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Kinetics of Slow Reversible Inhibition of Human Muscle Creatine Kinase by Planar Anions[1]

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The toxicity of NO_3^- and NO_2^- to mammals has been widely publicized. However, the kinetic mechanism of inhibition of human muscle creatine kinase by NO_3^- and NO_2^- has not been explored. The kinetic theory of the substrate reaction during the modification of enzyme activity previously described by Tsou (*Adv. Enzymol. Related Areas Mol. Biol.* 1988, 61, 381-436) has been applied to a study of the kinetics of slow reversible inhibition of human muscle creatine kinase by planar anions (NO_3^- and NO_2^-). The kinetic equation of the substrate reaction was derived from theoretical analysis and experimental data, then simplified. The microscopic rate constants for the reaction of the inhibitors with the enzyme were obtained from the simplified equation for the substrate reaction in the presence of the inhibitors. The results show that the apparent forward rate constant *A* is dependent on ATP concentration, indicating competition between the inhibitor (NO_3^- or NO_2^-) and ATP. The results also suggest that binding of creatine-MgADP and the anion with the enzyme is very tight, since their binding constants are much higher than those for normal substrates.

Key words: human creatine kinase, inactivation, kinetic analysis, phosphotransferase.

Creatine kinase (ATP: creatine *N*-phosphotransferase, EC 2.7.3.2) is an important enzyme which participates in cellular energy metabolism and catalyzes the reversible transfer of the phosphoryl group from MgATP to creatine, forming phosphocreatine and MgADP (*1*). The inhibition of rabbit muscle creatine kinase activity by simple inorganic anions is well known. Milner-White and Watts (*2*) reported that planar anions can form a stable and inactive quaternary enzyme-creatine-MgADP-anion complex. Formation of this complex, particularly in the forward reaction, can lead to marked inhibition of enzyme activity. They suggested that, in the course of the reaction, the tetrahedral phosphate-binding site for the transferable phosphoryl group of the substrate changes into a trigonal bipyramid site (also occupied by planar anions). A variety of anions can be used as probes in investigating the geometry of the anion-binding site and its relation to the mechanism of the enzyme action. Recently, Johnson *et al.* (*3*) observed that creatine kinase activity in the blood serum decreased rapidly after $NaNO_2$ was injected into the body of a cat. Matyushichev *et al.* (*4*) reported the effects of KNO_3 on the activity of creatine kinase in rat skeletal and kidney muscles after extended absorption of KNO_3. Kinetics analysis is an important tool in exploring the mechanism controlling enzyme activity. Recently, the kinetic theory of the substrate reaction during the modification of enzyme activity described by Tsou (*5-7*) has been applied to some complicated enzyme activity inhibition courses. Experimental studies have produced useful results not only for enzymes with a single substrate in both irreversible inhibition and slow reversible inhibition (*8, 9*), but also for enzymes involving two substrates in irreversible inhibition (*10, 11*). Since human enzymes are of more interest to medical science than enzymes from other creatures, HMCK (human muscle creatine kinase) was used in the present kinetic investigation. This paper studies the complete kinetics course of planar anion inactivation, in which the phosphorylation of creatine was monitored in the presence of planar anions (NO_3^- and NO_2^-). The simplified equation for the substrate reaction was used to determine the microscopic rate constants for the inhibition of enzyme activity by NO_3^- and NO_2^-. The mechanism of enzyme activity inhibition by planar anions is also discussed.

MATERIALS AND METHODS

The preparation of human muscle creatine kinase was as previously described by Ritter *et al.* (*12*), and the enzyme assay was as described by Yao *et al.* (*13*). The reaction of ATP with creatine, which results in the release of a proton, was followed by measurement of the absorbance change at 597 nm of the pH indicator thymol blue. The 1-ml reaction mixture contained 24 mM creatine, 4 mM ATP, 5 mM $MgAC_2$, and 0.01% thymol blue, in 5 mM glycine-NaOH buffer, pH 9.0. The reaction mixture was carefully adjusted to pH 9.0 before use, and a calibration curve was constructed to correlate the number of protons generated with the absorbance change at 597 nm under the above conditions. The enzyme concentration was determined by measuring

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Abbreviations: CK, creatine kinase; HMCK, human muscle creatine kinase; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid).

the absorbance at 280 nm with the absorption coefficient $A^{1\%}_{1cm} = 8.8$ (*1*). ATP was obtained from Serva, creatine from Fluka, and the other chemicals were local products of analytical grade.

