Investigation of Substrate Specificity of Creatine Kinase Using Chromium(III) and Cobalt(III) Complexes of Adenosine 5′-Diphosphate†

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ABSTRACT: The specificity for substrate binding to creatine kinase for metal–nucleotide complexes of the type Cr-$(H_2O)_{4-n}(NH_3)_n$ADP (where n = 0, 3, or 4) and Co-$(H_2O)_{4-m}(NH_3)_m$ADP (for m = 3 or 4) has been investigated over the pH range 5.5–7.8 with the Δ-α,β-bidentate diastereoisomers. These inert nucleotide complexes acted as competitive inhibitors vs. MgADP over this range. In addition, the pH dependence of the V, V/K, and K_m values for MgADP has been determined. Metal–nucleotide binding to the enzyme is strongest below an approximate pK of 6.45 but again becomes pH independent above pH 7. This pK is not associated with the metal–nucleotide complex. Instead, we conclude that the pK of the acid–base catalyst (thought to be histidine) is about 6.45 in the absence of nucleotide but is raised to 7.2 in its presence. This perturbation of the pK may result from a protein conformational change that allows a hydrogen bond to form between the phosphorylated nitrogen of phosphocreatine and the acid–base catalyst. The pK of the water in $Cr(H_2O)(NH_3)_3$ADP has been determined to be 6.6, and by comparison of the binding affinity of this complex with that of $Cr(NH_3)_4$ADP or $Cr(H_2O)_4$ADP, it can be deduced that the hydroxo species binds more strongly than the aquo complex. In general, chromium nucleotides are bound more strongly than cobalt complexes, and binding affinity increases as water replaces ammonia in the first coordination sphere of the metal. Both trends are a result of stronger hydrogen-bond interactions between the metal complex and protein.

Creatine kinase (EC 2.7.3.2) catalyzes the reversible transfer of a phosphoryl group from phosphocreatine to ADP, generating ATP and creatine. The kinetic mechanism has been studied extensively (Kenyon & Reed, 1983) by initial velocity and product inhibition techniques (Nihei et al., 1961; Morrison & James, 1965) and by isotope exchange studies (Morrison & Cleland, 1966). At pH 8, the kinetic mechanism appears rapid equilibrium random in both directions; however, at pH 7 and below, the forward reaction has a rapid equilibrium ordered mechanism, with MgATP binding first (Schimerlik & Cleland, 1973). More recently, on the basis of the pH variation of the kinetic parameters, Cook et al. (1981) suggested that a cationic acid group (probably histidine) acts as an acid–base catalyst in the reaction and that a neutral acid (probably a carboxyl group) is involved in binding the positively charged guanidinium group of either phosphocreatine or creatine. Moreover, isotope-trapping experiments demonstrated that at pH 7 phosphocreatine is sticky and reacts to give creatine 4–6 times faster than it dissociates from the enzyme–MgADP–phosphocreatine complex. These studies elucidated the kinetic mechanism of creatine kinase but did not address in detail the effect of the metal–nucleotide complex on the active site.

The selectivity of many enzymes for coordination isomers of metal–nucleotide complexes may be demonstrated by one of three general approaches. First, one may synthesize and resolve isomers of exchange-inert complexes of nucleotides by using Cr(III) and Co(III) ions in lieu of the exchange labile Mg(II) ion. These inert complexes may then be used as dead-end inhibitors in kinetic studies (Schimerlik & Cleland, 1973; DePamphilis & Cleland, 1973; Janson & Cleland, 1974; Danenberg & Cleland, 1975; Cornelius & Cleland, 1978; Cleland & Mildvan, 1979; Dunaway-Mariano & Cleland, 1980a,b). Second, one may exploit the different affinity for sulfur vs. oxygen coordination of hard [Mg(II)] vs. soft [Cd(II)] metal ions with nucleoside phosphorothioates (Eckstein, 1979; Knowles, 1980; Cohn, 1982). Third, the stereochemical configuration may be elucidated by paramagnetic metal complexes with electron paramagnetic resonance and chirally ^{17}O-labeled nucleotides (Leyh et al., 1982).

Using the pure α-phosphate epimers of ATPαS,[1] Burgers & Eckstein (1980) were able to show that creatine kinase used the Δ-screw-sense isomer of MgADP. By applying the Cr(III) nucleotide method, Dunaway-Mariano & Cleland (1980a,b) demonstrated that creatine kinase binds Δ-α,β-bidentate CrADP preferentially over either Λ-α,β-bidentate or β-monodentate CrADP. Presumably, creatine kinase, a physiologically reversible enzyme, requires the predominant form of MgADP in order for rephosphorylation of the nucleotide to occur when MgATP levels are already high.

