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Stereospecificity of the Metal–Adenosine 5′-Triphosphate Complex in Reactions of Muscle Pyruvate Kinase†

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ABSTRACT: Rabbit muscle pyruvate kinase in the presence of mono- and divalent cations catalyzes the phosphorylation of glycolate by $Cr^{III}ATP$. The product complex CrADP–glycolate-P is released slowly from the enzyme, and generally only a single turnover is observed for these reactions. The Λ β,γ-bidentate isomer and the four α,β,γ-tridentate isomers are all inactive as substrates in this reaction. Only the Δ isomer of β,γ-bidentate CrATP is active in the phosphoryl transfer reaction and is the most active isomer in the enolization of pyruvate catalyzed by pyruvate kinase. In contrast to the phosphoryl transfer reaction, all of the bidentate and tridentate CrATP isomers are effective in promoting the pyruvate kinase catalyzed enolization of pyruvate. The V_{max} of the most active isomer Δ β,γ-bidentate CrATP (Mn^{2+} and K^+; pH 5.7; 21 °C) is 6.0 μmol of pyruvate enolized per min mg of protein, and the K_m is 100 μM. In comparison the V_{max} of the Λ bidentate isomer under the same conditions is 3-fold lower and the K_m is 1.7-fold greater. The four tridentate isomers of CrATP do not differ greatly in their activity but are all less active than the bidentate isomers by more than 1 order of magnitude. The most active tridentate isomer at pH 5.7 has a V_{max} of 0.26 μmol of pyruvate enolized per min mg of protein and a K_m of 600 μM. Pyruvate kinase also requires an enzyme-bound divalent cation for activity. The order of activation by various enzyme-bound divalent cations in the phosphorylation of glycolate by CrATP is the same as it is in the CrATP-stimulated enolization of pyruvate: Mn(II) > Co(II), Zn(II) > Mg(II) ≫ Ca(II) ~ 0. The paramagnetic effects of the bidentate CrATP isomers on the relaxation rate of water protons are significantly lower than those of the tridentate isomers. Analysis of the temperature dependence of these effects indicates 1 order of magnitude slower escape of protons from the coordination sphere of bidentate CrATP (1.1×10^6 s^{-1}) than that from tridentate CrATP, suggesting an intramolecular hydrogen-bond interaction in the bidentate isomers but not in the tridentate isomers.

Rabbit muscle pyruvate kinase catalyzes the reversible phosphorylation of ADP by 2-phosphoenol pyruvate and, in a second step, the reversible protonation of the pyruvate enolate (Rose, 1960; Robinson & Rose, 1972). Previous studies of the role of metal ions in the pyruvate kinase reaction, by a variety of kinetic and magnetic resonance techniques, have revealed the requirement for two divalent cations per active site (Gupta et al., 1976a,b; Gupta & Mildvan, 1977). One of these divalent cations binds to the enzyme and forms second-sphere complexes with the bound substrates while the second binds directly to the triphosphate chain of the ATP (Gupta et al., 1976a,b; Gupta & Mildvan, 1977). The kinetic and magnetic resonance studies established coordination of the β- and γ-phosphoryl groups of enzyme-bound ATP by the nucleotide-bound metal but were inconclusive as to α-coordination (Gupta & Mildvan, 1977). The present studies were undertaken to resolve this ambiguity and to study, by an entirely independent method, the chelate structure and stereochemistry of the active metal–ATP complex on pyruvate kinase.

A direct and independent approach to this problem is that used previously by Cornelius & Cleland (1978) to define the active metal–ATP isomer in the yeast hexokinase reaction

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system and by Li et al. (1978) to define the active isomer in the phosphoribosylpyrophosphate (P-Rib-PP) synthetase system. The approach in general involves examination of the substrate specificity of the enzyme toward exchange-inert metal–ATP coordination complexes of known structure and stereochemistry (Cleland & Mildvan, 1979). In both the hexokinase and P-Rib-PP synthetase studies, the exchange-inert complex $Co(NH_3)_4ATP$ was used. With muscle pyruvate kinase, it has previously been shown that a mixture of the tridentate complexes of CrATP is highly active in promoting the enolization of pyruvate (Gupta et al., 1976a,b; Gupta & Mildvan, 1977).

In the present studies, purified CrATP isomers (Dunaway-Mariano, 1978; Cleland & Mildvan, 1979) were used to demonstrate that pyruvate kinase is specific for the Δ β,γ-bidentate CrATP isomer in the phosphoryl transfer reaction while it shows only a preference for this particular CrATP isomer in the enolization half-reaction. In addition, CrATP was also used in the studies reported below to examine the specificity of pyruvate kinase toward the enzyme-bound divalent cation activator in both the phosphoryl transfer and enolization reactions.

Materials and Methods

General. Rabbit muscle pyruvate kinase was purchased from Boehringer and prior to use chromatographed on a Sephadex G-200 column in the manner previously described (Gupta & Mildvan, 1977) to remove traces of contaminating adenylate kinase and ATPase. The pyruvate kinase containing eluate was concentrated in collodion bags by vacuum dialysis at 4 °C (final sp act. 250 units/mg). The three-tritiated sodium pyruvate was a generous gift from Dr. Irwin A. Rose. It was diluted with unlabeled pyruvate to a specific activity of 10^5 cpm/μmol for use in the tritium-exchange experiments. The concentration and purity of the pyruvate solutions were checked by standard enzymatic assay procedures using lactate dehydrogenase. The CrATP isomers were prepared in the manner described previously (Dunaway-Mariano, 1978; Cleland & Mildvan, 1979). The $Co(NH_3)_4ATP$ was prepared in the manner described by Cornelius & Cleland (1978). ATP, NADH, and Pipes[1] were purchased from Sigma Chemical Co. All other chemicals used were of the highest purity commercially available. [^{14}C]Glycolate was obtained from Amersham.

Enolization of Pyruvate. The tritium-exchange experiments used to determine the rates of the pyruvate enolization reactions were carried out in the manner previously described (Gupta et al., 1976a,b), using either K-Pipes buffer (pH 5.7 or 6.4) or Tris-HCl buffer (pH 7.7).