The kinetics of the inactivation reaction were followed by the substrate reaction in the presence of the modifier as described by Tsou (*5-7*). To 1 ml of the above reaction mixture containing different concentrations of NO_3^- or NO_2^- was added 5 μl of 54 μM human muscle creatine kinase. The progress curve for the substrate reaction was analyzed as discussed below to obtain the rate constants. All measurements were carried out with a Perkin-Elmer Lambda Biospectrophotometer at 25°C.

RESULTS

1. Reaction Mechanism of Human Muscle Creatine Kinase Catalysis in the Presence of NO_3^- or NO_2^-—The courses of inactivation of creatine kinase by NO_3^- (or NO_2^-) in the presence and absence of ADP were very similar, indicating that the amount of ADP generated in the initial phase of the reaction was enough to form the transition-state analogue of the quaternary complex. The time courses of the substrate reaction for HMCK in the presence of different concentrations of NO_3^- are shown in Fig. 1. At each concentration of NO_3^-, the rate of product formation decreases with increasing time until a straight line is approached. Therefore, the inhibition of creatine kinase activity by NO_3^- is a slow reversible inhibition. Milner-White and Watts (*2*) and Nihei *et al.* (*14*) both suggested that planar anions act in the quaternary complex by simulating the transferable phosphoryl group in the transition state of the reaction. Thus we conjectured that HMCK would not bind with ATP after binding with NO_3^- or ADP-NO_3^-. However, in the enzyme active site, the creatine-binding site is located at a position different from the NO_3^--binding site. Therefore, the presence of NO_3^- does not affect the binding of the enzyme with creatine. This conjecture was verified by the following experiments. With fixed concentrations of the inhibitors and the other components in the assay reaction mixture, decreasing the creatine concentration to half of its initial concentration did not change the rate of product formation. However, decreasing the ATP concentration caused the rate of product formation to decrease markedly. Figure 2 shows the effects of creatine and ATP concentration on the rate of substrate reaction for enzyme activity in the presence of NO_3^-. This activity-measuring method was developed by Yao *et al.* (*13*). The two substrate concentrations were much higher than the value of K_m, and control experiments showed that changes of either substrate concentration in the experiment did not affect the calibration. The result (Fig. 2) verified the conjecture that the presence of NO_3^- does not affect the binding of the enzyme with creatine. Similar results were obtained in the presence of NO_2^-, indicating that NO_2^- inhibits the enzyme similarly to NO_3^-. Therefore, the substrate reaction mechanism for enzyme activity in the presence of planar anions can be simplified. The reaction scheme can be written as in Scheme 1, where E, Y, and Cr are the enzyme, inhibitor, and creatine; P and Q are the products; and E·ATP, E·Cr, E·Y, E·Cr·Y, and E·ATP·Cr, are complexes.

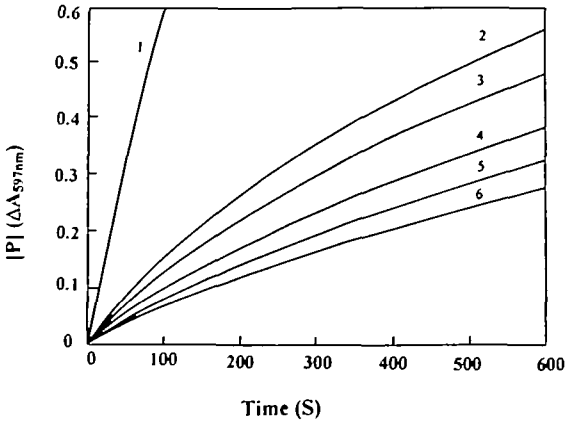


Fig. 1. **Courses of substrate reaction for human muscle creatine kinase in the presence of different concentrations of $NaNO_3$.** The final HMCK concentration was 0.27 μM. The mixtures were incubated at 25°C. The concentrations of $NaNO_3$ in the reaction mixture for curves 1-6 were 0, 52.5, 75, 112.5, 150, and 187.5 mM, respectively.

