Evidence for Conformers of Rabbit Muscle Adenylate Kinase*

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Changes of the apparent $M_r$ values and the circular dichroism patterns suggest the existence of three relatively stable conformers of rabbit muscle adenylate kinase (RMAK). The effects of dithiothreitol (DTT) which stimulates activity, pH, the substrates, and ATP on the $M_r$ value and the Stokes radius of RMAK were determined from gel filtration data, and apparent $M_r$ values near 22,000, 26,000, and 29,000 resulted. Substrates generated multiple $M_r$ values, suggesting the presence of multiple conformers of RMAK. The higher apparent $M_r$ values were obtained in the presence of DTT and at the higher substrate concentrations, indicating more open conformations. The effect of the substrates on the conformation of RMAK is discussed in relation to the kinetic mechanism of this random bi-reactant system.

Circular dichroism studies were undertaken in order to observe any changes in the secondary structures of RMAK in relation to changes of the $M_r$ values. The secondary structure composition of RMAK, determined under our conditions, does not agree with results determined from crystallographic studies. The gel filtration and the CD studies suggest that above pH 7 a more open conformation of RMAK obtains in the presence of DTT. The results of these studies are discussed with reference to the location of the active sites.

These studies derive from observations that crystalline porcine skeletal muscle AK' exists in more than one pH-dependent conformation (1, 2), our observation (3) that a single AK isozyme from cat heart showed multiple $M_r$ values by gel filtration (4), and that multiple $M_r$ values of AK were reported in the urine of patients with myocardial infarctions (5). The reported variations of conformations from chemical modifications of bovine serum albumin (6), detected as altered $M_r$ values, and Stokes radii, determined by gel filtration techniques, serve as a basis for these studies. The skeletal muscle AK isozymes are bilobe molecules consisting of two globular units joined by an unordered region forming a cleft (1, 2, 7-10). We studied the effect of pH, substrates, and DTT on the $M_r$ values of RMAK using Sephadex G-75 (4) and observed alterations of $M_r$ values and the estimated Stokes radii that suggest changing conformations. The detection of RMAK conformers in solution supports the view that changes in conformations observed in AK crystals due to pH are an inherent property (2).

CD studies also revealed alterations in the conformations of AK with changes of the pH and in the presence of DTT. In the absence of DTT and with changes in pH, there are very small changes in the $\alpha$-helix and the $\beta$-pleated sheet regions while the unordered region shows no variations. In the presence of DTT and with changes in the pH, there are increases in the $\alpha$-helix regions and concomitant decreases in the $\beta$-pleated sheet regions while the unordered region remains essentially constant. The more open configurations of RMAK obtain in the presence of DTT. The effect of the substrates on the apparent $M_r$ values suggest the promotion of open and closed conformations, depending upon conditions. Discussion is presented related to these observations and the enzymatic mechanism.

EXPERIMENTAL PROCEDURES

Materials—RMAK is the commercial preparation from Sigma, designated "myokinase, grade III from rabbit muscle." When subjected to isoelectric focusing, the RMAK showed a single isozyme with an isoelectric point (pI) value near 9 and gave a single protein band by acrylamide electrophoresis with the silver staining method (11). We used the commercial RMAK without further purification. The specific activity was in the range of 2000 enzyme units/mg under the conditions of our assay. The activity of RMAK in the presence of 1 mM DTT was 50-100% higher (12). Sephadex G-75 was obtained from Pharmacia LKB Biotechnology Inc.

AK Assay—We measured AK activity, AMP + ATP + Mg = ADP + ADP + Mg, according to Adam (13), and as modified elsewhere (14). The 1-nl assay mixture was 20 mM potassium phosphate buffer, pH 7.0 buffer, 0.3 mM phosphoenolpyruvate, 0.4 mM NADH, 8.0 mM of AMP and ATP each, 8.0 mM MgCl$_2$, and sufficient amounts of lactate dehydrogenase (EC 1.1.1.27) and pyruvate kinase (EC 2.7.1.40) so that the coupling system was not rate-limiting. Reactions were initiated by the addition of AK. All initial reaction rates were determined by measuring the decreased absorbance of NADH at 340 nm with time. The molar absorbitivity value of 6220 was used to convert the change of NADH absorbance to micromoles of product formed. One enzyme unit of activity is equivalent to the formation of 1 mol of ADP/min at 25 °C under the specified assay conditions.