In the present work, we have employed a series of Δ-bidentate[2] complexes of Cr(III) and Co(III)ADP that have varying degrees of ammine substitution for first coordination sphere water to probe the catalytic mechanism of rabbit muscle creatine kinase. These complexes were tested as competitive inhibitors vs. MgADP over the pH range 5.5–7.8. Additionally, the K_m, V, and V/K values for MgADP were also determined over this pH range. We will show the manner in

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; ATPαS, adenosine 5′-*O*-(1-thiotriphosphate); Mes, potassium salt of 2-(*N*-morpholino)-ethanesulfonate; Hepes, potassium salt of *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonate; CHpA, cycloheptaamylose cross-linked with epichlorohydrin; DEAE-Sephadex, diethylaminoethyl-Sephadex; CD, circular dichroism; NMR, nuclear magnetic resonance.

[2] The screw sense nomenclature is that of Cornelius & Cleland (1978). In this system, the reference axis is a line passing through the metal perpendicular to the chelate ring. The bond from the chelate ring to the rest of the molecule is the skew line defining either a left- (Λ) or a right-hand (Δ) helix.

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which the binding of nucleotides is affected by the protonation state of the acid–base catalytic group on the enzyme and how the pK of this group is affected by binding of nucleotide.

Materials and Methods

Rabbit muscle creatine kinase and ADP were from Boehringer-Mannheim. Yeast hexokinase, glucose-6-phosphate dehydrogenase, ion-exchange resins, phosphocreatine, NADP, and buffers were from Sigma, and $Cr(NO_3)_3 \cdot 9H_2O$ was from Mallinckrodt. The inorganic starting materials used were $[Cr(NH_3)_4(H_2O)Cl]Cl_2$ (Pfeiffer, 1905), $[Cr(NH_3)_3(H_2O)Cl_2]Cl$ (Werner, 1906), $[Co(NH_3)_4CO_3]NO_3$ (Schlessinger, 1960a), and $[Co(NH_3)_3(H_2O)Cl_2]Cl$ (Schlessinger, 1960b). Cycloheptaamylose gel (CHpA) was prepared by the method of Cornelius & Cleland (1978).

The CD and UV–vis spectra were measured on a Jasco J41 spectropolarimeter (equipped with a J-DPY data processor) and a Cary 118 spectrophotometer, respectively. Molar ellipticities are reported in deg cm^2/dmol. Creatine kinase inhibition studies were carried out at 25 °C by using a Beckman DU monochromator, a Gilford optical density converter, and a 10-mV recorder or a Cary 118 spectrophotometer. The complexes were quantitated by measuring absorbance at 259 nm, with the assumption that $\epsilon = 15\,400$ M^{-1} cm^{-1}. HPLC separations were carried out with a radial-compression, C_{18} reverse-phase column, with 10 mM potassium methanesulfonate, pH 2.5, as eluant. Detection was by refractive index.

Preparation of Complexes. The cobalt and chromium complexes of ADP were synthesized as described by Cornelius et al. (1977) and DePamphilis & Cleland (1973). The metal ion with the appropriate water and ammine stoichiometry to generate the desired ADP complex was dissolved in 150 mL of water to give a 10 mM solution. Enough solid Na_2HADP was then added to make the solution 10 mM in nucleotide, and the pH of the mixture was adjusted to 3 with KOH. The solution was heated to 80 °C for 10 min, filtered, and flash evaporated at 25 °C to a 15-mL volume. The sample was then charged to a CHpA column (245 × 1.3 cm) kept at 4 °C. The column was eluted with 10 mM Mes, pH 5.5, at a flow rate of 5 mL/h. The first ADP complex eluted from the column in each case had a CD spectrum identical with that reported for the Δ-isomer of that complex. The Δ-screw-sense isomers, which have been shown by Burgers & Eckstein (1980) to be the preferred substrate for creatine kinase, were used in the pH studies described below.

Ligand Hydrolysis Constants. The pK value for $Cr(H_2O)(NH_3)_3ADP$ was determined spectrophotometrically by monitoring the change in the d–d transitions as a function of pH. The complex was titrated with 3 M KOH to pH 7.6 and then back with 3 M HNO_3. The pH was determined on a Radiometer pH meter equipped with a Radiometer combination pH electrode. The data were analyzed by the method of Schwarzenbach & Schwarzenbach (1963). A graphical treatment of eq 1 yields K_a as the slope and $A_{\text{low pH}}$ as the

$$A = \frac{(A_{\text{high pH}} - A)K_a}{[H^+]} + A_{\text{low pH}} \quad (1)$$

vertical intercept for a one-proton process. In eq 1, A represents the absorbance at any pH, $A_{\text{high pH}}$ is the absorbance at the beginning of the titration where all of the $Cr(H_2O)(NH_3)_3ADP$ was in the high-pH form, K_a is the acid dissociation constant, and $A_{\text{low pH}}$ is the absorbance when all of the material was in the protonated form. The isosbestic points demonstrate that only two absorbing species are in solution, as indicated by eq 2. This procedure is especially useful with

