocity of [^{14}C]AMP $\rightleftharpoons$ ATP exchange measured in the presence of P_i and PP_i at pH 7.0 as a function of divalent cation (see Experimental Procedures) follows the order Mg^{2+} (110 μmol/(min·mg)) > Mn^{2+} (60 μmol/min·mg)) $\gg$ Co^{2+} (4 μmol/(min·mg)). On the basis of the observed sensitivity of the ATP/P_i partial reaction to the nature of the divalent metal ion cofactor, the use of alternate divalent metal ion cofactors was targeted as a possible means to manipulate and enhance the level of E-PP·AMP complex formation in the reaction of PPDK with ATP.

Accordingly, the formation of radiolabeled pyrophosphorylenzyme from the reaction of [γ-^{32}P]ATP with excess PPDK in the presence of NH_4Cl and $MgCl_2$, $CoCl_2$, or $MnCl_2$ was measured. The results obtained, illustrated as a plot of the amount of radiolabeled enzyme formed as a function of reaction time, are presented in Figure 2. As can be seen from Figure 2, the effect of the metal ion cofactor on the amount of E-PP·AMP observed at equilibrium is significant. A priori, the metal ion cofactor could affect the E-PP·AMP level by affecting the ATP binding affinity to the enzyme and/or by affecting the stability of the E-PP·AMP complex formed.

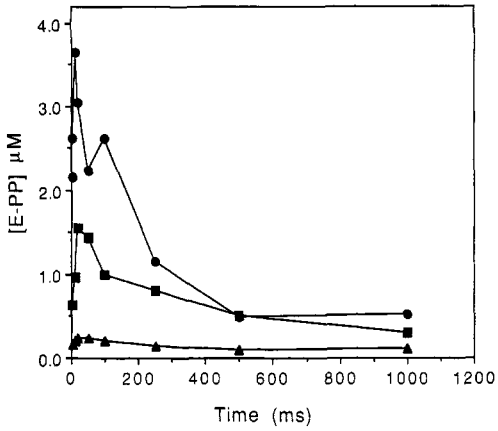

FIGURE 3: Time course profile for the pyrophosphorylenzyme intermediate formed from a single turnover of [γ-^{32}P]ATP, P_i, and pyruvate in the active site of PPDK (sp act. = 8 units/mg) activated by NH_4^+ and different divalent cations at 25 °C. The reactions contained 6 μM [γ-^{32}P]ATP, 34 μM PPDK active sites, 10 mM NH_4Cl, 2 mM P_i, 1 mM pyruvte, 50 mM K^+Hepes (pH 7.0), and 2.5 mM (▲) $MgCl_2$, (■) $CoCl_2$, or (●) $MnCl_2$.

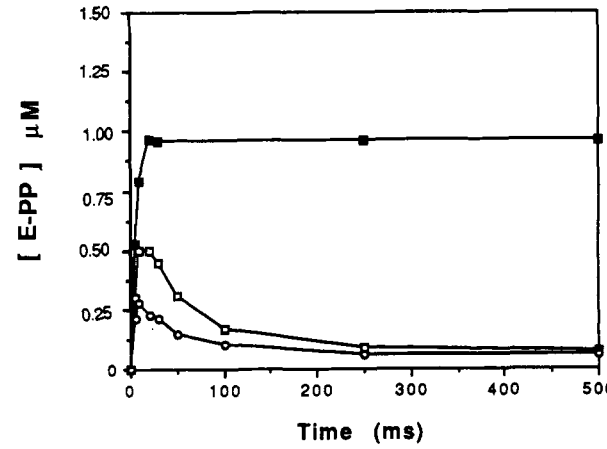

FIGURE 4: Time course profile for the pyrophosphorylenzyme intermediate formed from a single turnover of [γ-^{32}P]ATP and P_i in the active site of Co^{2+}/NH_4^+ activated PPDK (sp act. 8 units/mg) at 25 °C. The reaction contained 5.4 μM [γ-^{32}P]ATP, 36 μM PPDK active sites, 10 mM NH_4Cl, 2.5 mM $CoCl_2$, 50 mM K^+Hepes (pH 7.0), and (■) 0 mM P_i, (□) 2 mM P_i, or (○) 20 mM P_i.