Reaction of Glycolate with CrATP or $Co(NH_3)_4$ ATP in the Presence of Pyruvate Kinase. $Co(NH_3)_4ATP$. Reaction mixtures (0.5 mL) 1 mM in pyruvate kinase sites, 2.5 mM in [^{14}C]glycolate (10^5 cpm/μmol), 2 mM in $Co(NH_3)_4ATP$, 100 mM in KCl, 5 mM in $MnCl_2$, and 100 mM in K-Pipes (pH 6.7) or 50 mM Tris-HCl (pH 7.5 and 8.5) were incubated at 25 °C for 30 min. The reactions were terminated by adding 25 μmol of EDTA, 2 drops of CCl_4, and 50 μL of 70% $HClO_4$ to the vortexing solutions. The samples were then diluted to 5 mL with cold water and centrifuged at 15 000 rpm for 10 min. The supernatant solutions were adsorbed onto 1 × 4 cm Dowex 50 H^+ columns (100–200 mesh) at 4 °C. The columns were rinsed with 20 mL of 10 mM HCl to elute the glycolate, collecting 5-mL fractions, and then with 20 mL of 1 M HCl to elute the product. The relative amounts of glycolate and product present in the column fractions were determined by assaying the fractions for ^{14}C by using a scintillation counter. The results obtained are presented in the text. *CrATP.* The exact reaction conditions used in the CrATP experiments are specified in the text. In general, however, these reactions were carried out by incubation for a specified time at 25 °C of a 0.25-mL mixture containing K-Pipes and the other components as described above for $Co(NH_3)_4ATP$ with CrATP replacing $Co(NH_3)_4ATP$. The reactions were terminated as described above. After diluting to 1 mL with cold water, we filtered the samples through a glass wool plug and adsorbed them onto 25 × 0.5 cm Dowex 50 H^+ columns at 4 °C. The unreacted glycolate was eluted from the column with 50 mL of 10 mM HCl (collecting 5-mL fractions), and the CrADP–glycolate-P was eluted with 50 mL of 1 M HCl. The column fractions were then assayed for ^{14}C by using a scintillation counter.

Identification of the CrATP–Glycolate Reaction Product. A reaction mixture containing CrATP (as a mixture of isomers) (5.1 mM), [^{14}C]glycolate (2.5 mM), $MnCl_2$ (4.8 mM), KCl (110 mM), K-Pipes (84 mM, pH 6.7), and pyruvate kinase (1.1 mM sites) was incubated at room temperature for 30 min. The reaction was then terminated by precipitation of the enzyme in the manner previously described. The resulting solution was diluted to 3.2 mL with cold water and then centrifuged (10 min; 6000 rpm). The supernatant was applied to a Dowex 50 H^+ column at 4 °C. The column was washed with 10 mM HCl until no ^{14}C counts eluted (25 mL) and then with 1 M HCl until all of the product was eluted (25 mL). The product-containing fractions were combined (5.7 mL, 20 μM product), and a 0.7-mL aliquot was removed and diluted with "cold" phosphoglycolate to give a final phosphoglycolate concentration of 7.5 mM. The HCl was removed from this solution by repeated lyophilization. The final residue was dissolved in 0.5 mL of water, adjusted to pH 6.0 by using 1 M K_2CO_3, made 5 mM in EDTA, and then heated to 100 °C for 10 min. The resulting solution was concentrated in vacuo and then chromatographed on a Baker flex cellulose PEIF plate by using 1.2 M LiCl as the solvent (Randerath & Randerath, 1964). The adenine-containing compounds on the plates were detected by using shortwave UV light, phosphate-containing compounds were detected by using the procedure of Hanes & Isherwood (1949), and ^{14}C-containing compounds were detected by using radioautographic techniques (Du Pont Cromex 4 X-ray film; 1-month exposure at −70 °C).

Water Proton Relaxation Rate Measurements. The longitudinal relaxation rate ($1/T_1$) of water protons was measured at 24.3 MHz with an NMR Specialties PS60W spectrometer and at 100 MHz with a modified Varian XL-100-FT spectrometer, using the null-point method with a 180°–τ–90° pulse sequence. The addition of a timing circuit to the Varian system, in place of the computer-controlled timing, permitted direct null-point measurements of strong signals without Fourier transformation, resulting in a significant saving of time.[2] The transverse relaxation rate ($1/T_2$) was measured at 24.3 MHz by using the modified Carr–Purcell spin–echo method.

Results

Pyruvate Kinase Catalyzed Phosphorylation of Glycolate with Exchange-Inert ATP Complexes. Since the phospho-

[1] Abbreviations used: Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); NADH, reduced diphosphopyridine nucleotide.

[2] We are grateful to S. Altstein for making this modification.

Table I: Substrate Activities of CrATP Isomers in the Pyruvate Kinase Catalyzed Phosphorylation of Glycolate[a]

CrATP isomer(s)	[CrATP] (mM)	[enzyme sites] (mM)	incubation time (min)	pH	product (mM)
mixture[b]	5.33	0.56	10	6.5	0.33
mixture[b]	5.33	0.56	20	6.5	0.36
mixture[b]	5.33	0.56	30	6.5	0.38
tridentate	0.50	0.14	30	6.0	0.00
tridentate	0.50	0.14	30	6.7	0.00
tridentate	0.50	0.14	30	7.1	0.00
bidentate	0.63	0.11	30	6.0	0.11
Λ bidentate	0.25	0.17	30	6.0	0.00
Δ bidentate	0.46	0.07	4	6.0	0.06

[a] Each reaction contained $MnCl_2$ (5–10 mM), KCl (100–200 mM), [^{14}C]glycolate (2–10 mM; 10^5 cpm/μmol), and K-Pipes (30–120 mM) at 25 °C. The enzyme-site concentration was determined as previously described (Gupta et al., 1976a). The product was determined chromatographically as described under Materials and Methods. [b] Mixture of bidentate and tridentate isomers prepared by the method of Janson & Cleland (1974).

