and home-built 500-MHz NMR spectrometers. Finally, we also thank Man-Nie Kuo for her excellent technical help.

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Kinetic Evidence for Active Monomers during the Reassembly of Denatured Creatine Kinase†

Steven H. Grossman,* Jacqueline Pyle, and Raymond J. Steiner

ABSTRACT: Treatment of rabbit muscle creatine kinase, which normally exists as a dimer, with various denaturants leads to different states of unfolded protein. Studies of intrinsic fluorescence indicate the degree of denaturation is most pronounced with 8 M urea, followed by 4 M guanidine hydrochloride and then 1 M glycine–H_2PO_4, pH 2.3, buffer. Titration of creatine kinase with increasing concentrations of urea produces parallel changes in inactivation, denaturation (as measured by fluorescence changes and reacting sulfhydryl groups), and dissociation. Renaturation, achieved by dialysis or dilution, leads to 70% recovery of activity. The remainder is in the form of high molecular weight, inactive aggregates. The kinetics of reactivation of creatine kinase denatured in 8 M urea indicates that the rate and percent of reactivation are independent of enzyme concentration ($k_1 = 1.7 \times 10^{-3}$ s^{-1}, at 22 °C). The renaturation rate observed by measuring the decrease in intrinsic fluorescence is also independent of enzyme concentration. Here the kinetic profile exhibits a phase with a rate constant similar to that found for reactivation. Changes in fluorescence are complete after 30 min of initiating renaturation. Renaturation of creatine kinase treated with different denaturants exhibits very similar kinetic profiles and rate constants. Reassociation, as determined by competitive dimerization and hybridization followed by electrophoretic separation or by a selective ultrafiltration technique, reveals that dimerization ($k_2 = 1.8 \times 10^4$ M^{-1} s^{-1}) is still occurring after renaturation and reactivation are complete. The concentration independence of reactivation considered along with the kinetics and temporal relationship among reactivation, renaturation, and reassociation leads to the conclusion that the monomeric subunits of creatine kinase are active. Apparently dimerization is not an obligatory requirement for the expression of enzymic activity.

Numerous studies dealing with the reversibility of protein denaturation have demonstrated that the folding of a polypeptide chain into its native conformation is directly and only dependent upon its amino acid sequence and aqueous environment (Tanford, 1968; Wetlaufer & Ristow, 1973; Anfinsen & Scheraga, 1975). Oligomeric proteins also possess the information in their subunit structure to determine highly specific associations (Cook & Koshland, 1969; Teipel & Koshland, 1971a,b). The importance of association to enzyme function in vivo is based upon whether or not the subunits are intrinsically active. An impressive collection of examples indicates that in vitro native quaternary protein structure is a prerequisite for enzymic activity (Jaenicke & Rudolph, 1977; Gerschitz et al., 1978; Groha et al., 1978; Yamato & Murachi, 1979). These conclusions are based on studies employing a variety of physicochemical techniques (Friedman & Beychok, 1979) and include kinetic evidence from experiments demonstrating that a rate-limiting bimolecular reaction occurs during the reactivation of denatured enzymes (Jaenicke, 1978). These studies include an analysis of the temporal relationship among reactivation, renaturation, and reassociation. On the

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other hand, by a similar analysis, it was shown that aldolase, denatured with Gdn·HCl,[1] initially refolds in a first-order reaction to give active monomeric subunits which subsequently associate into dimers and tetramers (Chan et al., 1973). Rudolph et al. (1977a) have shown that full reactivation of aldolase is achieved only after formation of a tetrameric protein; however, the preparation of matrix-bound monomers of aldolase that exhibit enzymic activity (Chan & Mawer, 1972) adds to the evidence in favor of active subunits.

Matrix-bound monomers of the dimeric enzyme CK from rabbit muscle have been shown to possess full enzymic activity (Bickerstaff & Price, 1976). Studies have shown that Sepharose-linked monomers exhibit several properties similar to those of the dimeric form, including synergistic binding of substrates and formation of transition-state analogues (Bickerstaff & Price, 1978). Evidence derived from analysis of this monomeric, immobilized derivative of CK indicates that dimerization adds little to catalysis by the enzyme or influences its known regulatory properties. However, the degree to which this matrix-bound enzyme is structurally like the soluble enzyme is uncertain. The present study is an effort to provide independent evidence that unmodified, soluble monomeric subunits are active and that dimerization is not an obligatory step in the expression of enzymic activity. We have applied the kinetic approach of Jaenicke (1978) to the study of CK. His analysis depends upon a description of the native and variously denatured states, a demonstration of the reversibility of inactivation, denaturation, and dissociation, and physicochemical proof for the identity between the native enzyme and fully reconstituted enzyme.