While the dissociation constant for the E·ATP complex is not known, we can approximate it from the dissociation constant measured for the enzyme complex with the ATP analog AMPPNP. In the presence of Mg^{2+}/NH_4^+, the K_D = 50 μM and in the presence of Co^{2+}/NH_4^+, the K_D = 13 μM (Mehl, 1991). Using these values, we can calculate that under the reaction conditions (1.5 μM ATP, 40 μM PPDK) approximately 50% of the ATP would be enzyme bound prior to turnover in the presence of Mg^{2+}/NH_4^+ while approximately 80% would be bound in the presence of Co^{2+}/NH_4^+. Thus, while a portion of the equilibrium effect observed in Figure 2 may result from the metal ion effect on ATP binding, a majority appears to derive from a shift in the internal E·ATP ⇌ E-PP·AMP equilibrium wherein the E-PP·AMP complex is stabilized to the greatest extent by Mn^{2+} and the least extent by Mg^{2+}.

The effect of the divalent metal ion cofactor on the stability of the E-PP·AMP complex is also reflected in the level to which the E-PP·AMP intermediate accumulates during the single-turnover reaction of the enzyme with [γ-^{32}P]ATP and P_i. As shown in Figure 3, the maximum level of E-PP·AMP reached during turnover is greatest for Mn^{2+} and smallest for Mg^{2+}. The competence of the E-PP·AMP intermediate in the overall reaction is demonstrated by the rates of E-PP·AMP formation and disappearance which fall within those predicted by the turnover rate observed under steady-state conditions (viz., 16 s^{-1} for Mg^{2+}/NH_4^+ activated PPDK, 3 s^{-1} for Co^{2+}/NH_4^+ activated PPDK; see Experimental Procedures).

Reaction of P_i with E-PP·AMP. The data described in the previous section convincingly demonstrate that PPDK catalyzes E-PP·AMP formation from ATP in the absence of added P_i. This finding is in-line with Wood's proposed chemical mechanism of PPDK catalysis involving the intermediacy of a novel pyrophosphorylenzyme (Wood et al., 1977). We are still unable, however, to account for the high yield of E-PP·AMP generated from the equilibration of [γ-^{32}P]ATP with the Mg^{2+}/NH_4^+ activated enzyme that was reported by Milner et al. (1978). On the basis of the results presented in this paper, we suggest that the internal equilibrium constant limits E-PP·AMP formation from E·ATP in the presence of Mg^{2+}/NH_4^+ to ≤10%.

The issue that remains is that of the kinetic mechanism. The studies reported in Wang et al. (1988) demonstrated that the kinetic mechanism is, as shown in Scheme II, bi-bi-uni-uni and not tri-uni-uni (Scheme I) as reported in Milner et al. (1978). The bi-bi-uni-uni mechanism, however, must now take into account the present finding that E-PP·AMP forms from E·ATP in the absence of P_i. Specifically, the observed requirement of P_i for PPDK catalyzed [^{14}C]AMP ⇌ ATP exchange and $\alpha,\beta \rightarrow \alpha$ positional isotope exchange in [α,β-^{18}O,β,β-$^{18}O_2$]ATP (Wang et al., 1988) must be explained. One possible explanation is that P_i binding stimulates the rate of reaction of PPDK with ATP. The similar rates observed for E-PP·AMP formation from [γ-^{32}P]ATP and Mg^{2+}/NH_4^+ activated PPDK in the presence and absence of P_i (depicted in Figure 1), however, suggest that P_i does not significantly stimulate EPP·AMP formation. The independence of the pyrophosphorylenzyme forming step and the P_i binding step is clearly illustrated in Figure 4, wherein the formation and disappearance of pyrophosphorylenzyme is plotted as a function of the concentration of P_i present in the [γ-^{32}P]ATP + PPDK (Co^{2+}/NH_4^+) reaction mixture. These data show that the rate of E-PP·AMP consumption rather than formation increases as the concentration of P_i increases. If P_i binding to the E·ATP complex initiated pyrophosphorylenzyme formation, then the level and lifetime of the E-PP intermediate would increase as the concentration of P_i in the reaction mixture was increased.[3] Instead, inclusion of P_i in the ATP/PPDK reaction mixture decreases the amount of E-PP. This result (as do the data shown in Figure 5) confirms that P_i binding follows rather than precedes pyrophosphorylenzyme formation.