$$[CrADP]_t = [Cr(H_2O)(NH_3)_3ADP] + [Cr(NH_3)_3ADP(OH)] \quad (2)$$

systems that have the two species of interest present only over a small pH range with multiple, overlapping equilibria occurring for most of the titration curve. Due to problems of olation and polymerization, the pK for $Cr(H_2O)_4ADP$ could not be determined accurately by titration. However, we can place a lower limit of 7.5 on this pK. Similarly, we believe the pK for $Co(H_2O)(NH_3)_3ADP$ to be in the 7.0–7.5 range.

Initial Velocity Studies. The activity of creatine kinase was determined by assaying the formation of MgATP with hexokinase and glucose-6-phosphate dehydrogenase. Reaction mixtures contained 50 mM Mes (pH 5.5–6.7) or Hepes (6.8–8.2), 10 mM $MgCl_2$, 19 mM phosphocreatine, 1 mM NADP, 6 mM glucose, 1 mM citrate, 30 units/mL hexokinase, 15 units/mL glucose-6-phosphate dehydrogenase, 1.5 units/mL creatine kinase, and variable MgADP. At pH 6 and below, phosphocreatine, hexokinase, and glucose-6-phosphate dehydrogenase were added just prior to the addition of creatine kinase to avoid degradation of phosphocreatine and to ensure retention of coupling enzyme activity. The required levels of coupling enzyme were invariant over the pH range studied. The temperature of the assay mixtures was maintained at 25 °C with a circulating water bath. Both buffers were titrated with KOH. Sufficient overlap was obtained between the buffers to rule out buffer effects.

Isotope-Trapping Studies. A solution containing 1 mM creatine kinase [based on $\epsilon_{280}^{1\%} = 8.96$ (Cook et al., 1981)] and 1.1 mM Mg[^{14}C]ADP (42.2 Ci/mol) in 0.05 mL was added to 5 mL of a rapidly stirred solution of 50 mM Hepes, pH 7, 10 mM $MgCl_2$, 1 mM ADP, and 50 mM phosphocreatine. The reaction was stopped after 5 s by addition of enough 60% perchloric acid to reduce the pH to 1.9, followed by vortexing the solution. The solution was filtered, the volume increased to 100 mL with water, and the pH adjusted to 7.6 with KOH. This mixture was then charged to a DEAE-Sephadex column (2 × 20 cm) kept at 4 °C and eluted with a linear triethylammonium bicarbonate gradient (0.1–0.8 M, pH 7.6). The fractions were counted to determine the amount of [^{14}C]ATP formed; ADP was eluted at fraction 47, while ATP was centered at 57. The control experiment involved adding creatine kinase to a reaction mixture containing all of the components listed above, including [^{14}C]ADP. The reaction was stopped and analyzed in the same manner.

Data Processing. Reciprocal initial velocities were plotted against reciprocal substrate concentrations with all plots being linear. The data were fitted to the appropriate equations with the Fortran programs of Cleland (1979). The individual saturation curves used to obtain pH profiles were fitted to eq 3. Inhibition studies were analyzed by fitting data to eq 4.

$$V = V[A]/(K + [A]) \quad (3)$$

$$V = \frac{V[A]}{K(1 + [I]/K_i) + [A]} \quad (4)$$

$$\log y = \log [C/(1 + [H^+]/K_1)] \quad (5)$$

$$\log y = \log [C/(1 + K_2/[H^+])] \quad (6)$$

$$\log y = \log [C/(1 + [H^+]/K_1 + K_2/[H^+])] \quad (7)$$

$$\log y = \log [[y_l + y_h(K_1/[H^+])]/(1 + K_1/[H^+])] \quad (8)$$

The pH profiles for MgADP were fitted to eq 5, 6, or 7, after the exact levels of $MgADP^-$ present at a given pH were calculated from the levels of total Mg^{2+} and ADP present, with 5.12 and 6.68 as the pK values of MgHADP and $HADP^{2-}$ and

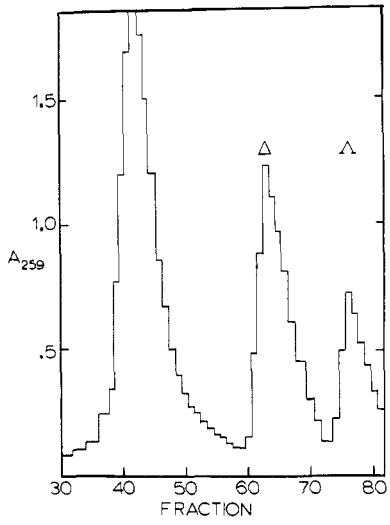


FIGURE 1: Elution profile for $Cr(H_2O)(NH_3)_3ADP$ on cycloheptaamylose gel. The major peak at the left represents unreacted starting material and polymers. The following two bands correspond to the pure Δ- and Λ-screw-sense isomers. Fractions were 3 mL.

