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CHARACTERISTICS OF RABBIT MUSCLE ADENYLATE KINASE INHIBITION BY SULFUR AND RECOVERY BY DITHIOTHREITOL

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Structure-function relationships of rabbit muscle adenylate kinase (RMAK) were studied by examining the characteristics of inhibitions by hydrophobic inhibitors and reactivations by sulfhydryl reagents. RMAK is inhibited by 1-butanol, N-ethylmaleimide (NEM) and elemental sulfur (S_8) with increasing effectiveness in the order of increasing hydrophobicity. Characteristics of these hydrophobic inhibitors are compared with inhibitors forming covalent bonds or reversible complexes.

A mechanism is proposed for hydrophobic inhibitors of RMAK that involves conformational changes promoted by interacting with hydrophobic regions. The reversal of RMAK inhibition by sulfhydryl compounds involves a conformational change that exposes hydrophobic regions and the inhibitor to water. Circular dichroism (CD) data show changes in the secondary structures of RMAK, indicating that the inhibitors and the sulfhydryl compounds promote conformational changes. The results of these studies show that the activity of a small enzyme can be controlled in a manner analogous to the allosteric control of larger enzymes.

KEY WORDS: Adenylate kinase, hydrophobic inhibitors, sulfur inhibition, conformations, DTT reactivations

INTRODUCTION

The inhibition of RMAK by S_8 was reported previously.[1] Elemental sulfur, S_8, is a hydrophobic, eight-membered ring that inhibits RMAK at micromolar concentrations.[1] Elemental sulfur specificity parallels those reported for other reagents interacting with sulfhydryl groups;[2] sulfhydryl compounds, such as DTT, also reverses S_8 inhibitions. We found also that glycerol kinases from *Bacillus stearothermophilus* and *Candida mycoderma* are completely inhibited by comparable concentrations of S_8 but were not reversible by DTT.[3]

The objective of this paper is to characterize the mechanism of RMAK inhibition by this potent, highly specific, hydrophobic inhibitor. The reactions[4] of sulfides with S_8 forming polysulfide complexes and the reversibility of the S_8 inhibition by DTT seem

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consistent with an inhibition mechanism involving interaction with free sulfhydryl groups of RMAK.[5] Titrations of RMAK against S_8 were, in contrast, consistent with reversible inhibition. This study presents inhibition kinetics and compares S_8 inhibition with inhibitions by other compounds having similar characteristics. We found that such varied chemical structures as S_8, NEM and the low molecular weight alcohols have common RMAK inhibition characteristics.

We propose a general mechanism of inhibition in which S_8, NEM, or low molecular weight alcohols interact with the hydrophobic cores of RMAK,[6–9] resulting in conformational changes that can be reactivated by DTT. This type of hydrophobic inhibition, as a control mechanism for the activity of small enzymes, is compared with allosteric modifications.

MATERIALS AND METHODS

Materials

Rabbit skeletal muscle adenylate kinase (RMAK, EC 2.7.4.3) is the commercial preparation from Sigma Chemical Co., St. Louis, MO 63178, designated "Myokinase, grade III from rabbit muscle". When some lots were subjected to isoelectric focusing, the RMAK showed a single isozyme with an isoelectric point value near 9 and gave a single protein band by polyacrylamide gel electrophoresis with the silver staining method.[10] Such commercial RMAK was used without further purification. The specific activity was in the range of 2,000 EU/mg under our assay conditions. The activity of RMAK in the presence of 1 mM DTT was 50 to 100 percent higher.[1]

The following chemicals were obtained from Sigma Chemical Co.: N-ethylmaleimide, NEM; dithiothreitol, DTT; 5,5′-dithio-bis (2-nitrobenzoic acid), DTNB. Illudin S, a sulfhydryl reagent and the natural product from the mushroom *Omphalotus illudens*,[11] was provided by Dr. Trevor McMorris of the University of California, San Diego Chemistry Department.

The elemental sulfur, S_8, used was designated "Sulfur, sublimed (powder)" from Mallinckrodt, Inc. and is indistinguishable as an inhibitor from a more purified preparation.[12] Stock solutions of S_8 were stored in absolute ethanol at −20°C. The concentrations of S_8 were determined spectrophotometrically, using a molar absorptivity of 6000 at 263 nm. Dilutions of the S_8 stock solutions for the purposes of the kinetic studies were with the appropriate buffer; dilutions were so large that turbidity was not a problem.[1]

Methods

AK standard assays. We measured AK activity, AMP + MgATP = ADP + MgADP, according to Adam,[13] and modified elsewhere.[2] The 1 mL assay mixture was 0.3 mM phosphoenolpyruvate; 0.4 mM NADH; 8.0 mM AMP and ATP each; 8.0 mM $MgCl_2$, and 20 mM potassium phosphate buffer, pH 8.0. The RMAK activity decreases very sharply in assay mixtures below pH 6.5. Sufficient amounts of lactate dehydrogenase, LDH, (EC 1.1.1.27) and pyruvate kinase, PK, (EC 2.7.1.40) were added so that the

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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 absorptivity value of 6,220 was used to convert the change of NADH absorbance to micromoles of product formed. One enzyme unit (EU) of activity is the formation of 1 μmole of ADP per min at 25°C.

