Investigations of Anion Binding Sites in Transition State Analogue Complexes of Creatine Kinase by Infrared Spectroscopy*

George H. Reed, Clyde H. Barlow,‡, and Ramon A. Burns, Jr.

From the Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

A specific class of anions inhibit creatine kinase by stabilizing the dead-end complex, enzyme•divalent cation•ADP•creatine (Watts, D. C. (1973) The Enzymes 8, 383-455). The inhibitory action of the anions is attributed to the ability of the anions to mimic the equatorial PO₃ plane formed by the migrating phosphoryl group in the transition state of the reaction. Infrared spectroscopy has been used to investigate the mode of binding of the inhibitory anions, thiocyanate, azide, and nitrate to the dead-end complex of creatine kinase. The infrared absorptions for these anions undergo characteristic changes in frequency or in multiplicity when the anions are liganded to various divalent cations, and infrared spectra for the enzyme complexes with the anions provide a means for recognizing anion-metal ion coordination at the active site of creatine kinase.

Infrared spectra for the complexes of the anions with enzyme•divalent cation•ADP•creatine species coincide with the vibrational spectra obtained for the simple anion-metal ion complexes in concentrated solutions of the metal-anion salts. These cation specific responses of the infrared absorptions for the enzyme-bound anions provide cogent evidence for liganding of the anions to the activating divalent cation. Since none of the anions investigated exhibit a high affinity for the cations, Mg(II), Mn(II), and Co(II) in free solution, protection of anion-metal ion coordination in the dead-end complex of creatine kinase connotes coordination of the metal ion to an oxygen atom of the equatorial PO₃ plane in the transition state of the reaction. Such a coordination scheme would be compatible with an in-line displacement mechanism for the reaction where the divalent cation activates through template, polarization, and charge shielding influences.

The mechanistic role of inorganic cations in the enzymic catalysis of phosphoryl transfer reactions has been of interest for a number of years. Several plausible schemes for activation of phosphoryl transfer by divalent cations have been proposed such as charge shielding, template effects, polarization, activation of a nucleophile, induction of strain, and coordination to a leaving group (1-5). In order to evaluate the potential contributions of such effects toward the enzymic catalysis of phosphoryl transfer it is necessary to have structural information which identifies the ligand donor groups on the substrates or enzyme or both which are bound to the metal ion in the activated complex. The labile coordination properties of the more common divalent activators of enzymic phosphoryl transfer, namely Mg(II), Co(II), Mn(II), and Ca(II), have hindered acquisition of requisite structural data for even simple enzyme-substrate complexes although considerable progress has been made (2, 6, 7). However, since the metal ions of interest may undergo ligand substitution reactions in the pathway to the transition state, the most insightful information should come from studies of complexes which approach the composition and structure of the activated state.

Creatine kinase is a good candidate for detailed studies of coordination of the divalent cation in an analogue of the transition state. Milner-White and Watts (8) found that a specific class of anions inhibit the enzyme by stabilizing the dead-end complex, enzyme divalent cation•ADP•creatine. The sizes and shapes of anions in this class suggested that their inhibitory action resulted from their ability to fit into the site normally occupied by the transferable phosphoryl group as it is distorted in the activated state of the reaction (8). The synergistic relationships in the binding of the anions, metal ions, and substrates to the enzyme have supported the idea that the inhibitory complex with the anions resembles the structure of the transition state (8-13). Magnetic resonance studies have revealed that binding of the anion to the dead-end complex with Mn(II) has a pronounced influence on the environment of the cation (9-11, 14). However, it has proven difficult to judge whether the anion effects on the metal ion arise directly from liganding of the anion to Mn(II) or are mediated indirectly through perturbations in the overall structure of the active site (10). Since possible coordination of the transferable phosphoryl group to the metal ion in the transition state has been a central mechanistic issue, knowledge of the mode of binding of the anions in the transition state analogue complexes is of considerable interest.

