Conformationally Restricted Creatine Analogues and Substrate Specificity of Rabbit Muscle Creatine Kinase†

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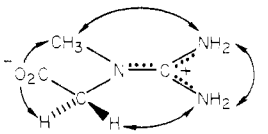
ABSTRACT: Several conformationally restricted analogues of creatine have been both synthesized and examined as potential substrates or inhibitors of rabbit muscle creatine kinase (EC 2.7.3.2). When an asymmetric center was included in a creatine analogue in the position α to the carboxyl group, the enzyme had a pronounced preference for the *R* enantiomer. Thus, whereas (*R*)-*N*-amidinoazetidine-2-carboxylic acid (**7**) has been shown to be a good substrate (K_s = 72 mM, K_m = 39 mM, and V_{max} = 29% relative to that of creatine) for creatine kinase, the corresponding *S* enantiomer **6** showed only barely detectable reactivity (V_{max}(rel) $\ll$ 1%). When the corresponding ring-opened analogue, *N*-methyl-*N*-amidinoalanine, was examined as a substrate, creatine kinase again showed a strong preference for the *R* enantiomer **9** [K_s = 94 mM, K_m = 82 mM, V_{max}(rel) $\simeq$ 10%]. The *R* enantiomer was approximately 7 times more reactive than its *S* enantiomer **8** when they were both examined as substrates at 40 mM in the presence of 4 mM ATP. On the other hand, the conformationally restricted creatine analogues *N*-[2-(4,5-dihydroimidazolyl)]sarcosine (**10**), 2-iminoimidazolidine-4-carboxylic acid (**11**), and 2-imino-3-methylimidazolidine-4-carboxylic acid (**12**) did not show detectable activity either as substrates or as inhibitors. Binding studies of the (*R*)-*N*-amidinoazetidine-2-carboxylic acid (**7**) and its corresponding *S* enantiomer **6** in dead-end complexes with MnADP and in anion-stabilized dead-end complexes revealed that the affinity of the enzyme for **7** was between one and two orders of magnitude greater than for its *S* enantiomer **6**. Unlike several other analogues of creatine, analogue **7** gave EPR spectra in the anion-stabilized complexes with MnADP that were virtually identical with those for creatine.

Creatine kinase (adenosine-5′-triphosphate:creatine phosphotransferase, EC 2.7.3.2) catalyzes the reversible transfer of a phosphoryl group from ATP to creatine. An important physiological function of the enzyme is the regeneration of ATP from phosphocreatine as the ATP is utilized during muscular contraction. It exists as a dimer of apparently identical subunits with a total molecular weight of 81 000 (Yue et al., 1967). Kinetic analyses have indicated that at pH 9, that used in this study, the reaction follows a rapid equilibrium, random, bimolecular, bimolecular scheme with phosphoryl transfer as the rate-limiting step (Morrison & James, 1965; Morrison & Cleland, 1966; Maggio et al., 1977).

In an attempt to provide information about the mechanism of action of creatine kinase at a molecular level, a series of analogues of creatine has been synthesized and examined as potential substrates for the creatine kinase catalyzed reaction (Rowley et al., 1971; McLaughlin et al., 1972). Substrate analogues can serve not only as probes of the active site to determine where steric bulk may or may not be tolerated, but also, by use of conformationally restricted analogues, information may be gained indicative of the preferred geometry of the substrate when bound to the active site of the enzyme. In conformationally restricted analogues, the different parts of the normal substrate molecule are tied together in rings, locking the molecules into particular conformations (Kenyon & Fee, 1973). In this study, we report the synthesis and the investigation of the interactions with creatine kinase of several conformationally restricted analogues of creatine. The four arrows in the structure shown below represent potential positions for the formation of rings to lock the basic creatine structure into particular conformations:



Results of kinetic studies with representatives of each of these four types of conformationally restricted analogues are presented. Results of electron paramagnetic resonance (EPR) studies of transition-state analogue complexes of the active and inactive enantiomers are also given, together with equilibrium binding measurements obtained by water proton relaxation enhancement titrations.

We also report a new high-yield synthesis of 2-(2-aminoethylamino)ethanoic acid, the synthetic precursor of the highly reactive, conformationally restricted creatine analogue, 1-carboxymethyl-2-iminoimidazolidine (**2**).

Experimental Procedures

Materials. ATP, creatine, and bovine serum albumin were all obtained from Sigma Chemical Co. Creatine kinase used in the analyses of compounds **7**, **11**, **12**, and **13** was isolated from fresh rabbit skeletal muscle as described by Kuby et al. (1954) and had a specific activity of ~110 μmol per min per mg of protein; the enzyme used in the analysis of compound **9** was purchased from Calbiochem, with a corresponding specific activity of ~60 μmol per min per mg of protein.

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The following synthetic procedures were used in the preparation of compounds utilized in this study. A carbon-13 NMR spectrum for each analogue, consistent with its proposed structure, is reported elsewhere (Dietrich et al., 1980).

