Comparison of the Rates of Inactivation and Conformational Changes of Creatine Kinase during Urea Denaturation†

Qi-Zhi Yao, Min Tian, and Chen-Lu Tsou*

ABSTRACT: The denaturation of creatine kinase in urea solutions of different concentrations has been studied by following the changes in the ultraviolet absorbance and intrinsic fluorescence as well as by the exposure of hidden SH groups. In concentrated urea solutions, the denaturation of the enzyme results in negative peaks at 285 nm with shoulders at 280 and 290 nm and positive peaks at 244 and 302 nm in the denatured minus native enzyme difference spectrum. The fluorescence emission maximum of the enzyme red shifts with increasing intensity in urea solutions of increasing concentrations. At least part of these changes can be attributed to direct effects of urea on the exposed Tyr and Trp residues as shown by experiments with model compounds. The inactivation of this enzyme has been followed and compared with the conformational changes observed during urea denaturation. A marked decrease in enzyme activity is already evident at low urea concentrations before significant conformational changes can be detected by the exposure of hidden SH groups or by ultraviolet absorbance and fluorescence changes. At higher urea concentrations, the enzyme is inactivated at rates 3 orders of magnitude faster than the rates of conformational changes. The above results are in accord with those reported previously for guanidine denaturation of this enzyme [Yao, Q., Hou, L., Zhou, H., & Tsou, C.-L. (1982) *Sci. Sin. (Engl. Ed.) 25*, 1186–1193] and can best be explained by assuming that the active site of this enzyme is situated near the surface of the enzyme molecule and is sensitive to very slight conformational changes.

Studies on the structure–function relationship have always been one of the central issues in the investigations of biological macromolecules. However, attempts to correlate structure modifications of proteins to changes in their biological activities have been hitherto largely confined to the modification of the covalent structure of the protein molecules by chemical modification of the side-chain functional groups or by proteolytic digestion of the peptide chain. Although the first approach has provided useful information and has become a routine method for the study of structure–function relationships of proteins (Thomas, 1974), the second approach has met with only very limited success (Maroux & Desnuelle, 1969; Richards & Wyckoff, 1971) in spite of the fact that for several decades many efforts have been made to obtain an active fragment of a protein molecule by proteolytic digestion (Tsou, 1951; Perlmann, 1954). It has now become clear that the functionally essential groups of a protein are usually scattered throughout the entire peptide chain and are brought together to carry out their biological role only when the whole peptide chain is suitably folded in its native conformational state.

An alternative approach to study whether the entire protein molecule or at least a substantial part of it is essential for its biological activity would be to study the correlation of activity changes to the conformational changes of a protein during unfolding and refolding of the molecule. Protein molecules unfold during denaturation by acid or heat treatment or by exposure to a number of agents such as urea, guanidine, and detergents, with loss of their biological activities (Tanford, 1968, 1970; Baldwin, 1975; Jaenicke, 1982). Upon neutralization, lowering of the temperature to ambient temperature, or diluting out the denaturants, it is sometimes possible to select suitable conditions that allow the protein molecules to return to their original native conformational state with full recovery of their biological activities. It has also been well recognized that during the course of the unfolding of the protein molecule upon denaturation, the native conformational state of a certain protein usually passes through several intermediary conformational states before reaching the final fully unfolded state (McCoy et al., 1980; Henkens et al., 1982; Kim & Baldwin, 1982; Jaenicke, 1982). However, although the unfolding and refolding of the protein molecules have been extensively studied, very few efforts have been made to correlate the conformational changes with the changes in the biological activities of the protein molecules concerned. The conventional way of following the activity changes during the denaturation or renaturation of a protein is to stop the denaturation or renaturation reaction at time intervals, take aliquots, and assay for biological activity. This not only is laborious but also would be most difficult when the activity change is very fast.