Materials and Methods

Crystalline rabbit muscle CK and partially purified rabbit brain CK were purchased from Sigma Chemical Co. (St. Louis, MO). CK was also obtained from Calbiochem-Behring (La Jolla, CA), as were substrates and coupling enzymes used in the assay of CK. Ultrapure urea and Gdn·HCl were products from Schwarz/Mann (Orangeburg, NY). Molecular Probes (Plano, TX) supplied the NDAZ and Ans.

All muscle CK preparations exhibited a single band upon staining for protein after disc electrophoresis in 7% polyacrylamide gels (Davis, 1964). When subjected to a similar test of purity in the presence of sodium dodecyl sulfate (Weber & Osborne, 1969), some samples contained a low molecular weight protein contaminant (approximately 35000), which was eliminated by permeation of the protein sample through a column of Sephadex G-150. When crystalline CK was used, stock solutions (2–5 mg/mL) were prepared in buffer A and incubated at 22 °C for at least 1 h before use to renature any denatured enzyme which was present. All preparations exhibited a specific activity of at least 200 units/mg according to the assay method of Rosalki (1967). Supplementation of the assay reagent with 2 mM DTT and maintenance of the enzyme in 2 mM DTT reduced the lag period in the assay to approximately 15 s. Protein concentrations were calculated from the absorbance at 280 nm by utilizing the extinction value of $E_{280}^{1\%} = 8.88$ (Kuby et al., 1962) or by the fluorescamine assay (Böhlen et al., 1973) with bovine serum albumin as a calibration standard. All molar concentrations were based on the molecular weight of 82000 for dimeric CK (Yue et al., 1967). Assays were performed by using a Gilford-modified Beckman DU spectrophotometer. The cuvette chamber was maintained at 22 °C by using a Polytemp circulating constant-temperature bath and KR refrigerated chiller from Polyscience Corp. (Niles, IL).

Denaturation was performed by incubating stock solutions of CK diluted at least 20-fold in buffer A containing 8 M urea or 4 M Gdn·HCl. Acid denaturation was achieved by adding a large excess of 1 M glycine–H_3PO_4 buffer, pH 2.3, plus 2 mM DTT to a stock solution of CK in buffer A. All denaturations were carried out for 50–60 min.

Quantification of reactive sulfhydryl groups was performed on DTT-free solutions of CK, according to the method of Ellman (1959). The total number of protein sulfhydryl groups was determined in the presence of sodium dodecyl sulfate as described by Habeeb (1972). Reaction of CK with NDAZ was accomplished by the method of Scouten et al. (1974). Isoelectric focusing of NDAZ-labeled CK was carried out as described previously (Grossman & Mollo, 1979). All preparations containing NDAZ-labeled CK were exhaustively dialyzed against buffer A to remove unreacted NDAZ. Binding of Ans to CK was measured in solutions containing 100 μM Ans and 12.4 μM CK in buffer A. All fluorescence measurements we made with an Aminco-Bowman spectrofluorometer equipped with a cuvette chamber maintained at 22 °C.

Molecular weights were estimated by gel permeation chromatography using Sephadex G-150 and buffer A. Calibration standards included aldolase, hexokinase, bovine serum albumin, and pepsin. Subunit molecular weight was determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Weber & Osborne, 1969), with bovine serum albumin, pyruvate kinase, aldolase, and cytochrome *c* used as calibration standards. When the physicochemical properties of denatured and renatured enzyme were compared, the renatured preparation was first centrifuged (5000*g*; 10 min) and then eluted through a column of Sephadex G-150 (2 × 15 cm), maintained in buffer A.

Reactivation of CK denatured in 8 M urea was carried out as follows: Samples of denatured CK were diluted at least 100-fold in buffer A and incubated at 22 °C. Aliquots were removed and added to the assay reagent. After a 15-s mixing and incubation period, the rate of change in absorbance at 340 nm was monitored for 30 s. A short assay time course was employed to minimize any changes in initial velocity which resulted from reactivation during the assay period. In some cases reactivation was followed by measuring initial velocity after introducing urea-denatured CK directly into the assay mixture. The increase in absorbance from 0 to 2.0 was recorded as a function of time. The slopes of tangents drawn to the resulting kinetic profiles were designated as initial velocities. Reactivation was considered complete when identical rates were observed for three consecutive 1-min time intervals.