(R)- and (S)-N-Amidinoazetidine-2-carboxylic Acid (**7** *and* **6**). (*R*)-Azetidine-2-carboxylic acid (0.5 g, 5 mmol) (Rodebaugh & Cromwell, 1969) and the corresponding *S* enantiomer (0.5 g, 5 mmol) (Sigma Chemical Co.) were each added to separate solutions of cyanamide (0.25 g, 6 mmol) dissolved in 0.4 mL of water followed by the addition of 2 drops of concentrated aqueous ammonia. After the solution was allowed to stand for 48 h at room temperature, the crystals that had formed were filtered to give 0.6 g (85% yield) of each enantiomeric product: mp 275–285 °C dec; $[\alpha]^{28.5}_D$ −321° (*S* enantiomer) and +318° (*R* enantiomer) (c = 2, water). Anal. Calcd for $C_5H_9N_3O_2$: C, 41.95; H, 6.34; N, 29.35. Found: (*S* enantiomer) C, 41.68; H, 6.27; N, 29.14; (*R* enantiomer) C, 41.96; H, 6.31; N, 29.22. The 100-MHz ^{1}H NMR spectrum (D_2O) showed peaks at δ 2.1–2.9 (2 H, complex multiplet), 4.04 (2 H, triplet, J = 7 Hz), and 4.81 (1 H, doublet of doublets, J = 5 and 6 Hz).

(R)- and (S)-N-Methyl-N-amidinoalanine (**8** *and* **9**). (*R*)- and (*S*)-*N*-methylalanine (Quitt et al., 1963) were treated exactly as described by Rowley et al. (1971) in the preparation of the racemic analogue. Recrystallization, however, was accomplished by careful precipitation of the final product from water by the addition of acetone. Each enantiomer was shown to have a 60-MHz ^{1}H NMR spectrum identical with that reported for the racemic compound, $[\alpha]^{28.5}_D$ −105° (*S* enantiomer) and +103° (*R* enantiomer) (c = 1, water). It should be noted that unless great care is taken in the purification of **8** and **9**, they will cyclize to the corresponding creatinine analogues (Rowley et al., 1971; Dietrich et al., 1980).

N-[2-(4,5-Dihydroimidazolyl)]sarcosine (**10**). Sarcosine (1.1 g, 12.4 mmol, Aldrich Chemical Co.) was ground to a fine powder with 2-methylmercapto-4,5-dihydroimidazole (2.9 g, 25 mmol), which in turn was prepared from the hydriodide salt (Aspinall & Bianco, 1951) by neutralization with sodium hydroxide and extraction of the free base into ethyl acetate. The mixture was then heated to 120 °C, and heating was continued for 0.5 h. The solid residue was triturated with acetone and filtered. The white solid was then dissolved in hot water, and acetone was added until turbidity was permanent. The white crystalline solid that formed was filtered to give 1.6 g (84% yield) of *N*-[2-(4,5-dihydroimidazolyl)]-sarcosine monohydrate, mp 240–250 °C dec (sinters 190–195 °C). Anal. Calcd for $C_6H_{11}N_3O_2 \cdot H_2O$: C, 41.13; H, 7.48; N, 23.99. Found: C, 41.15; H, 7.30; N, 23.98.

The 60-MHz ^{1}H NMR spectrum (D_2O) showed peaks at δ 3.06 (3 H, singlet), 3.77 (4 H, singlet), and 3.91 (2 H, singlet).

2-Iminoimidazolidine-4-carboxylic Acid (**11**). 2,3-Diaminopropionic acid hydrochloride (1.5 g, 10.6 mmol) (Dietrich et al., 1979) was dissolved in 3 mL of 7 N NaOH, and cyanogen bromide (1.3 g, 12.3 mmol) in 1.5 mL of absolute methanol was added dropwise to the stirred solution using a 2-mL syringe and a septum over a period of 1 h. After the solvent was stirred for an additional 8 h, it was removed in vacuo, and the solid residue was suspended in cold, concentrated ammonia. The white solid was filtered and crystallized by dissolution in the minimum amount of water, followed by the addition of 2 volumes of absolute ethanol. Filtration of the white crystalline solid gave 705 mg (52% yield) of 2-iminoimidazolidine-4-carboxylic acid, mp 275–285 °C dec. Anal. Calcd for $C_4H_7N_3O_2$: C, 37.21; H, 5.46; N, 32.54. Found: C, 37.27; H, 5.44; N, 32.63. The 60-MHz ^{1}H NMR spectrum (D_2O) showed an ABC pattern (δ 3.5–4.6) with a doublet of doublets centered at δ 4.48 (J = 6.5 and 10 Hz).

2-Imino-3-methylimidazolidine-4-carboxylic Acid (**12**). 3-Amino-2-methylaminopropionic acid monohydrochloride (1.0 g, 6.5 mmol) (Dietrich et al., 1979) was stirred in 2.0 mL of 6.5 N NaOH, and cyanogen bromide (0.7 g, 6.6 mmol) in 1 mL of methanol was added to the stirred suspension with a 2-mL glass syringe through a septum over a period of 1 h. Immediately after the cyanogen bromide addition was initiated, the stirred suspension was transformed into a yellow solution. As the addition was continued a precipitate appeared, and the suspension was stirred an additional 0.5 h after the last of the cyanogen bromide had been added. The precipitate was filtered and washed with methanol, cold concentrated ammonia, and again with methanol. The filtrate was concentrated in vacuo, suspended in cold concentrated ammonia, filtered, and washed with methanol. The two solids, identical according to their ^{1}H NMR spectra, were combined to give 573 mg (62% yield) of 2-imino-3-methylimidazolidine-4-carboxylic acid, mp 345–350 °C dec. Anal. Calcd for $C_5H_9N_3O_2$: C, 41.96; H, 6.34; N, 29.36. Found: C, 41.85; H, 6.30; N, 29.46. The 60-MHz ^{1}H NMR spectrum (D_2O) showed peaks at δ 3.0 (3 H, singlet) and a multiplet from δ 3.4 to 4.55 typical of an ABC pattern including a doublet of doublets centered at δ 4.37 (J = 7.0 and 10.5 Hz).