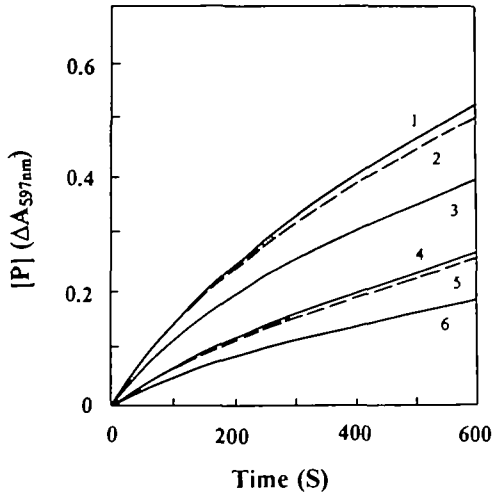
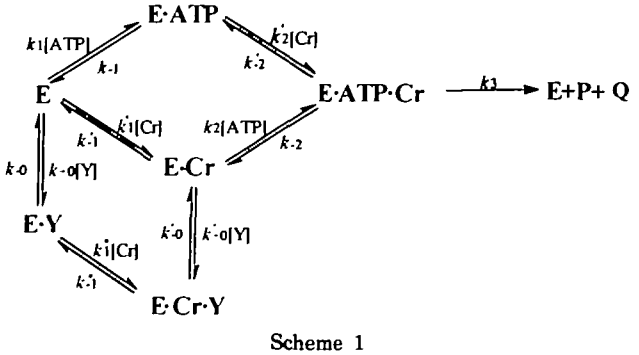


Fig. 2. **Effects of creatine and ATP concentrations on the progress courses of the substrate reaction in the presence of fixed $NaNO_3$ concentrations.** The experimental conditions were as for Fig. 1 except for the $NaNO_3$ concentrations. The $NaNO_3$ concentration was 52.5 mM for curves 1-3, and 187.5 mM for curves 4-6. The concentrations of creatine and ATP were respectively 24 and 4 mM for curves 1 and 4, 12 and 4 mM for curves 2 and 5, and 24 and 2 mM for curves 3 and 6.



Scheme 1

An enzyme reaction involving two substrates with random substrate binding sequence in rapid equilibrium for creatine kinase is shown in Scheme 1. The kinetic equation was deduced as briefly outlined in the Appendix. The product concentration is given by

$$[P]=\frac{Bv}{A[Y]+B}t+\frac{A[Y]v}{(A[Y]+B)^2}-\frac{A[Y]v}{(A[Y]+B)^2}e^{-(A[Y]+B)t} \quad (1)$$

where [P] is the product concentration formed at time t, v is the initial rate of the substrate reaction in the presence of inhibitors, and A and B are the apparent rate constants for the forward and reverse reactions between inhibitors and enzyme. [Y] is the concentration of the inhibitors.

2. Determination of Microscopic Rate Constants for the Reaction of Inhibitors with the Enzyme—In Fig. 1, the curves relating the product concentration and reaction time agree with Eq. 1. When the reaction time t is sufficiently long, the curves become straight lines:

$$[P]_{calc}=\frac{Bv}{A[Y]+B}t+\frac{A[Y]v}{(A[Y]+B)^2} \quad (2)$$

The slope, s, and the x-axis intercept, i, are

$s=vB/(A[Y]+B)$

$i=A[Y]/B(A[Y]+B)$

The apparent rate constants for the forward reaction, A, and the reverse reaction, B, can be obtained from the values of s and i at different inhibitor concentrations.

Alternatively, subtraction of Eq. 1 from Eq. 2 gives

$$[P]_{calc}-[P]=\frac{A[Y]v}{(A[Y]+B)^2}e^{-(A[Y]+B)t} \quad (3)$$

where $[P]_{calc}$ and [P] are the product concentrations extrapolated from the straight line portions of the curves as calculated from Eq. 2, and the actual observed values.

