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Phosphonate Analogue Substrates for Enolase†

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ABSTRACT: Phosphonate analogues in which the bridge between C-2 and phosphorus is a CH_2 group are slow substrates for yeast enolase. The pH variation of the kinetic parameters for the methylene analogue of 2-phosphoglycerate suggests that the substrate binds as a dianion and that Mg^{2+} can bind subsequently only if a metal ligand and the catalytic base are unprotonated. Primary deuterium isotope effects of 4–8 on V/K_{Mg}, but ones of only 1.15–1.32 on V for dehydration, show that proton removal to give the carbanion intermediate largely limits V/K_{Mg} and that a slow step follows which largely limits V (presumably carbanion breakdown). Since there is a D_2O solvent isotope effect on V for the reverse reaction of 5, but not an appreciable one on the forward reaction, it appears that the slow rates with phosphonate analogues result from the fact that the carbanion intermediate is more stable than that formed from the normal substrates, and its reaction in both directions limits V. Increased stability as a result of replacement of oxygen by carbon at C-2 of the carbanion is the expected chemical behavior.

When one is trying to determine the identity of enzymic acid–base catalysts by study of pH profiles, one obtains equivocal data if the substrates are "sticky", that is, if substrate release from the enzyme is slower than the rate of reaction and release of the first product. Consequently, it is desirable to use slow alternate substrates where the rate of the chemical reaction has become slower than both product and substrate dissociation rates. These conditions will normally hold if the maximum velocity is considerably smaller and the Michaelis constant is larger for the alternate substrate than for the normal one. This approach was fruitful in the study of serine as an alternate substrate for alanine dehydrogenase (Grimshaw et al., 1981).

Stubbe and Kenyon reported that MePEP[1] (the methylene-bridged analogue of phosphoenolpyruvate) was a slow alternate substrate for enolase. The maximum velocity was 1.6% of that with phosphoenolpyruvate, and K_{MePEP} was larger than K_{PEP}. We report in this paper the isolation of MePGA (the methylene analogue of 2-phosphoglycerate, and the product of the hydration of MePEP), the pH variation of the kinetic parameters, and the primary deuterium and D_2O solvent isotope effects with the methylene analogue substrates. We conclude that these phosphonate analogues are slow substrates because the intermediate carbanion is too stable, so that its decomposition is rate limiting in both directions.

Materials and Methods

Materials. Yeast enolase was obtained from Sigma as the lyophilized powder. MePEP was synthesized by the method of Stubbe and Kenyon (1972). The free acid, crystallized after removal of the 48% HBr hydrolysis solvent, was purified by dissolving 400 mg in 20 mL of water, titrating to pH 9.0 with KOH, loading this solution on a 2.5 × 25 cm column of Do-

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[1] Abbreviations: MePEP, the methylene-bridged analogue of phosphoenolpyruvate; MePGA, the methylene-bridged analogue of 2-phosphoglycerate.

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wex-1-X8-Cl, and eluting with a linear 0–0.3 M LiCl gradient. MePEP was preceded by a contaminant absorbing at 240 nm. The fractions containing MePEP were pooled, titrated to pH 10, and lyophilized, and LiCl was removed by solution in 5 mL of water and precipitation of the trilithium salt with 50 mL of MeOH. The salt was collected by centrifugation and dried in vacuo over P_2O_5. The 60-MHz proton NMR spectrum was consistent with that reported by Stubbe and Kenyon (1972). Titration of a 2 mM solution with HCl gave pK values of 8.25 and 4.6. For use in kinetic studies the trilithium salt was converted into the tripotassium salt by passage through a column of Dowex-50-K^+.

(2-Carboxy-3-hydroxypropyl)phosphonic acid (MePGA) was synthesized by adding 5 mg of enolase to 20 mL of 20 mM MePEP, 5 mM $Mg(OAc)_2$, 10 mM Taps, pH 9.0, and 0.1 mM EDTA. The approach to equilibrium was followed spectrophotometrically. After equilibrium was reached, the reaction was stopped by adding 5 drops of CCl_4 and vortexing vigorously. The reaction mixture was filtered through a 40-μm Millipore filter, placed on the previously described Dowex-1 column, and eluted with 20 mM HCl. Small aliquots of each fraction were added to an enolase assay, and the change in A_{240} was noted. MePGA (A_{240} increases with time in the assay) eluted after 100 mL, and MePEP (A_{240} decreases with time) eluted after 210 mL. The MePGA fractions were pooled and titrated to pH 10 with LiOH, and the tripotassium salt was obtained by the same procedure used with MePEP. The 270-MHz proton NMR spectrum in D_2O showed δ 1.70 (m, 2 H), 2.60 (m, 1 H), and 3.60 (octuplet, 2 H). Decoupling the δ 2.60 multiplet converted the δ 1.70 multiplet to an octuplet, J_{HP} = 17 Hz and J_{HH} = 15 Hz, and the δ 3.60 multiplet to a quartet, J_{HH} = 12 Hz. Titration with HCl gave secondary phosphonate and carboxyl pK values of 7.85 and 4.70, respectively.