Renaturation was monitored by recording the change in the intensity of the intrinsic fluorescence accompanying the addition of denatured CK to 3.0 mL of buffer A. The dilutions which were always at least 1 to 60 were followed by a mixing period lasting 3–5 s. Immediately afterward, fluorescence changes were recorded at excitation and emission wavelengths of 282 and 340 nm, respectively. All reactions occurred in cuvettes positioned in a thermostated chamber maintained at 22 °C. The excitation and emission slit widths were set with filters of 5.5- and 11-μm band-pass, respectively. The fluorescence intensities are relative and uncorrected. Emission wavelengths and intensities were standardized by using 1

[1] Abbreviations used: Gdn·HCl, guanidine hydrochloride; CK and CK-MM, muscle-type creatine kinase; CK-BB, brain isozyme of creatine kinase; Ans, 8-anilinonaphthalenesulfonate; NDAZ, *N*-dansylaziridine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); buffer A, 0.2 M Tris-HCl, pH 7.4, and 2 mM dithiothreitol; DTT, dithiothreitol.

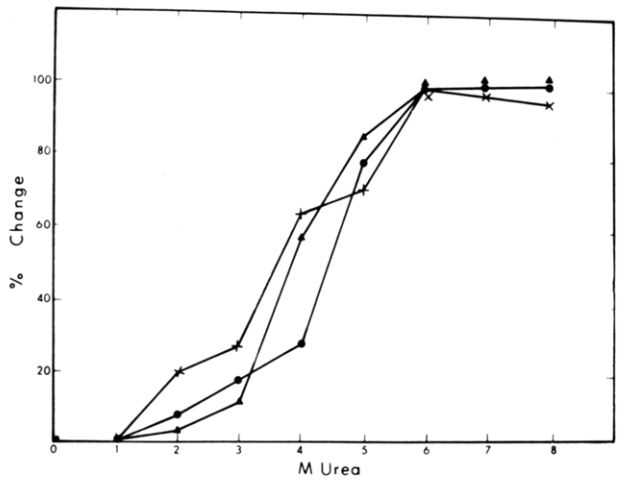


FIGURE 1: Inactivation and denaturation of CK at 22 °C as a function of urea concentration in buffer A. Denaturation: 1 h in urea. (▲) Activity measured after sampling 25 μM CK incubated in denaturant. Initial velocity was monitored for 30 s. (●) Reactive SH groups measured in solutions containing 25 μM CK incubated in denaturant. Changes in absorbance at 412 nm was measured 1 h after the addition of 0.1 mL of 0.4 mg/mL DTNB. Zero percent represents two SH and 100% represents six SH. (×) Emission wavelength maximum of intrinsic protein fluorescence measured in solutions containing 7.7 μM CK incubated in denaturant. Excitation wavelength was 282 nm. Zero percent represents a wavelength of 340 nm and 100% represents a wavelength of 352 nm.

μg/mL quinine sulfate in 0.1 N H_2SO_4 or calibrated solid standards of ovalene and naphthalene (Perkin-Elmer Corp., Norwalk, CT).

Reassociation was first measured by a method utilizing isozyme hybridization and competitive dimerization, followed by electrophoretic analysis. Denatured CK-MM was diluted with buffer A to initiate renaturation. At various intervals, denatured CK-BB was added to samples of the renaturing CK-MM. At least 1 h after the addition of the CK-BB isozyme, the samples were subjected to electrophoretic separation using the Corning ACI apparatus and CK detection reagent (Corning, Palo Alto, CA). We determined the limiting detectable concentration of purified CK by this procedure to be approximately 0.1 nM. The electrophoretogram was subjected to densitometric analysis using a Photovolt (New York, NY) densitometer, equipped with a UV (366 nm) light source.

The rate of reassociation was also monitored by use of membrane selective ultrafiltration. A 50 mL capacity ultrafiltration cell (Amicon, Lexington, MA) equipped with a YM-30 membrane was used to separate monomers from dimers during reassociation. A 1.0-mL sample of CK in 8 M urea was diluted into renaturation buffer (50 mL) in the ultrafiltration cell. Immediately a pressure of 18 psi was applied. The low positive pressure was used to produce continuous ultrafiltration slow enough to prevent a significant increase in concentration of the protein during reassociation. Fractions of filtrate (50 μL) were collected over a 1-h period and assayed. Control experiments showed that a continuous level of 5% of the total CK activity passed through the YM-30 membrane.

Results

Inactivation, Denaturation, and Dissociation. A comparison of the relative dependence of inactivation, denaturation, and dissociation on denaturant concentration indicates the extent to which enzyme activity depends upon native conformation and association. Inactivation was first observed after incubation in 2 M urea; complete inactivation was achieved after 1 h in 6 M urea (Figure 1). The dependence of the extent