It has now become possible, at least with enzymes, to follow the rapid changes in activity by measuring the substrate reaction under denaturation conditions as described by Tian & Tsou (1981) on the basis of the equations first proposed by Tsou (1965a,b) and subsequently by Laidler & Bunting (1973). In previous papers (Yao et al., 1982a,b), it has been reported from this laboratory that during the guanidine denaturation of creatine kinase, inactivation precedes significant conformational changes. Similar results have now been obtained with urea denaturation. At low urea concentrations, marked inactivation of the enzyme takes place without noticeable conformational changes as monitored by changes in the ultraviolet absorbance, fluorescence, and exposure of SH groups whereas at high urea concentrations, the rates of inactivation are at least 1000 times faster than the overall conformational changes of the enzyme molecule.

Materials and Methods

Creatine kinase was prepared and further purified as described previously (Yao et al., 1982a). The $A^{1\%}_{1cm}$ value of 8.8 (Noda et al., 1954) was used for protein concentration measurements. The final preparations used usually had a specific activity of 130–160 μmol min^{-1} mg^{-1} (Mahowald et al., 1962) and showed only one band by polyacrylamide gel electrophoresis. Enzyme assay was carried out by the thymol blue–pH spectrophotometric method described previously (Yao & Tsou, 1981). Urea solutions were always freshly prepared from a twice-recrystallized (Marangos & Constantinides,

† From the Institute of Biophysics, Academia Sinica, Beijing, China. *Received November 7, 1983.*

0006-2960/84/0423-2740$01.50/0

1974) local product of analytical grade. Other reagents were also local products of analytical grade.

Fluorescence measurements were made with a Hitachi MPF-4 spectrofluorometer. All the emission spectra measured were corrected spectra, and the excitation wavelength was usually 285 nm unless otherwise specified. When the fluorescence emission intensity was followed during urea denaturation of creatine kinase, an excitation wavelength of 300 nm was chosen so that the protein concentration used was close to that used in ultraviolet difference spectra measurements with an absorbance at this wavelength less than 0.05. The emission wavelength chosen to follow the flluorescence intensity changes was at the emission maximum for a particular urea concentration. This wavelength changes from 340 nm for 2 M urea to 350–352 nm at urea concentrations greater than 4 M. The changes in fluorescence intensity with time follow first-order kinetics, and the data were treated according to Guggenheim (1926) to obtain the first-order rate constant for the denaturation reaction.

Ultraviolet difference spectra were measured with a Cary 219 spectrophotometer with tandem cuvettes. In the reference beam, each compartment of the cuvette contained the enzyme and the urea solutions separately whereas in the sample cuvette these two solutions were mixed. When the rate of denaturation was measured, the absorbance change, usually at 287 nm, was followed, and the data obtained were treated the same as those obtained from the fluorescence studies.

Fast inactivation and denaturation reactions were measured with a Dionex D-115 stopped-flow apparatus. For fast inactivation measurements, one syringe contained both the denaturant and the reaction mixture of buffer, substrates, and thymol blue whereas the other syringe contained the enzyme. Upon mixing, the reaction was followed at 597 nm, and the results obtained were treated by Tsou's method (Tsou, 1965a,b; Tian & Tsou, 1982) to obtain the first-order rate constant for the inactivation reaction as described in a previous paper (Yao et al., 1982b). For denaturation reactions, the two syringes contained the enzyme and the denaturants, respectively, and upon mixing, changes in absorbance at 287 nm were followed. As has been shown previously for guanidine (Yao et al., 1982a,b), the presence of the substrates does not significantly protect the enzyme against denaturation by urea.

Results

Ultraviolet Absorbance. The ultraviolet difference spectra of creatine kinase denatured in urea solutions of low concentrations showed positive peaks in the 280-nm region. At urea concentrations higher than 3 M, negative peaks at 287 and 293 nm indicate the exposure of Tyr and Trp residues as in the case of guanidine denaturation (Yao et al., 1982a). Figure 1 compares the absorbance changes of the enzyme with those of the model compounds, acetyltyrosinamide and acetyltryptophanamide, in urea solutions of different concentrations. The increases in A_{287} for acetyltyrosinamide and in A_{294} for acetyltryptophanamide have been previously reported by Herskovits (1965). It appears that the increase in absorbance in the 280-nm region of the enzyme is due to direct effects of urea on the ultraviolet absorption of Tyr and Trp residues a few of which are, presumably, located on the surface region of the enzyme molecule.