Some of the inhibitory anions have infrared-active vibrational modes in regions of the spectrum which are observable in aqueous solution. The frequencies of these vibrations are altered in a characteristic fashion when the anions are liganded to metal ions (15), and these changes in absorption frequency can be diagnostic for binding of the anion to a given metal ion. The present paper reports the results of infrared spectral studies of the binding schemes for thiocyanate, azide, and nitrate in complexes with creatine kinase.

MATERIALS AND METHODS
Enzyme—Creatine kinase was isolated from rabbit skeletal muscle by Method B of Kuby et al. (16). The specific activities of the preparations were of the order of 50 IU in the coupled assay with pyruvate kinase and lactate dehydrogenase at 21°C (17). The enzyme...
appeared to be homogeneous on polyacrylamide gel electrophoresis in the system described by Davis (18). Substrates — Creatine was obtained from Pfentich Laboratories. ADP was from Sigma Chemical Co., and Tris (ultrapure) was from Mann Laboratories. A sample of enriched (95 atom %) K'NO₃ was purchased from Thompson Packard, Inc. Other chemicals were reagent grade.

Infrared Measurements — Infrared spectra were recorded with a Perkin-Elmer model 521 spectrophotometer. Demountable cells (Barnes Engineering) were used with CaF₂ windows. The cells were fitted to water-jacketed holders through which water was circulated for cooling. Because of the low transmittance of aqueous solutions, short path lengths, approximately 0.05 mm, were used. The path lengths were established with either Teflon or latex rubber spacers. The path lengths of the sample and reference cells were matched before each recording by tightening the cell with the longer path length to null a water absorption. Most of the spectra were recorded with a 1-cm slit width of the transmittance scale. For optimum signal-to-noise ratios a fixed slit width of 1 mm was used. The 1-mm slit width provided spectral resolution from 10 to 12 cm⁻¹ in both regions of the spectrum which were investigated. The resolution produces some broadening of bands for transitions of half-band width less than 50 cm⁻¹ but does not affect the areas of the bands. In some cases, the relative areas of absorption bands were estimated with a compensating polar planimeter. Approximately 125 μl of solution were required to fill each cell, and the cells were flushed with distilled water and air-dried under suction between samples. Raman spectra were recorded on a spectrometer system described by Adar and Srivastava (19).

Sample preparation for Infrared Measurements — Observation of the infrared absorptions for the enzyme-bound form of the anions requires high concentrations of protein. Creatine kinase was equilibrated by dialysis with a solution containing 10 mM Tris/Cl⁻, pH 7.9, and 50 mM creatine. The solution of enzyme was concentrated to ~270 mg/ml with an Amicon Minicon B device. ADP was added from a 100 mM stock solution so that the final concentrations of enzyme sites and of ADP were approximately equal and of the order of 6 mM. Additions of anions and of divalent cations were made from concentrated stock solutions with a microsyringe to minimize dilution effects.

The choice of a buffer and counter ion was important since it was desirable to avoid unnecessary absorptions in the two spectral regions of interest (i.e. 2400 to 2000 cm⁻¹ and 1500 to 1000 cm⁻¹) and to minimize competition between the anion of interest and counterions for binding to the enzyme. Although Cl⁻ is a member of the class of inhibitory anions (8, 9), the affinity of Cl⁻ for the dead-end complex is among the weakest of the class (i.e. the dissociation constant for Cl⁻ is -12 cm⁻¹, see below). Moreover, it was convenient to add the divalent metal ions as Cl⁻ salts so that the contributions of Cl⁻ from the buffer solutions about 30% of the total Cl⁻ in the solutions. For the weaker binding anions, e.g., SCN⁻ and N₂O₃, there was a reduction of the amount of anion bound to the enzyme because of the competition with ~17 mM Cl⁻ in the solution. This competition was considered in the semiquantitative aspects of the experiments.

Magnetic Resonance Measurements — Longitudinal relaxation times (T₁) of water protons were measured at 24.3 MHz with a pulsed NMR spectrometer as described previously (10).