Initial velocity studies It was determined that S_8 had no effect on the assay system and that the ethanol solvent had no measurable effect on RMAK at the dilutions used. When determining the effect on the initial velocity, S_8 at the proper concentration was in the assay mixture. Reactions were initiated by the addition of AK.

Inhibition assays The conditions for the inhibition of RMAK by S_8, the alcohols, and NEM were as follows unless stated otherwise. The RMAK, in 100 mM potassium phosphate buffer, pH 8.0, was incubated with the inhibitor under study for 0.5 h to 1.0 h and the activity remaining was then determined. For the concentrations of enzymes and inhibitors used in these studies, it has been determined that no additional significant inhibition occurred after 0.5 h of incubation.

Protein determinations The RMAK concentrations were determined spectrophotometrically using the absorbancy value E 0.1% = 0.53 at 279 nm.[7]

CD measurements The CD measurements were carried out on a modified Cary 61 spectropolarimeter[14] as described elsewhere.[15] The CD spectra were obtained by a signal averaging 10 scans, using a 0.1 mm cell. Each experiment had a baseline for that specific condition minus RMAK. All CD spectra were deconvoluted by the method of Compton and Johnson,[16,17] using a program given to the authors by Dr. Steven Koerber, Biosym, San Diego, CA. The buffer was 10 mM potassium phosphate at pH 8.0. The DTT was 1.0 mM and the S_8 was 7 μM when used. The RMAK was always 200 μg/mL.[15] The percentages of the secondary structures were also determined by using the following equation[18] with good agreement.

$$X = f_h X_h + f_\beta X_\beta + f_R X_R + f_t X_t$$

The X term is the total experimental molar ellipticity, $[\theta]$. The f_h, f_β, f_R and f_t terms are the fraction or percentage of α-helix, β-pleated sheet, unordered and β-turns, respectively. The X_h, X_β, X_R, and X_t terms are estimated values for the corresponding secondary protein structures.

RESULTS

Characteristics of S_8 Inhibition

A characteristic of the S_8 inhibitions of AKs from animal sources is the ability to recover the activity with sulfhydryl compounds.[3] The recovery of activity, greater than the control following DTT addition was reported and discussed earlier.[1,15] We report here on DTT effects exclusively, but it can be shown that mercaptoethanol, glutathione, and cysteine are equally effective. Table 1 shows that DTT recovers

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TABLE 1
Recovery of RMAK activity after inhibition by sulfhydryl reagents.

Inhibitor[a]	% inhibition[b]	% activity recovered[c]	
		5 mM DTT	5 mM KCN
None	4	143	92
50 μM $AgNO_3$	45	111	44
500 μM NEM	50	111	41
500 μM *p*-HMB	50	119	57
34 μM S_8	100	172	0
500 μM DTNB	98	102	97

[a]2 EU/mL of RMAK in 100 mM potassium phosphate buffer, pH 7.0, at 25°C was incubated for 1.5 h and the activity remaining was determined. [b]The activities relative to the zero-time control. The 96 percent of the activity remaining after 2.5 h incubation served as the base for % inhibition and the % activity recovered. [c]After 1.5 h incubation with inhibitors, 10 mM DTT or 10 mM KCN in 0.4% BSA was added, incubated an additional 1 h, and the activity remaining was determined.

the RMAK activity from a variety of sulfhydryl inhibitors. Table 1 also shows that KCN reverses the inhibition by DTNB, presumably by rupturing the mixed disulfide bonds formed, but does not reverse the inhibition of RMAK by S_8. This and other observations suggested to us that S_8 was not forming mixed sulfide bonds with cysteinyl residues, as does DTNB. The reversal of NEM inhibition by DTT is of particular interest because of its potential to form thioethers with the two cysteinyl residues of RMAK.[5] Thioethers are not generally ruptured by DTT.

Titration of SH Groups in the Presence of S_8

The sulfhydyl groups of RMAK were titrated in the presence and absence of S_8 to establish whether S_8 interacted with the CYS-25 or CYS-187 residues under conditions of the maximum exposure of the sulfhydryl groups in urea solutions. Urea also prevented the precipitation of the highly hydrophobic S_8. Bovine serum albumin (BSA) was used as a protein standard. The *p*-hydroxymercuribenzoate chloride (*p*-HMB) and NEM were sulfhydryl reagent standards for comparisons with S_8. Table 2 shows that S_8 has no effect on the number of detectable sulfhydryl groups in BSA or RMAK under similar conditions. On the other hand, *p*-HMB masks free sulfhydryl groups in both proteins. The NEM masks all the sulfhydryl groups in BSA but only half of the groups in RMAK. It is interesting that only one of the sulfhydryl groups of RMAK is available for interaction with NEM under these conditions. These results do not support a S_8 inhibition mechanism of RMAK involving formation of polysulfide bonds with RMAK sulfhydryl groups.

