Circular Dichroism of Cobaltous Complexes of Creatine Kinase†

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ABSTRACT: Circular dichroic spectra in the visible wavelength region were used to investigate the local environment of Co(II), an activating metal, at the active site of rabbit muscle creatine kinase. There was a small spectral change when $CoADP^-$ was bound to creatine kinase, no change when creatine was added, and another small change when NO_3^- was added to form the transition state analogue. Using matrix rank analysis to quantitate these small spectral changes, a titratable group with a $pK = 7.4$ was found which modified the enzyme-bound metal-nucleotide interaction. These data suggest that, throughout the enzyme's catalysis, the metal-nucleotide interaction remains very similar in structure to the complex not bound to the enzyme.

Creatine kinase (ATP:creatine *N*-phosphotransferase, EC 2.7.3.2) is one of the most thoroughly studied metal-requiring enzymes. It is a clear example of a system in which the M^{2+}-nucleotide complex is a substrate and direct interactions between the divalent cation and the enzyme are minimal (Watts, 1973). In studying the environment of the activating metal and its role in catalysis, the experimenter can make measurements on the M^{2+}-ADP complex bound to the enzyme, the quaternary complex including creatine, and finally the transition state analogue where anions (e.g., nitrate) mimic the phosphoryl group being transferred (Milner-White and Watts, 1971).

Most of the information about the interactions of the activating metal at the active site of creatine kinase has come from work in which Mn(II) was used as an active site probe in either PRR[1] or EPR studies (Cohn and Reuben, 1971; Reed and Cohn, 1972). Unfortunately, the interpretation of PRR or EPR experiments is frequently ambiguous because there are contributions from both static (i.e., average conformation) and dynamic (i.e., exchange or collision) effects which may not be separable.

Use of transition metals as optical probes is an alternative approach to studying metal interactions at the active sites of the enzymes. In particular, CD studies of Co(II) complexes with alkaline phosphatase (Taylor et al., 1973) and pyruvate kinase (Kwan et al., 1975) have demonstrated the sensitivity of the technique. Although quantitative interpretation of these data is much more difficult to make as compared with PRR or EPR, they do have the advantage that (since they are manifestations of electronic transitions) they are essentially independent of the dynamic effects which contribute to magnetic resonance properties. For these reasons, we have measured and interpreted the CD spectra of $CoADP^-$ complexes with rabbit muscle creatine kinase.

Experimental Procedure

Creatine kinase was isolated from frozen rabbit muscle using method B of Kuby et al. (1954). Creatine kinase used for spectroscopic studies had 55 to 65 units/mg at 25 °C using the coupled pyruvate kinase, lactate dehydrogenase assay (Tanzer and Gilvarg, 1959). Protein concentration was measured using $\epsilon_{280} = 0.87$ mL mg^{-1} cm^{-1} (Noda et al., 1954) and molar protein concentrations expressed in terms of binding sites were based on a subunit molecular weight of 41300 (Hooten, 1968).

The following reagents were obtained from Sigma: lactate dehydrogenase, creatine·H_2O, the tricyclohexylammonium salt of phosphoenolpyrovate, and the sodium salts of βNADH, ADP, and ATP. Rabbit muscle pyruvate kinase was a gift of Dr. Chiu-Yin Kwan. All other chemicals were reagent grade.

CD spectra were taken on a Durrum Jasco J-10 spectropolarimeter which was thermostated and calibrated as described before (Studdert et al., 1972). Spectra slit widths were always less than 3 nm. Absorption spectra and assays were performed on a Cary 15 spectrophotometer. CD measurements were made on approximately 1 mM creatine kinase solutions. If the solution was cloudy, it was centrifuged to clarify it before spectroscopic measurements were taken. CD samples were thermostated at 5 °C to minimize problems of enzyme denaturation and Co(II) oxidation. $CoADP^-$ stock solution (188 mM Na_2ADP and 282 mM $CoSO_4$) was made daily by dissolving solids in the appropriate buffer. A 5-μL aliquot of this stock solution in a total volume of 4.7 mL (5-cm cuvette) resulted in a final concentration of 0.2 mM ADP and 0.3 mM Co(II). Creatine was added as a solid directly into the cuvette. The Li^+ salt of NO_3^- was added from 5 M stock solutions.