MePGA-*2-d* was synthesized similarly, except that prior to the addition of enolase the reaction mixture was flashed to dryness, dissolved in 5 mL of 99.8% D_2O, redried, and dissolved finally in 20 mL of D_2O. Enolase was added as the lyophilized powder. The 270-MHz proton NMR spectrum was identical with that of MePGA with the δ 2.60 multiplet uncoupled, and no peak was seen at δ 2.60, indicating over 98% deuteration at C-2.

Methods. MePEP and MePGA concentrations were determined by assaying for total phosphate after ashing with $MgNO_3$ (Ames, 1966), using methylphosphonic acid as the standard. This assay confirmed the extinction coefficient for MePEP of 554 M^{-1} cm^{-1} at 240 nm and pH 6.8 reported by Stubbe and Kenyon (1972). Equilibrium constants were determined by observing the fraction of absorbance from MePEP that disappeared following the addition of enolase, or from the concentrations of MePGA-*2-d* and MePEP that produced no net reaction in the equilibrium perturbation experiments.

Steady-state assays contained magnesium acetate varying from 0.1 to 100 mM with EDTA maintained at 1% of the total Mg^{2+} concentration. Ionic strength was maintained at 0.3 M by addition of an appropriate amount of potassium acetate. The stock potassium acetate solution was filtered through a 1.5 × 15 cm column of Chelex (K^+ form) to remove contaminating heavy metal ions. Buffers used (pH values) were acetate (5.5), Mes (6.2), Mops (7.1), and Taps (8.0 and 9.0). These buffers were shown not to inhibit enolase. Free Mg^{2+} concentrations were calculated by assuming that all EDTA was present as a Mg^{2+} complex and that the magnesium monoacetate complex has a dissociation constant of 300 mM (O'Sullivan, 1969). Assays were monitored at 240 nm and performed in a Gilford spectrophotometer thermostated at 25 °C. The extinction coefficient for MePEP at each pH and Mg^{2+} concentration was determined with a standardized MePEP solution.

Equilibrium perturbations initially contained equilibrium concentrations of MePEP and MePGA-*2-d*, magnesium and potassium acetates, and buffer as above. The perturbations were initiated by the addition of enolase. The maximum perturbation from the initial and final absorbance at 240 nm was determined and the isotope effect calculated by comparison to an analytical solution of the differential equations (Bahnson & Anderson, 1989).

For measurement of D_2O solvent isotope effects all reagents were dissolved in D_2O except enolase, which comprised less than 1% of the final volume. The pD was calculated as pH_{app} + 0.4 (Schowen, 1977).

Data Analysis. Kinetic parameters were determined from nonlinear least-squares fits of the data to the equations below. V, V/K_{Mg}, and K_{ia} (the dissociation constant of the organic substrate) were obtained from eq 1 at noninhibitory levels of Mg^{2+} ($<0.3K_I$). In eq 1, A and M are the concentrations of

$$v = \frac{VMA}{K_{ia}K_{Mg} + MK_a + AK_{Mg} + AM} \quad (1)$$

the organic substrate and Mg^{2+}, respectively, and K_a and K_{Mg} are their Michaelis constants. The K_I for substrate inhibition by Mg^{2+} was obtained from eq 2. The pH variation of V/K_{Mg} was fitted to eq 3, which assumes two pK's on the acid side.

$$v = \frac{VMA}{K_{ia}K_{Mg} + MK_a + AK_{Mg} + AM(1 + M/K_I)} \quad (2)$$

$$\log (V/K_{Mg}) = \log \left[\frac{C}{(1 + H/K_1)(1 + H/K_2)} \right] \quad (3)$$

The pH variation of $K_{i\,MePGA}$ was fitted to eq 4, which assumes one pK on the basic side. The direct comparison isotope

$$pK_{i\,MePGA} = \log \left(\frac{C}{1 + K/H} \right) \quad (4)$$

effects obtained with MePGA-*2-d* were obtained from separate fits to eqs 5–7. Equation 5 assumes different isotope effects on V, V/K_{Mg}, and V/K_{MePGA}. Equation 6 assumes one isotope effect on V and a different, but identical, isotope effect on the two V/K values. Equation 7 assumes identical isotope effects on all three parameters. In all three equations, F_i is the fraction of deuterium in the labeled molecule, and E is the isotope effect minus one on the subscripted kinetic parameter.