Given that P_i does not stimulate E-PP·AMP formation from PPDK and ATP, its effect on the [^{14}C]AMP ⇌ ATP exchange and the $\alpha,\beta \rightarrow \alpha$ positional isotope exchange in [α,β-^{18}O,β,β-^{18}O]ATP must derive from its effect on the E-PP·AMP complex. If AMP release from E-PP·AMP is precluded and α-P torsional equilibration is restricted, P_i could enhance both exchange reactions by converting E-PP·AMP to E-P·AMP·PP_i

[3] The computer-based kinetics simulation program KINSIM (Barshop et al., 1983) as modified by Ken Johnson (Anderson et al., 1988) was used to stimulate curves describing the rate of accumulation and disappearance of the pyrophosphorylenzyme as a function of the P_i concentration in the reaction of PPDK, [γ-^{32}P]ATP, and P_i (conditions reported in Figure 4) using a kinetic model in which pyrophosphorylenzyme is formed only from the E·ATP·P_i complex. Contrary to what was found experimentally (Figure 4), this model predicted the highest level of pyrophosphorylenzyme to be observed at 200 mM P_i and the lowest at 2 mM P_i (Mehl, 1991).

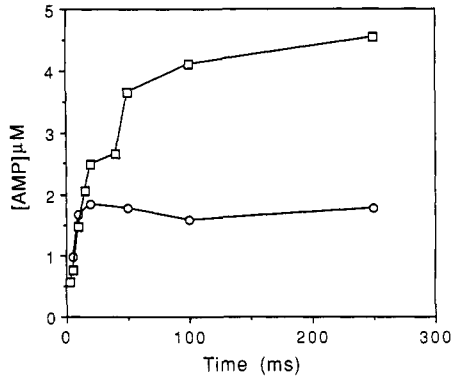

FIGURE 5: Time course profile for AMP formed from a single turnover of [^{14}C]ATP in the active site of Co^{2+}/NH_4^+ activated PPDK (sp act. = 8 units/mg) at 25 °C. The reaction contained 4.7 μM [^{14}C]ATP, 44 μM PPDK active sites, 10 mM NH_4Cl, 2.5 mM $CoCl_2$, 50 mM K^+Hepes (pH 7.0), and (○) 0 mM P_i or (□) 50 mM P_i.

and, hence, by ordered release of PP_i and AMP (Wang et al., 1988) to E-P. The conversion of E-PP·AMP to E-P·AMP·PP_i in the presence of P_i is apparent from Figures 1, 3, and 4 where the removal of the ^{32}P-radiolabeled from E-PP·AMP generated from reaction of [γ-^{32}P]ATP with PPDK is depicted and from Figure 5 where the single-turnover time course of the reaction of [^{14}C]ATP with excess PPDK (Co^{2+}/NH_4^+) in the presence and absence of P_i is depicted. As can be seen from Figure 5, inclusion of 50 mM P_i in the reaction increased the amount of AMP formed from ≈30% to ≈90% of the bound ATP by driving the conversion of the E-PP·AMP species to E-PP·AMP·P_i and hence to E-P·AMP·PP_i.

On the basis of these results, we conclude that the P_i required for the [^{14}C]AMP ⇌ ATP exchange (Wang et al., 1988) converts the E-PP·AMP complex (from which AMP is not released) to the E-P·AMP·PP_i complex for eventual AMP release. Similarly, the P_i required for the PPDK catalyzed $\alpha,\beta \rightarrow \alpha$ positional isotope exchange in [α,β-^{18}O,β,β-$^{18}O_2$]-ATP functions to convert the E-PP·AMP complex, in which torsional rotation about the P-O-(5′) bond is restricted, to the E-P·AMP·PP_i (or E-P·AMP) complex, in which torsional rotation is allowed. The kinetic mechanism depicted in Scheme II (Wang et al., 1988) is, in Scheme III, modified to reflect these findings and therefore, a more accurate picture of PPDK catalysis.[4]