Equation 3 can be rearranged to give:

$$\ln([P]_{calc}-[P])=\ln\left\{\frac{A[Y]v}{(A[Y]+B)^2}\right\}-(A[Y]+B)t \quad (4)$$

Transformation of the data in Fig. 1 into Fig. 3 was executed in three steps: (1) From Fig. 1, the curve becomes a straight line for large t, and this line can be extended back to the y-intercept. (2) Subtract the data on the kinetic curve from the data on the straight line to obtain $[P]_{calc}-[P]$. (3) Then plot $\ln([P]_{calc}-[P])$ *versus* t (Fig. 3). Plots of $\ln([P]_{calc}-[P])$ *versus* t give a series of straight lines at different concentrations of inhibitor Y with slopes $-(A[Y]+B)$, as shown in Fig. 3. Figure 4 shows plots of $(A[Y]+B)$ *versus* [Y], which can be used to determine the apparent forward and reverse rate constants A and B. The value of B directly gives the microscopic rate constant (k_{-0}) for the reverse reaction. The value of k_{+0} can be obtained from suitable plots of A *versus* [ATP]. Figure 4 shows that the apparent forward rate constant A is dependent on ATP concentration. A plot of $1/A$ *versus* [ATP], Fig. 5, gives a straight line with a positive intercept on the y-axis, indicat-

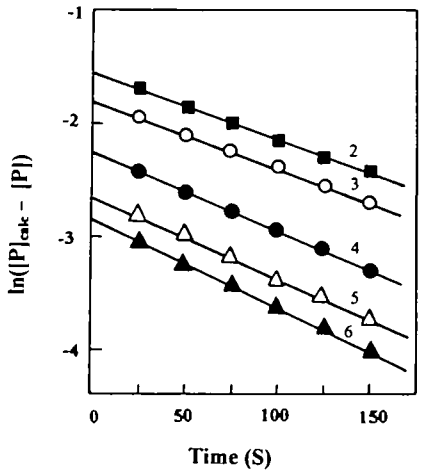


Fig. 3. **Semilogarithmic plots of ($[P]_{calc}-[P]$) *versus* time for the substrate reaction from curves 2-6 of Fig. 1.**

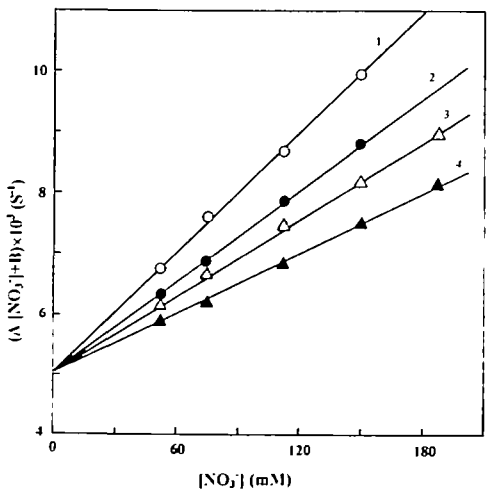


Fig. 4. **Determination of the microscopic rate constant k_{-0} for inactivation by $NaNO_3$.** The ATP concentrations were 1.0, 2.0, 3.0, and 4.0 mM for curves 1-4. The other conditions are given in Fig. 1. The intercept is k_{-0} and the slopes are the apparent rate constants, A.

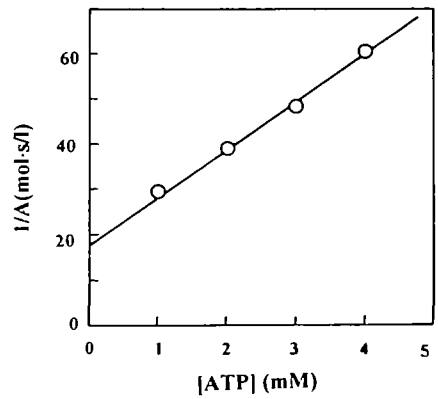


Fig. 5. **Determination of microscopic rate constant k_{+0} for inactivation by $NaNO_3$.** The intercept is $1/k_{+0}$.

TABLE I. **Microscopic rate constants for the reaction of creatine kinase with NO_3^- or NO_2^-.**

Inhibitor	k_{+0} ($s^{-1}\cdot M^{-1}$)	k_{-0} (s^{-1})
NO_3^-	0.056±0.002	0.005±0.0003
NO_2^-	0.250±0.006	0.005±0.0006

ing competition between NO_3^- and ATP. The microscopic rate constant can be obtained by plotting $1/A$ *versus* [ATP]. Similarly, the microscopic rate constant for NO_2^- can also be obtained. The results are in satisfactory agreement with those obtained by the plot of $1/A$ *versus* [Y], Table I.