Table II: Rates of Pyruvate Enolization by Pyruvate Kinase with Various Isomers of CrATP[a]

	pH 5.7			pH 6.4		
CrATP isomer	rel rate at 1 mM (%)	K_m (μM)	V_{max} [μmol/(min mg)]	rel rate at 1 mM (%)	K_m (μM)	V_{max} [μmol/(min mg)]
bidentate Δ	100	100	6.0			
bidentate Λ	30	170	2.0			
tridentate 1[b]	3.2			100		
tridentate 2	3.4			76		
tridentate 3	3.9	600	0.26	78	250	0.44
tridentate 4	1.7			64	240	0.34

[a] Each reaction contained $MnCl_2$ (2 mM), KCl (110 mM), K-Pipes (60 mM), [^{3}H]pyruvate (72 mM; 10^5 cpm/μmol), and pyruvate kinase (8–37 μM sites) at 21 °C. Incubation time was 15 min. [b] The tridentate isomers were isolated in the manner described by Cleland & Mildvan (1979).

rylation of pyruvate by metal-ATP at neutral pH is thermodynamically unfavorable, studies were carried out by using the known alternate substrate, glycolate (Kayne, 1974). Phosphorylation of the hydroxyl group of glycolate by ATP constitutes an energetically favorable reaction. The assay developed to study the pyruvate kinase catalyzed glycolate–$Co(NH_3)_4ATP$ and glycolate–CrATP reactions involved separation of unreacted [^{14}C]glycolate from the phosphorylation product $Co(NH_3)_4ADP$–[^{14}C]glycolate-P or CrADP–[^{14}C]glycolate-P on a Dowex 50 H^+ column and quantitation of the isolated substrate and product using a liquid scintillation counter.

Accordingly, the reaction of β,γ-bidentate $Co(NH_3)_4ATP$ with glycolate in the presence of Mn^{2+} and pyruvate kinase was attempted at pH 6.7, 7.5, and 8.5. With $Co(NH_3)_4ATP$, there was no detectable amount of product present in each of these reaction mixtures. On the other hand, CrATP, as a mixture of isomers, did show substrate activity (see Table I). The CrADP–[^{14}C]glycolate-P formed in the reaction was purified by column chromatography as described under Materials and Methods and then characterized by degradation to ADP and [^{14}C]glycolate-P by heating in the presence of EDTA. Thin-layer chromatography of the mixture revealed the presence of significant amounts of a component containing both ^{14}C and phosphorus but no UV-absorbing material, which comigrated with authentic P-glycolate (R_f = 0.35 ± 0.02 in 1.2 M LiCl). In addition, ADP (R_f = 0.22 ± 0.01) and AMP (R_f = 0.44 ± 0.02) were detected in the sample both by UV absorbancy and by phosphorus content. No residual ATP (R_f = 0.12 ± 0.01) was detected in the sample.

The following evidence thus indicates the formation of CrADP–[^{14}C]glycolate-P in the phosphorylation reaction. (1) After prior elution of free glycolate and glycolate-P with 10 mM HCl, the reaction product elutes as a single green, positively charged band from the cation-exchange column with 1 M HCl, showing the presence of Cr^{3+} in the complex. (2) The resulting product shows adenine absorption in the UV, indicating the presence of ADP, and, upon hydrolysis, yields glycolate-P which comigrates with authentic glycolate-P in thin-layer chromatography. The CrADP–[^{14}C]glycolate-P complex which we have detected could not have resulted from phosphorylation of glycolate by traces of free ATP.

An interesting feature of the pyruvate kinase catalyzed CrATP–glycolate reaction is that the product release step and not the catalytic reaction appears rate limiting. As indicated in Table I, the amount of product formed within a few minutes after initiation of the reaction is approximately stoichiometric with the pyruvate kinase active sites present. The product complex formed during the first turnover apparently remains tightly bound to the enzyme. Indeed, after a 60-min reaction period, the amount of product present is not significantly greater than expected for a single turnover (see Table I) which was reached within 10 min of the initiation of the reaction.

In order to determine which CrATP isomer is the active isomer in the phosphorylation reaction, we measured the substrate activities of purified α,β,γ-tridentate CrATP and β,γ-bidentate CrATP complexes with pyruvate kinase. The results obtained are shown in Table I. Tridentate CrATP was inactive at pH 6.0, 6.7, and 7.1. Bidentate CrATP, on the other hand, did effectively transfer a phosphoryl group to glycolate. Next, the pure bidentate screw sense isomers (Δ and Λ) were tested for substrate activity. While the Λ bidentate CrATP was not a substrate in the phosphorylation reaction, the Δ bidentate CrATP gave a single turnover of glycolate within 4 min.

Pyruvate Kinase Catalyzed Enolization of Pyruvate. CrATP has been previously shown to effectively promote the pyruvate kinase catalyzed enolization of pyruvate in the presence of monovalent and divalent cation activators (Gupta et al., 1976a,b; Gupta & Mildvan, 1977). The specificity of the enzyme toward the individual CrATP isomers in this partial reaction was examined by measuring the relative rates of pyruvate kinase catalyzed tritium exchange from labeled pyruvate to water solvent in the presence of the individual CrATP isomers, K^+, and Mn^{2+}. The tridentate CrATP isomers used in this study were prepared by using Dowex 50 H^+ column chromatography (Dunaway-Mariano, 1978; Cleland & Mildvan, 1979) and are numbered according to the order in which they were eluted from the column. The relative activities obtained for the CrATP isomers as activators of the enolization reaction are given in Table II. As indicated in Table II, the bidentate CrATP isomers are significantly more effective in promoting pyruvate enolization than the tridentate CrATP isomers. For example, at pH 5.7 the K_m of the most active bidentate isomer is 100 μM while that of the most active tridentate isomer is 600 μM, and the V_{max} of the bidentate isomer is 6 μmol/(min mg of protein), while that of the tridentate isomer is 0.26 μmol/(min mg of protein).

The Δ isomer was found to be the more active of the two bidentate isomers. At 21 °C it had a 3-fold greater V_{max} and a 1.7-fold lower K_m than the Λ bidentate isomer. As the data

Table III: Pyruvate Enolization-Temperature Dependence of V_{max} with Bidentate CrATP[a]

isomer and level	$V^{1°C}$	$V^{21°C}$	$V^{21°C}/V^{1°C}$
Δ (2 mM)	0.47	3.52	7.5
Δ (4 mM)	0.40		
Λ (2 mM)	0.03	1.10	31.5
Λ (4 mM)	0.04		

[a] The solutions contained 0.73 mg/mL pyruvate kinase (12.3 μM sites). Other components were as described in Table II. Incubation time was 15 min.

reported in Table III indicate, the activity of the Λ bidentate CrATP isomer is significantly more temperature dependent than that of the Δ isomer. From the data in Table III, the energies of activation were estimated to be 17 ± 1 kcal/mol for the Δ bidentate CrATP induced enolization and 28 ± 1 kcal/mol for the Λ bidentate CrATP induced enolization, indicating that the lower rate with the Λ isomer results from a larger enthalpy barrier.