Scheme III: Revised PPDK Reaction Sequence

$$E + ATP \rightleftharpoons E\cdot ATP \rightleftharpoons E\text{-}PP\cdot AMP \overset{P_i}{\rightleftharpoons} E\text{-}PP\cdot AMP\cdot P_i \rightleftharpoons$$

$$E\text{-}P\cdot AMP\cdot PP_i \underset{PP_i}{\rightleftharpoons} E\text{-}P\cdot AMP \underset{AMP}{\rightleftharpoons} E\text{-}P \overset{pyruvate}{\rightleftharpoons}$$

$$E\text{-}P\cdot pyruvate \rightleftharpoons E\cdot PEP \rightleftharpoons E + PEP$$

Formation of E-P. Previous work had shown that ^{32}P-radiolabeled enzyme could be formed by reaction of PPDK with either [β-^{32}P]ATP and P_i or [^{32}P]PEP (Milner et al., 1978). To test the kinetic competence of the radiolabeled enzyme as an intermediate in the overall reaction, we monitored E-P levels during a single turnover of [^{32}P]PEP in the presence and absence of AMP and PP_i. Enzyme, cofactors, and

[4] Comparison of the P_i binding rate (0.03 $\mu M^{-1}\,s^{-1}$) vs the ATP binding rate (4 $\mu M^{-1}\,s^{-1}$) and E·ATP → E-PP·AMP turnover (220 s^{-1}) suggests that under conditions of [ATP] ≈ [P_i], P_i is binding to E-PP·AMP rather than to E or E·ATP (Mehl, 1991).

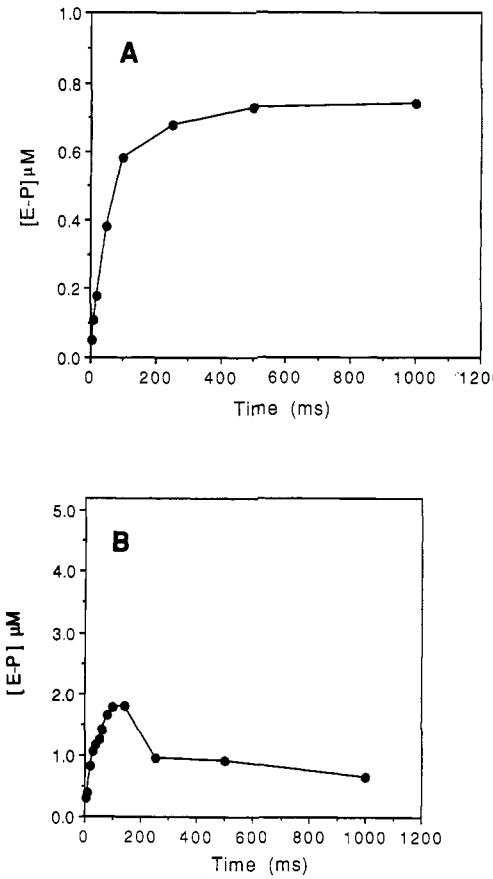

FIGURE 6: Time course profile for the phosphorylenzyme intermediate formed from a single turnover of [^{32}P]PEP in the active site of Mg^{2+}/NH_4^+ activated PPDK (sp act. = 8 units/mg) at 25 °C. The reaction contained 5 mM $MgCl_2$, 20 mM NH_4Cl, 50 mM K^+Hepes (pH 7.0), and (A) 1 μM [^{32}P]PEP and 20 μM PPDK active sites or (B) 4.8 μM [^{32}P]PEP, 32 μM PPDK active sites, 20 μM AMP, and 100 μM PP_i.

reactants were mixed with the [^{32}P]PEP in a rapid quench apparatus and after a specified time the reaction mixture was quenched with HCl. The ^{32}P-radiolabeled enzyme was precipitated from this mixture with organic solvent, collected, and then quantitated by liquid scintillation counting.

The time course for E-P formation during a single turnover of [^{32}P]PEP (1 μM) in the active site of Mg^{2+}/NH_4^+ activated PPDK (20 μM) is shown in Figure 6A. Approximately 70% of the ^{32}P-radiolabel was transferred from the [^{32}P]PEP to the enzyme at an observed rate of ~9 s^{-1} as compared to a 5 s^{-1} PEP turnover rate measured under steady-state conditions (see Experimental Procedures). In the presence of AMP (20 μM) and PP_i (100 μM) the E-P formed from 4.8 μM [^{32}P]PEP and 32 μM PPDK accumulated to a level of ~40% and then rapidly declined to an equilibrium level of ~15%. The observed rate of E-P formation and conversion falls within the time frame predicted by the steady-state turnover rate of enzyme demonstrating the competency of the E-P·pyruvate intermediate in the overall reaction (see Scheme III).