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TABLE 2
Determination of SH groups in the presence of S_8, NEM, and *p*-HMB.

	moles of SH groups/mole of protein			
	Inhibitor concentrations[a]			
Protein μM	NONE[b]	S_8 50 μM	*p*-HMB 1 mM	NEM 1 mM
RMAK 18.3 μM	1.86	1.74	0.07	0.77
BSA 20.4 μM	1.94	1.98	0.03	0.03

[a]All proteins were in 8 M urea and 0.1 M K-PO4 buffer, pH 8 at the concentrations given. [b]Incubated at 25°C for 2 h. The mixtures were then made 2 mM DTNB and the absorbance determined at 412 nm. An a_M value of 13,600 converted the absorbance to μmoles/L of titratable sulfhydryl groups.[29] Other conditions are in the text.

Effect of pH on recovery of RMAK activity by DTT

Since previous CD studies[15] showed RMAK conformation changes in DTT presence and absence were pH dependent, the effect of pH on RMAK stability, on the inhibited RMAK-S_8 complex, and on DTT recovery of activity were of interest. Figure 1 shows the effects of pH. The stability of RMAK declines slightly with increasing pH.

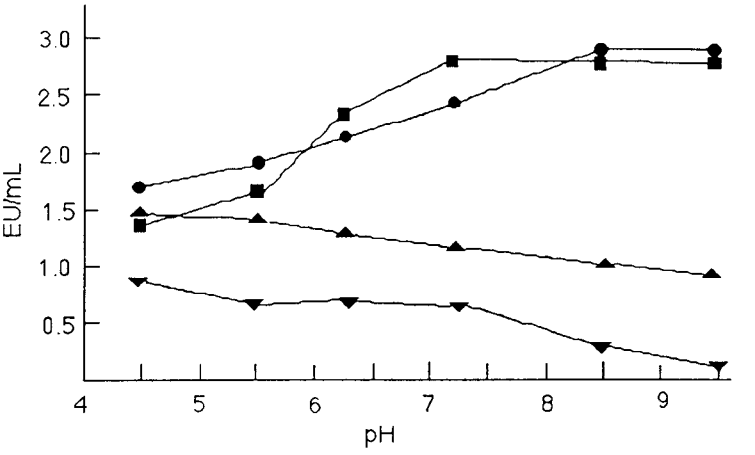

FIGURE 1 The effect of pH on S_8 inhibition and DTT activation of RMAK. All samples were at 25°C in 0.1% BSA and 100 mM potassium phosphate buffer. The incubations were at the pH value indicated and the activities measured at the times given below. The conditions are as follows: RMAK control with no additions (▲) and RMAK in 10 mM DTT (●) were incubated for 2.5 h. RMAK in 4.5 μM S_8 was measured at 1.5 h (▼), then made 10 mM DTT and the activity again measured at 2.5 h (■).

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TABLE 3
Comparison of inhibitor concentrations resulting in 50% inhibition of RMAK

Inhibitor	Concentration mM
methanol	7,300
ethanol	5,200
1-propanol	7,200
2-propanol	2,800
2-butanol	2,000
t-butanol	1,900
1-butanol	510
NEM	0.22
AP_5A	0.010
S_8	0.009

A twenty-fold range of concentrations of inhibitors indicated were incubated with 12 EU/mL of RMAK in 100 mM potassium phosphate buffer, pH 8.0 and 0.1 percent BSA at 25°C for 1 h. The activities were then determined. A plot of activity remaining vs. the inhibitor concentration was constructed to estimate the concentration resulting in a 50 percent inhibition.

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Similarly, the inhibition of RMAK by S_8 is more effective under alkaline conditions than under acid conditions. The recovery of activity by DTT is maximal above pH 7; below pH 7, recovery lessens considerably. The patterns of activity and DTT versus pH in Figure 1 are similar to previous patterns[15] of the pH versus apparent RMAK molecular weight values, M_r, and DTT, using molecular sieve methodology. We showed[15] that below pH 7 DTT had little or no effect on the conformation change as reflected by change in the M_r values but showed increases above pH 7.

Characteristics of S_8 and Other Inhibitors

Dithiothreitol recovers the activities of RMAK inhibitions by the following compounds; S_8, NEM, and the soluble alcohols. Alcohol inhibitions require very high concentrations compared to the other inhibitors. The highly specific AP_5A inhibitions[19] of AK-1 are not reversed by DTT. As shown in Table 3, we roughly estimated the potencies of the inhibitors by determining the concentration that results in a 50 percent inhibition against a standard concentration of RMAK. Alcohols inhibitions were interesting because they are reactivated by DTT and because 1-butanol showed some specificity relative to the other alcohols; methanol and ethanol require about 10 fold greater concentrations than butanol.