1 and 36 mM as the dissocation constants of $MgADP^-$ and MgHADP, respectively (Smith & Alberty, 1956). Data for the pH profiles of the inhibition constants were fitted to eq 8. In eqs 5–8, K_1 and K_2 represent the dissocation constants for groups on the enzyme, y is V, V/K, or $1/K_i$, and C is the pH-independent value of y. The parameters y_l and y_h represent the extrapolated values for $1/K_i$ at low and high pH, respectively.

Results

Separation of α,β-Bidentate $M^{3+}(H_2O)_n(NH_3)_mADP$. The Δ-screw-sense isomers of α,β-bidentate $M^{3+}(H_2O)_n(NH_3)_mADP$ were isolated in 10–30% yield from the synthetic procedure described above. The elution profile for the cycloheptaamylose chromatography of $Cr(H_2O)(NH_3)_3ADP$ is shown in Figure 1. This pattern is representative of the chromatography obtained for all of the ADP complexes synthesized. Two metal–nucleotide-containing bands are well resolved. The first of these bands (I) corresponds to the Δ-screw-sense isomer, while the second band (II) is the Λ-isomer.[3] The (chromium or cobalt)/adenine ratio for each isomer was 1:1. The front and back half of each band was examined by CD and visible spectroscopy, and the $Cr(H_2O)(NH_3)_3ADP$ was examined by HPLC, to determine if ring conformational isomers or, in the case of the mixed-ammine complexes, geometrical isomers were present. Each band appeared to be homogeneous by these methods. Thus, we must conclude either that only preferred conformational or geometrical isomers exist or that CHpA and HPLC chromatography cannot separate these complexes. Therefore, the pooled Δ-isomer peaks were used for these studies.

Physical Characteristics of Chromium- and Cobalt–ADP Complexes. The visible spectra for the Δ- and Λ-isomers were the same at pH 6, and with the exception of $Cr(H_2O)(NH_3)_3ADP$, there was no marked change for spectra measured at pH 3 (Table I). The substitution of ammonia ligands for water in the metal coordination sphere causes a blue shift to the visible spectrum, which is entirely consistent with the greater ligand field strength of ammonia than water and the consequent perturbation of $10D_q$ for the metal-based orbitals (Cotton & Wilkenson, 1972).

Table I: Physical Parameters of Inert ADP Complexes Used in This Work

complex	visible spectrum at pH 3.0 [nm (ε)]	CD spectra [nm ($[\theta]_M$)][a]
$Cr(H_2O)_4ADP$	598 (20), 428 (19)	580 (−100)
$Cr(H_2O)(NH_3)_3ADP$	543 (28), 400 (28) 566 (35),[b] 402 (38)[b]	508 (−148)
$Cr(NH_3)_4ADP$	516 (42), 382 (35)	505 (−180)
$Co(H_2O)(NH_3)_3ADP$	542 (25), 397 (37)	532 (−250)
$Co(NH_3)_4ADP$	512 (43), 380 (37)	507 (−370)

[a] Δ-Isomer principal band. [b] pH 7.5.

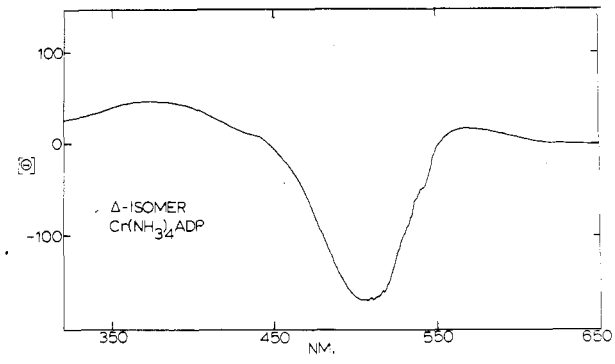


FIGURE 2: Circular dichroism spectrum of Δ-screw-sense isomer of $Cr(NH_3)_4ADP$. The Λ-isomer has a nearly mirror image CD with a positive peak at 505 nm. CD spectra were recorded at 22 °C in pH 5.8 Mes (10 mM) on a 1.3 mM solution of $Cr(NH_3)_4ADP$.

The circular dichroism spectra of the Δ-screw-sense isomer of $Cr(NH_3)_4ADP$ is shown in Figure 2. The CD peaks and molar ellipticities for the Δ-isomers of the remaining complexes are given in Table I.