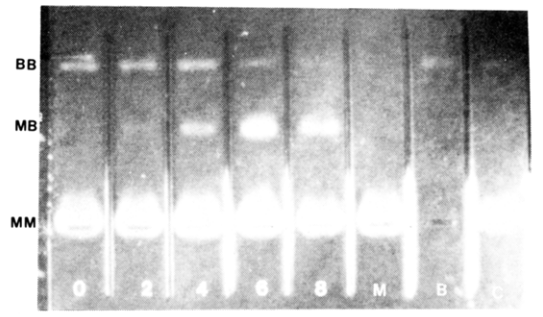


FIGURE 2: Dissociation of CK as a function of urea concentration in buffer A. Mixtures of 20 units of CK-MM and 24 units of CK-BB were incubated at 22 °C in denaturant. After 60 min, samples were diluted 1:100 with buffer A. After 60 min, 1-μL aliquots were subjected to agarose thin-film electrophoresis at pH 7.8 for 25 min, and activity was detected as described under Materials and Methods. Numbers in the channels indicate molarity of urea used for denaturation. Letters in the channels are for isozymes not exposed to urea. Channel M, MM; channel B, BB; channel C, MM plus BB.

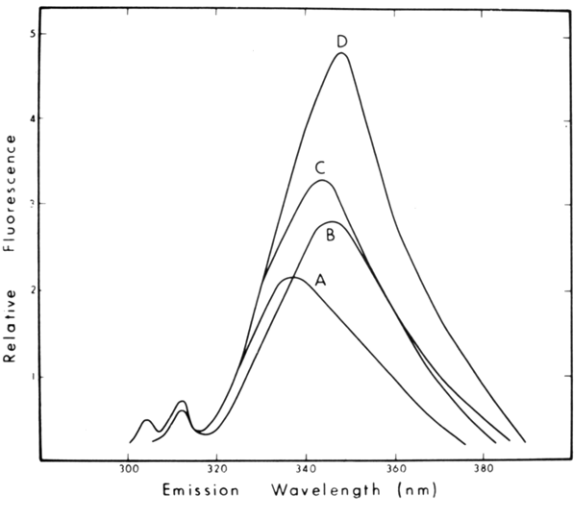


FIGURE 3: Intrinsic fluorescence of native and denatured CK. Excitation at 282 nm; enzyme concentration, 12.4 μM. (A) Buffer A; (B) 4 M Gdn·HCl; (C) 1 M glycine–H_3PO_4, pH 2.3; (D) 8 M urea.

of unfolding on urea concentration was monitored by recording the red shift in the maximum for the intrinsic fluorescence and by the increase in the fluorescence intensity (Figure 1 and see Figure 3 below). Unfolding in 8 M urea led to exposure of four more DTNB-reactive sulfhydryl groups (Figure 1). The six reactive sulfhydryl groups in CK observed after protein unfolding in 8 M urea should be compared with the eight reactive sulfhydryl groups exposed in 2% sodium dodecyl sulfate. Apparently, even in 8 M urea, CK retains some residual organized structure. Dissociation of CK-MM determined through its hybridization with CK-BB was initially detectable following renaturation from 2 M urea (Figure 2). Yue et al. (1967) have reported sedimentation analysis which shows that dissociation of CK into monomers is complete in 8 M urea. The data illustrated in Figures 1 and 2 suggest that increasing concentrations of urea produce parallel changes in inactivation, denaturation, and dissociation.

Denaturation of CK was also achieved by incubation of enzyme in 4 M Gdn·HCl in buffer A or in 1 M glycine–H_3PO_4 buffer, pH 2.3, supplemented with 2 mM DTT. Irrespective of the type of denaturant used, CK responded by displaying an increase in intrinsic fluorescence and a shift in the maximum of the fluorescence emission spectrum to longer wavelengths (Figure 3). CK in urea exhibited the most pronounced changes. While acid treatment led to an enzyme with greater intrinsic fluorescence than one treated with Gdn·HCl, the

Table I: Comparison of CK in Its Native, Renatured, and Denatured States

	native	renatured	denatured
M_r	82 000	82 000	41 500
pI	7.2	7.2	
K_m(C~P) (mM)	2.72	2.64	
V_{max}(C~P) (units/mg)	276	260	0
reactive SH (DTNB)	1.80	1.76	5.9[a] (7.7)[b]
fluorescence			
intrinsic			
$F_{rel,\ 340nm}$ (%)	100	98	205
λ_{max} (nm)	340	340	352
NDAZ labeled			
$F_{rel,\ 521nm}$ (%)	100	112	180
λ_{max} (nm)	521	517	498
Ans labeled			
$F_{rel,\ 482nm}$ (%)	100	95	
λ_{max} (nm)	482	482	

[a] Measured in 8 M urea. [b] Measured in 2% sodium dodecyl sulfate.

maximum for the emission spectrum of CK in the presence of Gdn·HCl was 5 nm closer to the red part of the spectrum than was that of the enzyme in acid. Significant differences in the maximum for the excitation spectra were not observed among native CK and CK treated with different denaturants; at a wavelength of 282 nm, the molar absorptivities differed by less than 5%.