Calibration of the Sephadex G-75 Column—The void volume of the column, $V_v = 56.0$ ml, was determined by using blue dextran 2000 from Pharmacia for each set of conditions and was found not to vary. The volume of unbound solute internal to the gel phase, $V_i = 87.8$ ml, was determined from $(V_T - V_v) = 0.95$ (6, 15), where $V_T = 143.8$ ml, the total volume of the Sephadex G-75 column. Using the distribution coefficient, $K_D$ as defined by Ackers (16), of ovalbumin as the standard, the pore radius for Sephadex G-75 determined by Equation 9 and Table III (16) was similar at all pH values tested and averaged $10.1 \pm 0.5$ nm. Based on the estimated pore radius of Sephadex G-75, the Stokes radii of the RMAK conformers were also determined by the method of Ackers (16). In order to determine the $K_D$ versus log molecular weight relationship (4), the molecular mass standards (bovine serum albumin, 45.0 kDa; cytochrome c, 12.5 kDa, from Sigma) were used to calibrate the column prior to and following each pH value set tested. The $K_D$ values of the molecular mass standards did not vary significantly over the pH range 4.5-9.0.
were carried out at 25 °C. The estimations of the M, value by gel filtration were similar to the method of Andrews (4), using a 0.8 × 73-cm column of Sephadex G-75, previously equilibrated for 24 h with 100 mM potassium phosphate buffers and 0.02% sodium azide. The flow rates were standardized to 22-24 ml/h, and 0.9-ml fractions were collected. All Kd value determinations of RMAK were performed in the absence of the molecular weight standards and with a 1.0 ml sample load of 10 μg/ml RMAK in order to avoid the possibility of polymer formation (17) or spurious protein-protein interactions. A minimum set of three separate M, value determinations of RMAK as routinely performed for each given condition. Recoveries of RMAK activities below 75% were not considered acceptable.

The Effect of pH and 1 mM DTT on Apparent M, Values and Stokes Radii of RMAK—The molecular mass of RMAK, based on amino acid analysis, is 21,600 Da (21). We observed that the M, values of the single peaks of RMAK, determined by gel filtration, vary with pH and with the presence of 1.0 mM DTT, as shown in Fig. 1. At the pH values between 6.0 and 7.0, the apparent M, values are near 26,000; at pH 5.0 and between 7.5 and 9.0, M, values near 22,000 obtain. We believe that these changes of the apparent M, values of RMAK are due to changes in the Stokes radius resulting from conformational changes.

The presence of 1.0 mM DTT shows little or no effect on apparent M, value as a function of pH up to a value of 7.0. At pH values above 7.0, the apparent M, values increase to near 29,000 at pH 9.0 which is consistent with a more open conformation. Table I gives the estimates of the Stokes radii of RMAK at different pH values based on gel filtration data. The minimum Stokes radius of 1.81 nm obtains at pH 5.0 in the absence or presence of DTT, and the maximum Stokes radius of 2.14 nm obtains at pH 9.0 in the presence of DTT. These observations and reports of conformational changes associated with substrate binding (1, 8, 22) suggested determinations of the M, values of RMAK at several DTT and substrate concentrations.

The Effect of Substrates on the Observed M, Values of RMAK at pH 6.5 and 8.0—We were interested in comparing the effects of the substrates on the apparent M, values with the effects shown by DTT. The pH values 6.5 and 8.0 were chosen because the high apparent M, value at pH 6.5 suggests a more open conformation unaffected by the absence or presence of DTT, and apparent M, values at pH 8.0 suggest more open configurations in the presence of DTT only. The elution patterns shown in Fig. 2 are typical for each condition. Table II gives the Stokes radii calculated from the gel filtration data shown in Fig. 2.

Some generalizations about the effect of the substrates can be derived from the elution patterns shown in Fig. 2. The presence of AMP, ATP, and ATP·Mg at 1.0 mM, at either pH value, resulted in changes of elution profiles that suggested more open and multiple conformations. The M, values were then determined at other concentrations of DTT, AMP, ATP, and ATP·Mg at pH 8.0.

The Effect of DTT on Apparent M, Values at pH g—Though not shown, DTT concentrations of 0.1, 1.0, and 2.0 mM at pH 8.0 result in single peaks with apparent M, values near 28,000 and an estimated Stokes radius of 2.10 nm.

The Effect of AMP on Apparent M, Values at pH 8—Above 0.1 mM AMP two peaks emerge corresponding to apparent M, values of 22,500 and 28,000 that shift toward higher apparent M, values with increasing AMP concentrations, as shown in Fig. 3.