In order to ensure that the activity observed for the Λ bidentate CrATP isomer in the pyruvate enolization reaction derived directly from this isomer and not via epimerization of the Λ to the Δ isomer while in the enzyme active site, we carried out preincubation studies. If under the reaction conditions the Λ bidentate isomer is being converted to the Δ isomer, the preincubation of the Λ isomer in the reaction mixture containing unlabeled pyruvate should result in the accumulation of the active Δ isomer. Hence, the Λ-isomer sample preincubated with this reaction mixture should give, after addition of labeled pyruvate, a faster enolization rate than an untreated Λ sample. Two studies were carried out at pH 5.7 and 21 °C: one in which the Λ bidentate CrATP was present in 230-fold excess of the pyruvate kinase [Λ bidentate CrATP (2 mM), pyruvate kinase (2.2 mg/mL), K-Pipes (118 mM), KCl (236 mM), $MnCl_2$ (4 mM), and pyruvate (114 mM)] and one in which the Λ bidentate was present only in a 5.6-fold excess of the pyruvate kinase [Λ bidentate CrATP (58 μM), pyruvate kinase (2.5 mg/mL), KCl (260 mM), $MnCl_2$ (4 mM), and pyruvate (127 mM)]. In both studies the preincubated Λ-isomer sample did not show enhanced activity over the corresponding untreated Λ-isomer sample. Specifically, the velocity measured for the first reaction was 0.042 mol/(min mg of protein). The velocity measured for the reaction after 1 h of incubation prior to addition of the labeled pyruvate was also 0.042 μmol/(min mg of protein). The velocity measured for the second reaction was 0.013 μmol/(min mg of protein), and that for a 30-min preincubated reaction mixture was 0.010 μmol/(min mg of protein).

Unlike the two bidentate CrATP stereoisomers, the tridentate CrATP stereoisomers showed virtually no difference in effectiveness in promoting the pyruvate kinase catalyzed enolization of pyruvate (see Table II). The activities of these isomers were measured at pH 5.7 and at pH 6.4. Not unexpectedly, the activities of the isomers were greater at the higher pH (K_m was 2-fold lower and V_{max} was 1.7-fold higher).

Specificity of Pyruvate Kinase toward Divalent Cation Activators. The data of Table IV show the essentiality of an enzyme-bound divalent cation for significant phosphoryl transfer from CrATP to glycolate. A similar requirement for an enzyme-bound divalent cation in the pyruvate enolization reaction (Gupta et al., 1976a,b; Gupta & Mildvan, 1977) is confirmed with the most active Δ bidentate CrATP isomer (Table V). As indicated by the data in Tables IV and V, pyruvate kinase shows the same specificity toward these cations in the phosphoryl transfer reaction as in the enolization reaction. Mn(II) is approximately twice as effective as Mg(II) in stimulating pyruvate enolization and glycolate phosphorylation. While Ca(II) does not form a catalytically active enzyme–metal complex, Zn(II) and Co(II) both have activities intermediate between those of Mn(II) and Mg(II).

Table IV: Specificity of Pyruvate Kinase toward Divalent Cation Activators in the CrATP-Glycolate Reaction[a]

divalent cation	[product][b] (mM)	rel act. (%)
none	0.04	8[c]
Ca(II)	0.04	8[c]
Mg(II)	0.20	42
Zn(II)	0.34	70
Co(II)	0.35	72
Mn(II)	0.48	100

[a] Reactions contained pyruvate kinase (ca. 0.9 mM sites), CrATP (4.6 mM; isomeric mixture), [^{14}C]glycolate (3.1 mM), divalent cations as their chlorides (1.6 ± 0.1 mM), 50 mM K-Pipes (pH 6.5), and KCl (100 mM) at 21 °C. Incubation time was 30 min. [b] Values corrected by the control value of 0.05 mM. The control reaction did not contain enzyme or divalent cation. [c] Incorporation of ^{14}C into product was less than twice the background level.

Table V: Specificity of Pyruvate Kinase toward Divalent Cation Activators in the Pyruvate Enolization Reaction Induced by Δ Bidentate CrATP[a]

divalent cation	rel act. (%)
none	0
Mn(II)	100
Zn(II)	73
Mg(II)	55
Ca(II)	3

[a] Reactions contained Δ bidentate CrATP (3 mM), divalent cations as their chloride salt [Mg(II) at 5 mM, others at 2 mM], KCl (110 mM), K-Pipes (60 mM, pH 5.7), [^{3}H]pyruvate (72 mM, 10^5 cpm/μmol), and pyruvate kinase (21 μM sites) at 21 °C. Incubation time was 15 min.

In the various reactions catalyzed by pyruvate kinase, Zn(II) has heretofore been found to activate only the phosphorylation of hydroxylamine (Kupiecki & Coon, 1959, 1960; Boyer, 1962). The ability of Zn(II) to function effectively as the enzyme-bound divalent cation in the phosphoryl transfer from CrATP to glycolate and in the CrATP-stimulated enolization of pyruvate led us to reexamine the activity of Zn(II) in the pyruvate kinase catalyzed phosphorylation of ADP by phosphoenolpyruvate and in the ATP-activated pyruvate enolization reactions. In the phosphorylation of ADP by phosphoenol pyruvate, no activity of $ZnCl_2$ (1.3 mM) was found at pH 7.5 (<0.2% of the rate with 4 mM $MgCl_2$), in agreement with previous results (Kupiecki & Coon, 1959, 1960). However, small but significant activation by $ZnCl_2$ (2.7% of the rate with $MgCl_2$) was found at pH 6.7 by using 50 mM K-Pipes buffer instead of Tris-HCl. The specific activity with $MgCl_2$ was 180 ± 10 units/mg over this pH range. In the ATP-stimulated enolization of pyruvate at pH 7.5, $ZnCl_2$ (1.3 mM) produced small but significant activation which amounted to 11% of the rate observed with 5 mM $MgCl_2$. The latter specific activity was 0.16 ± 0.005 μmol of pyruvate exchanged per min mg of enzyme. At pH 6.7, Zn(II) activated the enolization of pyruvate 2.3-fold more effectively than did Mg(II), due to a 2.3-fold increase in the specific activity with Zn(II) to a value of 0.041 ± 0.006 units/mg and a 9-fold decrease in the specific activity with Mg^{2+} to a value of 0.018 ± 0.006 units/mg.