DISCUSSION

The kinetic theory of the substrate reaction during the irreversible inhibition of enzyme activity described by Tsou (*5-7*) has been applied to study the kinetics of the course of slow reversible inhibition of an enzyme involving two substrates. Although the reaction scheme and kinetic equation for inactivation of HMCK are very complex, Tsou's kinetic theory can be satisfactorily applied with suitable simplifications. In the present paper, the kinetic equation of substrate reaction of HMCK in the presence of planar anions has been derived from the theoretical analysis and experimental results and then simplified. The microscopic rate constants for the reaction of the inhibitor with the enzyme were obtained through suitable plots. Therefore, Tsou's kinetic theory and method can be applied to the kinetic course of irreversible inhibition with a complex reaction mechanism by applying suitable simplifications.

Although NO_3^- and NO_2^- compete with ATP for the binding site at the enzyme active center, they are not simple competitive inhibitors of ATP. The results (Fig. 2) show that NO_3^- competes with MgATP for the binding site at the enzyme site and has no marked effect on the binding of creatine. At the start of the reaction, in the absence of the product ADP, the enzyme was inhibited by NO_3^-. However, the product MgADP can be bound into the active site of the enzyme to form an E-MgADP-NO_3^- complex easily and then form an E-MgADP-NO_3^--Cr quaternary complex. Formation of this complex can lead to nonlinear enzyme progress curves. It is known that transphosphorylation involves direct transfer of a phosphoryl group by an S_N2 type reaction, with a γ-phosphoryl group forming a planar sp^3d hybrid in the transition state (*2*). NO_3^- has a similar hybrid state. Thus the NO_3^- ion in combination with creatine and MgADP can inhibit enzyme activity by mimicking this transition state complex. The distance between creatine and the MgADP line is between 2.8 and 4.0 Å, which is approximately equal to the space required for a tetrahedral phosphoryl group to invert *via* the sp^3d hybrid and is approximately equal to the space required by NO_3^- and NO_2^- (*2*). The present results also show that the inhibition of enzyme activity by NO_2^- is stronger than this by NO_3^-. This can be expected since NO_2^- is smaller than NO_3^-, and thus steric hindrance by NO_2^- is smaller than that by NO_3^-. In addition, NO_2^- as an oxidizing agent can also lead to inactivation of the enzyme.

In the beginning phase of the reaction, the creatine concentration is high, whereas the MgADP concentration increases gradually. Therefore, the course of product formation can be plotted over time. The reaction rate gradually decreases with increasing reaction time. Because MgADP and MgATP compete for the same binding site on the enzyme, formation of the quaternary enzyme-creatine-MgADP-anion complex in the forward reaction is favored when the initial MgATP concentration is lowered. In this condition, the reaction curve has much greater curvature.

Many recent reports show that the binding of NO_3^-, creatine, and MgADP as components of the enzyme-creatine-MgADP-anion complex is much stronger than the binding of usual substrates. In the equilibrium state, the quaternary complex as a transition-state analogue occupies most of the enzyme binding sites (*15*). These facts show that the transition-state analogue simulates the transition-state complex between enzyme and substrate. Formation of this analogue could lead to conformational change of the enzyme molecules. For example, studies on creatine kinase from mitochondria have shown that the formation of the transition-state analogue of the enzyme by adding 4 mM ADP, 5 mM $MgCl_2$, 20 mM creatine, and 50 mM KNO_3 can lead to transformation of the mitochondrion enzymes from octamers to dimers (*16*). The formation of the quaternary complex can protect the enzyme from inhibition by DTNB and iodoacetamide (*2*). Price and Hunter (*17*) reported that although the reaction rates of two thiol groups of native creatine kinase with DTNB or iodoacetamide are the same, the modification reaction is biphasic in the presence of the transition-state analogue. In the present paper, the microscopic rate constants of reaction have been determined for human muscle creatine kinase with NO_3^- and NO_2^-. The results show that binding of creatine-MgADP and an anion with the enzyme is very tight, with much higher binding constants than with normal substrates.

