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INTERACTION OF BRAIN-TYPE CREATINE KINASE WITH ITS TRANSITION STATE ANALOG: KINETICS OF INHIBITION AND CONFORMATIONAL CHANGES

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The effects of components of the transition state analog (creatine, MgADP, planar anion) on the kinetics and conformation of creatine kinase isozyme BB from monkey brain was studied. From analysis of the reaction time course using the pH stat assay, it was shown that during accumulation of the reaction products (ADP and creatine phosphate), among several anions added, nitrate proved the most effective in inhibiting catalytic activity. Maximum inhibition (77%) was achieved with 50 mM nitrate. The K_m for ATP was 0.48 mM and in the presence of 2.5 mM nitrate, 2.2 mM; for ATP in the presence of the dead-end complex, creatine and ADP, the apparent K_m was 2.0 mM and the K_i was 0.16 mM; in the presence of the transition state analog, MgADP + NO_3^- + creatine, the K_i was estimated to be 0.04 mM.

Ultraviolet difference spectra of creatine kinase revealed significant differences only in the presence of the complete mixture of the components of the transition state analog. Comparison of gel filtration elution profiles for creatine kinase in the absence and presence of the complete mixture of components of the transition state analog did not reveal any differences in elution volume. Addition of components of the transition state analog to creatine kinase resulted in only a marginal change in intrinsic fluorescence. The presence of the components of the transition state analog increased the rate of reactivity of the enzyme with trinitrobenzenesulfonic acid from $k = 6.06 \pm 0.05\,M^{-1}min^{-1}$ to $6.96 \pm 0.11\,M^{-1}min^{-1}$.

This study provides evidence that, like the muscle isozyme of creatine kinase, the brain form is effectively inhibited by the transition state analog. However, the inhibition is accompanied by small changes in the overall conformation of the protein. This adds to the evidence that the functional differences of the isozymic forms of creatine kinase cannot be attributed to differences in kinetic properties.

KEY WORDS: Creatine kinase, transition state analog, conformational changes, brain enzyme, muscle enzyme.

INTRODUCTION

Creatine kinase (CK; creatine: ATP phosphotransferase, EC 2.7.3.2) occurs in three tissue specific isozymic forms (muscle: CK-MM[1]; brain: CK-BB; heart: CK-MB) and catalyzes the reversible phosphorylation between ATP and creatine phosphate. The functional significance for the occurrence of multiple forms has not been described. Numerous studies have shown that the inhibition of muscle CK by the dead-end complex of creatine and MgADP is potentiated by the presence of small planar anions such as chloride or nitrate.[1–5] It has been proposed that the presence of nitrate results in formation of a "transition state analog", with nitrate mimicking the transferable phosphate. Although several conformational analyses[4,6,7] have been reported, only

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small structural changes associated with the formation of the transition state analog for isozyme CK-MM have been observed.

The brain isozyme of CK is proposed to be a looser and more flexible protein than the muscle form.[8,9] Kinetic and spectroscopic studies suggest that the CK-BB conformation is more responsive than CK-MM conformation to substrate binding.[10] The effect of the transition state analog on the kinetics and conformation of CK from brain tissue has not been reported. In view of the present lack of understanding why multiple forms of creatine kinase occur, it was of interest to further investigate and characterize potential catalytic and conformational differences.

MATERIALS AND METHODS

Creatine kinase was purified from fresh frozen monkey brain as described previously[11] and the protein concentration determined using the extinction coefficient for the purified protein. Enzyme solutions were stored at 4°C in 0.1 M tris/acetate buffer, 1 mM EDTA and 2 mM dithiothreitol under a sealed atmosphere of dry nitrogen.

The pH stat assay was performed using a Type RTS 822 Autotitration assembly from Radiometer. The routine assay mixture contained 40 mM creatine, 5 mM magnesium acetate, 4 mM ATP and 2 mM dithiothreitol, pH 8.0 in a total volume of 2.0 ml. The stirred reaction vessel was maintained at 25°C with circulating thermostatted water and the atmosphere above the reaction solution continuously purged with dry nitrogen. The titrant used (5 mM NaOH) was calibrated each day against a standard solution of HCl. Enzyme was dialyzed against 0.01 M tris/acetate, pH 8.0 and 2 mM dithiothreitol for 6 h, then immediately used in the pH stat assay.