Effect of Bidentate and Tridentate Isomers of CrATP on the Relaxation Rate of Water Protons. Because of the large differences in the affinities and activities of the bidentate and

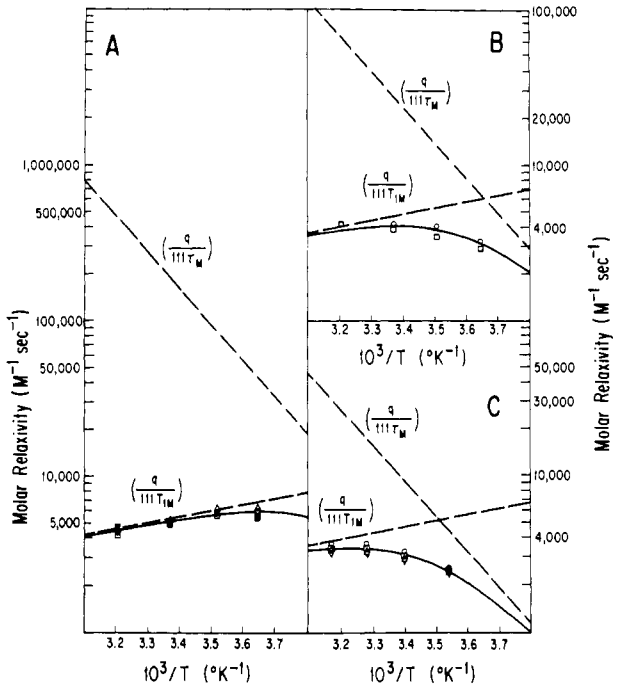

FIGURE 1: Temperature dependence of the paramagnetic effects of CrATP isomers on the longitudinal relaxation rate of water protons. (A) Molar relaxivity at 24.3 MHz of the tridentate CrATP isomers which elute from the cycloheptaamylose column in the order: isomer 1 (○) (2.2 mM); isomer 2 (□) (3.3 mM); isomer 3 (△) (2.4 mM); isomer 4 (▽) (5.1 mM). The samples also contained 10 mM K-Pipes, pH 5.6 ± 0.3. The solid curve through the data was obtained by using the equation molar relaxivity = $q/(111)(T_{1m} + \tau_m)$ (Luz & Meiboom, 1964), and the $q/(111)T_{1m}$ and $q/(111)\tau_m$ contributions are given by the dashed lines. The value 111 M is the total concentration of hydrogen nuclei in aqueous solutions. (B) Molar relaxivity at 24.3 MHz of the Δ (○) and Λ (□) bidentate CrATP isomers (2.5 mM). The pH was 5.4 ± 0.2. All other conditions and data analysis are as described in (A). (C) Molar relaxivity at 100 MHz of the bidentate CrATP isomers and conformers which elute from the cycloheptaamylose column in the order: Λ-A (○) (1.4 mM); Δ-A (□) (1.7 mM); Δ-B (△) (1.7 mM); Λ-B (▽) (1.8 mM). The pH was 3.5 ± 0.1. At this pH, two conformers of each isomer are detected (Dunaway-Mariano, 1978). All other conditions and data analysis are as described in (A).

tridentate isomers of CrATP with pyruvate kinase, nuclear relaxation studies of these isomers were made in an attempt to detect structural differences. Within our experimental error, no significant differences among the various bidentate isomers were found. Similarly, no differences among the tridentate isomers were detected. However, significant differences in the paramagnetic effects on water proton relaxation between the bidentate and tridentate isomers were observed. While for the tridentate isomers the relaxation rate is predominantly in the fast-exchange region, the relaxation rate of the bidentate isomers is partially exchange limited. This is shown as follows. At 23 °C and at 24.3 MHz the paramagnetic effects of the bidentate isomers (4000 ± 150 M^{-1} s^{-1}) are significantly lower than those of the tridentate isomers (5100 ± 150 M^{-1} s^{-1}). With the tridentate isomers the relaxation rate decreased with increasing temperature over the range 10–38 °C with an E_{act} = −1.8 ± 0.2 kcal/mol, indicating fast proton escape from the inner sphere of Cr(III) (Figure 1A). The fast exchange is consistent with the measured ratio $(1/T_{1p})_{protons}/(1/T_{1p})_{deuterons}$ of 37 ± 5 at 8 MHz and with the previously observed frequency dependence of $1/T_{1p}$ of protons below 8 MHz (Gupta et al., 1976a).

The somewhat lower $1/T_{1p}$ values of water protons observed with the bidentate isomers increased by 30% with temperatures between 1 and 23 °C and remained constant at higher temperatures up to 42 °C (Figure 1B,C). This behavior suggests partial exchange limitation. The alternative explanation of this small and positive temperature dependence of $1/T_{1p}$, fast exchange with $\omega_1^2\tau_c^2 \gg 1$, is ruled out by the observed equality at 21 °C and at 24.3 MHz of $1/T_{1p}$ and $1/T_{2p}$ and by the absence of a frequency dependence of $1/T_{1p}$ at 21 °C between 24.3 and 100 MHz (Figure 1B,C).

Assuming that in the fast-exchange region $1/T_{1p}$ of the bidentate isomers have the same E_{act} of −1.8 kcal/mol as the tridentate isomers, an E_{act} = 10.5 ± 0.5 kcal/mol was obtained for the escape rate of protons (q/τ_m) from the inner sphere of bidentate CrATP at pH 3.5. At 21 °C and pH 3.5 the absolute value of q/τ_m is 1.1 × 10^6 s^{-1} (Figure 1C) and increases to a value of 2.7 × 10^6 s^{-1} at pH 5.4 (Figure 1B) and to ~10^7 s^{-1} at pH 6.7, indicating base catalysis of proton escape. Similar values for the E_{act} (10 kcal/mol) and for q/τ_m at 21 °C (2.1 × 10^6 s^{-1}) were obtained for the escape of protons from $Cr(H_2O)_6^{3+}$ which has a 1.5-fold larger q value (Bloembergen & Morgan, 1961). At pH 4.0 and 22 °C no effect of ionic strength from 0.2 to 1.2 M NaCl on the $1/T_{1p}$ values of the bidentate CrATP isomers was observed, indiating no effects on q/τ_m.