N,N′-Dibenzoyl-2-(2-aminoethylamino)ethanol. 2-(2-Aminoethylamino)ethanol (10 g, 96 mmol, Eastman Organic Chemicals) and anhydrous sodium carbonate (25 g) in 250 mL of benzene were stirred in a three-necked flask equipped with a dropping funnel, a condenser, and a thermometer. The stirred suspension was cooled to 8 °C, and benzoyl chloride (25 mL, 30.3 g, 215 mmol) in 100 mL of benzene was added sufficiently slowly that the temperature did not rise above 10 °C (~2 h). The stirred suspension was warmed to room temperature, stirred for 2 h, and then heated at reflux for an additional 2 h. The warm suspension was filtered and the white precipitate washed with three 100-mL portions of $CHCl_3$. The combined filtrates were taken to dryness, and the white solid residue was recrystallized from benzene to give 26 g (87% of theoretical) of *N,N′*-dibenzoyl-2-(2-aminoethylamino)ethanol, mp 133–134 °C. Anal. Calcd for $C_{18}H_{20}N_2O_3$: C, 69.21; H, 6.45; N, 8.97. Found: C, 69.34; H, 6.50; N, 8.94. The 100-MHz ^{1}H NMR spectrum ($CDCl_3$) showed peaks at δ 1.84 (1 H, broad singlet), 3.2–4.1 (8 H, broad multiplet), and 7.2–7.9 (11 H, aromatic multiplets and amide NH). The chemical ionization mass spectrum (isobutane reagent gas) showed the following peaks greater than 10% of the base peak: m/e 313 (M + 1, base), 295, 269, 191, 123, 105.

N,N′-Dibenzoyl-2-(2-aminoethylamino)ethanoic Acid. Potassium permanganate (2.2 g, 13.9 mmol) in 60 mL of water was added dropwise over a period of about 1 h to a stirred solution of *N,N′*-dibenzoyl-2-(2-aminoethylamino)ethanol (2.5 g, 8.01 mmol) in 25 mL of glacial acetic acid. The reaction temperature was maintained at 40 °C during the addition by gentle heating, and heating was continued for 1 h after addition was complete. At this point the excess permanganate and MnO_2 were converted to soluble salts by the addition of about 2 g of sodium bisulfite. The reaction mixture was concentrated in vacuo to dryness, 200 mL of water was added, and the pH was adjusted to approximately 8. Chloroform extraction of the crude mixture was followed by acidification of the aqueous fraction with 3 N HCl. The aqueous portion was cooled, and

the crystalline product that precipitated was filtered to give 1.7 g (65% yield) of *N,N'*-dibenzoyl-2-(2-aminoethylamino)-acetic acid, mp 137–138 °C. Anal. Calcd for $C_{18}H_{18}N_2O_4$: C, 66.25; H, 5.56; N, 8.58. Found: C, 66.09; H, 5.57; N, 8.57. The 100-MHz ^{1}H NMR spectrum showed peaks at δ 3.2 (6 H, broad multiplet) and 7.0–8.1 (12 H, aromatic multiplet, amide NH, and COOH). The chemical ionization mass spectrum (isobutane reagent gas) showed the following peaks greater than 10% of the base: *m/e* 310, 309 (M + 1 − 18, base), 205, 161, 123, 105.

2-(2-Aminoethylamino)ethanoic Acid Dihydrochloride. *N,N'*-Dibenzoyl-2-(2-aminoethylamino)acetic acid (800 mg, 2.45 mmol) was suspended in 15 mL of 6 N HCl and heated at reflux for 18 h. After cooling to room temperature, the aqueous solution was extracted with three 25-mL portions of ether to remove benzoic acid, and the aqueous portion was concentrated to dryness in vacuo. The residual white solid product, obtained in quantitative yield, was identical with 2-(2-aminoethylamino)acetic acid dihydrochloride prepared by an alternative method (Rowley et al., 1971), which is used in the preparation of the creatine analogue 1-carboxymethyl-2-iminoimidazolidine (**2**).

Methods. ^{1}H NMR spectra were determined on either a Varian A60A spectrometer or a Varian XL-100 spectrometer operating in the pulse mode at 100.1 MHz (as indicated), in 2–10% solutions in D_2O or $CDCl_3$, and are reported relative to internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) for the D_2O solutions and relative to internal tetramethylsilane (Me_4Si) for $CDCl_3$ solutions. Mass spectral measurements were made on an Associated Electronic Industries MS-902 spectrometer. Melting points are uncorrected, and microanalyses were obtained from the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley, CA. Optical rotations were measured on a Perkin-Elmer Model 141 polarimeter. Water proton longitudinal relaxation times were measured with a pulsed NMR spectrometer as described previously (Reed & Cohn, 1972). EPR spectra were obtained at 9.1 GHz with a Varian E-3 spectrometer.