The stability of native CK was demonstrated by the persistence of full activity for at least 12 h at 22 °C and 72 h at 4 °C in the protein concentration range of 0.04–12.4 mM in buffer A. Furthermore, a linear relationship was observed between enzyme concentration (0.04–1 μM) and intrinsic protein fluorescence. Evaluation by gel permeation of the molecular weight of CK at an initial protein concentration of 106, 50, and 18 nM revealed molecular weights of 85 000 ± 4600. Mixing of 25 units of CK-MM and 20 units of CK-BB did not lead to detectable hybrids after electrophoretic analysis as described under Materials and Methods. These experiments provide evidence that inactivation, denaturation, and dissociation do not occur at low protein concentrations in the absence of denaturant.

Reactivated and Renatured CK. A demonstration of the identity between native and renatured CK is a prerequisite for a meaningful interpretation of the kinetics of renaturation. CK denatured in 8 M urea was reactivated by dialysis or dilution. The product obtained after removal by gel filtration of the insoluble irreversibly denatured protein exhibited the same K_m and V_{max} toward the substrate creatine as that of the native enzyme (Table I). In addition, the reactivated CK preparation possessed the same number of sulfhydryl groups reactive toward DTNB and the same molecular weight as were shown by the native enzyme (Table I). Further comparisons demonstrated that the native and reactivated enzymes displayed the same fluorescence intensity and emission maximum for intrinsic fluorescence and fluorescence due to interaction with Ans. However, after a cycle of denaturation–renaturation, CK labeled with the sulfhydryl-specific fluorescent reagent NDAZ displayed a slightly blue shifted emission maximum. On the other hand, the relative fluorescence intensities and the isoelectric points of the two preparations were the same (Table I).

The extent reactivation was between 70 and 80%, if the dialysis buffer contained 2 mM DTT; omission of DTT during reactivation was accompanied by a nearly complete loss of enzyme activity. Reactivation by dilution at 22 °C resulted in a recovery yield of 65–77%. The extent of reactivation was

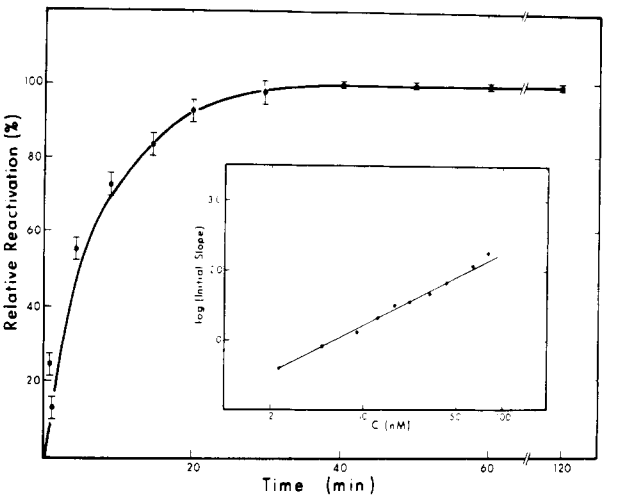


FIGURE 4: Effect of enzyme concentration on the kinetics of reactivation of CK after inactivation in buffer A containing 8 M urea. Inactivation: 60 min at 22 °C. Reactivation by 100-fold dilution in buffer A. Vertical bars indicate range of data points over the concentration range 2.4–96 nM, present during reactivation. Reactivation was measured by removing samples (discrete sampling) during reactivation and assaying as described under Materials and Methods. (Insert) First-order plot of reactivation data.

independent of the concentration of enzyme within the range of 0.4–250 nM; storage at 4 °C for 72 h did not significantly add to the extent of reactivation of CK by the dilution method. For the reactivation studies described below, we employed the dilution method since dialysis does not permit precise determination of reactivation intervals or concentrations.

Reactivation. The rate and concentration dependence of reactivation were monitored after diluting samples of urea-denatured CK into buffer A, followed by assaying portions at intervals thereafter. As illustrated in Figure 4, the rates of reactivation did not depend upon the concentration of enzyme in the range of 2.4–96 nM. Reactivation was complete within 20–25 min, and further incubation at 22 °C did not add to the extent of reactivation. It should be noted that the reactivation profile did not exhibit a sigmoidal region; this observation, combined with the absence of a concentration dependence for reactivation, indicated that, in the concentration range studied, reactivation rate was limited by a single, irreversible, unimolecular reaction (Jaenicke, 1978). The reactivation profile was described by the single, first-order rate constant, $k_1 = 1.7 \times 10^{-3}\ s^{-1}$. Based on initial rates from reactivation measurements, a reactivation reaction order of $n = 1 \pm 0.05$ was found (Figure 4, insert).