Fluorescence. A gradual increase in the fluorescence intensity (Figure 2) and a shift of the emission maxima from 330 to 352 nm were observed with creatine kinase in urea solutions of increasing concentrations. Moreover, at urea concentrations higher than 4 M, an emission shoulder appeared at 305 nm due most probably to the disruption of the radiationless energy transfer between Tyr and Trp residues and hence the appearance of a Tyr fluorescence peak at this wavelength (not shown). This increase in the fluorescence intensity of creatine kinase in urea can be explained, at least in part, by the direct effect of urea on the fluorescence of Trp and ionized Tyr residues in the 330–350-nm region as shown by model compounds. It can be seen from Figure 2 that a marked increase in fluorescence intensity was observed for both acetyltryptophanamide and ionized acetyltyrosinamide when the urea concentration was increased from 2 to 8 M. It is important to recall that some of the Tyr residues of this enzyme are most likely in the ionized state even at pH 9 (Yao et al., 1982a). On the other hand, guanidine increases the fluorescence of ionized Tyr at 345 nm to a much less marked extent,

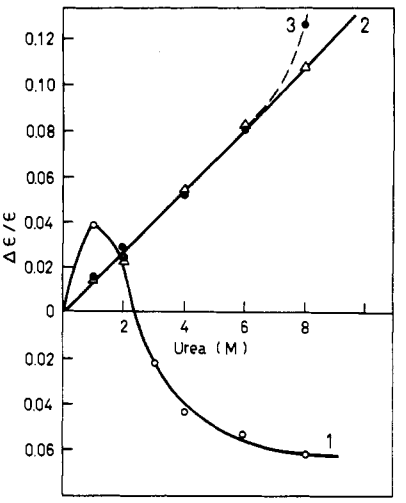

FIGURE 1: Absorbance changes of creatine kinase in urea solutions as compared with model compounds. Final concentrations were 12.2 μM creatine kinase, 0.1 M pH 9.0 glycine–NaOH buffer, and 1 mM EDTA with urea concentrations as indicated. The concentrations of the model compounds were selected so that the initial absorbance at 280 nm was approximately the same as that of the enzyme solution. Curve 1, creatine kinase, $\Delta\epsilon_{287}/\epsilon_{280}$; curve 2, acetyltryptophanamide, $\Delta\epsilon_{292}/\epsilon_{280}$; curve 3, acetyltyrosinamide, $\Delta\epsilon_{280}/\epsilon_{275}$.

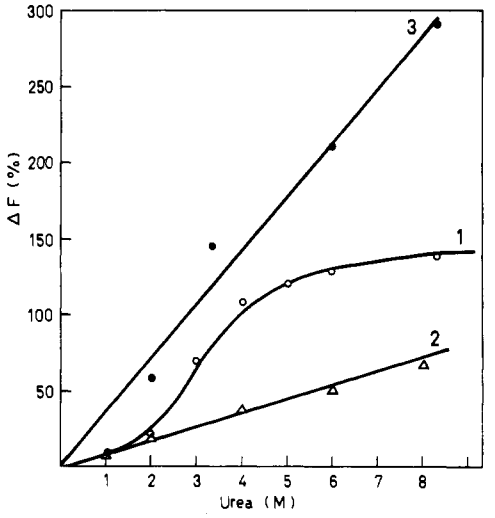

FIGURE 2: Fluorescence emission changes of creatine kinase in urea solutions as compared with model compounds. Corrected spectra of creatine kinase and the model compounds were measured with an excitation wavelength of 265 nm, and the changes in emission intensities were compared at 352 nm. Curve 1, creatine kinase, 1.1 μM in 0.1 M glycine–NaOH buffer, pH 9.0, containing 1 mM EDTA; curve 2, 8.8 μM acetyltryptophanamide in the same buffer as for curve 1; curve 3, 19.8 μM acetyltyrosinamide in 0.1 M KOH. The concentrations of the model compounds were selected so as to correspond to the amount of Trp and Tyr residues contained in 1.1 μM creatine kinase used for curve 1.