Creatine kinase assays were performed on a Radiometer TT2 pH Stat using a modification of the procedure of Mahowald et al. (1962) at 30 °C. The pH was maintained at pH 9.00 by the addition of 2.50 mM NaOH. The magnesium ion concentration in the assay solution was so adjusted that free Mg(II) was held constant at 1.0 mM, and the total ionic strength of the assay solution was held constant at 50 mM by addition of the appropriate amounts of sodium acetate. The creatine kinase concentration was determined spectrophotometrically at 280 nm by using the extinction coefficient $\epsilon_{280} = 7.1 \times 10^4$ M^{-1} cm^{-1} (Noda et al., 1954).

The initial velocity data for the creatine analogues were analyzed by using the computer program SEQUEN generously supplied by Dr. W. W. Cleland and modified as described previously (Maggio et al., 1977).

The kinetic inhibition studies were carried out by comparing the observed initial rate for 15 mM creatine and 4 mM ATP in the presence and absence of 30 mM analogue.

Results

Kinetic Studies. The kinetic parameters for a series of conformationally restricted creatine analogues are summarized in Table I. For those analogues in which an asymmetric center has been included in the molecule α to the carboxylate function, and substrate activity has been retained, creatine kinase shows a strong preference for the *R* enantiomer. Compounds **4** and **6** both react more than 100 times slower than their corresponding *R* enantiomers, whereas **9**, which does not contain a ring but which does include an asymmetric center, was shown to react approximately 7 times faster than its *S* enantiomer **8** under identical conditions. Two other analogues including asymmetric centers (**11** and **12**) were shown to be ineffective as either substrates or inhibitors of rabbit muscle creatine kinase when examined as racemic mixtures. Compound **10** also acted neither as a substrate nor an inhibitor.

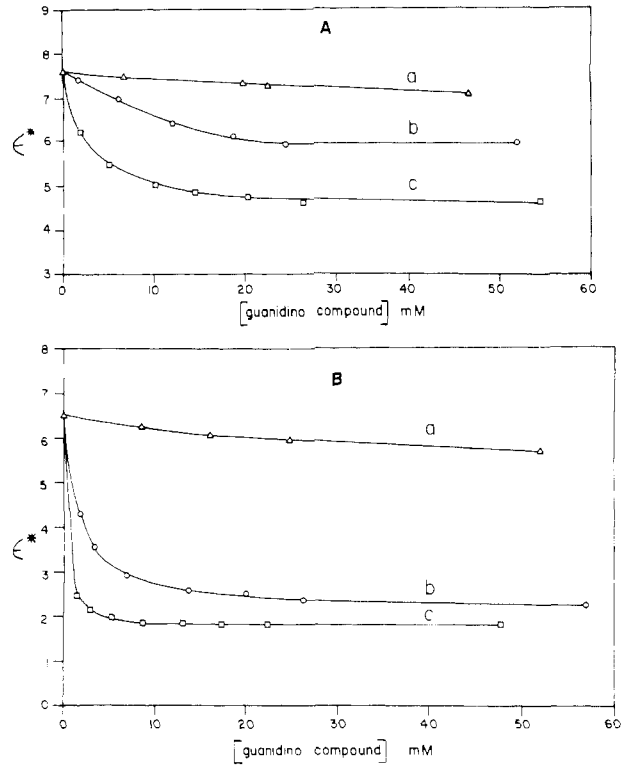


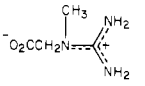
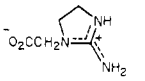
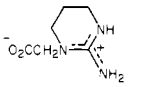
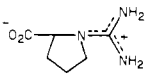
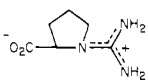
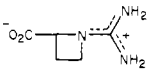
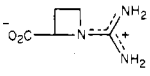
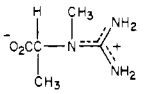
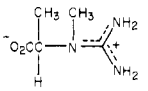
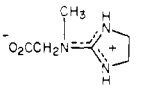
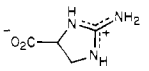
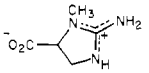
FIGURE 1: Water proton relaxation enhancement titrations for solutions of creatine kinase and MnADP titrated with a guanidino compound. In group A acetate was the counteranion, and in group B 33 mM KSCN was present as an additional component. The titrations were performed at 24.0 MHz at 5 °C. All solutions contained 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid–KOH, pH 8.0, 19 mg/mL creatine kinase, 270 μM ADP, and 100 μM Mn(acetate)$_2$. The solid curves were sketched through the data points: (a) titration with compound **6**; (b) titration with compound **7**; (c) titration with creatine.

The values of K_m reported for compounds **7** and **9** were determined using the computer program SEQUEN as described under Experimental Procedures. While the errors reported for the K_m values of **7** and **9** calculated from the computer program were quite large, ±39 and ±15%, respectively, a least-squares graphical approach yielded K_m values identical with those determined by the computer program.