Difference spectra were obtained using an IBM Model 9420/9430, double beam spectrophotometer and a matched set of 1 cm tandem cuvette cells. The reference and sample cuvettes each contained separated solutions for enzyme and transition state analog components. The cuvettes were balanced against each other at 350 nm and then scanned, with the difference stored in the instrument's memory. It should be noted that at any point over the spectral range, the two cuvettes with the identical components did not exhibit more than a 0.002 A difference nor baseline drift. The components in the separated chambers of the sample cuvette were then mixed, and the difference spectrum recorded. The difference spectrum obtained before mixing of the sample cuvette was subtracted from the difference spectrum obtained after mixing the sample cuvette.

Intrinsic protein fluorescence spectra were recorded with an SLM Model 8000 Spectrofluorometer in the ratiometric mode. Inner filter effects due to absorption of incident light by solution components were corrected by determining their effect on a solution of *N*-acetyltyrosinamide and *N*-acetyltryptophanamide, that was stoichiometric with the concentrations of tyrosine and tryptophan in CK-BB.[11] Spectra were also corrected for fluorescence contributions from the solvent and transition state analog components.

Reactivity with trinitrobenzene sulfonic acid (TNBS; Sigma Chem. Co.) was measured kinetically[12] using a Gilford Model 250 recording spectrophotometer. Details are given in the text and a description of data analysis provided in the Appendix.

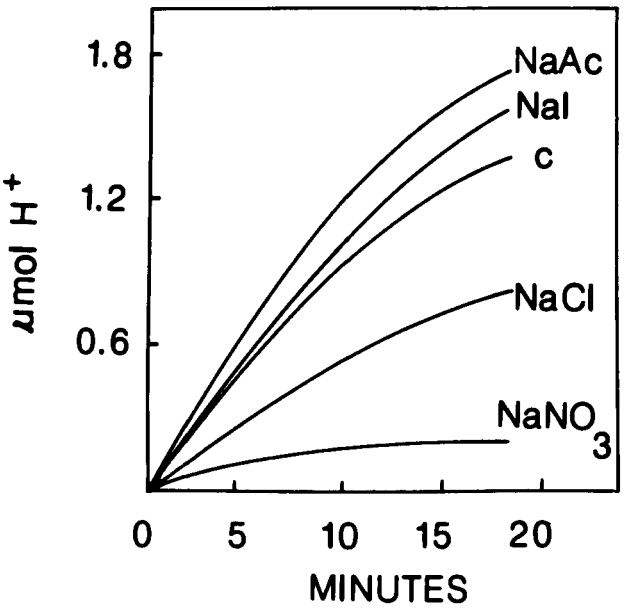

FIGURE 1 Progress curves for effect of anions on CK-BB activity measured by the pH stat assay. Assay conditions were 2.0 ml of solution containing 40 mM creatine, 4 mM ATP, 5 mM magnesium acetate, 2 mM dithiothreitol, and 50 mM sodium salt of the indicated anion, pH 7.80. The reaction mixture was maintained at 25°C, and continuously stirred under a stream of dry nitrogen. The assay was initiated by addition of 50 μg of CK-BB in 0.1 ml of 5 mM tris/acetate (with 1 mM dithiothreitol, pH 8.0).