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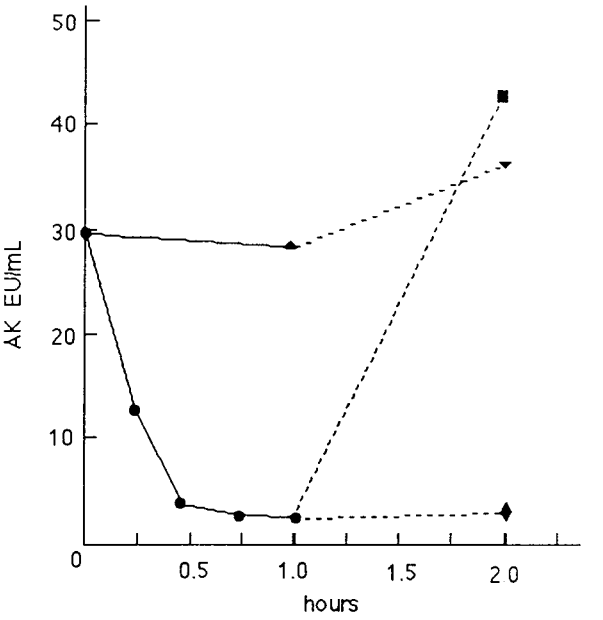

FIGURE 2 Reversing S_8 inhibition with DTT. The concentration of S_8 of 16.2 μM was incubated with 12 EU/mL of RMAK in 100 mM potassium phosphate buffer, pH 8.0 and 0.1% BSA at 25°C up to 1 h. The activity remaining was measured at the times indicated (●). One portion of the reaction mixture was diluted 30 fold, incubated an additional hour and the activity then determined (♦). Another portion was made 10 mM DTT, incubated for 1 h and the activity then determined (■). The control, containing no S_8, was incubated for 1 h and the activity measured (▲). Then the control was made 10 mM DTT, incubated an additional hour and the activity then measured (▼).

The recoveries of activity from complete inhibitions by NEM and the alcohols require dilution, which does not restore activity, followed by DTT addition or alternatively a dilution with a DTT solution. Dilution is not required for the recovery of activity after inhibition by S_8. We believe that the dilution requirement is due to the high concentrations of the other inhibitors that compete with DTT to promote conformational change. CD data indicates that 1-butanol nearly obliterates the α-helices of RMAK, accompanied by an increase of β-pleated sheets.[3] The similarities of the RMAK inhibition characteristics of such different chemicals as S_8, NEM and alcohols are consistent with the view that all inhibit by effecting conformational changes.

As shown in Figure 2, the dilution of the RMAK-S_8 complex did not reverse the inhibition. The alcohol inhibitions behaved similarly. If an inhibited complex is readily reversible, then dilution favors dissociation and a decreased inhibition.[20,21] It was of interest to establish whether S_8 inhibitions were reversible in the sense that no covalent bonds were formed.

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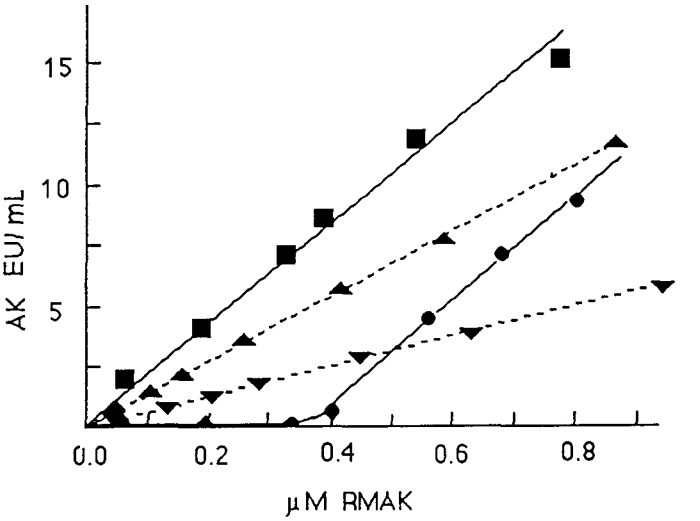

FIGURE 3 Titrations of RMAK against constant S_8, AP_5A and DTNB concentrations. Solutions of RMAK in 100 mM potassium phosphate buffer pH 7.0, at concentrations indicated above, were incubated for 1 h at 25°C with the inhibitors and the RMAK activity remaining was then determined. The figure is a composite of several experiments with the data normalized to the ± DTNB conditions. The concentrations of the inhibitors were as follows: no inhibitors, (■); 9 μM S_8, (▲); 7 μM AP_5A (▼); and 200 μM DTNB (●). A ratio of DTNB/RMAK = 1.8±0.3 was determined from this and other data. For similar experiments with the inhibitor NBD-Cl, a ratio of 0.9±0.4 was obtained.