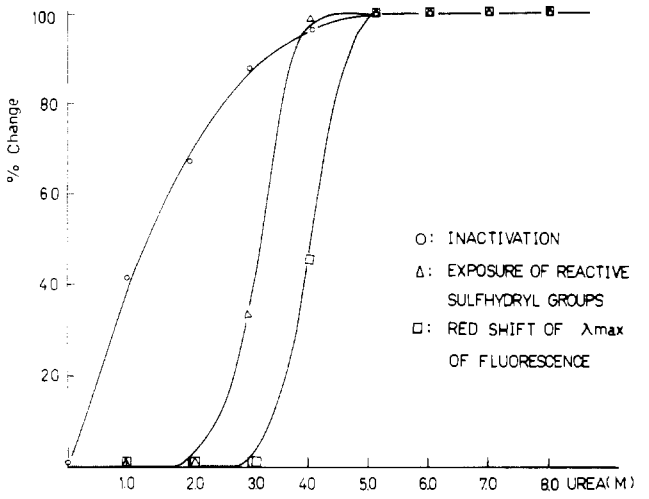

FIGURE 3: Inactivation and denaturation of creatine kinase in urea solutions of different concentrations. The enzyme solutions were incubated with urea for 24 h before determination of the remaining activity (○), the exposure of SH groups (△), and the red shift of fluorescence emission maxima (□). Final concentrations of enzyme were 0.2 μM for the activity assay, 1.1 μM for fluorescence measurements, and 5.8 μM for SH determinations.

and it has little effect on the fluorescence of Trp (Cowgill, 1972). The above results can no doubt account for the difference in fluorescence behavior of this enzyme in urea and in guanidine solutions. The fact that the increase in fluorescence intensity was greater in 8 M urea than in 4 M guanidine can best be explained by the direct effect of urea on the fluorescence of exposed Tyr and Trp residues rather than by a more extensive conformational change during urea denaturation as suggested by Grossman et al. (1981).

Inactivation of Creatine Kinase in Urea. The extent of inactivation of creatine kinase increased with increasing urea concentrations. This was compared with the exposure of SH groups and the red shift of the fluorescence emission maxima in different urea concentrations. The results obtained, shown in Figure 3, are also different from those reported by Grossman et al. (1981) in that a greater extent of enzyme inactivation rather than exposure of both the SH groups and the aromatic residues was observed. Because of the direct effects of urea on both the absorbance and the fluorescence intensity of the aromatic residues, the red shift of the fluorescence maxima would be a more reliable measure for the exposure of these residues.

The rates of inactivation of creatine kinase in urea solutions are very fast reactions and hence cannot be measured by the conventional method of taking aliquots at different time intervals after mixing and assay for enzyme activity. The kinetics of the inactivation reaction can, however, be followed in a stopped-flow apparatus by observing the substrate reaction in the presence of denaturant (Tsou, 1965a,b; Tian & Tsou, 1982). A typical result showing the course of inactivation in 4 M urea is given in Figure 4. The first-order rate constant for the inactivation reaction can be obtained from the results thus obtained as has been shown in a previous paper on the guanidine inactivation of this enzyme (Yao et al., 1982b). The rate constants for the inactivation reaction in different urea concentrations are summarized in Table I.