$Cr(H_2O)(NH_3)_3ADP$ Acid Dissociation Constant. The titration of $Cr(OH)(NH_3)_3ADP$ with nitric acid causes a shift in the λ_{max} of the visible spectrum from 566 to 543 nm as the hydroxo ligand is protonated. This is consistent with the greater ligand field strength of water over hydroxide. In addition, two isosbestic points are observed at 476 and 540 nm, demonstrating that there are only two absorbing metal-containing species in the pH region studied. These data may be interpreted by the method of Schwarzenbach & Schwarzenbach (1963). The graphical representation of the Schwarzenbach analysis for two wavelengths is shown in Figure 3. The slope of the line directly yields the K_a of the aquo complex. Least-squares analysis gave a value of 6.6 ± 0.1 for the pK.[4]

Initial Velocity Studies Using MgADP. The pH profile for the maximal rate is very similar to that previously reported by Cook et al. (1981). As shown in Figure 4, V decreases below a pK of 5.7 ± 0.1 and above a pK of 8.1 ± 0.1. V/K_{MgADP} decreases above a pK of 7.6 ± 0.1 but does not decrease at low pH when correction is made for the actual levels of $MgADP^-$ present at the lower pH values. To test whether MgADP might be a sticky substrate, as is phosphocreatine (Cook et al., 1981), an isotope-trapping experiment (Rose et al., 1974) was carried out. The nucleotide was found not to be sticky, since no ADP could be trapped. The pH profile for the binding of MgADP is shown in Figure 5. The Michaelis constant for MgADP (corrected for actual $MgADP^-$ levels at

[3] These assignments are based on the CD spectra by analogy with $Cr(H_2O)_4ADP$ isomers separated by Dunaway-Mariano & Cleland (1980a,b).

[4] By contrast, the pK of the water in $Cr(H_2O)(NH_3)_3ATP$ is 6.97 (V. L. Pecoraro, unpublished experiment); the elevation by nearly 0.4 pH unit results from the extra negative charge in the complex.

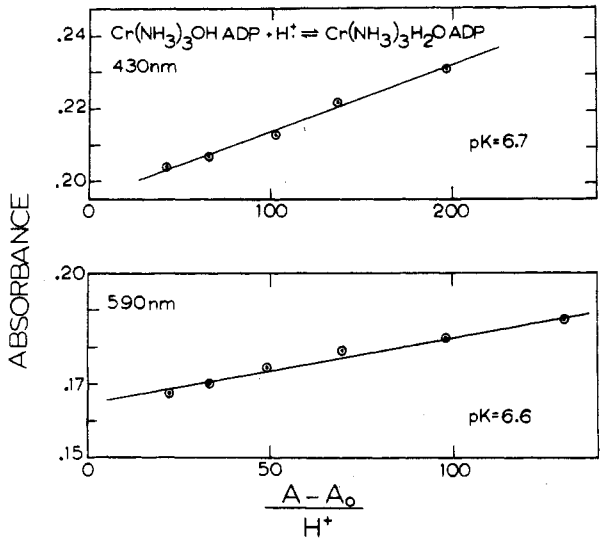


FIGURE 3: Schwarzenbach analysis of spectral changes observed at two wavelengths in the titration of $Cr(H_2O)(NH_3)_3ADP$ at 24 °C. The slope of the line gives the K_a for the complex. The concentration of $Cr(H_2O)(NH_3)_3ADP$ was 6.5 mM, and ionic strength was maintained at 0.1 M with KNO_3.

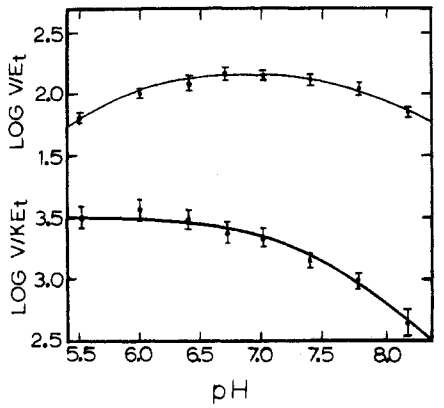


FIGURE 4: log $V/[E_t]$ and log $V/(K[E_t])$ vs. pH profiles for the creatine kinase reaction with MgADP as substrate. The concentration of phosphocreatine was maintained at 19 mM. The units for $V/[E_t]$ and $V/(K[E_t])$ are s^{-1} and mM^{-1} s^{-1}.

low pH) undergoes a change over this pH range with an apparent pK of 6.7 ± 0.1 when fitted to eq 8, with nucleotide binding being strongest at low pH.