^{31}P NMR Analysis of the Pyrophosphoryl and Phosphorylenzyme. E-PP. Because of the favorable equilibrium between E-PP·AMP and E·ATP observed in the presence of Co^{2+} or Mn^{2+} and NH_4^+ (Figure 2), it is in principle possible to generate enough pyrophosphorylenzyme for structural characterization. In practice, however, the technical difficulties have thus far proved insurmountable. First, the pyrophosphorylated enzyme was found to be too unstable at neutral pH to allow for concentration of the enzyme sample and data collection to be made ($t_{1/2}$ at pH 7.0 and 7 °C < 10

min as measured from the rate of loss of radiolabel from E-PP prepared from [γ-^{32}P]ATP (see Experimental Procedures)). In fact, ^{31}P NMR analysis of a 2 mM enzyme sample generated by concentrating a 9-mL reaction solution of 100 μM PPDK, 100 μM ATP, 0.5 mM $CoCl_2$, 11 mM NH_4Cl, and 50 mM K^+Hepes (pH 7.0), quenched with 1.5 mM EGTA and 5 M urea, to 0.3 mL using an Amicon device revealed a 2:1 ratio of inorganic phosphate (+2.3 ppm) and phosphorylenzyme (–3.9 ppm; see below). Similar results were obtained using reaction solutions quenched only with EGTA. The E-PP was observed to be comparatively more stable at low pH ($t_{1/2}$ = 18 h at 7 °C in 0.4 M HCl); however, concentration of the enzyme–ATP reaction mixture quenched with 0.4 M HCl in an Amicon cell resulted in extensive precipitation of the enzyme. The search for a useful protocol is continuing.

E-P. In contrast to the pyrophosphorylenzyme, the phosphorylenzyme proved amenable to ^{31}P NMR analysis. Three issues addressed by ^{31}P NMR analysis are the position of N-phosphorylation on the catalytic histidine, the environment of the phosphorylated histidine, and metal cofactor binding to the phosphorylated histidine.

The chemical shift measured (at 162 MHz) for PEP-phosphorylated PPDK (see Experimental Procedures) denatured in 5 M urea (pH 7.0) is, as shown in Figure 7, –3.9 ppm. The ^{31}P NMR spectrum measured for independently prepared 3-*N*-phosphohistidine and 1-*N*-phosphohistidine (Gassner et al., 1977) in 5 M urea at pH 7 showed phosphorus signals from these compounds at –3.9 and –4.7 ppm, respectively. These results suggest that the PEP-phosphorylated PPDK contains a 3-*N*-phosphohistidine residue. This finding is in agreement with the 3-*N*-phosphohistidine assignment that was made earlier (Spronk et al., 1976) on the basis of the paper chromatographic retention time of the phosphohistidine residue obtained from phosphorylated PPDK digested in aqueous base.

The ^{31}P NMR resonance measured at 162 MHz for native PEP-phosphorylated PPDK[5] at pH 7.0 occurs at –4.0 ppm (Figure 7). Although the chemical shift of the resonance from native phosphorylenzyme is almost identical to that of the resonance from the denatured phosphorylenzyme (viz. –3.9 ppm)[6] the line width is significantly broader (45 Hz vs 7 Hz). The broader line width would suggest a more motion restricted environment for the phosphohistidine residue of the native vs unfolded phosphorylenzyme.

The environment of the phosphohistidine residue in the native enzyme was probed further by measuring the field dependence of the line width of its ^{31}P resonance (Figure 7). In order to separate the contributions from the field-independent dipole–dipole couplings and field-dependent chemical shift anisotropy (CSA), a plot of the observed line width vs the square of the magnetic field strength was made. Figure 8 shows a linear dependence of field strength and line width of the phosphoryl resonance which is diagnostic for CSA relaxation (Vogel et al., 1982). The dashed line in Figure 8 represents the intercept for no applied field, approximately