$$v = VAM/[K_{Mg}(K_{ia} + A)(1 + F_iE_{V/K_{Mg}}) + MK_a(1 + F_iE_{V/K_{MePGA}}) + AM(1 + F_iE_V)] \quad (5)$$

$$v = \frac{VAM}{[K_{Mg}(K_{ia} + A) + MK_a](1 + F_iE_{V/K}) + AM(1 + F_iE_V)} \quad (6)$$

$$v = \frac{VAM}{[K_{Mg}(K_{ia} + A) + MK_a + AM](1 + F_iE_V)} \quad (7)$$

The Mg^{2+} dependence of $^D(Eq.P.)_{MePGA}$ (the isotope effect calculated from an equilibrium perturbation experiment) was fitted to eq 8, where IE_0 is the limiting isotope effect at low Mg^{2+} and K is the concentration of Mg^{2+} that gives an isotope effect equal to $(IE_0 + 1)/2$.

$$^D(Eq.P.)_{MePGA} = \frac{IE_0 + M/K}{1 + M/K} \quad (8)$$

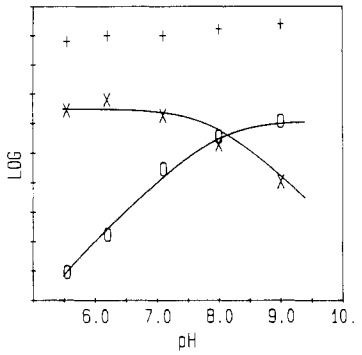

FIGURE 1: The pH dependence of log V (+), log V/K_{Mg} (O), and p$K_{i\,MePGA}$ (×) with MePGA as substrate. The ordinate units have the same spacing as the pH scale. V for MePGA was 0.4 s^{-1} at pH 7.1 (0.6% of the value with 2-P-glycerate as substrate). The solid lines are the best fits of the data to eq 3 for V/K_{Mg} (pK's of 5.0 and 8.0) and to eq 4 for p$K_{i\,MePGA}$ (pK 7.9). The limiting values for V/K_{Mg} and $K_{i\,MePGA}$ were 3×10^3 M^{-1} s^{-1} and 77 μM, respectively.

The solvent isotope effects in both directions were obtained by fitting the data obtained in water or D_2O to eq 9, where F_i is the fraction of D_2O and different isotope effects on V/K_{Mg} and V are assumed.

$$v = \frac{VAM}{K_{Mg}(K_{ia} + A)(1 + F_iE_{V/K_{Mg}}) + MK_a + AM(1 + F_iE_V)} \quad (9)$$

The affinity of Mg^{2+} for MePGA and MePEP was obtained from a fit to eq 10, where K_{eq}^{3-} is the equilibrium constant for the uncomplexed trianions, H and M are concentrations of H^+ and Mg^{2+}, respectively, K_a values are acid dissociation constants, and K_d values are dissociation constants of the Mg^{2+} complexes.

$$\text{app } K_{eq} = K_{eq}^{3-}\frac{(1 + H/K_{a\,MePEP} + M/K_{d\,MePEP})}{(1 + H/K_{a\,MePGA} + M/K_{d\,MePGA})} \quad (10)$$

RESULTS

pH Variation of Kinetic Parameters. When the levels of MePGA and Mg^{2+} were varied, intersecting initial velocity patterns were seen at all pH values. Uncompetitive substrate inhibition by Mg^{2+} was seen only at levels of 60 mM or above, and data used to obtain the pH variation of the kinetic parameters were limited to noninhibitory Mg^{2+} levels (this was no problem as K_{Mg} was <1 mM at pH 7.1). The pH variation of V_{max}, p$K_{i\,MePGA}$, and V/K_{Mg} are shown in Figure 1. p$K_{i\,MePGA}$ decreases above a pK of 7.9 ± 0.2. Because the pH profile for V/K_{Mg} with 2-P-glycerate as substrate clearly showed that two groups with pK's of 7.5 and 6.0 had to be unprotonated (Anderson, 1981), two ionizable groups were assumed for the V/K_{Mg} profile in the present work. The resulting pK values from a fit to eq 3 were 8.0 ± 0.2 and 5.0 ± 0.7. V_{max} and V/K_{MePGA} (data not shown) were pH independent. The pH variation of the kinetic parameters in the reverse reaction is similar, but the maximum velocity is 20% that in the forward direction.