Inhibition by Cobalt- and Chromium–ADP Complexes. The Δ-screw-sense isomers of $Cr(H_2O)_4ADP$, $Cr(H_2O)(NH_3)_3ADP$, $Cr(NH_3)_4ADP$, $Co(H_2O)(NH_3)_3ADP$, and $Co(NH_3)_4ADP$ were used as competitive inhibitors vs. MgADP of creatine kinase. All five complexes acted as competitive inhibitors over the entire pH range investigated. The data plotted in Figure 5 were calculated from fits to eq 4. In every case, a change analogous to that observed for MgADP was seen, with nucleotide binding being tighter at low pH. The individual pK values are $[Cr(H_2O)_4ADP]$ 6.3 ± 0.2, $[Cr(H_2O)(NH_3)_3ADP]$ 6.47 ± 0.1, $[Cr(NH_3)_4ADP]$ 6.35 ± 0.2, $[Co(H_2O)(NH_3)_3ADP]$ 6.5 ± 0.17, and $[Co(NH_3)_4ADP]$ 6.61 ± 0.2. The average pK value is 6.45 ± 0.15. The apparent K_i appears to decrease as the degree of aquo ligation to the metal increases. Similarly, chromium complexes have slightly higher affinity for the enzyme than the corresponding cobalt nucleotides.

Discussion

MgADP Profiles. The pH profile for V/K_{MgADP} in Figure 4 supports the conclusions of Cook et al. (1981) that there is

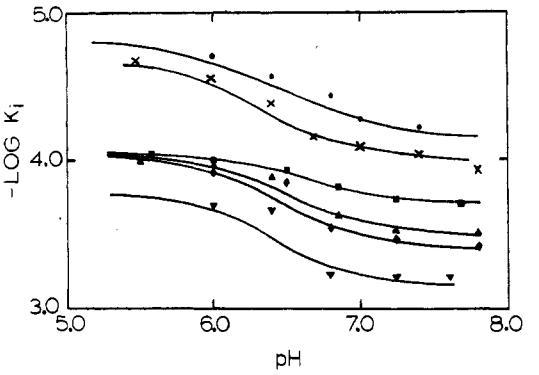
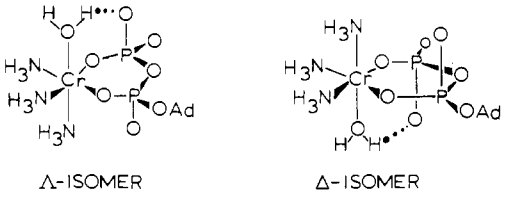


FIGURE 5: pH variation of pK_i or pK_m for $Cr(H_2O)_4ADP$ (●), MgADP (×), $Cr(H_2O)(NH_3)_3ADP$ (■), $Co(H_2O)(NH_3)_3ADP$ (▲), $Cr(NH_3)_4ADP$ (♦), and $Co(NH_3)_4ADP$ (▼). These complexes acted as competitive inhibitors vs. MgADP, and the data were fitted to eq 4 to obtain K_i values. Solid lines are fits to eq 8 with the assumption of an average pK of 6.45.

a cationic acid group with a pK near 7 that acts as an acid–base catalyst. This group, most likely a histidine, must be protonated for the phosphorylation of MgADP. The correct pK for such a group is seen only when the variable substrate is not sticky [a sticky substrate reacts to form product faster than it dissociates from the central complex (Cleland, 1977)]. Indeed, phosphocreatine is sticky, reacting 4–6 times faster than it dissociates from the ternary complex (Cook et al., 1981). However, when we used the isotope-trapping method of Rose et al. (1974) to determine whether MgADP was sticky, the lack of observation of [^{14}C]ATP demonstrated that nucleotide dissociation from the central complex is fast compared to catalysis. Therefore, the K_m of MgADP is its dissociation constant, and the pK of 7.4 ± 0.1 in the V/K_{MgADP} profile is the correct value for the E–phosphocreatine complex.

Structure of the Complexes. After Burgers & Eckstein (1980) showed that the Δ-isomer of MgADP was the substrate, Dunaway-Mariano & Cleland (1980a,b) demonstrated that creatine kinase exhibited a strong preference for binding of the Δ-screw-sense isomers of α,β-bidentate cobalt– or chromium–ADP. Regardless of the metal ion used, the Δ-screw-sense isomer eluted first from cycloheptaamylose and had a prominent negative band in the CD spectrum. We have extended this correlation to the mixed aquo–ammine complexes reported herein.