Determining the reversibility of inhibitors

Reversible and irreversible inhibitors yield distinguishing pattern plots when enzymes are titrated against a constant inhibitor concentration.[22] Figure 3 shows plots of RMAK titrated against the constant concentrations of three inhibitors, S_8, AP_5A, and DTNB. The features of plots indicating reversible inhibitions are the converging of the lines at the origin and the slope changes with varying inhibitor concentrations. This reversible pattern obtains for RMAK titrated against AP_5A and S_8. Though not shown here, butanol, other alcohol inhibitors, and NEM show similar patterns, indicating they are reversible inhibitors also. Kinetics studies below provide additional evidence for the reversible character of S_8 inhibition by a mixed-type inhibition against ATP (Table 4, Figure 4). An irreversible inhibitor plot obtains when RMAK is titrated against DTNB. The features of irreversible inhibitions are intersections of rectilinear plots with the abscissa and the parallelism of the plots with and without the inhibitors, as shown. DTNB is a reagent that forms covalent mixed disulfide bonds with free sulfhydryl groups in proteins.[23] Dithiothreitol restores the activity of DTNB inhibitions of RMAK by rupturing the disulfide bonds and restoring the free sulfhydryl groups.

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TABLE 4
K_i and V_{max} values of S_8 (pH 8).

Substrate	K_m μM	K'_m μM	V_m[c] EU/mL	$V_{m'}$[c] EU/mL	$[S_8]$ μM	K_i[d] μM	Inhibition Type
AMP	110±5[a]	103±4[a]	3.1	1.4	2.0	1.8	NC
MgATP	69±4[b]	95±5[b]	3.3	1.5	2.0	1.4	Mixed

Final concentration was 2.0 EU/mL. Constant substrate concentrations were 1, 0.25, and 0.1 mM. [a]K_m values are not significantly different at $p > 0.1$ (df = 13). [b]K_m values are significantly different at $p < 0.001$ (df = 26). [c]Estimations by extrapolations of $V_{m'}$ value constant substrate concentrations. [d]Estimations of K_i values given in the text.

Initial Velocity Patterns

We studied the patterns derived from initial velocity data to obtain information concerning the mechanism of S_8 inhibition. Treatments of the data by either the double reciprocal plots[24] or the direct linear plots[25] are consistent with reversible inhibition kinetics. Though not presented, double reciprocal plots show patterns consistent with S_8 as a mixed inhibitor of MgATP and a non-competitive inhibitor of AMP. Figure 4 shows the direct linear plots of our data. We found this method more useful than the double reciprocal plots for estimating the reliablity of our data, the kinetic constants, and the inhibition types in this two substrate system. When AMP is the variable, direct linear plots show no significant difference between the K_m and the $K_{m'}$ values of the uninhibited and inhibited plots, respectively, as given in Table 4.

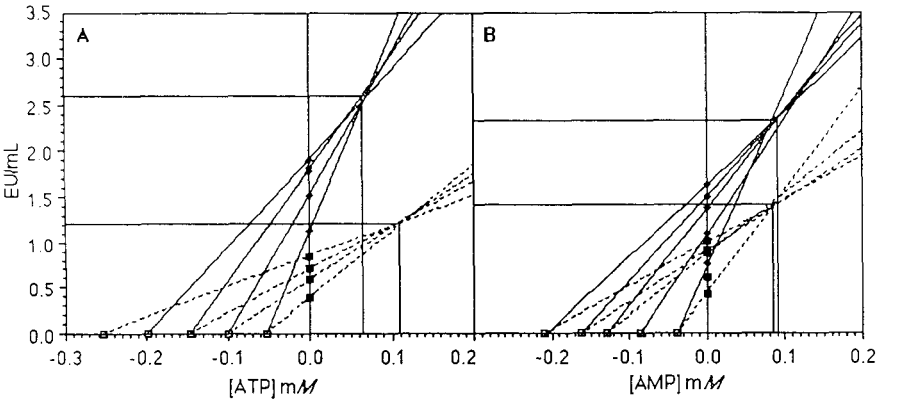

FIGURE 4 Inhibition kinetics of S_8. Except for the concentrations of the substrates and the presence of S_8, the conditions and measurements of activity were the same as that given in Materials and Methods. The presence of S_8 is indicated by (■) and the absence by (♦). In (A), the AMP constant concentration was 0.25 mM and the S_8 concentration was 2.0 μM. In (B), the ATP constant concentration was 0.25 mM and the S_8 concentration was 2.0 μM.

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TABLE 5
Effect of DTT on kinetic constants.

	pH 7		pH 8	
	K_m μM	V_m[a] EU/mL	K_m μM	V_m[a] EU/mL
ATP	60±4[b]	3.2	74±3[b,c]	2.5
ATP + DTT	58±7	3.2	56±4[c]	2.8
AMP	105±6[d]	3.6	120±5[d,e]	3.3
AMP + DTT	100±9	3.5	97±8[e]	3.7

[a]Determined by extrapolation of apparent V_m values at 1, 0.25, and 0.1 mM fixed substrate to infinite fixed substrate concentration. [b]K_m values are significantly different at $p < 0.05$ (df = 27). [c]K_m values are significantly different at $p < 0.05$ (df = 23). [d]K_m values are not significantly different, $p > 0.05$ (df = 18). [e]K_m values are significantly different at $p < 0.05$ (df = 18).