Oxidation of Co(II) to Co(III) in the presence of air was a potential problem. At pH 8, solutions of creatine kinase plus $CoADP^-$ became visibly brownish overnight, indicating Co(III) formation. At pH 9, the solution turned brown in only 30 min. Since it required about 30 min to record a CD spectrum, precautions were taken to exclude oxygen from samples at pH $\geq$8. N_2 gas, scrubbed by a V(II) solution–zinc amalgam mixture, was bubbled through buffer solutions to remove dissolved O_2. These buffers were used to dissolve $CoSO_4$, ADP, and lyophilized creatine kinase inside a glove bag with a N_2 atmosphere. CD cuvettes were filled and stoppered before removal from the bag. Taking these precautions, we found no evidence of Co(III) formation during the measurements reported here.

Results

The visible absorption spectra of the Co(II) complex of ADP, either bound to creatine kinase or free in solution, is weak

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[1] Abbreviations used: PRR, proton relaxation rate; EPR, electron paramagnetic resonance; CD, circular dichroism.

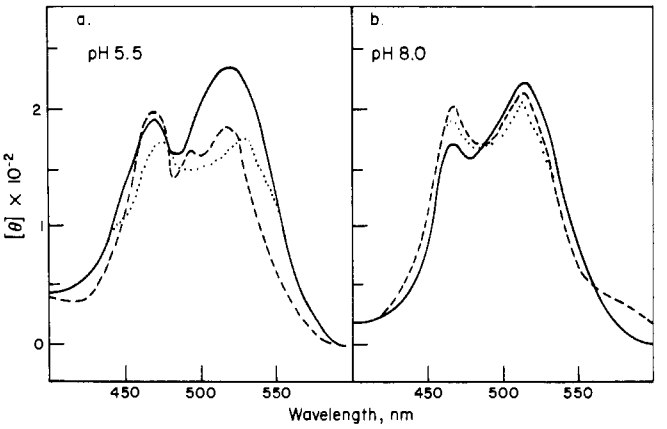

FIGURE 1: CD spectra of $CoADP^-$ and its complexes with creatine kinases at 5 °C. The solvents are (a) 50 mM K^+ acetate buffer, pH 5.5; and (b) 100 mM Tris-HCl, pH 8.0. [θ] is calculated on the basis of ADP concentration. $CoSO_4$, 3 mM; ADP, 2 mM (—). This spectrum is unchanged by adding 20 mM creatine and/or 8 mM $LiNO_3$. The previous spectrum plus 0.5 mM creatine kinase (- - -). This spectrum is unchanged by adding 20 mM creatine. The previous spectrum plus 20 mM creatine and 8 mM $LiNO_3$ (·····).

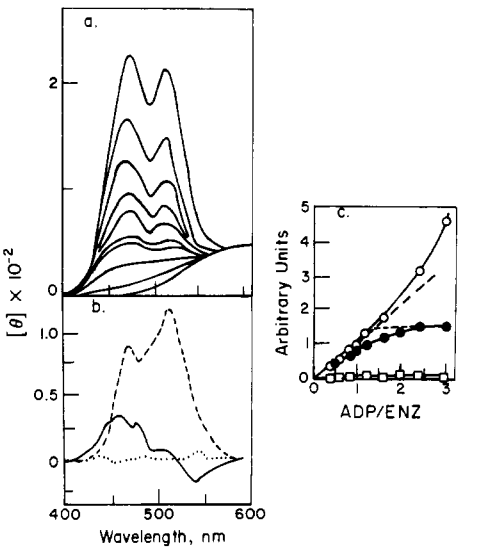

FIGURE 2: $CoADP^-$ titration of creatine kinase, 100 mM Tris-HCl, pH 8.0, 5 °C. [θ] is calculated on the basis of creatine kinase concentration. Co(II)/ADP ratio is 1.5. (a) CD spectra creatine kinase 1.0 mM spectra of increasing magnitude have ADP concentrations of 0, 0.4, 0.6, 0.8, 1.0, 1.2, 1.6, 2.0, 2.4, and 3.0 mM. (b) Basis spectra from matrix rank analysis. A_1 (- - -); A_2 (—); A_3 (····). (c) Coefficients a_1 (○), a_2 (●), and a_3 (□) a function of ADP/creatine kinase.