Binding Studies. Binding of creatine, the *R* enantiomer **7**, and the *S* enantiomer **6** to the MnADP complex of creatine kinase was followed in titration experiments by the change in the enhancement[1] of the water proton relaxation rate upon formation of the dead-end complex. Representative results of such titration experiments are given in Figure 1. The titration curves were analyzed by a least-squares method (Reed et al., 1970), and the dissociation constants are collected in Table II.

[1] The observed enhancement, ϵ^*, is defined as the ratio of the paramagnetic contribution to the longitudinal relaxation rate of water protons in the presence of complexes of Mn(II) to the corresponding rate in the absence of complexing agents (Mildvan & Cohn, 1970).

Table I: Comparison of Kinetic Parameters of Conformationally Restricted Creatine Analogues

no.	substrate	structure	rel V_{max}	K_m[a] (mM)	K_s[a] (mM)
1	creatine		(100)[b]	8.61 ± 0.57	24.4 ± 5.5
2	1-carboxymethyl-2-iminoimidazolidine		90[c]	25	
3	1-carboxymethyl-2-iminohexahydropyrimidine		n.d.[d,e]		
4	(*S*)-*N*-amidinoproline		n.d.[c]		
5	(*R*)-*N*-amidinoproline		0.9[c]	100	
6	(*S*)-*N*-amidinoazetidine-2-carboxylic acid		n.d.		
7	(*R*)-*N*-amidinoazetidine-2-carboxylic acid		29.0 ± 1.5	39.0 ± 5.8	71.6 ± 25.1
8	(*S*)-*N*-methyl-*N*-amidinoalanine		*e*		
9	(*R*)-*N*-methyl-*N*-amidinoalanine		10.0 ± 0.5	82.2 ± 32.8	93.5 ± 18.4
10	*N*-[2-(4,5-dihydroimidazolyl)] sarcosine		n.d.		
11	(*R,S*)-2-iminoimidazolidine-4-carboxylic acid		n.d.		
12	(*R,S*)-2-imino-3-methylimidazolidine-4-carboxylic acid		n.d.		

[a] K_m and K_s are the binding constants to the enzyme in the presence and absence of ATP, respectively (Morrison & James, 1965). [b] From Maggio et al. (1977), pH 9.00 at 30 °C. [c] From McLaughlin et al. (1972), pH 9.0 at 1.0 °C; error limits not reported. [d] n.d. = nondetectable either in coupled assay (for discussion of this assay, see McLaughlin et al. (1972)) or pH stat assay (see Experimental Procedures). [e] Compounds **3** and **8** were shown to be substrates in initial rate studies, but of such low reactivity that kinetic parameters were not determined.

EPR Studies. The EPR spectrum of Mn(II) in the anion-stabilized dead-end complexes with creatine kinase has been shown to be a sensitive indicator of structural perturbations at the active site of the enzyme (Reed & Cohn, 1972; Reed & McLaughlin, 1973; McLaughlin et al., 1976). In particular, well-resolved spectra that were characteristic of the complexes with creatine were not obtained with a series of analogues of creatine, some of which had substantial substrate activity (McLaughlin et al., 1976). EPR spectra for solutions of the nitrate and of the thiocyanate complexes of the three guanidino compounds are compared in Figure 2. With both anions the active *R* enantiomer **7** gives an EPR spectrum which is very similar to that of the corresponding complex with creatine. In contrast, a significant change in the EPR line shape occurs only with thiocyanate when the essentially inactive *S* enantiomer **6** is present. The results with thiocyanate demonstrate that the inactive *S* enantiomer **6** can form a complex with the enzyme; however, the spectroscopic properties of this complex differ from those for the complexes with viable substrates.

Discussion

As can be seen in Table I, a wide variety of substrate analogue activities is observed from relatively minor changes in the structure of the normal substrate, creatine. This wide range of activities not only can permit predictions about the three-dimensional structure of creatine bound to the active site of creatine kinase, but also can give information about bulk tolerances of various regions of the creatine binding site.

Compound **2** was shown by McLaughlin et al. (1972) to be a very good substrate for creatine kinase, with a V_{max} 90% that of creatine and a relative V_{max}/K_m about 3 times lower than that of creatine. The addition of a single methylene group, expanding the five-membered ring to a six-membered ring, almost completely destroys activity. Rowley et al (1971) show that **3** reacts considerably slower than **2** in initial rate studies, indicating very little tolerance for extra bulk about the planar guanidinium group.

Comparison of the *R* enantiomers of the four- and five-membered ring analogues shows a similar sensitivity to the addition of steric bulk in the region of the creatine molecule between the α carbon and the *N*-methyl group. (*R*)-*N*-Amidinoazetidine-2-carboxylic acid **7** was shown to have a relative V_{max}/K_m about 15 times smaller than that of creatine, but, in turn, approximately 80 times larger than that of the corresponding five-membered ring analogue, compound **5**.