We also examined reactivation as it occurred continuously in the enzyme assay mixture (Waley, 1973). Preliminary experiments revealed that reactivation was complete before significant substrate depletion or product accumulation occurred. Initial velocities were equivalent to the values of the tangents to the continuously increasing activity profile. Complete reactivation was signaled when the rates remained unchanged for three consecutive 1-min intervals. Since the range of enzyme concentrations studied by this procedure was restricted by the response rate of the instrumentation, the concentration studied was lower than that employed in the previously described sampling method. Again, results indicated that the rate of reactivation did not depend upon the enzyme concentration. A value of $n = 1 \pm 0.1$ was obtained for the slope of a double-logarithmic plot of concentration against initial rate. The first-order rate constant, $k_1 = 3.1 \times 10^{-3}\ s^{-1}$, was higher than that obtained in experiments using the sampling procedure.

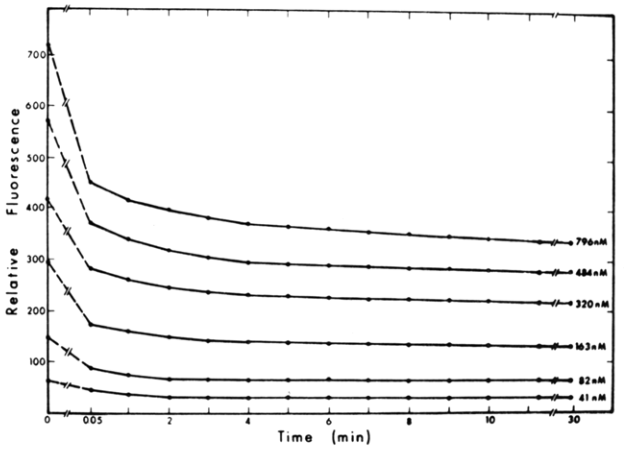


FIGURE 5: Change in intrinsic fluorescence as a function of time after dilution into buffer A of urea-denatured CK. Denaturation: CK (24.7 μM) in buffer A with 8 M urea. Renaturation: addition of denatured CK to 3.0 mL of buffer A in a spectrofluorometer cuvette. Excitation, 282 nm; emission, 340 nm. Dashed lines represent extrapolation of data to include changes in the first 3 s of the renaturation period.

Renaturation. The kinetics of renaturation, measured from changes in intrinsic protein fluorescence, were studied by using CK denatured in 8 M urea, 4 M Gdn·HCl, or 1 M glycine–H_3PO_4 buffer, pH 2.3. It is clear from the data illustrated in Figures 3 and 5 that an extremely rapid initial decrease in fluorescence must occur during the 3-s mixing period after the addition of the urea-denatured enzyme to the renaturation buffer. The analysis of this phase was not subject to study with the instrumentation at hand. Considering the first experimentally detectable fluorescence intensity, we derive a first-order rate plot, which exhibits two notable features: first, a more rapid decrease in fluorescence lasting approximately 3 min, followed by a slower decrease, which was complete within 30 min; second, the rates of these two phases did not depend upon protein concentration. Furthermore, the renaturation of CK denatured in Gdn·HCl or acid determined in this way was independent of protein concentration. The first-order rate constants for the more rapid, initial detectable phase were similar to that observed with urea [k_1 = 5.17 ($\pm$1.02) $\times$ 10^{-3} s^{-1} with acid; k_1 = 3.6 ($\pm$0.6) $\times$ 10^{-3} s^{-1} with Gdn·HCl; k_1 = 4.7 ($\pm$0.7) $\times$ 10^{-3} s^{-1} with urea]. If one considers the expected fluorescence at time zero, then the difference between the first and second observable kinetic phases become considerably less distinct and indeed may consist of a single kinetic phase. The data indicate that the initial reordering of the unfolded monomers is a unimolecular process. The similarities in the rate constants for renaturation of CK unfolded by different denaturants indicate that the monomer passes through a common intermediate during refolding, regardless of the initial state and mode of denaturation.

Reassociation. The rate of reassociation was monitored by examining the formation of the hybrid MB form of CK. The rationale for this approach is that the B subunit of CK will hybridize with an M subunit only if the M subunit is in its monomeric state. The concentration of urea present during the hybridization (0.1 M) was well below the concentration which induces dissociation (see Figure 2). Therefore, the period of time after which hybridization did not occur was the interval in which dimerization was complete since M subunits were no longer in a dissociated state. As shown in Figure 6a,b after 40 min of renaturation, hybrid formation was still detected, proving that reassociation of denatured M subunit was still proceeding at that time. However, when isozyme subunit M was renatured for 60 min and then mixed with denatured