Deuterium Isotope Effects. The data obtained by varying both Mg^{2+} and MePGA-*2-d* concentrations should yield isotope effects on all of the kinetic parameters. One of the key arguments we make is that ^DV is significantly less than $^D(V/K_{MePGA})$ and $^D(V/K_{Mg})$. To establish this, we made the three different assumptions about the independence of the three isotope effects reflected in eqs 5–7, and the resulting fits are shown in Table I. Examination of the standard errors of the fits to eq 5 suggests that $^D(V/K_{Mg}) > {}^DV$. Additionally, the increase in σ when ^DV, $^D(V/K_{MePGA})$, and $^D(V/K_{Mg})$ are assumed to be equal (eq 7) supports our conclusion that eq 5 best represents the data.

Table I: Primary Deuterium Isotope Effects on the Dehydration of MePGA[a]

pH	data fitted to eq	^DV	$^D(V/K_{Mg})$	$^D(V/K_{MePGA})$	σ
9.0	5	1.15 ± 0.15	6.8 ± 2.2	1.7 ± 0.4	0.0084
	6	1.07 ± 0.10	2.6 ± 0.4	2.6 ± 0.4	0.0091
	7	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	0.010
7.1	5	1.32 ± 0.15	11.6 ± 2.6	4.4 ± 1.1	0.0039
	6	1.26 ± 0.13	7.8 ± 1.2	7.8 ± 1.2	0.0042
	7	2.3 ± 0.2	2.3 ± 0.2	2.3 ± 0.2	0.007

[a] Isotope effects measured by comparison of data with MePGA-*2-d* and unlabeled MePGA. The concentrations of both MePGA and Mg^{2+} were varied. Equation 5 assumes different isotope effects on all parameters. Equation 6 assumes the same isotope effect on both V/K values, but a different one on V. Equation 7 assumes equal isotope effects on all parameters.

Table II: Variation of ^{D}(Eq.P.) with pH and [Mg^{2+}]

pH	60 μM Mg^{2+}	17 mM Mg^{2+}	60 mM Mg^{2+}	K^a (mM)
5.5	nd[b]	3.7	4.2	nd
6.3	4.2	3.7	2.3	31
7.0	3.7	2.6	2.0	18
8.1	4.1	2.1	1.8	11
9.0	2.8	1.6	1.4	9

[a] Concentration of Mg^{2+} that halves [^{D}(Eq.P.) + 1]. [b] nd, not determined.

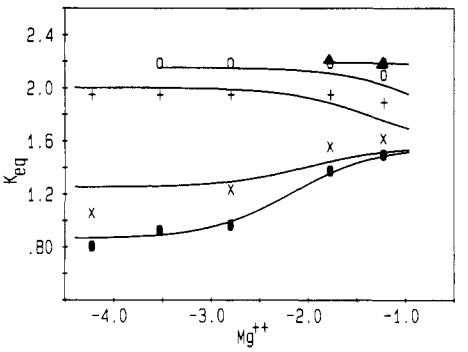

FIGURE 2: Equilibrium constant for the dehydration of MePGA to MePEP as a function of pH and Mg^{2+} concentration. Equilibrium constants were determined from the concentrations of MePGA and MePEP used to generate equilibrium perturbations in H_2O at pH 9.0 (●), 8.1 (×), 7.0 (+), 6.3 (O), and 5.5 (▲). The solid lines are calculated from eq 10 with the parameters given in the text.

The isotope effects from equilibrium perturbations with MePGA-*2-d* and H_2O, or unlabeled MePGA and D_2O, as perturbants are shown as a function of pH and Mg^{2+} concentration in Table II. $^D(Eq.P.)_{MePGA}$ decreases as the Mg^{2+} level increases. The concentrations of Mg^{2+} required to halve the value of [$^D(Eq.P.)_{MePGA}$ + 1] in the absence of Mg^{2+}, determined by fits to eq 8, are also included in Table II. The equilibrium constant, determined during the course of the equilibrium perturbations in H_2O with MePGA-*2-d*, is both pH and Mg^{2+} dependent as shown in Figure 2, varying from 1.0 at pH 9.0 to 2.2 at pH 5 in the absence of Mg^{2+}.

Solvent Isotope Effects. The D_2O solvent isotope effect was 1.1 ± 0.1 on V for the reaction of MePGA to MePEP. The solvent isotope effect on the maximum velocity of the reverse reaction from a fit to eq 9 was 5.1 ± 0.6.