APPENDIX

The inactivation of creatine kinase by NO_3^- or NO_2^- appears to be a slow reversible inhibition. This can be written as in Scheme 1.

$$[E_T] = [E] + [E\cdot ATP] + [E\cdot Cr] + [E\cdot ATP\cdot Cr]$$

$$[E_T^*] = [E\cdot Y] + [E\cdot Y\cdot Cr]$$

$$[E_0] = [E_T] + [E_T^*]$$

It is assumed that the steady state of the substrate reaction is rapidly established and that both [S] and [Y] $\gg$ [E]. The inactivation is apparently monophasic. The following relations can be obtained:

$$[E_T] = \frac{e^{-(A[Y]+B)t} + \dfrac{B}{A[Y]}}{1 + \dfrac{B}{A[Y]}} \cdot [E_0] \qquad \text{(A1)}$$

The apparent rate constant for the forward reaction, A, and reverse reaction, B, are

$$A = \frac{k_{+0} + k'_{+0}\dfrac{[Cr]}{K_{Cr}}}{1 + \dfrac{[ATP]}{K_{ATP}} + \dfrac{[Cr]}{K_{Cr}} + \dfrac{[Cr]\cdot[ATP]}{K_{Cr}\cdot K_{Cr\cdot ATP}}} \qquad \text{(A2)}$$

$$B = \frac{k_{-0} + k'_{-0}\dfrac{[Cr]}{K^*_{Cr}}}{1 + \dfrac{[Cr]}{K^*_{Cr}}} \qquad \text{(A3)}$$

The rate of the substrate reaction in the presence of Y is

$$\frac{d[P]}{dt} = k_3 \cdot [E \cdot ATP \cdot Cr] = k_3 \cdot \frac{[Cr] \cdot [ATP]}{K_{Cr} \cdot K_{Cr \cdot ATP}} \cdot \left(\frac{[E_T]}{1 + \frac{[ATP]}{K_{ATP}} + \frac{[Cr]}{K_{Cr}} + \frac{[Cr] \cdot [ATP]}{K_{Cr} \cdot K_{Cr \cdot ATP}}} \right)$$

The product concentration at time t is

$$[P] = \frac{Bv}{A[Y]+B} t + \frac{A[Y]v}{(A[Y]+B)^2} - \frac{A[Y]v}{(A[Y]+B)^2} e^{-(A[Y]+B)t} \quad \text{(A4)}$$

where [P] is the concentration of the product formed at time t and v is the initial rate of the substrate reaction in the presence of Y.

When t is sufficiently long, the curves become straight lines

$$[P]_{calc} = \frac{Bv}{A[Y]+B} t + \frac{A[Y]v}{(A[Y]+B)^2}$$

$$\ln([P]_{calc} - [P]) = \ln\left\{\frac{A[Y]v}{(A[Y]+B)^2}\right\} - (A[Y]+B)t \quad \text{(A5)}$$

Plots of $\ln([P]_{calc} - [P])$ *versus* t give a series of straight lines at different concentrations of Y with slopes $-(A[Y]+B)$. A plot of $A[Y]+B$ *versus* [Y] gives a straight line, indicating that A is independent of [Y]. Its slope and intercept are A and B, respectively.

The experimental results suggest that $k_{+0} = k'_{+0}$, $k_{-0} = k'_{-0}$. Therefore, Eq. A2 can be simplified to

$$A = \frac{k_{+0} + k_{+0}\frac{[Cr]}{K_{Cr}}}{1 + \frac{[ATP]}{K_{ATP}} + \frac{[Cr]}{K_{Cr}} + \frac{[Cr] \cdot [ATP]}{K_{Cr} \cdot K_{Cr \cdot ATP}}}$$

$$\frac{1}{A} = \frac{1}{k_{+0}} + \frac{\frac{1}{K_{ATP}} + \frac{[Cr]}{K_{Cr} \cdot K_{Cr \cdot ATP}}}{k_{+0}\left(1 + \frac{[Cr]}{K_{Cr}}\right)} \cdot [ATP] \quad \text{(A6)}$$

At a fixed creatine concentration, a plot of $1/A$ against [ATP] gives a straight line and k_{+0} can be obtained from the intercept.

Similarly, Eq. A3 can be simplified to

$$B = \frac{k_{-0}\left(1 + \frac{[Cr]}{K^*_{Cr}}\right)}{1 + \frac{[Cr]}{K^*_{Cr}}} = k_{-0} \quad \text{(A7)}$$

The value of B is therefore equal to the microscopic rate constant, k_{-0}.

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