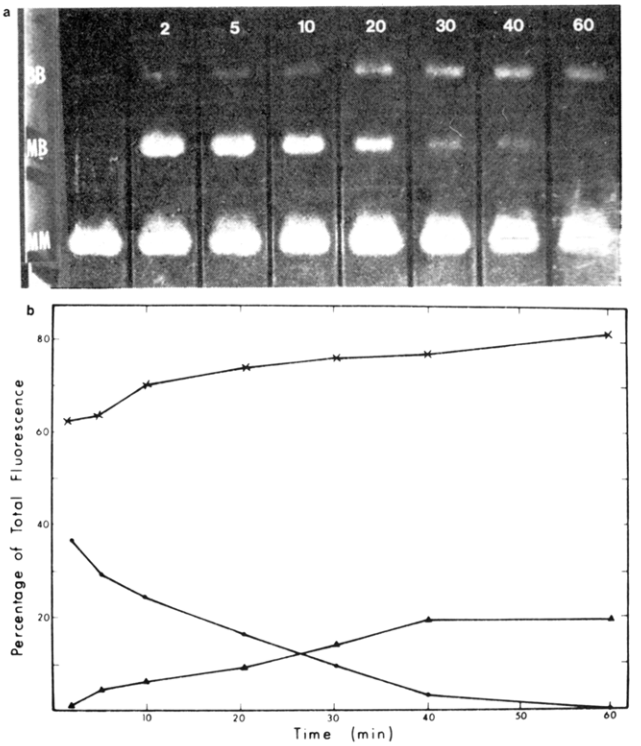


FIGURE 6: (a) Reassociation of urea-denatured CK as determined by competitive dimerization. At time zero, 5.0 mL of buffer A was added to 0.6 mL of 8 M urea in buffer A containing 2 units of CK-MM. At the indicated intervals (minutes), 0.5 mL of the reassociating CK-MM was mixed with 0.01 mL (0.2 unit) of urea-denatured CK-BB. One hour after the last test point, samples were subjected to agarose thin film electrophoresis as described under Materials and Methods. (b) Fluorescence intensity of each isozyme as percentage of total intensity in each channel in (a) determined densitometrically. (×) MM; (▲) MB; (●) BB.

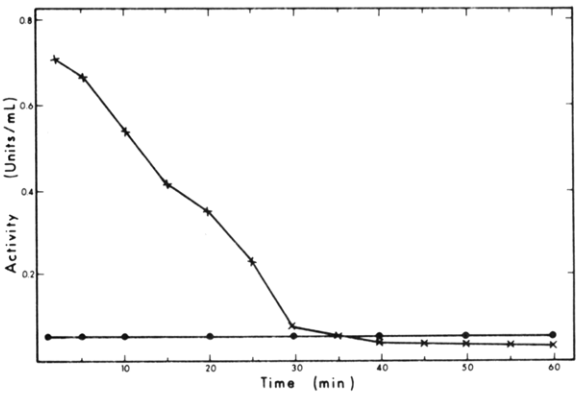


FIGURE 7: Reassociation of urea-denatured CK as determined by selective ultrafiltration. Fifty units of CK in 8 M urea was added to the ultrafiltration cell and treated as described under Materials and Methods. (×) Urea-denatured CK; (●) native CK. Activity was measured 1 h after collection of each filtrate.

isozyme BB (B subunit), no hybrids were detected. This suggests that reassociation was complete within this time period.

The time course of reassociation was also monitored by a technique involving selective ultrafiltration. Denatured monomers of CK were added to a large excess of renaturation buffer in an ultrafiltration cell equipped with a membrane of nominal molecular weight cutoff of 30 000. As illustrated in Figure 7, activity ceased to filter through the membrane at approximately 40 min after initiation of renaturation. Over the same 60-min time interval, 95% native, undenatured CK was rejected by the membrane. A plot of the reciprocal of

concentration of monomers remaining as a function of time is linear and leads to a value of $k_2 = 1.8 \times 10^4$ M^{-1} s^{-1} for the second-order rate constant.

Discussion

The utility of the kinetic method for describing the reconstitution of denatured proteins depends upon a description of the native state and the denatured state as well as a demonstration of the identity between the native protein and the reactivated, renatured, reassociated protein (Jaenicke, 1978). Furthermore, the processess of reactivation, renaturation, and reassociation must be analyzed, where possible, over a similar range of protein concentrations and in an identical aqueous environment.

The products of protein denaturation depend upon the denaturant used (Tanford, 1968, Gerschitz et al., 1977; Hibbard & Tulinsky, 1978). Results of fluorescence analysis of CK demonstrate that among the denaturants studied, urea leads to the most complete denaturation. However, some residual structure remains intact, in view of the greater exposure of cysteine residues in sodium dodecyl sulfate, when compared with that of enzyme in urea. Apparently, considerable structure is retained in 4 M Gdn·HCl. In acid, where the maximum in the intrinsic fluorescence spectrum is closest to that of the native enzyme, it is possible that subunit conformation is largely retained. Stellwagen & Schachman (1962) reported that acid-dissociated aldolase still retains considerable helical structure. Rudolph et al. (1977b) have also reported that appreciable residual conformation is retained by lactate dehydrogenase denatured in acid.