and difficult to measure accurately. Both spectra have maxima near 500 nm with $\epsilon_{max} \sim 15\ M^{-1}\ cm^{-1}$. On the other hand, CD spectra of these complexes can be measured rather accurately without much difficulty. Visible CD spectra of creatine kinase are shown at pH 5.5 and pH 8.0 in the presence of Co(II), ADP, creatine, and the stabilizing anion, NO_3^- (Figure 1). At each pH value, the addition of Co(II) to creatine kinase resulted in a CD spectrum identical to the metal-free apoenzyme (i.e., baseline). A CD spectrum different from that of the apoenzyme was the result of the addition of 2 mM ADP to the solution of creatine kinase plus Co(II) (3 mM). This spectrum was similar to that of free $CoADP^-$ in buffer indicating that the environment of the metal in the $CoADP^-$ complex was not greatly changed by the presence of the enzyme. The addition of 20 mM creatine to the solution of creatine kinase + $CoADP^-$ (at either pH) resulted in no change in the CD spectrum. However, the further addition of 8 mM $LiNO_3$ resulted in a spectrum very similar in shape, but with a slight decrease in intensity, to the spectrum in the absence of NO_3^- ions. This slight decrease in spectral intensity was not seen in the control experiment (i.e., adding NO_3^- to a solution containing Co(II), ADP, and creatine in the absence of enzyme) and was attributed to the formation of the transition state analogue, creatine kinase-$CoADP^-$-NO_3^--creatine.

In order to clearly define the distortion of the free $CoADP^-$ spectrum caused by the presence of the enzyme and other species binding at the active site, a matrix rank analysis procedure was used (McMullen at al., 1967). Each experimental spectrum was considered to be a row vector consisting of observed ellipticity values at various wavelengths, **S**, with a constant wavelength interval between readings. An entire series of spectra (e.g., creatine kinase plus increasing quantities of $CoADP^-$) forms a matrix, with each row of the matrix representing a spectrum. Each row vector or spectrum, **S** is considered to be a linear combination of component spectra or basis vectors, $\mathbf{A}_1, \mathbf{A}_2, \ldots \mathbf{A}_n$; that is:

$$\mathbf{S}_i = a_{i1}\mathbf{A}_1 + a_{i2}\mathbf{A}_2 + \ldots + a_{in}\mathbf{A}_n$$

Spectral components with the same shape and varying intensities will not all be members of the basis; only one representative component will appear as a member of the basis set. In this way, the number of species contributing spectra with different shapes (i.e., linearly independent) can be defined.

$\mathbf{A}_1$ was the most intense spectrum and was chosen to correspond to $CoADP^-$ in the absence of creatine kinase. The matrix of spectra to be analyzed correspond to a titration of creatine kinase with increasing concentrations of $CoADP^-$ under one of a variety of conditions. The second component of the basis coming from the matrix rank analysis, $\mathbf{A}_2$, corresponded to the perturbation of the $CoADP^-$ visible CD spectrum caused by binding to creatine kinase. In all cases, the third component, $\mathbf{A}_3$, was small, irreproducible, and corresponded to noise.

Figure 2 shows the analysis of the CD titration of creatine kinase with $CoADP^-$. The first component of the basis set corresponds to the $CoADP^-$ CD spectrum. Its coefficient, a_1, increases approximately linearly with increasing $CoADP^-$ concentration. The coefficient of the second basis spectrum, a_2, levels off at high ADP/creatine kinase ratios indicating the enzyme has become saturated. The stoichiometry of ADP binding to each subunit of creatine kinase, 1.3 ± 0.2, is defined by the intersection of the tangents to the curve at total ADP/creatine kinase equal to zero and infinity, respectively. The number is more than 1.0 because the coordinate is defined in terms of the concentration of ADP and (in spite of the 50% excess of Co(II) over ADP) not all ADP is complexed with Co(II) (Khan and Martell, 1967). Therefore, free ADP is competing with $CoADP^-$ for the enzyme, but not giving a visible CD spectrum. As the Co(II) and ADP concentrations are increased, the fraction of free ADP decreases and the enzyme finally does become saturated with CoADP.