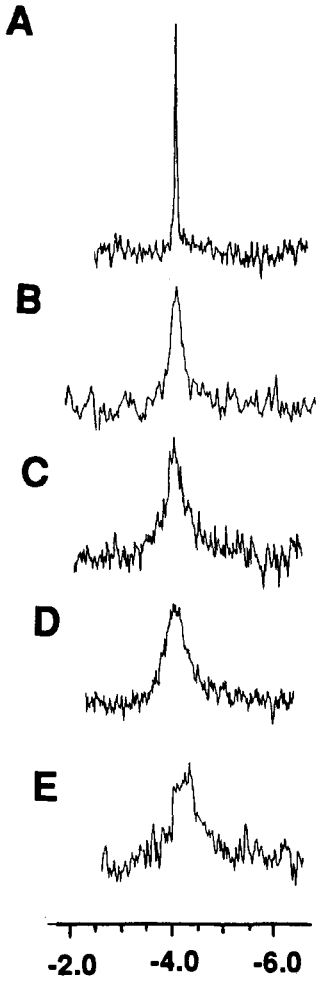

FIGURE 7: ^{31}P NMR spectra at 27 °C of phosphorylated PPDK prepared as described in Experimental Procedures. Spectra were plotted with an instrumental line broadening of 3 Hz, unless otherwise noted. Samples contained 30% v/v D_2O, 1 mM EGTA, and phosphorylated enzyme in 50 mM K^+Hepes, pH 7.0, with the following concentrations and number of scans: (A) 400 μM denatured E-P in 5 M urea, 21 699 scans at 162 MHz; (B) 700 μM native E-P, 23 595 scans at 81 MHz; (C) 600 μM native E-P, 28 961 scans at 162 MHz; (D) 600 μM native E-P, 15 000 scans at 203 MHz; (E) 800 μM native E-P, 5 mM $MgCl_2$, no EGTA, 25 000 scans at 162 MHz; plotted with 5 Hz instrumental line broadening.

6 Hz, which is the field-independent line width contribution from diple–dipole interaction.

The anisotropy value for the 3-*N*-phosphohistidine of native E-P was calculated from the observed line width minus the dipolar contribution (6 Hz) ($\Delta\nu$) according to eq 2 (Shindo, 1980; Hull & Sykes, 1975) where ω_p is the magnetic field strength for phosphorus, $(\Delta\sigma)^2(1 + (\eta^2)/3)$ is the anisotropy term (where $\Delta\sigma$ is the anisotropy of the chemical shift and η is the asymmetry term), and τ_c is the rotational correlation time. Substituting the line width (observed minus dipolar

$$\pi\Delta\nu = 4/45\omega_p^2(\Delta\sigma)^2\left(1 + \frac{\eta^2}{3}\right)\tau_c \qquad (2)$$

contribution), the corresponding field strength, and a rotational correlation time of 55 ns calculated for PPDK,[7] we arrived at the value of 156 ppm. This value is somewhat lower than that reported for the model compound, imidazole diphosphate, 187 ppm, and considerably lower than the values determined

[5] The catalytic viability of the native E-P used in the ^{31}P NMR studies was demonstrated by treating the E-P sample with AMP, PP_i, $MgCl_2$, and NH_4Cl and then analyzing the resulting mixture at pH 7 by ^{31}P NMR. The E-P signal at –4.0 ppm was replaced by signals at –18.5, –9.9, and –4.8 ppm deriving from ATP.

[6] For comparison, the 3-*N*-phosphohistidine of phosphorylated histone H_4 is shifted 2.5 ppm upfield in the native vs denatured state (Fujita et al., 1981) and the 1-*N*-phosphohistidine of HPr is shifted 1.6 ppm downfield in the native vs denatured state (Gassner et al., 1977) while the 3-*N*-phosphohistidine of the ATP-citrate lyase resonates at –3.7 ppm both in the native and in the denatured state (Williams et al., 1985).