RESULTS AND DISCUSSION

Binding of Thiocyanate to Creatine Kinase — Thiocyanate is a member of the class of anions which stabilize the dead-end complex of creatine kinase (10). The thiocyanate ion gives a strong infrared absorption in the "triple bond" region of the spectrum due to the stretching vibration, υ₃, of the carbon-nitrogen bond (15). Fronaeus and Larsson (20) have made a thorough study of competition between SCN⁻ and metal ions of the first transition series taking advantage of the characteristic changes in the frequency of the C-N stretching mode which occur upon binding of SCN⁻ to the metal ions. The absorption for SCN⁻ in aqueous solution occurs at 2066 cm⁻¹, and the absorptions for the anion bound to Mn(II) (Mn(H₂O)₆NCSC⁻) and to Co(II) (Co(H₂O)₆NCSC⁻) are at 2093 cm⁻¹ and 2112 cm⁻¹, respectively (20). The complexes of SCN⁻ with Mn(II) and Co(II) are weak with dissociation constants of 230 mM and 110 mM, respectively (20), and high concentrations of SCN⁻ and metal ions are therefore required to obtain an appreciable extent of complex formation in free solution. The weak interaction of SCN⁻ with Mn(II) and Co(II) in free solution simplifies the interpretation of results for the enzymic complexes since there is a negligible amount of the simple metal ion SCN⁻ complex present at the concentration of metal ions and SCN⁻ used in the experiments with enzyme.

The infrared spectrum for solutions of KSCN and difference spectra for SCN⁻ obtained in the presence of enzyme, metal ions, and substrates are shown in Fig. 1. In a solution of KSCN, enzyme, MnCl₂, ADP, and creatine an absorption band appears at 2093 cm⁻¹ (the frequency where SCN⁻ absorbs when bound to Mn(II)) (20). For a similar solution with CoCl₂ replacing MnCl₂ the new band appears at 2109 cm⁻¹. Although the absorption maximum for the Co(II) enzymic complex is ~3 cm⁻¹ below that for the simple complex of Co(II) and SCN⁻, the slight shift is likely a perturbation due to the other ligands to the Co(II) (15). The close correspondence of the frequencies of the bands for the enzymic complexes with those for the respective metal SCN⁻ complexes and the dependence of the absorption frequency on the species of metal ion which is present make it likely that the shift in frequency for the enzyme-bound thiocyanate arises from a direct binding of SCN⁻ to the metal ion at the active site of the enzyme.

To confirm that the new absorption bands found in the presence of the enzymic complex with Mn(II) and SCN⁻ represents virtually all of the bound SCN⁻, longitudinal proton relaxation rate measurements were carried out at the temperature used for the infrared experiments to determine the dissociation constant for SCN⁻ from its complex with the
Infrared Studies of Anion Binding to Creatine Kinase

8
7
6
5
4
3
2
1
0

FIG. 2. Proton relaxation rate titration for dead-end complex containing Mn(II) with KSCN as titrant. \( r^* \) is the enhancement in the proton relaxation rate of water due to binding of Mn(II) to the enzyme substrate complex. The titration was performed at a resonance frequency of 24.3 MHz at 22°C. Concentrations of enzyme sites (500 µM), MnCl₂ (130 µM), ADP (630 µM), and creatine (50 mM) were constant throughout. The buffer was 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/K⁺ at pH 7.9.

The enzyme MnADP·creatine species (10). Data from a titration are shown in Fig. 2. An approximate dissociation constant of 7 mM is obtained from the half-saturation point in the titration. For the determination of a dissociation constant for SCN⁻ from the dead-end complex with Mn(II). From the infrared spectrum for the Mn(II) enzymic complex the ratio of free SCN⁻ to bound SCN⁻ estimated from the areas of the bands centered at 2066 cm⁻¹ and 2093 cm⁻¹ is 3.2. This value correlates favorably with a ratio of 3.5 calculated from the composition of the solution and the dissociation constants for SCN⁻ and Cl⁻.