Rate of Denaturation As Followed by Fluorescence Changes. The increase in fluorescence intensity upon mixing creatine kinase with urea solutions was very fast, too fast to be followed by manual mixing, and a subsequent much slower change was observed, as shown in Figure 5 for 4 M urea. The fast phase is no doubt due to the direct effect of urea on the

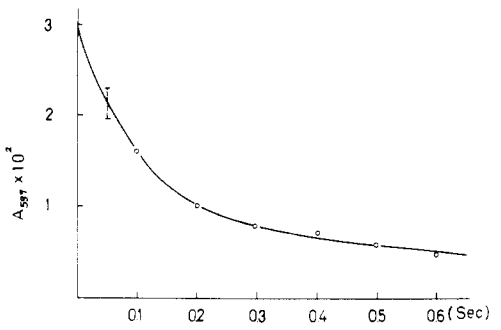

FIGURE 4: Rapid inactivation of creatine kinase in 4 M urea. In one syringe of the stopped-flow apparatus was placed the enzyme solution and in the other the assay mixture in urea. Final concentrations were 2 μM enzyme and 4 M urea, and the assay mixture was as described under Materials and Methods.

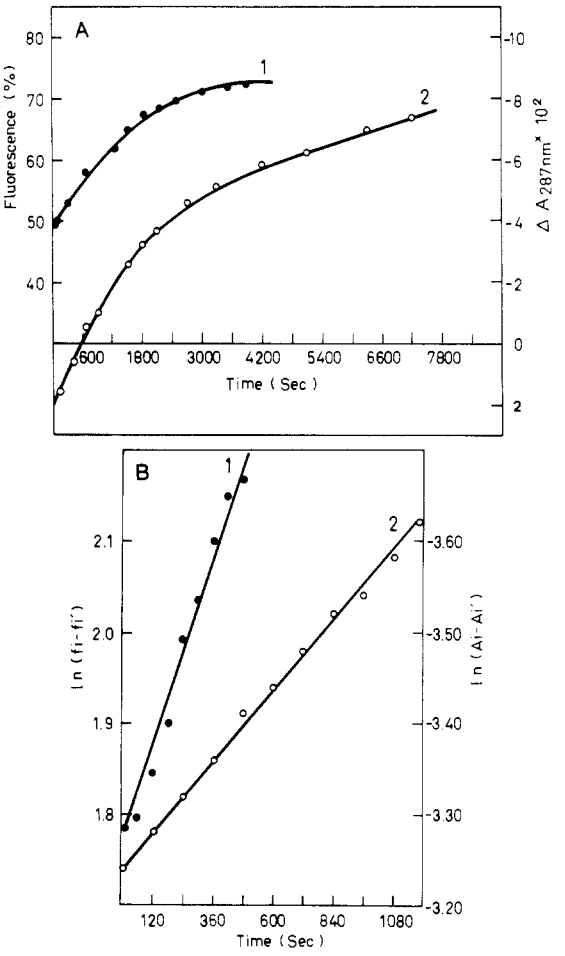

FIGURE 5: Denaturation rates of creatine kinase in 4 M urea as followed by fluorescence and absorbance changes. (A) The denaturation solution contained 4 M urea in 0.1 M glycine–NaOH buffer, pH 9.0, at a temperature of 25 °C. Both fluorescence and absorbance changes were recorded 15 s after mixing. Curve 1, fluorescence at 352 nm with an excitation wavelength of 300 nm; curve 2, absorbance at 287 nm. Enzyme concentrations were 3.9 μM for curve 1 and 11 μM for curve 2. (B) Guggenheim plots of data shown in (A) for the determination of the rate constants.

fluorescence intensity of Tyr and Trp residues located in the surface region of the enzyme molecule, and the slow increase is due to the exposure of internal aromatic residues brought about by conformational changes of the enzyme in urea solutions. At this urea concentration, the direct effect of urea on the fluorescence intensity of the aromatic residues represented about 50% of the total fluorescence change observed. The rate constants of the denaturation reactions in urea solutions of different concentrations can then be obtained by