In theory, triammine complexes may exist as geometrical as well as screw-sense isomers. One possible orientation is facial, where all three ammines are mutually cis and occupy one face of an octahedron. The other geometry is meridinal, where two of the ammines are trans. It does not appear that CHpA or HPLC with a C_{18} reverse-phase column is effective in resolving these isomers if they exist, since we observed only one Δ- and one Λ-isomer. We cannot conclusively state which geometrical isomer we have isolated, but two pieces of circumstantial evidence suggest that these are facial complexes. First, the ^{1}H NMR and CD spectra of tridentate $Co(NH_3)_3ATP$ demonstrate that this complex exists as the facial isomer (Pecoraro & Cleland, 1982). Second, the starting material $[Co(NH_3)_3(H_2O)Cl_2]Cl$ is facial (V. L. Pecoraro and W. W. Cleland, unpublished results) so that a reorientation of the ammine ligands would be required during the synthesis of meridinal $Co(H_2O)(NH_3)_3ADP$. We suspect that each of the isomers we have isolated has the water cis to both phosphates and in the position that allows hydrogen bonding from this water to a β-phosphate oxygen when the adenosine is in the equatorial position, since such isomers would be the most stable possible:



Inhibition Studies. The pK_i profile for each of the five metal complexes tested parallels that observed for the pK_m of MgADP. Binding of the inhibitor is strongest at low pH and then undergoes a change to a new plateau value at higher pH. The pK value obtained from analysis of these curves is in the range 6.4–6.5. Although $Cr(H_2O)(NH_3)_3ADP$ has a pK of 6.6, neither $Cr(NH_3)_4ADP$ nor $Co(NH_3)_4ADP$ is expected to have pK values less than 20 (Pearson & Basolo, 1956; Jorgenson, 1956), and protonation of the adenine ring occurs only below pH 4. Therefore, the observed pK of 6.4 can unequivocally be assigned to a group on the enzyme rather than the metal–nucleotide complex.

In previous pH studies on creatine kinase (Cook et al., 1981), the level of nucleotide was saturating. Under these conditions the pK for the acid–base catalyst is 7.2. In contrast, we have kept phosphocreatine at saturating levels and varied the nucleotide concentration, so that the K_i values represent the situation in which the nucleotide is absent from the active site. In this case, the pK of the acid–base catalyst decreases to 6.4.

A rationale for the perturbation of the acid–base catalyst pK may be made by using Figure 6. In the absence of nucleotide, the acid–base catalyst (His) does not interact strongly with the phosphorylated nitrogen of phosphocreatine, resulting in a low pK for this group. Upon nucleotide binding, a protein conformation change occurs, causing the histidine and phosphorylated nitrogen to move closer together and form a hydrogen bond. This hydrogen bond would serve to stabilize the ternary complex and cause an elevation of the pK of the acid–base catalyst. This would explain why nucleotide binding at low pH was strongest, since as the pH is decreased the percentage of histidines that are protonated increases.

With the triammine complexes one can observe the effect of deprotonating the water in the inner coordination sphere. By subtracting the apparent pK_i values for a triammine complex from those of $Cr(NH_3)_4ADP$, which has no pK in the neutral pH range, one can determine the effect of water ionization. In Figure 7, the top curve represents the difference between the pK_i values of the tetraaquo and tetraammine CrADP compounds. A straight line is observed, since neither complex undergoes ionization over this pH range. In contrast, a change is observed in the bottom curve [where $Cr(H_2O)(NH_3)_3ADP$ is used in place of $Cr(H_2O)_4ADP$] with a pK of 6.8, which is similar to the pK of 6.6 determined from the triammine complex. Apparently, the $Cr(NH_3)_3ADP(OH)$ complex is bound more strongly than the corresponding aquo complex. The higher affinity may be due to a decrease in the overall charge of the complex, making it isoelectronic with the normal substrate MgADP. The effect is small, however, suggesting that the bound hydroxo ligand is facing away from the active site toward bulk water.

A distinct trend toward stronger binding is observed as water replaces ammonia in the first coordination sphere. This effect may be explained by the better hydrogen bond donating character of coordinated water than that of coordinated ammonia, so that aquo ligands interact better with binding groups on the enzyme than do the poor hydrogen bond donating ammine ligands. Similarly, the water molecules coordinated

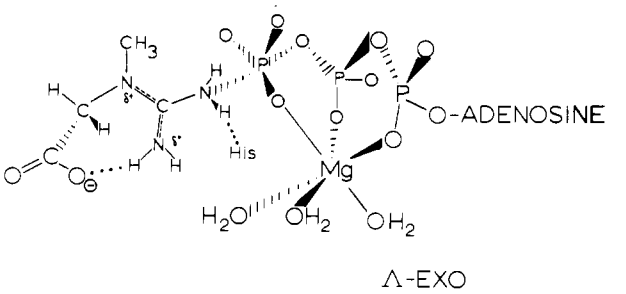


FIGURE 6: A possible representation of the creatine kinase transition state as modified from Cook et al. (1981). His is the acid–base catalyst. The conformation of the metal–nucleotide is based on the work of Dunaway-Mariano & Cleland (1980a,b) and Burgers & Eckstein (1980). Binding of the nucleotide complex results in a hydrogen bond forming between the phosphorylated nitrogen of phosphocreatine and the acid–base catalyst.