Creatine kinase was then titrated with $CoADP^-$ at pH 5.5 where the reverse reaction was 100% active. As was the case of pH 8, only two components, $\mathbf{A}_1$ and $\mathbf{A}_2$ (those above noise level), contributed to the forming of the observed spectra (Figure 3). The break in the graph of a_2 vs. [ADP]/[creatine kinase] was at 1.5. This was due to the fact that ADP^{3-} is protonated ($pK = 6.4$) preferentially to $CoADP^-$ ($pK = 4.2$) (Khan and Martell, 1962, 1967), thereby decreasing the fraction of ADP complexed to Co(II).

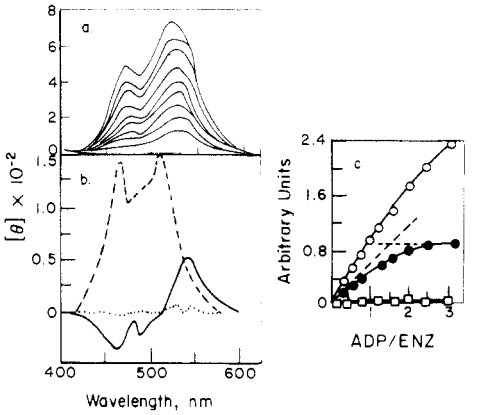

FIGURE 3: $CoADP^-$ titration of creatine kinase, 50 mM acetic and KOH, pH 5.5, 5 °C. [θ] is based on creatine kinase concentration. Co/ADP ratio is 1.5. (a) CD spectra, creatine kinase 1.0 mM. Spectra of increasing magnitude have ADP concentrations of 0, 0.4, 0.6, 0.8, 1.0, 1.2, 1.5, 1.8, 2.4, 3.0. 3.6, and 4.5 mM. (b) Basis spectra from matrix rank analysis A_1 (- - -); A_2 (—); A_3 (· · · ·). (c) Coefficients a_1 (○), a_2 (●), and a_3 (□) as a function of ADP/creatine kinase.

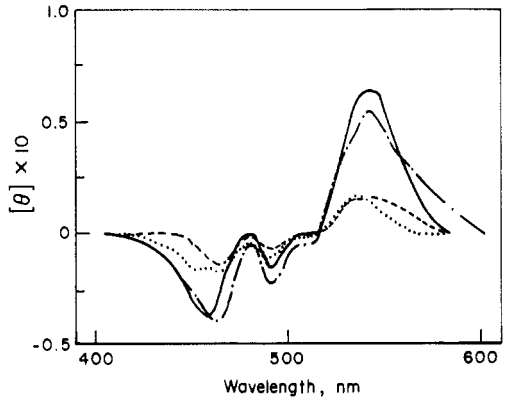

FIGURE 4: Comparison of the A_2 components for $CoADP^-$ titrations of creatine kinase 50 mM acetic acid-KOH, pH 5.5, 5 °C. Creatine kinase (1 mM) (— · —); creatine kinase (1 mM) and creatine (10 mM) (—); creatine kinase (1 mM), creatine (10 mM), and $LiNO_3$ (10 mM) (- - -); creatine kinase (1 mM), creatine (10 mM), and LiHCOO (10 mM) (· · · · ·).

The A_2 components, representing the distortion of the free $CoADP^-$ spectrum due to the enzyme, differed at pH 8 and pH 5.5. At pH 8, a positive peak at 460 nm and a negative peak at 540 nm appeared, while, at pH 5.5, a negative peak at 460 nm and a positive peak at 545 nm were observed. Although one component spectrum seemed to be the mirror image of the other, the A_2 component at pH 5.5 was twice as intense as the pH 8 A_2 component.

The ternary complex, creatine kinase-creatine-$CoADP^-$, was studied by titrating creatine kinase and 10 mM creatine with increasing amounts of $CoADP^-$ at pH 5.5. The A_2 component derived from the titration in the absence of creatine (Figure 4) was observed.