The proton escape rate (q/τ_m) from the coordination sphere of the tridentate isomers of CrATP is approximately 1 order of magnitude greater than that of the bidentate isomers, although the precise value of q/τ_m and its activation energy for the tridentate isomers could not be determined due to the predominance of fast exchange. Assuming an activation energy of 10.5 kcal/mol for the exchange process, the small curvature at the lowest temperature could be fit, yielding an extrapolated value of q/τ_m of 1.8 × 10^7 s^{-1} for the tridentate isomers at 21 °C, pH 5.6 (Figure 1A), which is 6.7-fold greater than that of the bidentate isomers under identical conditions. The number of exchangeable inner-sphere water protons on Cr(III) (q) would approach maximum values of 8 and 6, respectively, for the bidentate and tridentate isomers of CrATP at pH values below 3. The lower escape rate of water protons (q/τ_m) from the bidentate CrATP isomers, despite their larger q values, may be due to intramolecular hydrogen bonding within the polyphosphate chain.

Discussion

The stereochemical and structural specificity of rabbit muscle pyruvate kinase toward its metal–ATP substrate was investigated by using the exchange-inert complexes Co$(NH_3)_4$ATP and CrATP. The overall reaction shown in eq 1

$$\text{ATP} + \text{pyruvate} \xrightleftharpoons{M^{2+},\, M^{+}} \text{ADP} + \text{phosphoenolpyruvate} + H^{+} \quad (1)$$

may be divided into the two half-reactions shown in eq 2 and 3.

$$\text{pyruvate} \xrightleftharpoons{\text{ATP},\, M^{2+},\, M^{+}} \text{pyruvate enolate} + H^{+} \quad (2)$$

$$\text{pyruvate enolate} + \text{ATP} \xrightleftharpoons{M^{2+},\, M^{+}} \text{phosphoenolpyruvate} + \text{ADP} \quad (3)$$

The phosphorylation partial reaction (eq 3) was studied by using glycolate as the phosphoryl acceptor. We have previously found no phosphoryl transfer to pyruvate from a mixture of tridentate and bidentate isomers of CrATP (Gupta & Mildvan, 1977). Similarly, only 1.5% of a single-turnover phosphoryl transfer to pyruvate has been detected by using the pure Δ

isomer of β,γ-bidentate CrATP[3] which is active in the phosphorylation of glycolate.

In contrast to the phosphorylation of pyruvate, the phosphorylation of glycolate by ATP at neutral pH is thermodynamically favorable. With Mg^{2+} as the activator, the equilibrium shifts toward phosphoenolpyruvate and α,β-bidentate MgADP on the enzyme (Nageswara Rao et al., 1979). However with β,γ-bidentate CrATP and pyruvate, the inability of Cr(III) to dissociate from the transferred phosphoryl group and to coordinate the α-phosphoryl group may account for the unfavorable equilibrium on the enzyme.

As the data reported in Tables I and IV clearly indicate, CrATP (as a mixture of isomers) is a substrate for pyruvate kinase in the glycolate phosphorylation reaction. This reaction requires an enzyme-bound divalent cation and shows the same metal ion specificity (Table IV) as that of the transfer from ATP to glycolate (Kayne, 1970). The present data thus establish the requirement for an enzyme-bound divalent cation in the phosphoryl transfer reaction, in addition to the previously demonstrated requirement for a nucleotide-bound metal (Gupta & Mildvan, 1977). Both have also been shown earlier to be necessary for the enolization of pyruvate (Gupta et al., 1976b).

Interestingly, the product, CrADP–glycolate-P, is released from the enzyme so slowly that only single-turnover phosphoryl transfers are observed for these reactions. In order to determine which of the CrATP isomers is responsible for the observed substrate activity, the purified bidentate and tridentate isomers were individually tested as substrates. These studies were carried out at pH 6 with the bidentate isomers since they racemize rapidly at higher pH. The only species of CrATP which functions in the phosphoryl transfer reaction of eq 3 is the Δ stereoisomer of the β,γ-bidentate chelate (Table I). This result is consistent with the preferential formation of the A isomer of ATPβS from ADPβS with Mg(II)-activated pyruvate kinase (Jaffe & Cohn, 1978). The Δ isomer of β,γ-bidentate CrATP is also the most effective activator of the partial reaction of eq 2, the enolization of pyruvate (Tables II and III). However, all of the bidentate and tridentate isomers of CrATP studied can bind to the active site as indicated by their activation of the enolization of pyruvate (Table II). Presumably, only the Δ stereoisomer of β,γ-bidentate CrATP correctly positions and aligns this substrate for phosphoryl transfer and for the optimal rate of enolization. Figure 2A shows the conformation and arrangement of substrates and essential cations at the active site of pyruvate kinase, consistent with the present data and with *ten* metal–substrate, *six* intersubstrate, and *three* intermetal distances determined by NMR (Mildvan et al., 1976; Hutton et al., 1977; Gupta, 1977; Gupta & Benovic, 1978).

Figure 2B shows the active site with the Λ isomer of bidentate CrATP, inactive in the phosphoryl transfer, and Figure 2C shows the structure of the enzyme complex of Δ bidentate CrATP which is most active in the enolization of pyruvate. The lower activity of the Λ bidentate isomer in the enolization reaction may be due to its less favorable interaction with the enzyme, as reflected in a larger K_m, and with the carbonyl group of bound pyruvate, as reflected in both a lower V_{max} and in a higher activation energy for V_{max} by 11 ± 2 kcal/mol. It is of interest that this difference in activation energy agrees with the energy of activation (10.5 ± 0.5 kcal/mol) for the slower escape of water protons from the hydration sphere of Cr(III) in the bidentate CrATP complexes. Hence, the breaking of additional internal hydrogen bonds in the enzyme-bound bidentate CrATP complex may be a necessary prerequisite for pyruvate enolization. The order of magnitude lower activity of the tridentate complexes may be due to the greater distance of the nucleotide-bound Cr^{3+} from the carbonyl group of the enzyme-bound pyruvate, since the maximal rate of enolization has previously been correlated with the electrophilicity of the nucleotide-bound metal (ρ = 0.2) (Gupta et al., 1976b). In addition, other unfavorable geometric effects may be operative to increase the K_m and decrease the V_{max} of pyruvate enolization with the tridentate CrATP isomers. Although the "cup-shaped" tridentate complexes can be accommodated by the active site, the most active conformation of the polyphosphate chain of the ATP for binding and catalysis may well be an extended one, as suggested by X-ray studies of bound MgATP on phosphoglycerate kinase (Blake & Evans, 1974; Watson et al., 1977) and hexokinase (Steitz et al., 1977).