Table I: Comparison of Denaturation and Inactivation Rates of Creatine Kinase in Urea Solutions

[urea] (M)	denaturation rate[a] ($s^{-1} \times 10^3$) absorbance	fluorescence	SH exposure	inactivation rate[a] ($s^{-1} \times 10^3$)	inactivation residual activity (%)
1	*b*	*b*	*b*	4000	59
2	*b*	*b*	*b*	7000	32
3	0.24	0.24	0.41	10000	14
4	0.32	0.60	3.83	13000	5
5	2.86	1.02	5.75	*c*	0

[a] First-order rate constant. [b] No detectable change; for details, see text. [c] Not determined.

Guggenheim plots of the second part of the curve; these are also summarized in Table I.

Changes in the Ultraviolet Difference Spectra during Denaturation. A repeated spectral scan between 240 and 350 nm during the denaturation of creatine kinase in 4 M urea is shown in Figure 6. During the first scan which was completed 1.5 min after mixing, a difference spectrum with positive peaks at 278, 287, and 295 nm was obtained, and this, in the course of a few hours at 25 °C, gradually changed to the final spectrum for the enzyme denatured at this urea concentration with positive peaks at 244 and 302 nm, negative peaks at 285 nm, and negative shoulders at 280 and 290 nm.

The denaturation reactions at different urea concentrations have also been followed by absorbance changes at 287 nm (Figure 5). Upon mixing of the enzyme with the urea solutions, A_{287} also showed a very fast increase followed by a slow decrease approaching completion in the course of a few hours. Stopped-flow experiments have shown that the fast increase in absorbance at 287 nm takes place before the dead time of mixing the urea solution with the enzyme. This again shows that the increase in A_{287} is a direct effect of urea on the aromatic residues and has nothing to do with conformational changes. Treatment of the slow part of the curve shown in Figure 5 gives the rate constant as shown in Table I.

Discussion

Urea and guanidine are two of the most commonly used denaturants for protein denaturation, and they are believed to have a similar mode of action (Lapanje, 1978). A comparison of the results presented in this paper for the urea denaturation of creatine kinase with those reported previously for guanidine denaturation (Yao et al., 1982a,b) shows that although these two denaturants are qualitatively similar in bringing about the exposure of the aromatic residues as well as the SH groups of the enzyme, there are, however, significant quantitative differences. Guanidine is much more effective in the unfolding of the enzyme molecule in the sense that 0.3 M is already effective in bringing about the exposure of part of the buried SH groups, and in 0.5 M guanidine, the exposure of buried SH groups is essentially complete (Yao et al., 1984). Urea concentrations of 3 and 4 M respectively, are required, to effect similar changes (Figure 3).

A comparison of the effects of urea and guanidine on the intrinsic fluorescence of creatine kinase is somewhat more complicated. It has been pointed out previously in this paper that the greater increase in fluorescence intensity in urea solutions is due to a direct effect of urea on the exposed Tyr and Trp residues. It is to be noted that under similar conditions, the emission maximum was at 355 nm in 3 M guanidine and at 352 nm in 8 M urea. Furthermore, a comparison of the fluorescence emission spectra of creatine kinase in guanidine and urea solutions shows that the 305-nm emission