The Effect of ATP on Apparent M, Values at pH 8—The elution profiles in the presence of AMP are more complex than in the presence of AMP, as shown in Fig. 3. At concentrations of 0.1, 0.5, and 1.0 mM ATP (Fig. 2), three peaks appear with apparent M, values near 22,000, 26,000, and 29,000. As with AMP, there is a shift toward an apparent M, value of 29,000 as the ATP concentration increases and a single peak obtains at 4.0 mM ATP.

The Effect of Both ATP·Mg on Apparent M, Values at pH 8—The pattern in Fig. 3 shows two peaks at ATP·Mg containments.
Fig. 2. The effect of DTT, AMP, ATP, and ATP-Mg at pH 6.5 and 8.0 on the elution profiles of RMAK from G-75. The conditions were similar to those given in Fig. 1, except that the equilibration and elution solutions contained combinations of 1.0 mM DTT, AMP, ATP or ATP-Mg as indicated.

Table II

The effect of substrates and DTT on the apparent molecular weight and Stokes radius of RMAK.

| Additions (1 mM) | pH 6.5 | pH 8.0 |
|-----------------|--------|--------|
|                 | $M_\text{r}$ | Stokes radius | $M_\text{r}$ | Stokes radius |
| None            | 26,500 | 2.04 | 22,500 | 1.86 |
| DTT             | 25,600 | 2.00 | 28,100 | 2.10 |
| AMP             | 27,300 | 2.06 | 28,500 | 2.12 |
| ATP             | 22,500 | 1.86 | 22,500 | 1.86 |
| ATP-Mg          | 27,500 | 2.08 | 29,200 | 2.14 |
|                 | 21,500 | 1.83 | 26,100 | 2.02 |
|                 | 26,700 | 2.04 | 27,200 | 2.16 |
|                 | 21,600 | 1.83 | 21,500 | 1.83 |

Fig. 3. The effect of substrate and ATP concentrations on the $M_\text{r}$ value. The conditions are the same as in Fig. 3, except that the concentrations of the adenosine nucleotides are indicated as the concentrations of the adenosine nucleotides are indicated in Fig. 2. Other details are the same as those given in Table I.

centrations below 1.0 mM, but in this instance the area under a peak corresponding to an apparent $M_\text{r}$ value of 21,500 is greater than that corresponding to 26,000 at 0.1 mM ATP-Mg. At 1.0 (Fig. 2), 2.0, and 4.0 mM ATP-Mg, a single peak obtains with an apparent $M_\text{r}$ value near 26,000. Though not shown, 1.0 mM DTT under similar conditions had little or no effect on either the patterns or the apparent $M_\text{r}$ values in the presence of the substrates.

The effect of Substrates at Equilibrium on Apparent $M_\text{r}$ Values at pH 8—We also determined the $M_\text{r}$ values when RMAK was equilibrated and eluted with an equilibrium mixture of its substrates and magnesium chloride. The Sephadex G-75 column was equilibrated and RMAK was eluted with the following solution: 1.0 mM ATP, 0.5 mM ADP, 1.0 mM AMP, and 2.0 mM magnesium chloride, which were determined as the observed equilibrium concentrations at pH 8.0 and 25 °C. An apparent $M_\text{r}$ value near 21,500 for the single peak (not shown) obtains for these conditions.

Circular Dichroism Studies—In order to better understand the changes of the apparent $M_\text{r}$ values, we studied the effects of pH and DTT on the circular dichroic patterns. Fig. 4A shows typical CD patterns when pH is varied, and Fig. 4B shows an example of the differences in the patterns in the presence and absence of DTT at pH 8. Using circular dichroic data from pH 5 to 9 and in the absence and presence of DTT, Fig. 5 shows the percent the secondary structures determined by using the following equation (23),

$$X = f_\alpha X_\alpha + f_\beta X_\beta + f_\pi X_\pi + f_\delta X_\delta,$$

where $X$ is the total of the secondary structures and $f_\alpha$, $f_\beta$, etc., are the fraction or percent of $\alpha$-helix, $\beta$-pleated sheet, unordered, and $\beta$-turns, respectively, and $X_\alpha$, $X_\beta$, $X_\pi$, and $X_\delta$ are estimated values for the corresponding secondary protein structures. The CD spectra were deconvoluted by the method of Compton and Johnson (24, 25) with a program made available to the authors by Dr. Steven Koerber, Biosym, San Diego, CA. The results shown in Fig. 5 indicate little changes in the secondary structure between pH 5 and 9 in the absence of DTT, the $\alpha$-helix ranging between 10 and 15%, the $\beta$-pleated sheet decreasing from 55 to 48%, and the unordered and $\beta$-turns being near 30 and 5%, respectively. These results are in agreement with the values obtained from the crystalline structure of RMAK (2, 6). In the presence of DTT over the same pH range, the percent of the $\alpha$-helix fraction increases from 9 to about 30%; the $\beta$-pleated sheet fraction decreases from 55 to about 30%; and the unordered and the $\beta$-turn fractions show less than a 10% change.