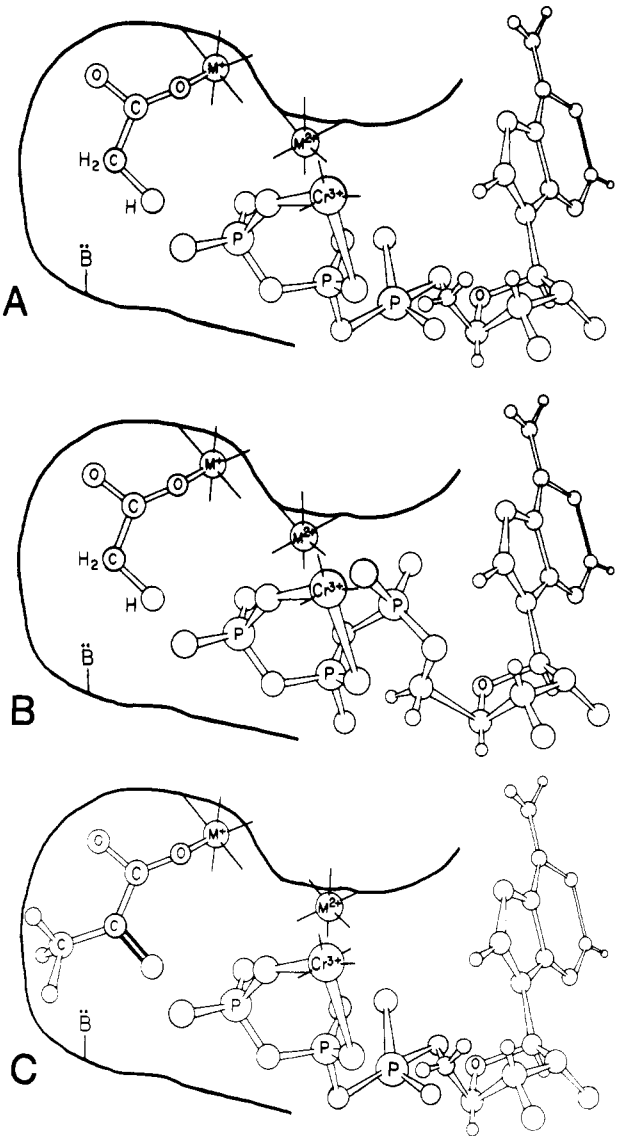

FIGURE 2: Structure and stereochemistry of CrATP isomers in pyruvate kinase complexes consistent with present data and previous NMR studies. (A) Δ β,γ-bidentate CrATP–glycolate complex active in phosphoryl transfer to glycolate. (B) Λ β,γ-bidentate CrATP–glycolate complex inactive in phosphoryl transfer. (C) Δ β,γ-bidentate CrATP–pyruvate complex most active in enolization of pyruvate.

The substrate activity of the β,γ-bidentate $Co(NH_3)_4ATP$ complex with pyruvate kinase was also examined. Interestingly, while this complex shows activity with both yeast

[3] D. Dunaway-Mariano, unpublished observations.

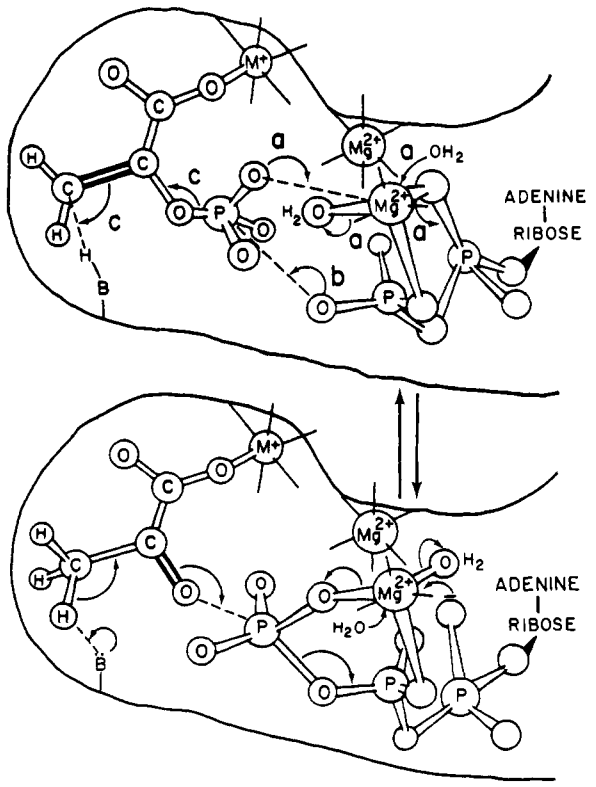

FIGURE 3: Minimal mechanism of pyruvate kinase consistent with the structures of Figure 2 and the present data. In step a, Mg(II) migrates from α,β-ADP coordination to β-ADP, phosphoenolpyruvate coordination by two ligand substitution reactions which may be separate or concerted. In step b, the phosphoryl group is transferred, yielding Δ β,γ-bidentate MgATP and the enolate of pyruvate. In step c, the enolate of pyruvate is protonated and ketonized.

hexokinase (Cornelius & Cleland, 1978) and P-Rib-PP synthetase (Li et al., 1978), it shows little or no activity with pyruvate kinase. Specifically, in the presence of Mn(II) the complex only weakly stimulates pyruvate enolization (Gupta & Mildvan, 1977), and we have found that in the presence of Mg(II) neither the resolved Δ isomer nor the mixture of bidentate isomers induces enolization. Furthermore, the present data indicate that $Co(NH_3)_4ATP$ does not transfer a phosphoryl group to pyruvate kinase bound glycolate. Presumably, the ammonia ligands of the $Co(NH_3)_4ATP$ complex inhibit its proper binding to the active site of the enzyme as suggested by the greater distances observed[4] between the adenosine H_2, H_8, and H_1' atoms of $Co(NH_3)_4ATP$ and enzyme-bound Mn(II) compared with the corresponding distances between the bound Mn^{2+} and the same protons of bound ATP (Sloan & Mildvan, 1976).