The band widths at half-maximum, \( \Delta v_{1/2} \), of the absorption for free SCN⁻ and for the enzymic complex are approximately 38 cm⁻¹ and 19 cm⁻¹, respectively. Part of the reduction in band width for the enzymic complex is attributable to binding of SCN⁻ to the metal ion since Fronaeus and Larsson (20) report a \( \Delta v_{1/2} \) of 31 cm⁻¹ for the Mn(H₂O)₅NCS⁻ complex. The other factor which contributes to the reduction in band width for the enzyme-bound SCN⁻ is probably a change in the solvent environment of the anion (see below).

Binding of Azide to Creatine Kinase—Although it has not been reported previously, N₃⁻ binds to the dead-end complex with Mn(II) present and exerts a pronounced influence on the environment of Mn(II) as reflected by the EPR spectrum of Mn(II) and by the influence of Mn(II) on the T₁ relaxation rate of water protons. A proton relaxation rate titration gives a dissociation constant of -600 pM for N₃⁻ from its complex with enzyme. MnADP·creatine. Like the other anions in the series, there is no measurable effect of N₃⁻ on the enzyme-MnADP complex in the absence of creatine.

Azide exhibits a strong absorption in the triple bond region of the infrared spectrum. The frequency of this absorption changes in a characteristic manner upon binding of azide to

metal ions (15). The interaction between azide and Mn(II) is weak in aqueous solution, and high concentrations of the order of 0.1 to 1 M of metal ion and ligand are required to promote an appreciable extent of complexation. Infrared spectra for concentrated solutions of KN₃ and MnCl₂ at two different ratios of metal to ligand are shown in Fig. 3A. The free ion exhibits a maximum absorbance at 2045 cm⁻¹ whereas

...
N\textsuperscript{-} complexed to Mn(II) absorbs at 2074 cm\textsuperscript{-1}. Incomplete resolution of the bands for free and bound azide complicates a quantitative evaluation of the dissociation constant for the complex. However, an approximate value of 0.1 M is obtained from the relative absorbances at 2045 cm\textsuperscript{-1} and 2074 cm\textsuperscript{-1} at various ratios of metal ion to ligand.

Spectra for azide in the presence of creatine kinase, MnADP\textsuperscript{-}, and creatine are shown in Fig. 3B. The band observed for the enzyme-bound anion appears at 2074 cm\textsuperscript{-1} which is the position of the absorption for N\textsuperscript{-} when bound to Mn(II).

The Co(II) complex with azide in free solution also gives an absorption maximum at 2074 cm\textsuperscript{-1}. However, experiments with the Co(II) enzymatic complex with anion, CoADP\textsuperscript{-}, and enzyme sites at equimolar concentration show only the band characteristic of free azide. Since the affinity of azide for the Co(II) complex with the enzyme is not known, the infrared results suggest a weaker affinity of azide for the Co(II) complex with the enzyme than for the corresponding Mn(II) species.

**Binding of Nitrate to Creatine Kinase**—As might be expected from its composition and geometry, nitrate is one of the more potent of the class of inhibitory anions (8, 9). A proton relaxation rate titration gives a dissociation constant for nitrate from its complex with enzyme MnADP\textsuperscript{-} creatine of 130 \textsuperscript{-1}. Upon binding to a metal nitrate salt, the DH\textsuperscript{h} symmetry of the free ion is lowered to C\textsubscript{2v} or C\textsubscript{1}, and the degeneracy of the mode is lifted.\textsuperscript{7} Hence one expects a splitting of the v\textsubscript{3} absorption when nitrate binds to a metal ion (15), and splitting has been observed in solid state infrared measurements of metal complexes which contain coordinated nitrate (22).