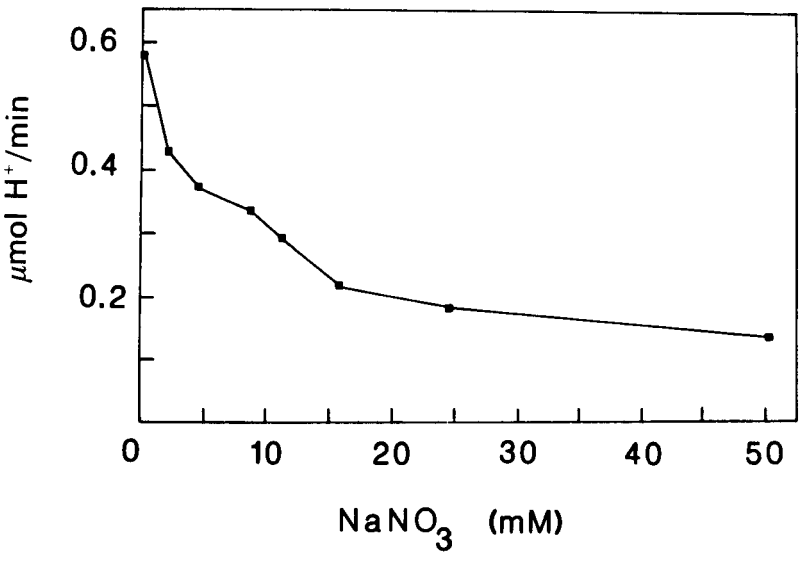

FIGURE 2 Effect of nitrate anion concentration on the initial velocity of the creatine kinase reaction. Assay conditions are the same as those given in Figure 1 except 12.5 μg of enzyme in 0.025 ml dilute buffer was used to initiate the reaction. Nitrate was added as aliquots from a stock solution of 1 M sodium nitrate.

RESULTS

Time course plots (Figure 1) illustrate the effect of several anions on CK-BB activity, measured in the forward direction. Under the conditions used, the maximum inhibition (77%) was exhibited by nitrate, whereas acetate (as sodium acetate) activated CK-BB by approximately 38%.Iodide anion had a slight (7%) activating effect, whereas chloride produced a 38% inhibition.

As shown in Figure 2, 12 mM $NaNO_3$ is sufficient to inhibit the initial velocity of CK-BB by 50%. Maximum inhibition was achieved by 50 mM $NaNO_3$ and similar results were obtained with KNO_3.

The curvature of the time course for the titrimetric assay of CK-BB suggested accumulation of an inhibitor, likely ADP, as reported for the forward reaction of the muscle isozyme.[1] When the assay was allowed to procede until 0.144 mM ADP had accumulated and then $NaNO_3$ (50 mM) added to the assay, total inhibition was observed, while a similar addition of sodium acetate produced a 40% activation, and Na_2SO_4 or NaI did not alter the time course relative to the assay without added anion.

The K_m for MgATP was 0.48 mM (Figure 3). In the presence of 2.5 mM $NaNO_3$ the K_m for MgATP increased to 2.2 mM. When 0.5 mM MgADP was added to the assay mixture, the apparent K_m for MgATP was 2.0 mM and the K_i obtained was 0.16 mM. When both MgADP and nitrate anion were present, the K_i could only be estimated at a value of approximately 0.04 mM.

Difference spectra in the ultraviolet are illustrated in Figure 4. Very little difference

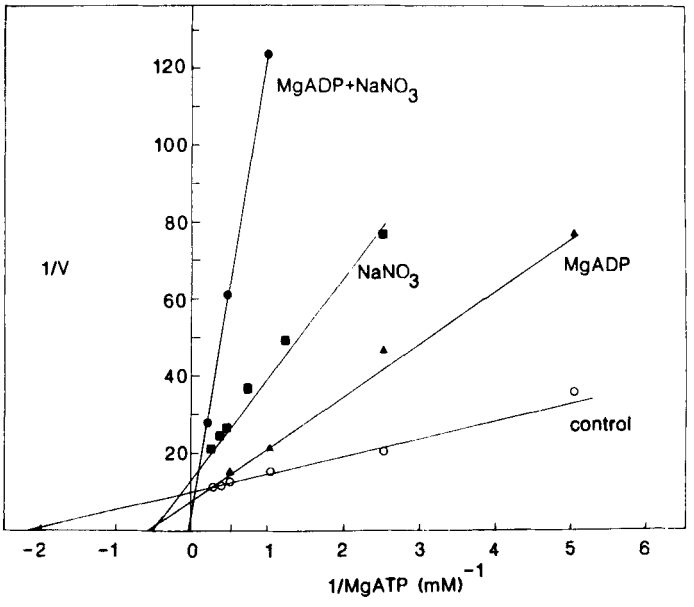

FIGURE 3 Inhibition of CK-BB by MgADP and nitrate anion. The pH stat assay contained 40 mM creatine, 5 mM magnesium acetate, 4 mM ATP and 2 mM dithiothreitol, pH 7.8. Reaction was initiated by the addition of 50 μg of enzyme in 0.1 ml of 5 mM tris/acetate pH 8.0 containing 2 mM dithiothreitol. (○) No additions. (▲) 0.5 mM MgADP. (■) 2.5 mM sodium nitrate. (●) 0.5 mM MgADP and 2.5 mM sodium nitrate.