Correlation of DTT Activation with Molar Ellipticity—We had determined previously that the enhancement of activity by the presence of DTT was measurable with time (12). Fig. 6, A and B, shows a preliminary study of the changes of RMAK activity at pH 8.0 and the changes of the molar ellipticity with time under similar conditions. Though 222 nm is generally associated with the $\alpha$-helix region and 206 nm with the $\beta$-pleated regions, these measurements at only two wavelengths with time do not permit an estimation of the changes in the secondary structures of RMAK.

Discussion

These studies suggest RMAK can exist in solution as three relatively stable conformers that are resolvable by gel filtration techniques (3, 4, 26). The occurrence of several RMAK conformers in solution at different pH values supports the view that the pH-dependent conformations observed in porcine skeletal muscle AK crystals are an inherent characteristic (1, 2) even though such changes may not apply to skeletal muscle AK from all sources (27). The $M_\text{r}$ values calculated...
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FIG. 4. The effect of pH and DTT on CD patterns. The conditions are the same as those given under "Experimental Procedures," except for the pH which is shown in A and in B, a pH of 8.0 in the presence (—) and absence (—) of 1.0 mM DTT.

AMP and ATP-Mg concentrations are consistent with a shift toward a single conformer as active sites approach saturation. At the concentrations of the substrate ATP-Mg studied, a single peak obtains above 0.5 mM. In kinetics experiments shown in this study, ATP-Mg cannot be shown to act as an inhibitor at high concentrations and presumably cannot bind to both substrate sites as can AMP and ATP (19, 26, 31–34). The Stokes radius of 2.00 nm estimated for the single peak in the presence of ATP-Mg is intermediate to the extremes determined in this study.

The equilibrium mixture of substrates also gave rise to a single peak but with a Stokes radius of 1.84, equivalent to a $M_r$ value of 21,500, indicating a very closed conformation. Such a closed conformation would not seem to permit the placement of the active site of either of the substrates in a cleft and suggests that the active sites may be more superficial than those that have been proposed (2, 31, 35–37, 50). The identities of the active sites of the AKs are still controversial, and it has been pointed out that despite very similar enzyme kinetics, there appears to be dramatic changes of amino acid composition from various sources (50) around what is considered the active sites of AKs. The isolation and identification of two peptides that bind either ATP-Mg or AMP seem to have established the major amino acid sequences constituting the active sites of calf muscle and RMAK (19). Recent studies with a synthetic peptide of positions 1–45 of RMAK that binds ATP-Mg showed secondary structures calculated from CD data (34) similar to what we found for RMAK in solution. We are presently attempting to study the effect of substrates on the secondary structures of RMAK.

The opening and closing of the cleft induced by the presence of the substrates is consistent with the kinetic mechanism. The RMAK is a random Bi Bi system (28, 38). Holding one of the substrates constant and at relatively high concentrations while varying the second results in a family of rectilinear lines that pass through a coordinate above the reciprocal substrate concentrations axis and to the left of the reciprocal initial velocities axis (28). Such a family of plots is interpreted as showing that the binding of the first substrate increases the affinity of the enzyme for the second substrate (39). The mechanism we propose for RMAK is that the initial addition of either AMP or ATP-Mg to RMAK induces the formation of a more open conformation that allows an easier binding of the second substrate; when the second substrate adds, a very closed conformation then obtains. The enhancement of

from these gel filtration studies are near 21,500, 26,000, and 29,000, equivalent to estimated Stokes radii (16) of 1.81, 2.02, and 2.14 nm, respectively. We interpret the variations of the Stokes radii as conformational changes because of alterations of intramolecular charge distributions induced by substrates, pH, and DTT. The structural features of mammalian skeletal muscle AK (6, 21, 22) permit alterations of the effective molecular radius by opening and closing of a cleft formed by two globular domains connected by an unordered region.