DISCUSSION

Enolase Equilibrium. Stubbe and Kenyon (1972) synthesized MePEP as a phosphoenolpyruvate analogue and noted that it appeared to be hydrated by enolase. We have isolated

the product of the reaction and unambiguously identified it as (2-carboxy-3-hydroxypropyl)phosphonic acid (MePGA). The equilibrium constant is pH and Mg^{2+} dependent. The 2.2-fold change from low pH to high pH at low Mg^{2+} concentrations is consistent with the 0.4 pH unit difference in the secondary phosphonate pK values of MePGA and MePEP. MePEP has a higher pK than MePGA, so the variation in K_{eq} with pH is in the opposite direction from that with the normal substrates. The equilibrium constant between the fully ionized forms is 0.8, compared to 6.3 for 2-P-glycerate and PEP (Wold & Ballou, 1957), indicating that the methylene substitution stabilizes the tetrahedral carbon with respect to the trigonal one at C-2 of these molecules.

The increase in equilibrium constant with increasing Mg^{2+} concentration at pH 9 requires that the affinity of MePEP for Mg^{2+} be greater than that of MePGA. The variation in Figure 2 is accurately described by eq 10 with $K_{d\,MePGA} = 6.4 \pm 1.5$ mM, and $K_{d\,MePEP} = 3.2 \pm 1.0$ mM.

pH Profiles. The pH variation of V/K_{Mg} and p$K_{i\,MePGA}$ seen in Figure 1 is similar to what was seen by Anderson (1981) with 2-P-glycerate, except that the pK values are somewhat increased. The pK for the p$K_{i\,MePGA}$ profile is 0.8 pH unit higher than seen with 2-P-glycerate and presumably corresponds to the increase in secondary phosph(on)ate pK from 7.0 in 2-P-glycerate (Wold & Ballou, 1957) to 7.85 in the methylene analogue. These data suggest that only the dianionic form of the substrate binds tightly in this direction. Interestingly, V/K_{MePGA} is nearly pH independent, suggesting that the rate constant for dissociation of MePGA increases when the phosphonate group ionizes, rather than the rate constant for association (which is the V/K value in an ordered mechanism) being affected.

The maximum velocity is pH independent, showing that the protonation states of MePGA and critical enzyme groups are locked in place once the substrate and Mg^{2+} have both combined. The most striking pH dependence is that of V/K_{Mg}, which decreases a factor of 10 per pH unit below a pK of 8.0, and then a factor of 100 per pH unit below a second pK of 5. Thus two groups must be unprotonated for binding of Mg^{2+} and then cannot be protonated once it does bind. At least one of these groups is probably a ligand of the Mg^{2+}, while one may be the putative base which is needed to remove the hydrogen from C-2 to give the carbanion intermediate, the existence of which is supported by exchange studies (Dinovo & Boyer, 1971; Stubbe & Abeles, 1980) and the very strong inhibition by carbanion analogues reported by Anderson et al. (1984). This base was postulated by Weiss et al. (1987) to be a cysteine, on the basis of an isotope effect of 0.41 on the dissociation constant of tartronate semialdehyde phosphate deuterated at C-2. Recent X-ray evidence suggests that this is not correct and that some other grouping with a low fractionation factor is involved (Stec & Lebioda, 1990).

The simplest kinetic mechanism consistent with all of these data and the isotope effects reported here is

$$\begin{aligned} E + MePGA \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\text{-}MePGA \underset{k_4}{\overset{k_3[Mg]}{\rightleftharpoons}} E\text{-}MePGA\text{-}Mg \underset{k_6}{\overset{k_5}{\rightleftharpoons}} \\ EH\text{-}carbanion\text{-}Mg \underset{k_8}{\overset{k_7}{\rightleftharpoons}} EH\text{-}MePEP\text{-}MgOH_2 \underset{k_{10}[Mg]}{\overset{k_9}{\rightleftharpoons}} \\ EH\text{-}MePEP \overset{k_{11}}{\rightarrow} EH + MePEP \end{aligned} \quad (11)$$

To match the pH profiles, we will assume that k_1 is not affected by the state of protonation of MePGA, of the base on the enzyme, or of the metal ligand, but that k_2 is increased by full deprotonation of the phosphonate group. The value of k_3 is pH dependent, requiring both the catalytic base and the metal ligand to be ionized. Once Mg^{2+} is bound, the pK of bound MePGA shifts to much higher pH, so that the phosphonate group stays protonated (this proton clearly is hydrogen bonded to something to accomplish this; it is tempting to postulate that it is the proton transferred to the 3-hydroxyl to give $MgOH_2$ in the step represented by k_7). Rate constants k_4–k_9 are probably all pH independent (or are not at all rate limiting if they are, since V_{max} is pH independent).