The v\textsubscript{3} absorption of nitrate occurs in a region of the spectrum where the background absorbance from water is considerable but manageable. However, various functional groups from the protein and substrates (notably carboxylate groups) also give absorptions in this region. Infrared difference spectra for nitrate in solutions of creatine kinase, ADP, and creatine and Mg(II) and Mn(II) and without divalent cations are shown in Fig. 4. A number of prominent, sharp bands appear in the difference spectra upon addition of metal ions to the sample solution. In order to identify those absorptions which are due to the bound nitrate, similar measurements were made with \textsuperscript{15}NO\textsubscript{3}\textsuperscript{-}, where the heavy isotope substitution produces a regular shift to lower frequency of the absorptions which belong to nitrate. Spectra for the \textsuperscript{15}NO\textsubscript{3}\textsuperscript{-} complexes are compared with those obtained for \textsuperscript{14}NO\textsubscript{3}\textsuperscript{-} in Fig. 4. The cross-hatching identifies the location of peaks which are sensitive to the isotopic substitution. There is a significant change in the pattern of the difference spectra for the enzymatic complexes upon substitution of \textsuperscript{15}NO\textsubscript{3}\textsuperscript{-} for \textsuperscript{14}NO\textsubscript{3}\textsuperscript{-}, although the presence of other bands which appear to be sensitive to the presence of the metal ion leaves some doubt as to the proper identification of the bands due to enzyme-bound nitrate.

To resolve the ambiguities in assigning the bands for the enzyme-bound nitrate it was desirable to know the characteristic frequencies of nitrate absorption when the ion is liganded to Mg(II) or Mn(II). Unfortunately, the affinity of nitrate for these metal ions is very weak in aqueous media. Although one might enhance the affinity of the metal ions for nitrate in a solvent of lower dielectric constant than water, virtually all of the solvents in which the metal salts are soluble have interfering absorptions in the region of interest. An appreciable extent of complex formation does occur in highly concentrated aqueous solutions of the metal nitrate salts. Because of the high absorbance of nitrate in such solutions, it was convenient to obtain the vibrational spectra by Raman spectroscopy. The results of these experiments are summarized in Fig. 5, where Raman bands for aqueous solutions of KNO\textsubscript{3} (\textsuperscript{14}N and \textsuperscript{15}N) are compared with those for concentrated solutions of the metal nitrate salts. The dashed vertical lines in Fig. 5 locate the frequencies of peak intensity for the cross-hatched bands in the infrared spectra for the respective enzymic complexes (see Fig. 4). The coincidence of the bands for the metal nitrate complexes in free solution with the isotopically shifted bands for the enzymic complexes indicates that nitrate is bound to the divalent cations at the active site of the enzyme. There is a slight but noticeable difference in the magnitude of the splitting for the v\textsubscript{3} band of nitrate for the complexes of Mn(II) and Mg(II) in free solution and at the active site of the enzyme. The variation in the splitting with species of metal ion is further evidence that the splitting for the enzymic complexes is due to coordination between nitrate and the metal ions at the active site.

Infrared results for Co(II) enzymic complexes with nitrate are similar to those shown for Mg(II) and Mn(II). However, Co(II) perturbs an enzymic chromophore, possibly a carbox-

---

\textsuperscript{7} It appears that the degeneracy of the v\textsubscript{3} mode is partly lifted with solution of the nitrate anion in water as the absorption band for this mode is broad and asymmetric. A mirror image asymmetry for this band is observed in Raman spectra for aqueous solutions of nitrate (21).
The linear metal-anion linkage in the enzymic complex of SCN may be partly responsible for the nearly native-like EPR spectrum which is obtained for the Mn(II) complex with the \(-\text{SCH}_2\) modified form of creatine kinase (25). In this modified enzyme all of the anions bind to the dead-end complex, but only SCN gives a response in the EPR spectrum of Mn(II) which is similar to that obtained for the native enzyme (25).

**Other Binding Determinants for the Anions**—The anions are known to bind to the enzyme-ADP-creatine complex in the absence of divalent metal ions, albeit with a considerably diminished affinity (8, 13). Hence, the metal ion is not the sole determinant of anion interaction at the active site. For example, the nuclear Overhauser effects observed for the formate complex of creatine kinase indicate the proximity of an e-amino group from a lysine residue to the formate (26).