When ATP is the variable, direct linear plots show significant differences (Table 4) between both the K_m and the $K_{m'}$ values and between the V_m and $V_{m'}$ values consistent with a mixed inhibition and inconsistent with an irreversible inhibition. Table 4 gives the average K_i values based on estimates using the equations $V_{m'} = V_m/(1 + i/K_i)$ in the case of noncompetitive inhibition, where $K_m = K_{m'}$. In the case of mixed inhibition, we used the equations $V_{m'} = V_m/(1 + i/K_{i'})$ and $V_{m'}/K_{m'} = (V_m/K_m)/(1 + i/K_{i'})$ for both noncompetitive and mixed inhibitions.

Effect of DTT on Kinetic Constants

Since DTT enhances the activity of RMAK, its effect on the K_m values is of interest. We studied the DTT effects on RMAK activity at pH 7, where there is little or no effect, and at pH 8, where activity is enhanced. Table 5, shows that at pH 7 neither the K_m values for AMP nor ATP change in the presence of DTT. At pH 8, an analysis of variance of the means (ANOVA) shows a significant decrease ($p < 0.05$) for the K_m values of both ATP and AMP in the presence and absence of DTT. ANOVA indicates that the K_m values for ATP is significantly higher at pH 8 compared to the K_m value at pH 7. The K_m values for AMP are not significantly different at pH 8, than at pH 7. At pH 8, the presence of DTT decreases the K_m value of AMP to a value that is not significantly different from the K_m values at pH 7 in the presence or absence of DTT.

Relationship of Inhibitor Site to Active Sites

To support or to eliminate the possibility that both active sites of RMAK were involved in the inhibition by S_8, we compared the results with inhibition kinetics AP_5A and 2′-AMP. Both AP_5A and 2′-AMP have been proposed as adenine nucleotide substrate analogs reacting at both sites.[19,26] Figure 5 shows the patterns obtained when MgATP and AMP were varied at a 1:1 ratio in the absence and presence of AP_5A and, under

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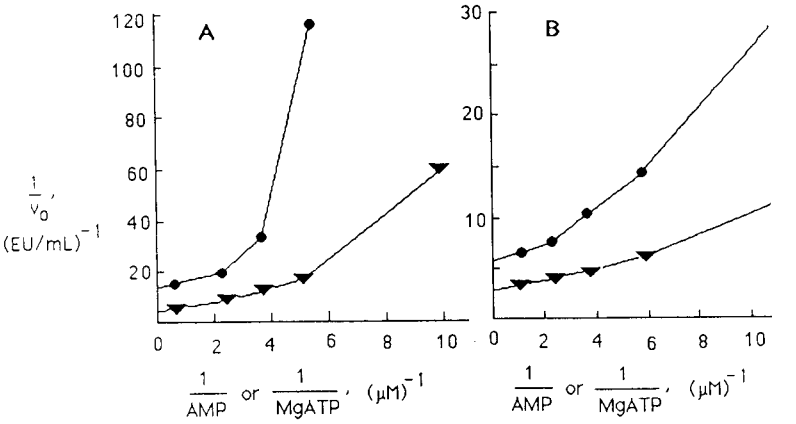

FIGURE 5 Initial velocity pattern of AP_5A and S_8 inhibitions at a constant substrates' ratio. (A) Double reciprocal plots in the absence (▼) and presence of 0.3 μM AP_5A (●) with the concentration of AMP and MgATP held constant at a ratio of 1:1. (B) Double reciprocal plots in the absence (▼) and presence of 0.5 μM S_8 (●) with the concentration of AMP and MgATP held constant at a ratio of 1:1.

similar conditions, in the absence and presence of S_8. It was shown previously[26] that when the inhibitor 2′-AMP binds at both substrate sites, a treatment of the initial velocity data as shown in Figure 5, results in a convergence of the curvilinear lines at the vertical axis. As shown in Figure 5B, the lines do not converge at the vertical axis but intersect the vertical axis at 5.9±0.17 μmoles min^{-1} $unit^{-1}$ mL^{-1} in the presence of S_8 and at 3.8±0.06 μmoles min^{-1} $unit^{-1}$ mL^{-1} in its absence. The pattern is consistent with S_8 affecting only one substrate site. Figure 5A shows that the two curves also do not converge when AP_5A is the inhibitor. The plot of initial velocity data in the presence of AP_5A intersects the vertical axis at 11.78±0.19 μmoles min^{-1} $unit^{-1}$ mL^{-1} and in the absence of AP_5A at 6.90±0.11 μmoles min^{-1} $unit^{-1}$ mL^{-1} of enzyme. The pattern is also consistent with the view that AP_5A binds at only one substrate binding site.[27]

Circular Dichroism Studies

In a previous study,[15] we estimated the changes in the secondary structural features under a variety of conditions, using CD methodology. Using similar estimates under conditions of inhibition by S_8, CD changes in the α-helix regions of RMAK were estimated. The conformational changes show a decrease in the α-helix regions that is not statistically significant with our small sample, as given in Table 6. The conformational changes resulting from addition of DTT to RMAK show an increase in the α-helices. As shown in Figure 6B and 6C, when activity is fully recovered from S_8 inhibition, the CD spectrum of RMAK + S_8 + DTT is not similar to

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TABLE 6
Estimation of the α-helix composition of RMAK from CD data.