This kinetic mechanism is essentially that proposed by Faller et al. (1977) on the basis of Mg^{2+} binding data. It predicts an intersecting initial velocity pattern when the concentrations of MePGA and Mg^{2+} are varied, with uncompetitive substrate inhibition by Mg^{2+} resulting from the combination of Mg^{2+} with EH–MePEP if k_{11} is small enough with respect to earlier steps. Such patterns were seen by Anderson (1981) with 2-P-glycerate, and Mg^{2+} inhibition has been reported by Faller et al. (1977) for the yeast enzyme and by Wang and Himoe (1974) for rabbit muscle enolase. The methylene substitution has decreased V 170-fold, while large isotope effects are seen on V/K_{MePGA} and V/K_{Mg} at pH 7.1, implying that the rate constants for dissociation of substrate and Mg^{2+} are large. Substrate inhibition by Mg^{2+} is only just visible at 60 mM Mg^{2+}, compared to a K_I of 10 mM with 2-P-glycerate as substrate. The decreased substrate inhibition by Mg^{2+} and the nearly equilibrium ordered initial velocity pattern ($K_{MePGA} \ll K_{i\,MePGA}$) argue that Mg^{2+} and product dissociation steps, k_9 and k_{11}, do not limit the maximum velocity. This is appealing because there is no physical rationale why the methylene substitution should dramatically decrease the dissociation rate of Mg^{2+} or product. The increase in K_I for Mg^{2+} substrate inhibition is consistent with the decreased V for the alternate substrate arising from a slower chemical reaction.[2] The comparable rate constants in the reverse direction, k_2 and k_4, are probably also fast with respect to the chemical interconversion steps k_5–k_8. While the available kinetic data do not unequivocally rule out a degree of randomness in the reactant addition and release steps of mechanism 11 (but see below our discussion of the isotope effects on V/K_{MePGA}), their inclusion would not qualitatively affect the interpretation of the observed isotope effects.

Deuterium Isotope Effects. The only steps in mechanism 11 that should show a primary deuterium isotope effect are formation or protonation of the carbanion (k_5 and k_6). The observed isotope effects will then be related to the intrinsic one on the bond breaking step ($^Dk_5$) by

$$^D(V/K) \text{ or } ^D(\text{Eq.P.}) = \frac{^Dk_5 + c_f + c_r{}^DK_{eq}}{1 + c_f + c_r} \quad (12)$$

$$^DV = \frac{^Dk_5 + c_{Vf} + c_r{}^DK_{eq}}{1 + c_{Vf} + c_r} \quad (13)$$

In eqs 12 and 13 c_f and c_r are forward and reverse commitments, while c_{Vf} is a rate ratio which compares k_5 to other forward rate constants. If we confine ourselves to levels of Mg^{2+} below those giving substrate inhibition, the value of c_{Vf} is given by

$$c_{Vf} = k_5\left[\frac{1}{k_7}(1 + k_8/k_9) + 1/k_9 + 1/k_{11}\right] \quad (14)$$

In direct comparison experiments:

[2] There is also an increase in K_I when 2-P-glycerate-2-*d* is used to decrease V at pH 7 (Anderson, 1981).

$$c_r = \frac{k_6}{k_7}\left[1 + \frac{k_8}{k_9}\left(1 + \frac{k_{10}[Mg]}{k_{11}}\right)\right] \quad (15)$$

but the last term can be neglected if we avoid substrate inhibitory levels of Mg^{2+}. The value of c_f for $^D(V/K_{Mg})$ is given by eq 16, while that for the apparent value of $^D(V/K_{MePGA})$ is given by eq 17. Since the limiting value of V/K_{MePGA}

$$c_{f\,Mg} = k_5/k_4 \quad (16)$$

$$c_{f\,MePGA} = \frac{k_5}{k_4}\left(1 + \frac{k_3[Mg]}{k_2}\right) \quad (17)$$

corresponds to infinite $[Mg^{2+}]$, $c_{f\,MePGA}$ should be infinity, and no isotope effect should be observed on V/K_{MePGA}. The fits to eq 5 in Table I suggest that the isotope effect on V/K_{MePGA} is not unity. However, the data used to obtain these values were limited to noninhibitory Mg^{2+} concentrations, and the data from the equilibrium perturbations obtained over a wider range of Mg^{2+} concentrations are more informative.

In equilibrium perturbation experiments, the perturbants between which label is exchanged are MePGA and water. In this case c_f is given by eq 17 and c_r by eq 15. These equations predict that ^{D}(Eq.P.) will decrease to unity with increasing $[Mg^{2+}]$, in agreement with the data in Table II. The difference between the ^{D}(Eq.P.) values extrapolated to infinite $[Mg^{2+}]$ and unity is a measure of residual randomness in what must be a largely ordered kinetic mechanism as given in eq 11.