The conformation of $CoADP^-$ in the transition state analogue was examined by titrating creatine kinase, 10 mM creatine, and 10 mM NO_3^- with increasing concentrations of $CoADP^-$. The A_2 component observed upon the addition of NO_3^- to the abortive complex was similar in shape but less intense than the A_2 component in the absence of the stabilizing anion (Figure 4). The same spectral component was observed when the formate anion was used to stabilize the abortive complex and form the transition state analogue. Stoichiometry for these complexes was 1.0 nucleotide/subunit.

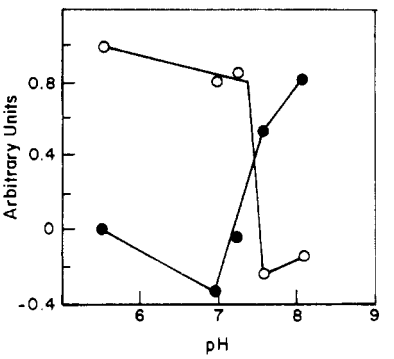

FIGURE 5: pH dependence of the coefficients produced by matrix rank analysis of the normalized spectra A_2'. (○) Low pH form; (●) second coefficient.

Additionally, we analyzed the pH dependent CD titrations of $CoADP^-$ onto creatine kinase-NO_3^--creatine to form the dead end complex which is a transition state analogue. In all experiments, only two components above noise level were observed, A_1 (free $CoADP^-$) and A_2 (the distortion on binding). In order to compare the change in shape of A_2 and quantitate the pH change, we first normalized each A_2 spectrum

$$A_2' = A_2/(A_2 \cdot A_2)^{1/2}$$

and then used these A_2' spectra (each at a diffirent pH) to carry out another matrix rank analysis. The set of A_2' spectra could be fit by a linear combination of the high and low pH forms, with a pK_a of the titratable group being equal to 7.4 (Figure 5). The smooth titration curve reflects the reliability of the measurement and analysis. Independent measurements at different pHs generated A_2 spectra which were reproducible and changed smoothly with the titration of the ionizable group, showing the reproducibility of our data.

Discussion

The visible absorption and CD spectra of $CoADP^-$ bound to creatine kinase or free in solution are not unusual for six coordinate Co(II). The absorption and CD near 500 nm with $\epsilon \sim 15\ M^{-1}\ cm^{-1}$ are typical for $^4T_{1g} \rightarrow {}^4T_{1g}(P)$ (plus possibly $^4T_{1g} \rightarrow 4T_{2g}$) transitions of octahedral Co(II) (Lever, 1968) as is the asymmetry factor, $g = \Delta\epsilon/\epsilon$ of ~0.01 (Michailidis and Martin, 1969).

In order to extract more information about changes in the local environment of Co(II) bound to ADP at the active site of creatine kinase, the small changes in the visible CD spectra must be considered. Since these changes are small, it is important that artifacts such as creatine kinase-Co(II) complexes, Co(II)-ADP complexes with stoichiometries other than 1:1, and Co(III) complexes be ruled out. Fortunately, the matrix rank analysis enables us to do exactly that. The fact that we always found only two components of the basis set above noise level and that these components changed in a reasonable manner as a function of pH exclude the possibility that more than two different spectral components were being observed. Since these two species are $CoADP^-$ on and off creatine kinase, we can rule out artifactual contributions from other Co(II) or Co(III) complexes. This is also reflected in the uncomplicated titration as monitored by the pH dependence of A_2' spectra in the presence of creatine and NO_3^-.

Changes in visible CD spectra can be summarized by considering three equilibria:

$$E + CoADP^- \rightleftarrows E\text{-}CoADP^- \quad (A)$$

$$E\text{-}CoADP^- + \text{creatine} \rightleftarrows E\text{-}CoADP^-\text{-creatine} \quad (B)$$

$$E\text{-}CoADP^{-}\text{-creatine} + NO_3^{-} \rightleftarrows E\text{-}CoADP^{-}\text{-}NO_3^{-}\text{-creatine} \quad (C)$$