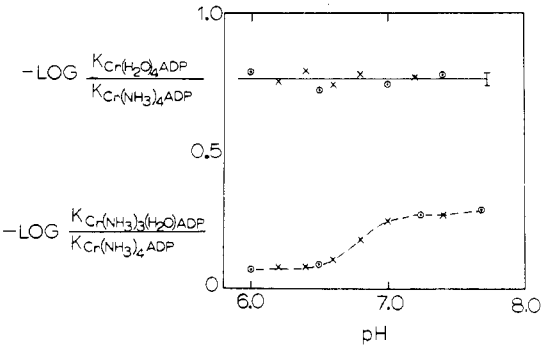


FIGURE 7: Difference in apparent pK_i for different metal complexes as a function of pH. The top curve is for two complexes that do not change protonation state over this pH range. The bottom curve involves $Cr(H_2O)(NH_3)_3ADP$, which has a pK of 6.6. At pH values where experimental data were obtained for both complexes, the difference is represented by (⊙), while (×) corresponds to differences obtained from pK_i values calculated from fits to eq 8.

to chromium(III) should be slightly more acidic than those coordinated to cobalt(III) [e.g., $Cr(NH_3)_5(H_2O)^{3+}$ has a pK of 6.2 while the $Co(NH_3)_5(H_2O)^{3+}$ pK is 6.7 (Broomhead et al., 1964)]. Chromium nucleotides show a 2-fold greater binding affinity to creatine kinase than the corresponding cobalt ones, while a difference in pK of 0.5 represents a 3-fold change. Thus, this polarizability difference between chromium(III) and cobalt(III) may explain why chromium complexes bind more tightly than the cobalt analogues.

Registry No. Δ-$Cr(H_2O)_4ADP$, 73037-58-8; Δ-$Cr(H_2O)(NH_3)_3ADP$, 87984-20-1; Δ-$Cr(NH_3)_4ADP$, 73175-88-9; Δ-$Co(H_2O)(NH_3)_3ADP$, 87938-87-2; Δ-$Co(NH_3)_4ADP$, 63937-09-7; MgADP, 7384-99-8; creatine kinase, 9001-15-4.

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High Plasmalogen and Arachidonic Acid Content of Canine Myocardial Sarcolemma: A Fast Atom Bombardment Mass Spectroscopic and Gas Chromatography–Mass Spectroscopic Characterization†

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ABSTRACT: Canine myocardial sarcolemma was purified, and its phospholipid constituents were determined by gas chromatography–mass spectrometry, fast atom bombardment mass spectrometry, and conventional techniques. Canine myocardial sarcolemma contained 2.7 μmol of lipid P_i/mg of protein which was comprised predominantly of choline glycerophospholipids (47%), ethanolamine glycerophospholipids (28%), and sphingomyelin (11%). Sarcolemmal phospholipids contained 40% plasmalogen which was quantitatively accounted for by choline (57% of choline glycerophospholipid) and ethanolamine (64% of ethanolamine glycerophospholipid) plasmalogens. Choline plasmalogens contained predominantly the vinyl ether of palmitic aldehyde though ethanolamine plasmalogens were composed predominantly of the vinyl ethers of stearic and oleic aldehydes. The majority of sarcolemmal ethanolamine glycerophospholipids (75%) contained arachidonic acid esterified to the *sn*-2 carbon. Sphingomyelin was composed predominantly of long-chain saturated fatty acids (stearic and arachidic) as well as substantial amounts (8%) of odd chain length saturated fatty acids. The possible functional role of these unusual phospholipid constituents is discussed.

Although it has been over 50 years since Feulgen's initial description of plasmalogens in biological tissues (Feulgen et al., 1924), their functional role has not been elucidated. The vinyl ether content of electrically active tissues such as brain (Scott et al., 1967; Wuthier, 1966; Freysz et al., 1968), peripheral nerve (Sheltawy & Dawson, 1966), or myocardium (Scott et al., 1967; Owens, 1966; Dawson et al., 1962) is 15–35% while other tissues such as liver (Scott et al., 1967; Dawson et al., 1962) and kidney (Scott et al., 1967) have a low content of plasmalogens (2–10%). Plasmalogens are distinguished from conventional diacyl phospholipids by the lack of an oxygen atom and the presence of two sp^2 carbons at the *sn*-1 position, which alter the molecular geometry and dynamics near the hydrophobic–hydrophilic interface (Paltauf, 1983). Furthermore, the *sn*-2 hydroxyl of plasmalogens is usually esterified to highly unsaturated fatty acids which would also contribute to altered molecular dynamics of plasmalogens in comparison with diacyl phospholipids in biologic membranes.

To identify the major phospholipid constituents of the electrically excitable membrane of myocardium, highly purified preparations of canine myocardial sarcolemma were analyzed with fast atom bombardment mass spectrometry, gas chromatography–mass spectrometry (GC–MS),[1] and conventional

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