The concentration of Mg^{2+} that halves $[^D(Eq.P.) + 1]$ qualitatively should be related to the observed uncompetitive substrate inhibition K_I for Mg^{2+}, since they both arise from the same physical phenomenon, i.e., by prevention of the dissociation of the substrates and/or products from the enzyme as shown in mechanism 11. However, the K from eq 8 and the K_I are not necessarily identical. Intuitively this can be understood since in the dehydration reaction only prevention of MePEP from dissociation results in substrate inhibition, while prevention of MePGA dissociation will reduce $^D(Eq.P.)_{MePGA}$ as well by increasing $c_{f\,MePGA}$ as predicted by eq 17. This is consistent with our ^{D}(Eq.P.) data in Table II, which are diminished by Mg^{2+} levels well below the K_I for Mg^{2+}.

The limiting ^{D}(Eq.P.) values seen at low Mg^{2+} range from 2.75 at pH 9 to around 4 at pH 6–8. By contrast, the values of $^D(V/K_{Mg})$ obtained by direct comparison were 7–8. The results from equilibrium perturbation may be too low, since the equations used for analysis assume that the distribution of the enzyme forms present does not change appreciably during the perturbation, which is true for small isotope effects but not for large ones. In any case, the large isotope effects on V/K_{Mg} suggest that they are close to the intrinsic one on k_5 and thus that $k_4 > k_5$ and $k_6 < k_7/(1 + k_8/k_9)$.

In contrast to the large isotope effects on V/K_{Mg}, ^DV is only 1.15 at pH 9 and 1.32 at pH 7.1. This requires that c_{Vf} be 46 at pH 9 and 21 at pH 7.1 (assuming $^Dk_5$ to be 8) while $c_{f\,Mg}$ must be small. Comparison of eqs 12 and 13 shows that this situation requires k_5 to be considerably larger than either k_9, k_{11}, or $k_7/(1 + k_8/k_9)$. The decreased Mg^{2+} substrate inhibition and nearly equilibrium ordered initial velocity pattern argue that Mg^{2+} and product dissociation steps (k_9 and k_{11}) do not limit the maximum velocity. Consequently, it must be $k_7/(1 + k_8/k_9)$ that is only 2–5% of k_5. A small c_r requires that $k_7/(1 + k_8/k_9)$ be more than k_6, implying that k_5 is much larger than k_6 and that the equilibrium constant for deprotonating enzyme-bound MePGA to form the carbanion is favorable. The subsequent breakdown of the carbanion to MePEP appears to be largely rate limiting for V. Thus the major effect of the methylene substitution has been to stabilize the carbanion so that its breakdown limits V and produces a slow substrate.

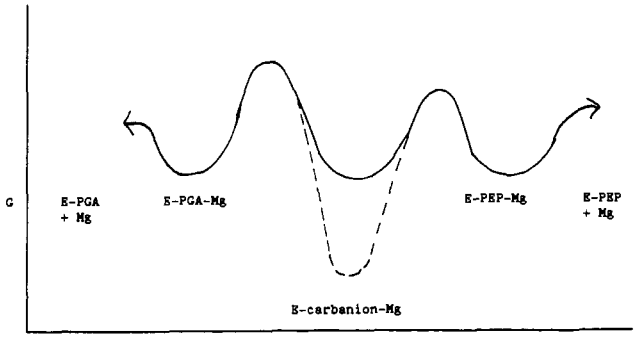

FIGURE 3: Free energy profiles for the interconversion of enolase substrates. (---) MePGA and MePEP; (—) 2-P-glycerate and phosphoenolpyruvate.

This is the expected effect of replacing oxygen with carbon in the bridge between C-2 and phosphorus, since substitution of fluorine (and by analogy, oxygen) for hydrogen in nitroalkanes decreases their acidity (Adolph & Kamlet, 1966), while alkyl substitution for hydrogen increases the acidity of nitroalkanes (Nielsen, 1969). A similar phenomenon makes chlorofumarate and 3-chloromalate slow substrates for fumarase (Tiepel et al., 1968; Blanchard & Cleland, 1980); the carbanion intermediate is stabilized by chlorine substitution at C-3 so that its subsequent reaction is slow in either direction of the reaction.