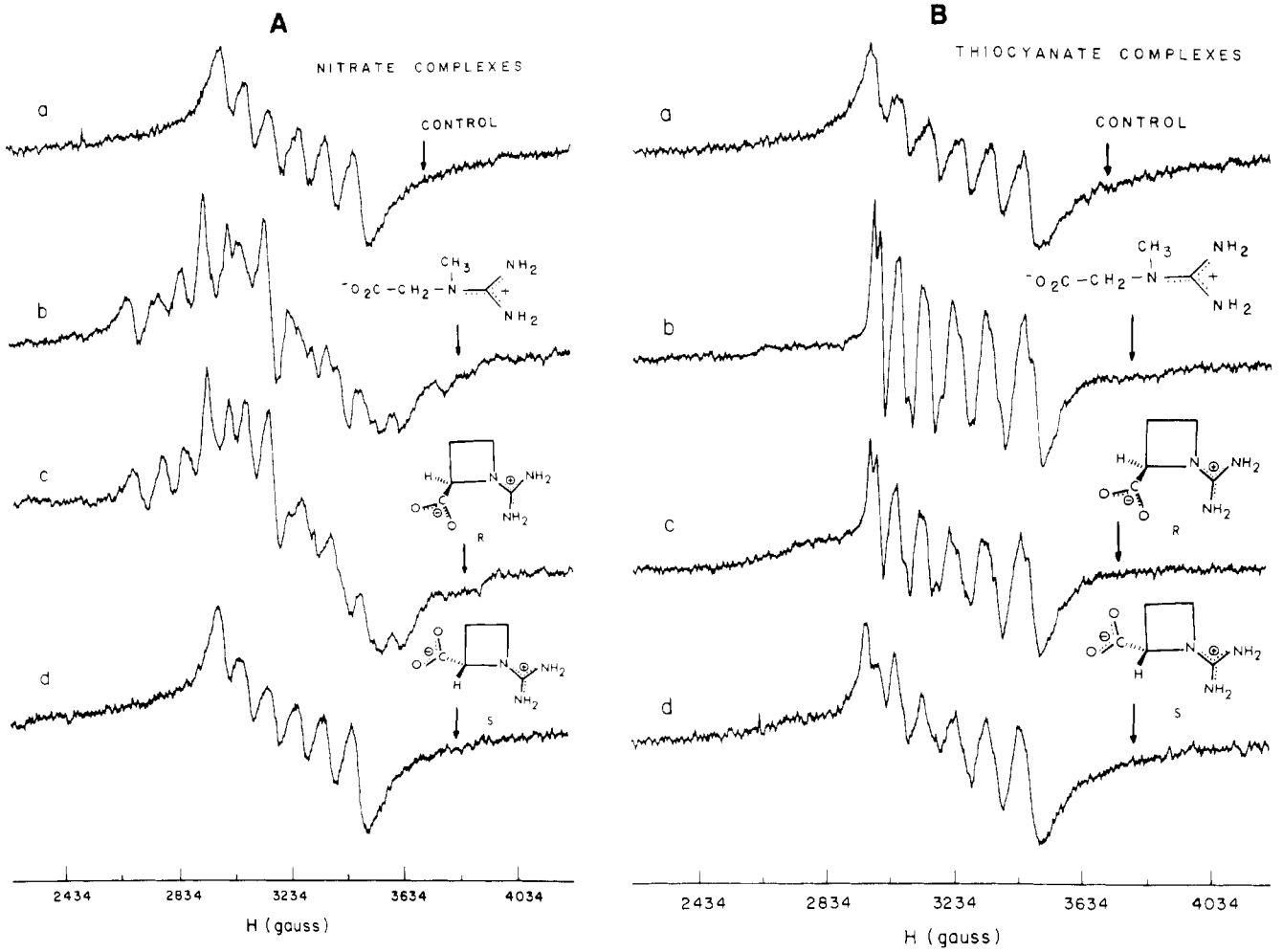


FIGURE 2: EPR spectra for solutions of creatine kinase and MnADP with and without guanidino compounds present. The solutions were buffered with 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid–KOH, pH 8.0. Spectra were recorded at 3 °C. All solutions contained 75 mg/mL creatine kinase, 1.9 mM ADP, and 0.5 mM $Mn(acetate)_2$. The potassium salts of nitrate and thiocyanate were present at 25 mM in groups A and B, respectively. The guanidino compounds were present in amounts sufficient to give a saturated solution (100 mM). Part a in each case shows the spectrum of a solution of enzyme·MnADP in the presence of the anion but in the absence of a guanidino compound (control).

Even more interesting is the stereospecificity observed for the *R* enantiomers **5** and **7**. Rowley et al. (1971) report that **5** reacts about 100 times faster than its corresponding *S* enantiomer **4**, and we found the selectivity for the *R* enantiomer of the azetidine analogue to be even greater. Detection of product formation for **6** was possible only by using the extremely sensitive poly(ethylenimine)-cellulose thin-layer chromatographic assay devised by Rowley & Kenyon (1974). In both cases, the low reactivity observed for the *S* enantiomer **6** might be due to a small amount of contamination (<1%) by the corresponding *R* enantiomer **7**. This pronounced selectivity for the *R* enantiomer of these two analogues considerably limits the number of potential conformations for creatine when bound to creatine kinase.