Conditions	% α-helix
RMAK[a,c]	11[b]
RMAK + DTT[a,d]	17
RMAK + S_8	10[b]
RMAK + DTT + S_8[c,d]	15

The estimates were derived from the average data of at least three runs similar to that shown in Figure 6 as described under "Experimental Procedures". The concentrations were as follows: RMAK, 200 μg/mL; 1 mM DTT; and 7 μM S_8, which resulted in a 90% inhibition of RMAK. The estimates have standard deviations of about ±1%. [a]Differences are significant at $p < 0.01$ (df = 9). [b]Differences are not significantly different (df = 9). [c]Differences are significantly at $p < 0.05$ (df = 9). [d]Differences are not significantly different (df = 9).

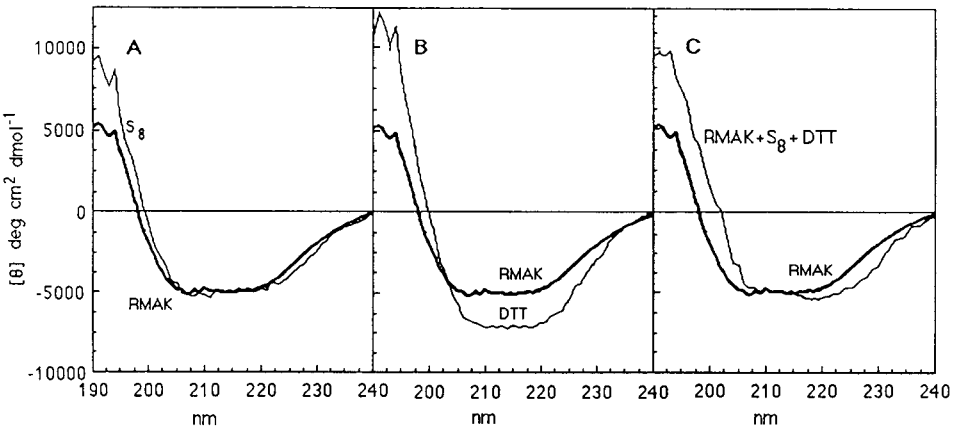

FIGURE 6 The effect of DTT and S_8 on CD patterns. The CD measurements were carried out as described under "Experimental Procedures." The CD spectra were obtained using a 0.1 mm cell. The buffer was a 100 mM potassium phosphate at pH 8.0. The RMAK was always 200 μg/mL.[15] The darkened line is the average RMAK control from the experiments. Other conditions are as follows: (A) 7 μM S_8; (B) 1.0 mM DTT, and (C) 7 μM S_8 and 1.0 mM DTT.

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RMAK + DTT. Table 6, on the other hand, shows that the percentages of the α-helix in RMAK + S_8 + DTT and RMAK + DTT are the same. No differences in these spectra were expected because 1 mM DTT was in such great excess of the 7 μM S_8. The possibility that RMAK forms complexes with some product(s) of the S_8 and/or DTT remains.

DISCUSSION

We propose that RMAK inhibitions by S_8, NEM, and 1-butanol and their reversals by DTT proceed through mechanisms involving conformational changes.[28,29] We propose that conformational change is promoted by these inhibitors interacting with the hydrophobic domains of RMAK,[6,8,9,30] such that the domains with the inhibitors are removed from the water environment, creating conditions that repress inhibitor dissociation. The proposed mechanism of inhibition reversal by sulfhydryl reagents also involves a conformational change[15] such that hydrophobic domains and inhibitors are exposed to the water enviroment, creating conditions that support the dissociations of inhibitors and the restoration of RMAK activity. The CD spectra and estimates of secondary structural changes in the presence of DTT support significant structural change. The presence of S_8 produced no significant change in the estimated secondary structure. This was not true of butanol inhibition which all but obliterates the α-helices of RMAK.[3]

While sites of inhibitor interactions are undefined, our results do not support a S_8 inhibition mechanism involving formation of covalent polysulfide bonds. Elemental sulfur does not mask RMAK sulfhydryl groups; under similar conditions *p*-HMB masks RMAK sulfhydryl groups. Titrations of RMAK against S_8 result in reversible inhibitor patterns.[22] Titrations against DTNB or *p*-HMB, inhibitors that form covalent bonds with RMAK sulfhydyl groups, show typical irreversible inhibitor patterns.[22] Additionally, KCN does not reverse inhibitions by S_8, unlike DTNB inhibitions. Kinetic studies show that S_8 is a mixed inhibitor of ATP, which is incompatible with an irreversible inhibition. All evidence supports a reversible inhibitor characteristic for S_8. Though not detailed here, similar characteristics obtain for NEM and 1-butanol.