This situation corresponds to the free energy profile shown in Figure 3. In short, with the methylene analogue the enzyme falls down a hole and cannot rapidly escape in either direction. This mechanism predicts that the slowest step in the hydration of MePEP will be proton transfer to the carbanion intermediate, which should be deuterium-sensitive. The D_2O solvent isotope effect of 5 on the maximum velocity for hydration of MePEP, while no appreciable solvent isotope effects were seen in the forward reaction with MePGA as substrate, confirms the partitioning of the carbanion as limiting the maximum velocities in both the hydration and dehydration reactions. These data clearly illustrate the principle enunciated by Albery and Knowles (1976) that for maximum catalytic efficiency all intermediates in an enzyme-catalyzed reaction should have nearly equal stability. If one intermediate is much more stable than the others, there will be a deep hole in the free energy profile from which it is difficult to escape in either direction of the reaction. Enolase has clearly evolved to deal with a rather unstable carbanion containing a destabilizing oxygen bridge attached to C-2, so that when presented with a more easily ionized substrate, it forms too stable an intermediate. This phenomenon has been demonstrated with enolase with the even more stable carbanion of (3-hydroxy-2-nitropropyl)phosphonate, which binds with a K_i of 6 nM and does not undergo dehydration at a significant rate (Anderson et al., 1984). Similar examples of enzymes tricked into forming overly stable intermediates include the already mentioned case of chloromalate with fumarase and the phosphorylation of glucose by CrATP catalyzed by hexokinase (Dunaway-Mariano & Cleland, 1980), where the CrADP–glucose-6-P complex formed is so stable that its slow release from the enzyme becomes totally rate limiting for the reaction.

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Kinetics of Oxidative Phosphorylation in *Paracoccus denitrificans*. 1. Mechanism of ATP Synthesis at the Active Site(s) of F_oF_1-ATPase†

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ABSTRACT: (1) The rate of ATP synthesis during NADH-driven aerobic respiration has been measured in plasma membrane vesicles from *Paracoccus denitrificans* as a function of the concentration of the substrates, ADP and inorganic phosphate (P_i). In both cases, the response of the reaction to changes in the degree of saturation of the F_oF_1-ATPase generated a perfect Michaelian dependence which allowed the determination of the corresponding Michaelis constants, K_m^{ADP} and $K_m^{P_i}$. (2) These kinetic parameters possess a real mechanistic significance, as concluded from the partial reduction of the rate of phosphorylation by the energy-transfer inhibitor venturicidin and the consequent analysis of the results within the framework of the theory of metabolic control. (3) The same membrane vesicles, which catalyze very high rates of ATP synthesis, have been shown to support much lower rates of the exchange ATP $\rightleftharpoons$ P_i and negligible rates of ATP hydrolysis. Under similar conditions, the preparations are also capable of generating phosphorylation potentials, ΔG_P, of 60–61 kJ·mol^{-1}. (4) These properties have allowed analysis of the synthetic reaction in the presence of significant concentrations of the product, ATP, using integrated forms of the Michaelis–Menten rate equations. (5) It has been shown that ATP produces pure competitive product inhibition of the forward reaction with a value of $K_i^{ATP} = 16 \pm 1$ μM, thus indicating that the affinity of the nucleotide for the active site(s) of the F_oF_1-ATPase, during net ATP synthesis, is significantly higher than previously thought. (6) The order of binding of the substrates, ADP and P_i, to the active site(s) has been determined as random. (7) At very low concentrations of ADP, a second and much smaller Michaelis constant for this substrate has been identified, with an estimated value of $K_m^{ADP} \simeq 50$ nM, associated with a maximal rate of only 2% of that measured at a higher range of concentrations. (8) The results obtained are discussed in relation to the presence of two or three equivalent catalytic sites operating in the cooperative manner explicitly described by the binding change mechanism.

The ubiquitous enzyme F_oF_1-ATPase[1] completes the process of oxidative phosphorylation or photophosphorylation in mitochondria, chloroplasts, and many bacteria by catalyzing the synthesis of ATP from ADP and inorganic phosphate (P_i) driven by the energy stored in the protonmotive force, Δp [for review, see Senior (1988)]. Given the central role of this enzyme in energy transduction, an understanding of the mechanism of ATP synthesis and of the events that take place at the active site(s) of this enzyme seems essential. There are two important questions concerning ATP synthesis which demand a precise answer: (1) What is the order of binding for the substrates, ADP and P_i, at the active site(s) of the F_oF_1-ATPase? (ii) What is the affinity of the active site for the product, ATP, during net phosphorylation?

With regard to the second question, the binding change mechanism proposed for this enzyme [e.g., Kayalar et al. (1977), Gresser et al. (1982), and Boyer (1989)] indicates that

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[1] Abbreviations: Δp, protonmotive force or transmembrane electrochemical proton gradient expressed in millivolts; $\Delta\psi$, transmembrane electrical potential; ΔpH, transmembrane pH gradient; P_i, inorganic phosphate; G6P, glucose 6-phosphate; HK, hexokinase; PK, pyruvate kinase; PEP, phosphoenolpyruvate; F_oF_1-ATPase, H^+-translocating ATPase type F_oF_1 (ATP-synthase; EC 3.6.1.3); F_1, soluble catalytic sector of F_oF_1-ATPase; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone.

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