While compounds **8** and **9**, the enantiomers of *N*-methyl-*N*-amidinoalanine, are not conformationally restricted analogues of creatine in the usual sense since they contain no rings, it is interesting to compare them to the four-membered ring analogues **6** and **7**, which contain the same number of carbon atoms. Once again, the *R* enantiomer is more reactive than the *S* counterpart, but, in this case, the difference is not nearly so great as in the case of the azetidines. At 40 mM analogue concentration, the *R* enantiomer **9** was shown in initial rate studies to react approximately 7 times faster than its *S* counterpart. When the relative V_{max}/K_m values of the *R* enantiomers **7** and **9** are compared, the rigid, four-membered ring analogue was shown to be a better substrate by a factor of about six. The added flexibility of **8** and **9** decreases the relative selectivity for the *R* enantiomer, as compared to the other two active analogues with asymmetric centers. The higher K_m value of **9** (82.2 mM compared to 39.0 mM for **7**) may be a reflection of the higher energy of the conformer of **9** in which the two methyl groups are eclipsed. This eclipsed conformer would have a three-dimensional structure closest to that of (*R*)-*N*-amidinoazetidine-2-carboxylic acid **7**, but, because of steric repulsions of its methyl groups, might represent only a small percentage of the total conformational population present in solution.

It is also interesting to compare the synergistic effects of metal–nucleotide binding for the normal substrate, creatine, and analogues **7** and **9**. This synergism is reflected by the ratios of the K_s and K_m values, the binding constants in the absence and presence of nucleotide (ATP), respectively (Morrison & James, 1965). For creatine binding, K_s/K_m is 2.8, indicating a strong synergistic effect. The K_s/K_m values for **7** and **9**, respectively, are only 1.8 and 1.1. Thus, while compound **7** is still strongly affected by ATP binding, and vice versa, compound **9** is nearly unaffected. Presumably, the conformational change produced upon substrate binding (Reed & Cohn, 1972) is not so effectively produced by the poorer

Table II: Dissociation Constants for Guanidino Component of Dead-End Complexes with Creatine Kinase and MnADP[a]

compound	anion	K_D (mM)[b]	ϵq[c]
creatine (1)	acetate[d]	2.0	4.5
	nitrate	0.06	2.0
	thiocyanate	0.2	1.8
(*R*)-amidinoazetidine-2-carboxylic acid (7)	acetate[d]	6.7	5.7
	nitrate	0.7	2.0
	thiocyanate	1.8	1.8
(*S*)-amidinoazetidine-2-carboxylic acid (6)	acetate[d]	>75[e]	*e*
	nitrate	>75[e]	*e*
	thiocyanate	>75[e]	*e*

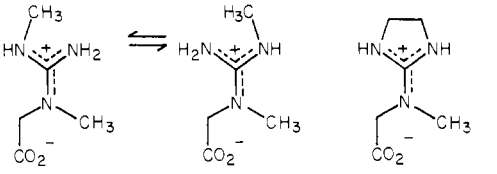
[a] Measurements were at 5 °C. The compositions of the solutions for the titrations with acetate present and with thiocyanate present are given in the legend for Figure 1. Solutions for the titrations in the presence of nitrate were identical except that 33 mM KNO_3 replaced KSCN. [b] Error in the dissociation constant is estimated to be of the order of ±20%, since changes of this order led to deviations between theoretical and experimental points that were outside of experimental uncertainties. [c] ϵq is the characteristic enhancement factor for water proton relaxation in the presence of the complex. The standard error of the mean of ϵq from the least-squares fit was less than 1% where values are given. [d] Acetate ion does not stabilize the dead-end complex. [e] The changes in ϵ^* upon addition of this compound to solutions of enzyme MnADP were too small to obtain a reliable estimate of this parameter.

substrate. It should also be noted that a similar loss of synergism in substrate binding was observed when the kinetic consequences of the presence of a CH_3S-blocking group on the active sulfhydryl group of the enzyme was examined (Maggio et al., 1977).

Results of the NMR binding studies and of EPR measurements confirm the high degree of selectivity of creatine kinase for the *R* enantiomer of *N*-amidinoazetidine-2-carboxylic acid. Although the *R* enantiomer **7** binds to the enzyme with a lower affinity than does creatine, the water proton relaxation enhancement factors and EPR spectra for the anion-stabilized complexes of creatine and of compound **7** are nearly identical. These observations point to a high degree of homology among the enzymatic complexes of the two compounds. In fact, compound **7** is unique among the several analogues of creatine that have been investigated thus far in giving an EPR spectrum for the nitrate complex that is virtually identical with that of the corresponding complex with creatine. However, this apparent structural homology between creatine and compound **7** does not correlate directly with the activity of the analogue as a substrate, since compound **2**, which has higher substrate activity than **7** (McLaughlin et al., 1972), gives an EPR spectrum in the nitrate-stabilized complex in which there is much poorer resolution of individual transitions (Reed & Cohn, 1972).