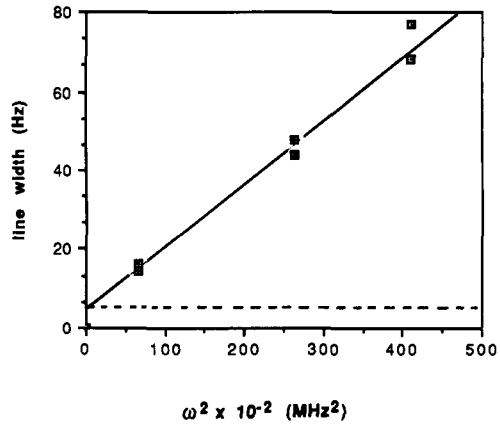

FIGURE 8: Plot of the line width of the phosphorus resonance from E-P vs the square of the magnetic field strength (the data were taken from Figure 7).

for the proteins HPr of the PEP: sugar phosphotransferase system (228 ppm) and succinyl-CoA synthetase (232 ppm) (Vogel et al., 1982), for which immobilization of the phosphohistidine residue in an electronically asymmetric environment has been proposed.

Previous studies have shown that phosphorylation of PPDK with PEP requires divalent and monovalent metal ion cofactors (Micheals et al., 1978; Moskovitz & Wood, 1978). In order to determine whether the divalent metal ion cofactor interacts with the phosphohistidine residue of E-P, the effect of Mg^{2+} and Mn^{2+} complexation to E-P on its ^{31}P NMR resonance was measured. Complexation of Mg^{2+} (5 mM) with the phosphorylenzyme (0.8 mM) ($K_D \approx 0.7$ mM; S. H. Thrall and D. Dunaway-Mariano, unpublished results) resulted in a 0.3 ppm upfield shift of the phosphorus resonance without extensive line broadening (Figure 7). Complexation of (paramagnetic) Mn^{2+} (0.14 mM) with the phosphorylenzyme (0.7 mM) in 50 mM K^+Hepes (pH 7) resulted in such extensive line broadening that the ^{31}P NMR signal was not visible. At 30 μM Mn^{2+} a 2-fold broadening effect was observed. Together, these results suggest that the divalent metal cofactor interacts with the phosphoryl group of E-P.[8]

The ^{31}P NMR spectrum of phosphorylated PPDK (0.67 mM) measured at 162 MHz in the presence of $MgCl_2$ (5 mM) and oxalate (1.3 mM) in 50 mM K^+Hepes (pH 7) showed no detectable phosphorus signal from E-P. A reasonable explanation for the oxalate effect can be made on the basis of the EPR studies carried out by Reed and co-workers (Michaels et al., 1975; Kofron et al., 1988). These workers showed that the phosphoryl group in the E-P·Mn·oxalate complex (a mimic of the intermediate state E-P·Mn·pyruvate (enolate)) is coordinated to the Mn(II) which in turn is coordinated to the oxalate. By analogy, the phosphoryl group of the E-P·Mg·oxalate complex would be immobilized in a highly assymetric environment, thus resulting in a larger correllation time ($\tau_c \approx 55$ ns) and most probably, in an enhanced CSA.

Conclusions. The transient kinetic experiments presented in this paper provide conclusive evidence for the intermediacy of the pyrophosphoryl- and phosphorylenzyme complexes during turnover in the PPDK active site. Furthermore, they have allowed us to refine the bi-bi-uni-uni kinetic mechanism proposed in Wang et al. (1988) to that shown in Scheme III.

The ^{31}P NMR studies of the phosphorylenzyme have provided evidence of a 3-*N*-phosphohistidine residue which appears to become immobilized in an electronically assymetric environment upon cofactor (Mg^{2+}) and substrate analog (oxalate) binding. The question of whether or not acquiring this new environment involves translocation of the phosphohistidine residue, as Wood's "swinging arm" model (Wood et al., 1977) might suggest, will require further study.

[7] Calculated on the basis of the rotational correlation time of a sphere, having a radius of 40 Å, as deduced from the molecular weight (MW): $r = [3MW\bar{v}/(4\pi N)]^{1/3}$ where $\bar{v}$ is the partial specific volume (0.73 mL/g) and N is Avogadro's number. Three angstroms were added to this radius to account for hydration of the protein. The overall correlation time was calculated from the Stokes-Einstein equation: $\tau_c = (4\pi\eta r^3)/(3kT)$ where η is the viscosity (0.8513 cP at 27 °C), T = 300 K, and k is Boltzmann's constant.

[8] These metal effects are analogous to those reported for phosphorylated succinyl-coenzyme A synthetase where interaction between the metal ion and the phosphoryl group was indicated (Vogel & Bridger, 1982).

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