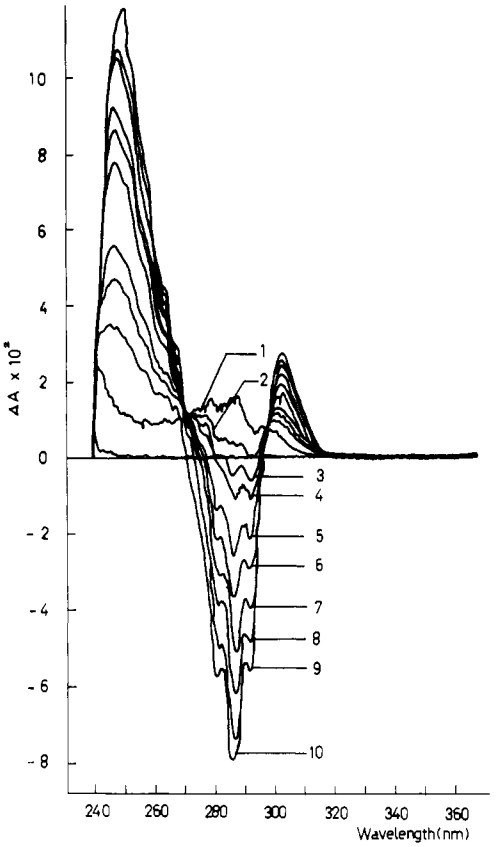

FIGURE 6: Repeated spectral scan during the denaturation of creatine kinase in 4 M urea. The enzyme concentration was 11.9 μM in 0.1 M glycine–NaOH buffer, pH 9.0. The scanning times were 1.5, 7, 11.5, 15, 25, 35, 55, 85, 120, and 140 min after mixing for curves 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10, respectively. T = 25 °C.

shoulder is more marked in 3 M guanidine than in 8 M urea. In contrast to the conclusion reached by Grossman et al. (1981), it seems clear that the unfolding of the enzyme molecule is more extensive in 3 M guanidine than in 8 M urea.

It has been shown in a previous paper (Yao et al., 1982b) that the rates of inactivation of creatine kinase at low guanidine concentrations are much faster than the rates of conformational changes as followed by absorbance and fluorescence changes as well as by changes in the ellipticity and the exposure of SH groups (Yao et al., 1984). Three possibilities have been considered for the difference in the inactivation and denaturation rates observed. First, guanidine may act as a competitive inhibitor; second, the inactivation may have been caused by the rapid dissociation of the active enzyme dimer into the inactive monomers followed by a slow unfolding of the monomers; third, a small conformational change, not detectable by the methods employed, may have brought about the inactivation of the enzyme.

The first possibility can be excluded as it has now been shown that in urea solutions the inactivation rates of the enzyme are also much faster than the denaturation rates as followed by similar methods (Table I). Moreover, it is known that the on rate for the binding of a reversible competitive inhibitor to the enzyme usually has a half-time in the microsecond range (Careri et al., 1975) whereas the half-time for the rapid inactivation of the enzyme in either urea or guanidine has been found to be in the range of 0.1–1 s. This is several orders of magnitude slower than the expected binding rate for a reversible competitive inhibitor.

The possibility that the inactivation of creatine kinase is due to a rapid dissociation of the active dimer into the inactive monomer still has to be seriously considered. Although the

subunit of this enzyme has been reported to be active (Bickerstaff & Price, 1976; Grossman et al., 1981), the recent elegant study of Degani & Degani (1980) would seem to suggest that the enzyme has to be in the dimeric state to be active. However, the renaturation and reactivation rates of the guanidine-denatured enzyme are both independent of enzyme concentration within a certain range (Hou et al., 1983). This would seem to argue against the contention that dissociation and association of the enzyme are responsible for its activity changes in guanidine and urea solutions. Nevertheless, direct measurements to detect whether rapid dissociation of the enzyme takes place in urea or guanidine solutions with rates compatible to the rapid inactivation rates observed would be required to resolve this point.

The most likely explanation for the rapid inactivation as compared to the relatively slow denaturation observed is that the conformational state required for the catalytic activity of the enzyme molecule is very sensitive to slight changes brought about by the denaturants. The active site of the enzyme could be situated within a restricted fragile region of the molecule very sensitive to denaturants, or alternatively, the overall conformation of the enzyme molecule must remain intact for its activity and small disturbances in conformation, too small to be detected by the methods employed in the present series of investigations, could lead to serious consequences in the catalytic activity of the enzyme.

Registry No. Creatine kinase, 9001-15-4; urea, 57-13-6.

References

Baldwin, R. L. (1975) *Annu. Rev. Biochem. 44*, 453.