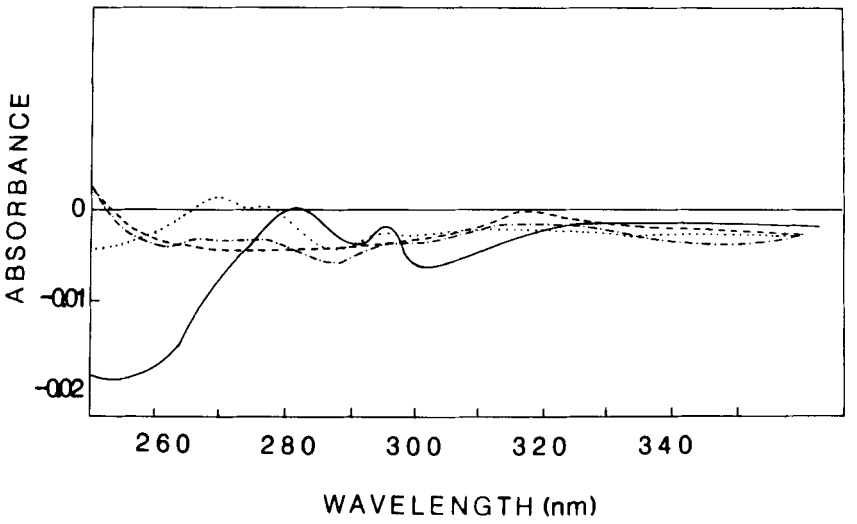

FIGURE 4 Ultraviolet difference spectra Tandem cuvettes were loaded with the following: front chamber 0.9 mL of CK-BB (8.7 μM); rear chamber 0.9 ml of a mixture of 2 mL of creatine (80 mM), buffer and the following: (– – –) $NaNO_3$ [0.1 ml of 2.4 M]; (–·–·–) $NaNO_3$ + ADP[0.05 ml of 10 mM]; (····) ADP + MgAcetate [0.2 ml of 50 mM]; (——) MgAcetate + ADP + creatine.

was exhibited by the exposure of the enzyme (with creatine) to nitrate only or nitrate plus ADP. A broad, shallow decrease for the enzyme (with creatine) was observed in the presence of nitrate only over the range of 260 to 310 nm. In the presence of creatine, ADP and nitrate, the enzyme exhibited a small minimum at 289 nm. When MgADP was mixed with CK-BB (and creatine), a small maximum was seen at 270 nm and a small minimum seen at 289 nm. In the presence of the complete transition state analog (creatine, MgADP and nitrate), minima were observed at 300, 291 and 257 nm. The spectra were recorded one minute after mixing and did not exhibit subsequent time-dependent changes.

Enzyme subjected to gel filtration through a column of Sephadex G-150, did not exhibit a change in elution volume (relative to void volume) upon inclusion of the components of the transition state analog in the equilibration and elution buffer.

Kinetics of reactivity of CK-BB with TNBS in the presence of components of the transition state analog are shown in Figure 5a. The only significant change was an increase in reactivity when CK-BB was exposed to TNBS in the presence of the complete components of the transition state analog. The changes observed with less than complete combinations of components of the transition state analog were essentially superimposible with the reaction rate for the control, and consequently these progress curves are illustrated as a single curve. The rate constants were determined (see Appendix) and found to be $6.06 \pm 0.05\,M^{-1}\,min^{-1}$ and $6.96 \pm 0.11\,M^{-1}\,min^{-1}$, in the absence and presence of the transition state analog, respectively. Applying the value $\varepsilon = 14{,}500\,M^{-1}\,cm^{-1}$ for the trinitrophenylation, we illustrate (Figure 5b) the fraction of lysines reacted in the absence and presence of the transition state analog, showing for example, that after 20 minutes reaction time, 4.0 and 4.5 lysines, respectively, have reacted.

Upon addition of the components of the transition state analog, changes in intrinsic protein fluorescence were very small. Whereas creatine and/or nitrate had no effect on