There is spectroscopic evidence that the anions, nitrate and thiocyanate, are bound to Mn(II) in the dead-end complexes of creatine kinase (Reed et al., 1978). Thus, the electronic environment of the Mn(II) is directly influenced by the anion. Based on NMR relaxation measurements (McLaughlin et al., 1976), it does not appear that the guanidino substrate has a direct interaction with the divalent cation. Hence, the influence of changes in the molecular structure of the guanidino substrate on the quality of the EPR spectra for Mn(II) in the anion-stabilized complexes is probably an indirect effect that is mediated by the protein. Broadening of the EPR signals for Mn(II) in such complexes is likely a result of a microheterogeneity of the sample population. Thus, there is a distribution of zero-field-splitting parameters (which determine the resonance positions) for Mn(II), which stem from subtle variations in coordinate bond lengths or bond angles of first-sphere ligands (Meirovitch & Poupko, 1978). The high quality of spectra for the anion-stabilized complexes with compound **7** may result in part from the lack of conformational flexibility of the molecule as well as from a close resemblance of the molecule to the active conformation of the natural substrate, creatine.

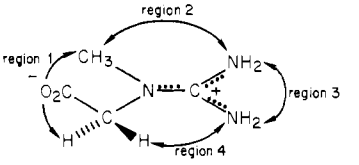
Compounds **10**, **11**, and **12** showed no detectable activities as substrates for creatine kinase when examined using the highly sensitive poly(ethylenimine)-cellulose thin-layer chromatography assay, which is able to detect product formation for analogues that react ~10000 times more slowly than creatine (Rowley & Kenyon, 1974). The fact that compound **10** is neither a substrate nor an inhibitor of creatine kinase is in agreement with similar findings (Rowley et al., 1971) for *N*-methylcreatine (*N*-methylamidino-*N*-methylglycine, **13**). In this compound, the methyl group was shown (Kenyon et al., 1976) largely to occupy the positions analogous to the two ring methylenes of **10**:



13 **10**

The other two conformationally restricted analogues that do not function as either substrates or inhibitors, **11** and **12**, indicate an additional region around the normal substrate where steric bulk is not tolerated. Addition of a single carbon atom and the formation of a five-membered ring on the side of the molecule opposite the *N*-methyl group of creatine completely eliminate observable binding to creatine kinase.

From these data, we are able to propose what we believe to be a reasonable three-dimensional picture of creatine as it



is bound to creatine kinase, and, moreover, we can pinpoint regions where steric bulk, in the form of methylene groups, can and cannot be tolerated. It should be emphasized that this picture represents an absolute stereochemical projection.

When single methylene groups are added to regions 1 and 2, and four- and five-membered rings are formed, good substrate activity is retained. The addition of methylene groups to regions 3 and 4 destroys all detectable substrate binding.

These results are in agreement with the very tight steric requirements in the region of the active site for the binding of creatine proposed by Rowley et al. (1971). Once the crystal structure of creatine kinase is determined, these analogues should be valuable in the investigation of the binding of creatine to the enzyme and, ultimately, in the elucidation of the mechanism of the transfer of the phosphoryl group from ATP.

Acknowledgments

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Mechanism of Action of Plasma Amine Oxidase Products Released under Anaerobic Conditions†

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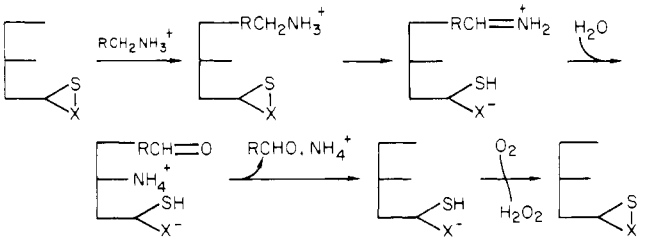
ABSTRACT: A mechanism that we have proposed for bovine plasma amine oxidase predicts that under anaerobic conditions (single turnover) 1 mol of benzaldehyde and 1 mol of NH_4^+ are produced from benzylamine. Other works have reported experiments with amine oxidases from other sources that show that NH_4^+ is not released under anaerobic conditions. The amount of NH_4^+ and benzaldehyde released when plasma amine oxidase acts on benzylamine under anaerobic conditions has been determined to resolve this discrepancy. It was found that 1 mol of NH_4^+ and 1 mol of benzaldehyde are released per active site. This result is consistent with the mechanism that we have proposed but is inconsistent with other mechanisms that invoke pyridoxal as an active-site component of amine oxidase.

Bovine plasma amine oxidase, a Cu^{2+}-containing protein, catalyzes the oxidation of primary amines (eq 1). Similar

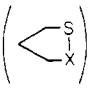
$$RCH_2NH_3^+ + O_2 + H_2O \rightarrow RCHO + H_2O_2 + NH_4^+ \quad (1)$$

enzymes have also been isolated from other sources. It is generally agreed that the reaction is a two-step process involving, first, the reduction of the enzyme by the substrate and then the oxidation of the reduced enzyme by O_2. There is, however, considerable disagreement concerning the immediate product that results from the oxidation of the substrate and the nature of the group on the enzyme that becomes reduced. We have proposed a mechanism for plasma amine oxidase, as shown in Scheme I (Suva & Abeles, 1978). The active site

Scheme I



of the enzyme contains an as yet incompletely identified structure



which can function as an electron acceptor. The sulfur component of this structure is provided by cysteine, which is bonded to an unidentified atom. Reaction of the amine substrate with enzyme results in the reduction of the prosthetic group and formation of an imine. The imine is hydrolyzed on the enzyme

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