The CD spectra associated with Co(II) (and therefore, its immediate environment) change slightly in reactions A and C but not observably in reaction B. These results complement PRR and EPR studies and help remove some of the ambiguities of their interpretations. The fact that the EPR spectrum of $MnADP^{-}$ is essentially unchanged on binding the nucleotide to creatine kinase (the analogue to reaction A) shows that there is no significant change in the immediate environment of the Mn(II) (Reed and Cohn, 1972). Our experiments have shown a small change in the CD of $CoADP^{-}$, demonstrating some distortion in its ligation. However, this is a very small change compared with substrate-induced CD changes of Co(II)-enzyme complexes where there is evidence for substantial reorganization at the active site (Taylor et al., 1973; Kwan et al., 1975). For equilibrium B, magnetic resonance studies show that there are major changes at the active site which include an immobilization of substrates, a reduction of water exchange between active site and bulk solvent, and possibly a change in the average conformation of the activating metal (Reed and Cohn, 1972; Markam et al., 1977). Because PRR and EPR spectra depend on both dynamic and time averaged conformational factors, it was impossible to establish changes in the average environment of activating divalent metal ion. The lack of observable change in the CD spectrum of creatine kinase-$CoADP^{-}$ on binding creatine strongly suggests that there is no meaningful change in the equilibrium local environment of the activating metal for B. The change taking place seems to involve shutting off the active site from the bulk solvent without important perturbation of the Co-ADP^{-} conformation. Further changes in EPR and PRR are recorded for the Mn(II) analogue to reaction C which was interpreted as a probable incorporation of ligands from the protein in the first coordination sphere of the Mn(II) (Reed and Cohn, 1972). Although we found changes in CD spectrum of $CoADP^{-}$ in reaction C, they were very small, comparable to changes on binding $CoADP^{-}$ to creatine kinase. If there is a ligand substitution in the first coordination sphere of Co(II), it is one which barely changes its ligand field.

The fact that the $pK = 7.4$ of the ionizable group, found in the quantitation of the pH dependence of the visible CD spectrum of $CoADP^{-}$ with creatine kinase, creatine, and NO_3^{-}, does *not* correspond to a feature of the pH-rate profile of creatine kinase (Noda et al., 1960) suggests that this conformational change is not related to the enzyme's catalysis. Since the change in CD spectra associated with this pH dependence is comparable to the other minor spectral changes observed, they may very well all not be associated with catalytically important events. The important point is that the metal-nucleotide complex throughout the enzyme's catalysis probably remains very similar in structure to the complex not bound to the enzyme.

References

Cohn, M., and Reuben, J. (1971), *Acc. Chem. Res. 4,* 214.

Hooten, B. T. (1968), *Biochemistry 7,* 2063.

Khan, M. M. T., and Martel, A. E. (1962), *J. Am. Chem. Soc. 84,* 3037.

Khan, M. M. T., and Martel, A. E. (1967), *J. Am. Chem. Soc. 89,* 5585.

Kuby, S. A., Noda, L., and Lardy, H. A. (1954), *J. Biol. Chem. 209,* 191.

Kwan, C. Y., Erhard, K., and Davis, R. C. (1975), *J. Biol. Chem. 250,* 5951.

Lever, A. B. P. (1968), Inorganic Electronic Spectroscopy, New York, N.Y., Elsevier.

McMullen, D. W., Jaskunas, S. R., and Tinoco, I., Jr. (1967), *Biopolymers 5,* 589.

Markham, G. D., Reed, G. H., Maggio, E. T., and Kenyon, G. L. (1977), *J. Biol. Chem. 252,* 1197.

Michailidis, M. S., and Martin, R. B. (1969), *J. Am. Chem. Soc. 91,* 4683.

Milner-White, E. J., and Watts, D. C. (1971), *Biochem. J. 122,* 727.

Noda, L., Kuby, S. A., and Lardy, H. A. (1954), *J. Biol. Chem. 209,* 203.

Noda, L., Nihei, T., and Morales, M. (1960), *J. Biol. Chem. 235,* 2830.

Reed G. H., and Cohn, M. (1972), *J. Biol. Chem. 247,* 3073.

Studdert, D. S., Patroni, M., and Davis, R. C. (1972), *Biopolymers 11,* 761.

Tanzer, M. L., and Gilvarg, C. (1959), *J. Biol. Chem. 234,* 3201.

Taylor, J. S., Lau, C. Y., Applebury, M., and Coleman, J. E. (1973), *J. Biol. Chem. 248,* 6216.

Watts, D. C. (1973), *Enzymes, 3rd Ed. 8,* 383.