The planar nitrate anion bears a greater resemblance to the planar PO$_4$ part of the trigonal bipyramid formed by the phosphoryl moiety and its entering and leaving groups in the transition state of the reaction (8). The higher affinity of nitrate for the enzymic complex probably reflects the ability of this anion to interact favorably with most of the groups which stabilize the transition state of the phosphoryl transfer event. Hence, although nitrate has the weakest interaction with the metal ions in free solution, this deficiency is compensated through stabilizing interactions with the additional binding determinants at the active site.

The reduced band widths in the infrared absorptions for the enzymic complexes of the anions indicate that the anions are at least partially screened from the solvent in the enzyme-bound state. Polar solvents broaden infrared absorptions through dipole-dipole interactions with the chromophore which preferentially stabilize the excited vibrational state (27); the randomness of such solvent-solute interactions produces a spread of absorption frequencies. NMR and EPR data (9, 10) indicate that the divalent cation, Mn(II), is not accessible to the bulk solvent in the anion complexes with the enzyme. Most of the inhibitory anions are lipophilic (I), and a region of lowered dielectric constant at the active site would also promote anion binding to the metal ions.

**Mechanistic Implications**—The infrared data for the thio-cyanate, azide, and nitrate complexes with the dead-end complex of creatine kinase show that the complex promotes a binding of these anions directly to the divalent cation despite the intrinsically weak affinities of these anions for the metal ions in free solution. This feature of the active site would also strongly favor a binding of the transferable phosphoryl group to the divalent cation in the transition state of the reaction. The metaphosphate anion, which would be a plausible intermediate if the phosphoryl transfer proceeded by a dissociative (S$_{61}$) pathway, is an electron-deficient species (I). Coordination of metaphosphate to the metal ion would not be expected to stabilize this already electron-deficient species. Hence, binding of the metal ion to the transferable phosphoryl group would favor an in-line displacement or S$_{62}$ pathway for this phosphoryl transfer where the metal ion would be attached to one of the in-plane oxygens of the trigonal bipyramid in the transition state (see Fig. 6). Following the arguments summarized by Cooperman (4), this coordination scheme could promote catalysis in the forward and reverse directions through charge shielding, polarization, and template effects. An examination of molecular models indicates that for the structure given in Fig. 6, the template effect of the metal ion would be especially relevant to catalysis. In such a model the oxygen atom which forms the bridge between the $\beta$ and $\gamma$ phosphorus atoms (i.e., the leaving group atom in phosphocreatine formation and the attacking nucleophile in ATP formation) should be most favorably oriented to enhance the reaction rate.
formation) moves in-line toward and away from the phosphorous atom of the transferable phosphoryl group when the chelate ring formed by the metal ion and the α and β phosphoryl groups undergoes a puckering motion.

Equilibrium binding studies have shown that the divalent cation binds to the enzyme with the nucleotide substrate (28). Thus prior to or coincident with the release of either MADP- or phosphocreatine in the forward direction, coordination between the metal ion and the phosphoryl group of phosphocreatine would be broken.

The present model for the transition state of the creatine kinase reaction differs from that proposed earlier (9, 14) in that the migrating phosphoryl group is bound to the metal ion. Previous EPR studies with Mn(II) complexes of the creatine kinase reaction differs from that proposed earlier (9, 14) in

REFERENCES


Acknowledgments—We are grateful to Drs. George D. Markham and Frances Adar for helpful discussions. We also thank Dr. Adar for assistance with the isotope measurements and Dr. S. Walter Engleman for providing access to the infrared spectrophotometer.

Fig. 6. Schematic drawing of the activated complex of creatine kinase.
Investigations of anion binding sites in transition state analogue complexes of creatine kinase by infrared spectroscopy.
G H Reed, C H Barlow and R A Burns, Jr