Bickerstaff, G. F., & Price, N. C. (1976) *FEBS Lett. 64*, 319–322.

Careri, G., Fasella, P., & Gratton, E. (1975) *CRC Crit. Rev. Biochem. 3*, 141–164.

Cowgill, R. W. (1972) *Biochemistry 11*, 4532–4539.

Degani, C., & Degani, Y. (1980) *J. Biol. Chem. 255*, 8221–8228.

Grossman, S. H., Pyle, J., & Steiner, R. J. (1981) *Biochemistry 20*, 6122–6128.

Guggenheim, E. A. (1926) *Philos. Mag. 2*, 538–541.

Henkens, R. W., Kitchell, B. B., Lottich, S. C., Stein, P. J., & Williams, T. J. (1982) *Biochemistry 21*, 5918–5923.

Herskovits, T. T. (1965) *J. Biol. Chem. 240*, 628–638.

Hou, L., Zhou, H., Yao, Q., & Tsou, C.-L, (1983) *Sheng Wu Hua Hsueh Yu Sheng Wu Wu Li Hsueh Pao 15*, 393–397.

Jaenicke, R. (1982) *Biophys. Struct. Mech. 8*, 231–256.

Kim, P. S., & Baldwin, R. L. (1982) *Annu. Rev. Biochem. 51*, 459–490.

Laidler, K. J., & Bunting, P. S. (1973) *The Chemical Kinetics of Enzyme Action*, 2nd ed., pp 175–180, Clarendon Press, Oxford.

Lapanje, S. (1978) *Physicochemical Aspects of Protein Denaturation*, pp 272–296, Wiley, New York.

Mahowald, T. A., Noltmann, E. A., & Kuby, S. A. (1962) *J. Biol. Chem. 237*, 1535–1540.

Marangos, P. J., & Constantinides, S. M. (1974) *Biochemistry 13*, 904–910.

Marous, S., & Desnuelle, P. (1969) *Biochim. Biophys. Acta 181*, 59–78.

McCoy, L. F., Jr., Rowe, E. S., & Wang, K. P. (1980) *Biochemistry 19*, 4738–4743.

Noda, L., Kuby, S. A., & Lardy, H. (1954) *Methods Enzymol. 2*, 605–610.

Perlmann, G. E. (1954) *Nature (London) 175*, 406–407.

Richards, F. M., & Wyckoff, H. W. (1971) *Enzymes, 3rd Ed. 4*, 669–704.

Tanford, C. (1968) *Adv. Protein Chem. 23*, 121–282.

Tanford, C. (1970) *Adv. Protein Chem. 24*, 1–95.

Thomas, J. O. (1974) in *Companion to Biochemistry* (Bull, A. T., Lagnado, J. R., Thomas, J. O., & Tipton, K. F., Eds.) pp 87–138, Longman, London.

Tian, W. X., & Tsou, C.-L. (1982) *Biochemistry 21*, 1028–1032.

Tsou, C.-L. (1951) *Biochem. J. 49*, 362–367.

Tsou, C.-L. (1965a) *Sheng Wu Hua Hsueh Yu Sheng Wu Wu Li Hsueh Pao 5*, 398–408.

Tsou, C.-L. (1965b) *Sheng Wu Hua Hsueh Yu Sheng Wu Wu Li Hsueh Pao 5*, 409–417.

Yao, Q., & Tsou, C.-L. (1981) *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Jin Zhan No. 3*, 52–54.

Yao, Q., Hou, L., Zhou, H., & Tsou, C.-L. (1982a) *Sci. Sin. (Engl. Ed.) 25*, 1186–1193.

Yao, Q., Zhou, H., Hou, L., & Tsou, C.-L. (1982b) *Sci. Sin. (Engl. Ed.) 25*, 1296–1302.

Yao, Q., Tian, M., & Tsou, C.-L. (1984) *Sci. Sin. (Engl. Ed.)* (in press).