The effectiveness of the inhibitors is in the order of their hydrophobicity — S_8 $\gg$ NEM $\gg$ 1-butanol. The inhibiting alcohol concentrations are too high to be considered specific. Still, these alcohol inhibitions were interesting because 1-butanol showed a specificity relative to the other alcohols. The concentration levels for 50 percent inhibition by methanol and ethanol were 7.2 M and 5.2 M, respectively, compared to 0.5 M for 1-butanol; under similar conditions, butyric acid was as effective. This suggests that the butyryl group is responsible for their relative effectiveness as inhibitors.

Inhibitions by NEM are generally considered the result of covalent, thioether bond formations with cysteinyl residues. The inhibitions of RMAK by NEM are reactivated by DTT and thioether bonds are not usually reactivated by sulfhydryl compounds.[31] There is evidence that NEM can function as an inhibitor without forming a covalent bond,[32] as we believe is the instance for NEM inhibitions of RMAK. By comparison,

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we can show that rabbit skeletal muscle creatine phosphokinase (CK) inhibition by NEM is not reactivated by DTT and when CK is titrated against NEM, an irreversible inhibition pattern obtains similar to DTNB and unlike RMAK.[1]

The hydrophobic inhibitors, S_8, NEM, and 1-butanol, have some characteristics that appear inconsistent with reversible systems. Unlike reversible systems, extensive dilutions do not result in dissociation of inhibitors.[20,21] The mechanisms of inhibition of RMAK by S_8, NEM or 1-butanol and reactivations by DTT are proposed as competitions between two sites or domains, one interacting with hydrophobic inhibitors and another interacting with DTT. Each site potentially promotes a different conformational change. We speculate that the additions of hydrophobic inhibitors promote inversions of RMAK molecules that remove the hydrophobic cores with the hydrophobic inhibitor from the water environment. Diluting the inhibited complex provides an even more polar environment that favors inversion and opposes dissociation of hydrophobic inhibitors. In the reactivation process, the interaction with DTT promotes an eversion of RMAK that exposes the hydrophobic regions and the inhibitor to the water environment. Such exposure then permits partitioning of the inhibitors between the large water phase and the hydrophobic core of RMAK. The proposed mechanism is consistent with the inability of KCN to reverse S_8 inhibition while readily reversing DTNB inhibition under similar conditions. Potassium cyanide reacts rapidly and quantitatively with S_8 to form KCNS (which is not inhibitory) and with DTNB. We envision S_8 in the inhibited complex as internally situated and inaccessible to cyanide ions which have no effect on the conformation. The conformational changes induced by DTT allow an access to S_8. By contrast, the mixed disulfide bonds of DTNB inhibition are accessible to KCN.

Using kinetics methodology, we attempted to ascertain where S_8 and DTT interacted relative to the active sites. The double reciprocal data plotted at a constant substrates ratio[26] indicate S_8 interacts at only one substrate site. Our kinetic data suggest that S_8 inhibitions are noncompetitive with AMP and mixed with MgATP at pH 8. The mixed inhibition pattern is kinetic evidence for a reversible inhibition.

DTT appears to have a significant effect on the K_m of both ATP and the K_m of AMP (Table 5) at pH 8. Taking these results into account, we propose that DTT acts to effect both substrate sites.[5,6,33] On the other hand, S_8 appears to effect the ATP site only. We previously suggested[15] the Cys-25 or Cys-187 residues[7,29,34–37] as likely sites of interaction with DTT, but they do not appear to be very reactive groups. In this regard, we found it interesting that Illudin S, a powerful sulfhydryl reagent derived from mushroom,[11] has no inhibitory effects on RMAK under a variety of conditions.

Presently, it is not known how common hydrophobic inhibitors with the described characteristics are, but we do not think them unique to skeletal muscle RMAK. Other studies in progress show that glycerol kinases (ATP: glycerol 3-phosphotransferase; EC 2.7.1.3) from rabbit muscle and from *C. mycoderma* are extensively inhibited by a few μmoles/L of S_8, but are not reactivated by DTT.

In summary, from these studies and others,[15,38] we propose that RMAK has four sites capable of promoting conformational change with proper modifiers. Either of the two active sites bound with the substrates promotes an open configuration,[15] the hydrophobic inhibitor site(s) promotes a closed conformation, and a site interacting

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with DTT promotes an open conformation.[15] We raise the possibility that the hydrophobic domains of some small proteins function as sites that control the activity of an enzyme by changes of conformation when complexed with low molecular weight hydrophobic metabolites. Sulfhydryl compounds, such as glutathione and cysteine, may also serve as modifiers of conformations and activity. Such control of enzyme activity would enhance the importance of AK as a putative control center for the energy charge of cells[39,40] with AK-1 isozymes.[2,41] It appears, then, that an enzyme of small dimensions has the possibility of the regulation of its activity in a manner analogous to allosteric modification.

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