The addition of substrates, AMP and ATP-MG, and ATP show pronounced complex effects on the gel filtration elution patterns of RMAK. The multiple peaks obtained probably relate to the multiple species of RMAK-adenosine nucleotide complexes evident from kinetic studies that show ATP or AMP can bind at both active sites, as a substrate and to the other site as an inhibitor (2, 8, 28–30). The shifts toward a higher apparent $M_r$ value and a single peak at the higher $M_r$ value of 21,500, indicating a very closed conformation. Such a closed conformation would not seem to permit the placement of the active site of either of the substrates in a cleft and suggests that the active sites may be more superficial than those that have been proposed (2, 31, 35–37, 50). The identities of the active sites of the AKs are still controversial, and it has been pointed out that despite very similar enzyme kinetics, there appears to be dramatic changes of amino acid composition from various sources (50) around what is considered the active sites of AKs. The isolation and identification of two peptides that bind either ATP-Mg or AMP seem to have established the major amino acid sequences constituting the active sites of calf muscle and RMAK (19). Recent studies with a synthetic peptide of positions 1–45 of RMAK that binds ATP-Mg showed secondary structures calculated from CD data (34) similar to what we found for RMAK in solution. We are presently attempting to study the effect of substrates on the secondary structures of RMAK.

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FIG. 5. The effect of 1.0 mM DTT on the molar ellipticity, ($	heta$), at 206 nm (A) and 222 nm (B) and activity with time. The conditions are the same as in Fig. 4B, except that in A measurements were taken at the times indicated in the presence of 1.0 mM DTT and in B the activity of the sample was measured for enzyme activity at the times indicated in the presence (△) and absence (●) of 1.0 mM DTT.
RMAK activity by DTT is also interpreted to be due to a more open and therefore more active conformation.

The presence of DTT has little effect on the apparent $M_c$ values up to pH 7.0. Above pH 7.0, the apparent $M_c$ values increase to a maximum of 29,000 and a maximum Stokes radius of 2.14 nm. A pH value of 7.5 is in the region of the pK value for the sulfhydryl group at Cys-25 (45), sulfhydryl groups generally requiring ionization for chemical interactions. The more reactive Cys-25 residue and the Cys-187 residue, common to most mammalian muscle AK (40-45), are likely sites of interaction with DTT. Either one or both sulfhydryls of RMAK could form a transient intermediate with DTT, leading to more open conformations. The opening and closing of the cleft may involve cis-trans isomerizations (46-48) among the 6 prolyl residues in RMAK. The observed protection and enhancement of RMAK activity by DTT and other sulfhydryl reagents appears to be related to the formation of more stable or more enzymatically active conformers rather than a protection from sulfhydryl oxidations or the prevention of disulfide bonds (38).

Under the conditions of these experiments, the estimations of the secondary structural composition from the CD data do not agree with the results obtained from crystallographic studies (1, 2, 6) as do the CD studies of the AK from Escherichia coli (49). The estimations, at pH 8 in the absence of DTT, are about 15% $\alpha$-helix fraction, 50% $\beta$-pleated sheet, 25% unordered region, and 6% $\beta$-turns; as shown in Fig. 5, the reverse of the ordered secondary compositions derived from crystallographic studies. Though not given, when CD data from these studies were applied to equations developed earlier (51, 52) for estimating the percent of $\alpha$-helix, these results agreed with estimates presented here within a few percent. The differences from the crystallographic data can derive from any of several possibilities. Among those worthy of study are that the structure of RMAK in solution is indeed different from that in the crystal or that the CD spectrum of RMAK is not compatible with the assumptions of the equations (23-25) developed for the estimates of secondary structures in proteins.

The pH alone did not have a large enough effect on the ordered regions to correspond to the changes of the Stokes radii, but we recognize also that changes in the unordered regions are not likely to be detected by CD under our conditions. In the presence of DTT, the changes of the secondary structures observed by CD do appear to reflect changes in the apparent $M_c$ values. Up to pH 7, the presence of DTT appears to have little or no effect on the $\alpha$-helix region; above pH 7 the percent of $\alpha$-helix structure increases. The $\beta$-pleated sheet region decreases with increasing pH from 5 to 9. Over the pH range tested the apparent $M_c$ values in the presence of DTT suggest that as the pH increases a more open conformation results, characterized by an increase in the $\alpha$-helix region and a concomitant decrease in the $\beta$-pleated sheet region.

Our preliminary studies showed that the rates of the enhancement of activity by DTT within the first minutes parallel increases of the $\theta$ at 222 nm while no changes are occurring at 206 nm. At present, it is not possible to associate the kinetics of $\theta$ changes at specific wavelengths with specific secondary structural changes. The kinetics of secondary structure changes as measured by CD techniques are the focus of present studies.

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