With the aid of CrATP we were able to further examine the specificity of pyruvate kinase toward the enzyme-bound divalent cation, in both the phosphoryl transfer and pyruvate enolization rections. As indicated in Tables IV and V the observed relative effectiveness of the metal as an activator of the phosphoryl transfer is Mn(II) > Co(II), Zn(II) > Mg(II) $\gg$ Ca(II) $\sim$ 0 and as an activator of the catalyzed enolization of pyruvate is Mn(II) > Zn(II) > Mg(II) $\gg$ Ca(II) $\sim$ 0. Not only was the order of activity the same for both reactions, as indicated above, but the relative degree of activation by the divalent cations is comparable. The same ordering of divalent cation activation [Mn(II) > Co(II) > Mg(II)] was observed in the pyruvate kinase catalyzed phosphorylation of glycolate by ATP (Kayne, 1974), a more complicated system in which the added divalent cations function at both sites. This order differs from that found in the phosphorylation of fluoride by ATP [Mg(II) > Mn(II)] (Tietz & Ochoa, 1958) and in the phosphorylation of hydroxylamine by ATP [Co(II) > Zn(II) > Mn(II) > Mg(II)] (Kupiecki & Coon, 1959, 1960), suggesting differing rate-limiting steps.

The present data also show that Zn^{2+} functions effectively as the enzyme-bound activating cation for pyruvate kinase reactions but only poorly as the nucleotide-bound metal. The latter may be due to the strong tendency of Zn^{2+} to coordinate to the adenine ring nitrogens of ATP (Cohn & Hughes, 1962). At lower pH this ring interaction may be diminished, permitting Zn^{2+} to function more effectively as the nucleotide-bound metal activator as well. Of the divalent cations tested, only Ca(II) did not form a kinetically active enzyme–metal complex, although it binds to the active site of the enzyme (Mildvan & Cohn, 1965).

On the basis of results from the present and earlier studies, some conclusions can be drawn regarding the mechanism of the pyruvate kinase catalyzed phosphorylation of pyruvate by MgATP (Figure 3). These conclusions depend on the reasonable assumption that the mechanism of the more rapid phosphorylation reaction with substitution-labile MgATP is the same as that with the exchange-inert CrATP complex. The present results indicate that the Δ isomer of the β,γ-bidentate chelate of MgATP is the active complex in the phosphoryl transfer reaction of pyruvate kinase, although a mixture of bidentate and tridentate MgATP may be detected by NMR on pyruvate kinase (Gupta & Mildvan, 1977). Since the major species of enzyme-bound MgADP is the α,β-bidentate chelate (Gupta & Mildvan, 1977) and the active form of enzyme-bound MgATP is the β,γ-bidentate chelate, Mg(II) migration from the γ-phosphate of the ATP to the α-phosphate must occur as ATP transfers its phosphoryl group. Furthermore, the inability of the exchange-inert complex, α,β-bidentate CrADP, to undergo pyruvate kinase catalyzed phosphorylation by phosphoenol pyruvate (Gupta & Benovic, 1978) indicates that the Mg(II) migration occurs after the phosphoryl transfer from MgATP to the enolate of pyruvate and before the phosphoryl transfer from phosphoenolpyruvate to ADP. Such a stepwise mechanism for the pyruvate kinase reaction is shown in Figure 3.

Acknowledgments

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Thermodynamic Model for Micelle Formation by Phosphatidylcholines Containing Short-Chain Fatty Acids. Correlations with Physical–Chemical Data and the Effects of Concentration on the Activity of Phospholipase A_2[†]

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ABSTRACT: A theoretical model of short-chain (C_6–C_8 fatty acids) lecithin aggregation was developed to determine if a change in form or concentration of aggregate species would be predicted which could explain the parabolic kinetic behavior observed near the critical micelle concentration with phospholipase A_2 [Wells, M. A. (1974) *Biochemistry 13*, 2248]. In order to satisfactorily predict the physical properties of the micelles (critical micelle concentration, aggregation number, and molecular weight heterogeneity) as reported by Tausk et al. [Tausk, R. M. J., Kaomiggelt, J., Oudschoorn, C., & Overbeek, J. Th. G. (1974) *Biophys. Chem. 1*, 175; Tausk, R. M. J., Van Esch, J., Karmiggelt, J., Voordouw, G., & Overbeek, J. Th. G. (1974) *Biophys. Chem. 1*, 184; Tausk, R. M. J., Oudshoorn, C., & Overbeek, J. Th. G. (1974) *Biophys. Chem. 2*, 53], the ^{13}C NMR data of Schmidt et al. [Schmidt, C. F., Barenholz, Y., Huang, C., & Thompson, T. E. (1977) *Biochemistry 16*, 3948], and the kinetic data, we developed a two micelle model. In this formulation the first micelle is formed at lecithin concentrations near the critical micelle concentration and the second micelle arises from the first at higher concentrations of lecithin. It was shown that a satisfactory fit to the kinetic data as well as ^{13}C NMR data was achieved, assuming that the second micelle is the form of the substrate responsible for the large rate enhancement observed above the critical micelle concentration as well as the substrate form responsible for the majority of the ^{13}C NMR shift above the critical micelle concentration. It was suggested that dehydration of the carbonyl groups of the substrate could account for both the ^{13}C NMR shifts and the enhanced activity as a substrate for phospholipase A_2.

The kinetics of hydrolysis of lecithins containing short-chain fatty acids by phospholipases often show anomalous regions in velocity vs. substrate concentration plots. In these regions the plots are parabolic rather than hyperbolic (Allgyer and Wells, unpublished experiments). In the case of phospholipase A_2, this region occurs near the critical micelle concentration (cmc)[1] (Wells, 1974a), but it occurs well below the cmc in the case of phospholipase D (Allgyer and Wells, unpublished experiments). It has been proposed that this parabolic region represents a range of concentration of substrate over which the properties of the micelle are changing (Wells, 1974a), although the nature of the change has not been defined. In order to more completely understand these kinetic "anomalies" it is necessary to know the properties and concentration of the various species which may exist in solution.

A theoretical model of lecithin aggregation was developed to determine if a change in form or concentration of aggregate species would be predicted which could explain the parabolic kinetic behavior observed near the cmc with phospholipase A_2.

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[1] Abbreviations used: cmc, critical micelle concentration; DiC_4, 1,2-dibutyryl-*sn*-glycero-3-phosphorylcholine; DiC_5, 1,2-divaleryl-*sn*-glycero-3-phosphorylcholine; DiC_6, 1,2-dihexanoyl-*sn*-glycero-3-phosphorylcholine; DiC_7, 1,2-diheptanoyl-*sn*-glycero-3-phosphorylcholine; DiC_8, 1,2-dioctanoyl-*sn*-glycero-3-phosphorylcholine; rmse, root mean square error.

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