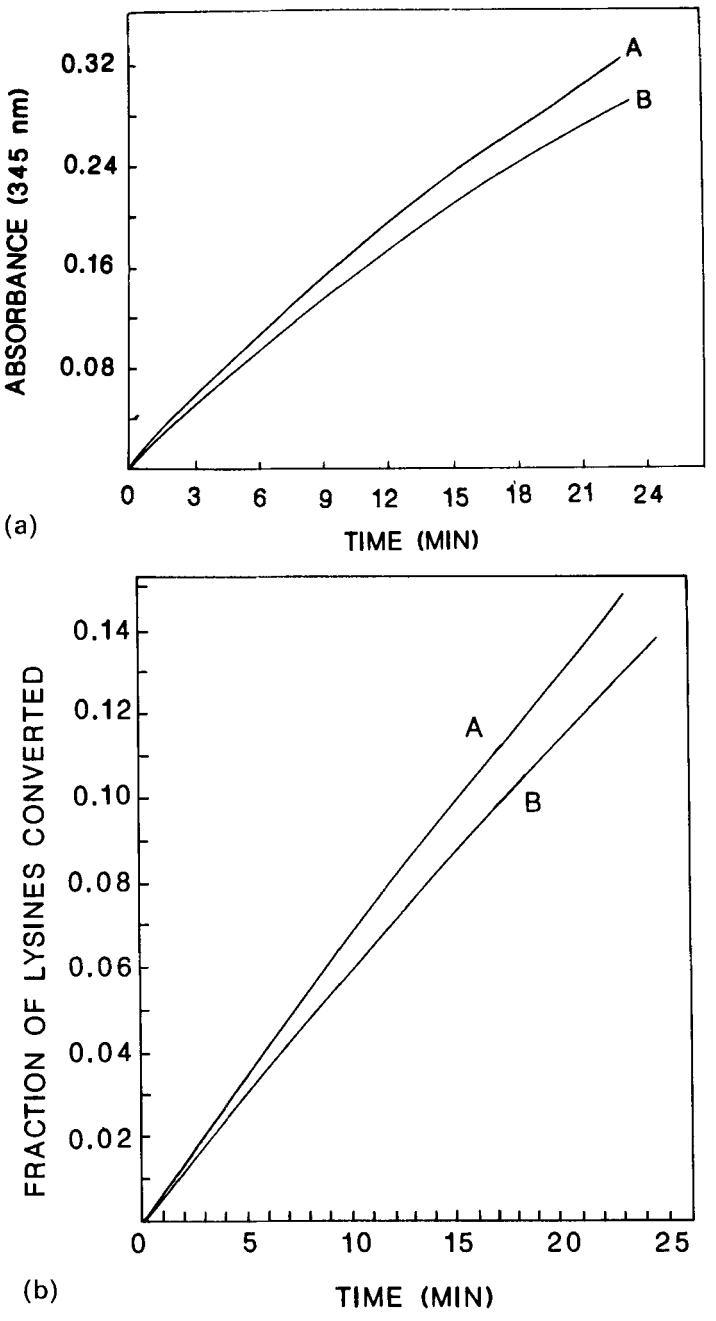

FIGURE 5 Reaction of CK-BB with TNBS in the presence of components of the transition state analog. CK-BB (4.4 μM) in 0.05 M potassium HEPES buffer (pH 7.9) supplemented with components of the transition state analog was treated with 0.1 ml of TNBS (from 10 mM stock solution) in a total reaction volume of 1.1 ml and change in absorbance (345 nm) recorded. (a) A: 20 mM creatine + 5 mM MgAcetate + 4 mM ADP + 0.1 M $NaNO_3$. B: no additions, creatine + MgAcetate + ADP, creatine + $NaNO_3$, or creatine + ADP + $NaNO_3$. (b) as in (a) with the number of lysines reacted as a function of time. The total number[11] of lysines in CK-BB is 35.

the fluorescence emission at 337 nm (excitation at 285 nm), addition of MgADP (with creatine) or MgADP and nitrate (with creatine) produced only a marginal increase of about 4%, after correction for the self-absorption of the nucleotide.

DISCUSSION

Certain monovalent anions such as chloride and nitrate when added to creatine/MgADP/CK-MM significantly protect the highly reactive sulfhydryl of CK against reaction with sulfhydryl reagents.[1] The dissociation constants of creatine and ADP are also decreased by the presence of nitrate anion.[1] It has been proposed[1] and supported by numerous studies (e.g. references 4, 5) that the abortive quaternary complex between MgADP/creatine/CK is stabilized by nitrate anion by virtue of the formation of a transition state analog in which nitrate mimics the transferable phosphate.

The present study demonstrates that the activity of creatine kinase from monkey brain is profoundly inhibited by interaction with creatine/MgADP/nitrate and that the interaction is accompanied by small differences in polypeptide chain conformation. Consequently, like the muscle type isozyme, brain creatine kinase responds to interaction with the components of the transition state analog by a limited change in tertiary structure, with subtle changes in the active site region accounting for the pronounced inhibitory effect.

The kinetic characteristics of the anion inhibition of the brain-type isozyme and muscle-type isozyme are similar. The progress curves, the effect of anions and the effects of inhibitors on the kinetics appear similar to those reported for the rabbit muscle isozyme.[1] The inhibition constant for MgADP and the brain type isozyme is lower than that reported for the muscle isozyme. Although essentially quantitatively indeterminate, the inhibition constant for the complete transition state analog containing nitrate, appears to be the lowest yet reported for a creatine kinase.

In considering the sensitivity of the catalytic reaction to the transition state analog and the previous finding of a small but significant change in the rotational relaxation time of CK-BB when binding MgADP,[10] it seemed that CK-BB might exhibit conformational changes accompanying interaction with the transition state analog. Experiments using absorption difference spectroscopy, fluorescence spectroscopy and reactivity with TNBS showed small changes accompanying treatment of CK-BB with MgADP/creatine/nitrate. No significant changes in any of the parameters was noted if nitrate was omitted. One interesting feature of the reaction of CK-BB with TNBS is that the presence of the transition state analog actually caused an increase in the reactivity. It is known that dimeric CK-MM contains two reactive lysines near the active site[13] and like CK-MM,[13] CK-BB can be specifically labelled with two moles of dansyl moieties per mole of protein.[10] One might expect that the transition state analog would inhibit reactivity of CK towards TNBS, by obstructing access to the reactive lysines. The fact that the transition state analog enhances the reactivity of CK-BB with TNBS suggests that the conformational change that does occur is accompanies by a net increase in the exposure of the lysine residues.

The present study suggests that both isozymes of creatine kinase undergo substantial inhibition of activity due to the presence of transition state analog. Considered with other kinetic data, this suggests that functional differences are not based on catalytic differences. Furthermore, if substrate or inhibitors induce conformational

changes, these changes appear to be most pronounced in the region of the active site and are of limited magnitude for the overall tertiary structure. We have shown too, that the mechanisms by which the CK isozymes refold from the denatured to the native states are similar.[9,14] On the other hand, in the native conformation and in the absence of substrate, CK-BB appears to have less packing density than CK-MM.[9,10] This appears true for the active site, where cooperativity in substrate binding has been reported, and for the tertiary structure, where studies of intrinsic fluorescence and susceptibility to tryptic hydrolysis, suggest a looser, more open conformation.[9,10] The biochemical significance of the isozymic differences in conformation remains to be established, and may be linked to some property other than catalysis, such as tissue specific compartmentation.[15]

Appendix

The reaction between TNBS and CK-BB (C) can be expressed as a second order differential equation for which there is a known analytical solution:

$$C = C_0 (1 - e^{\alpha})/(M - e^{\alpha}) \qquad \text{(A-1)}$$

where $M = TNBS_0/C_0$ and $\alpha = C_0 (M - 1)kt$

The subscript (o) indicates initial conditions, k is the second order rate constant and t is the reaction time in minutes. Using the treatment of Muhlrad and Takashi[12] if the total number of lysines is divided into fast reacting (m) and slow (n), the mass balance on the lysyls can be expressed as

$$\begin{aligned} du/dt &= k_1 (mC_0\text{-}u) (TNBS_0\text{-}u\text{-}w) \\ dw/dt &= k_2 (nC_0\text{-}w) (TNBS_0\text{-}u\text{-}w) \end{aligned} \qquad \text{(A-2)}$$

where u and w are the concentrations of fast and slow lysines reacted after time t and k_1 and k_2 are the corresponding constants.

In order to determine if the data could be interpreted in terms of fast and slow reacting lysine residues of which CK-BB contains[11] a total of 35 , the models represented by equations A-1 and A-2 were fitted to the data using a non-linear least squares routine based on Marquardt-Levenberg's algorithm. The integration of the set of equations A-2 was accomplished using a 4th order Runge-Kutta algorithm. Typical total measurement errors were estimated to be ± 0.005 absorbance unit. The estimated error was assumed to be constant and as such it was used to test for model adequacy. On the basis of the observed experimental error, Eqn. A-1 proved to be adequate for describing the behavior shown in Figure 5. [The rate constants are given in the text.]

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