Renatured CK, after removal of aggregated protein, is, with one exception, indistinguishable from the native dimer according to available physicochemical criteria (Table I). The spectral difference with NDAZ may reflect a small conformational difference in the region of the sulfhydryl-containing active site (Mahowald et al., 1962), the persistence of urea near the catalytic site, or formation of aggregated protein or irreversibly denatured monomers. Such aggregates do exhibit blue spectral shifts and, at high concentration, considerable enhancement in fluorescence.

Reactivation of CK denatured in 8 M urea is independent of enzyme concentration over the range studied. The first-order rate constant is significantly, but not dramatically, higher for the reactivation occurring in the assay mixture. The half-time for reactivation of CK directly in the assay mixture is approximately three-fourths of the value obtained in the reactivation kinetics by using the sampling method. Possibly, the "working enzyme" (Milner-White & Kelly, 1976), consisting of a conformational isomer of CK, is formed in the presence of substrates or induction of nucleation centers by the substrate occurs.

Using two different methods, we have demonstrated complete reactivation occurs in less than 30 min. Bickerstaff & Price (1977) found that complete reactivation of Gdn·HCl-denatured CK took approximately 19 h. This difference may be attributed to the use of a different denaturant or different denaturation and renaturation conditions. The presence of Gdn·HCl in low concentrations was reported to retard the rate and extent of reactivation of CK (Bickerstaff et al., 1980) as it does also for the reactivation of lactate dehydrogenase (Zettlmeissl et al., 1979). The origin of this discrepancy remains to be explored, but structural homology between creatine and guanidine may be relevant.

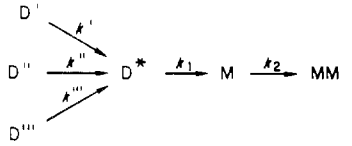
The initial recorded fluorescence after addition of denatured CK to renaturation buffer is approximately 35% less than would be predicted from the relative fluorescence of native and denatured CK, indicating that considerable structural change takes place in the 2–3-s mixing period. Rapid initial phases in renaturation profiles are also noted for staphlococcal nuclease (Epstein et al., 1971), malate dehydrogenase (Jaenicke et al., 1979), and alcohol dehydrogenase (Gerschitz et al., 1978). The first recordable kinetic phase of renaturation can be described by a first-order rate constant which is comparable to that for reactivation. It is possible then that the major recovery of structure and formation of a fully functional active site are controlled by the same isomerization step. In view of evidence for a rapid renaturation step prior to the first observable phase, nonlinearity in a derived first-order plot of the data shown in Figure 5 may be attributed to the last portion of this very rapid initial reaction. However, an analysis of the data in terms of the expected initial fluorescence of denatured CK still displays a biphasic first-order plot, albeit the slopes are somewhat closer and one cannot say unequivocally that these are two independent reactions. It is noteworthy that fluorescence changes are still occurring after complete reactivation.

The first-order kinetics of the reactivation process prove isomerization, and not association, to be the rate-limiting step in the process of reactivation. At least three mechanisms are consistent with this observation: First, a rapid association of denatured monomeric subunits occurs, followed by a rate limiting reorganization of the dimer into the native, active conformation. The second is a slow refolding of the monomers, followed by a rapid association which leads to activity. The third is the possible reorganization of the denatured monomer into an active subunit, followed by a dimerization which does not affect activity. Studies of changes in molecular size and competitive dimerization indicate that association continues after completion of reactivation. Therefore, full recovery of enzyme activity precedes complete reassociation. The use of the hybridization procedure for monitoring reassociation requires that the amount of activity generated during renaturation not be below the limit of detection of the electrophoretic procedure, that hybridization under nondenaturing conditions does not occur, and that fully reassembled isozyme MM does not hybridize with denatured or native isozyme BB. We did not observe hybridization under nondenaturing conditions until storage had been extended to 72 h at 4 °C. Yue et al. (1967) have remarked on the strong affinity that M subunits of CK display for each other.

While the present study was in progress, Bickerstaff et al. (1980) reported that reassociation of CK denatured in 3 M Gdn·HCl, as determined by cross-linking with dimethylsuberimidate, was complete in 15 min. Studies with other denaturants were not reported, but we have shown that in 4 M Gdn·HCl, CK retains a greater degree of residual structure than that found with 8 M urea. Subunit recognition sites may already be available or form more rapidly during renaturation from Gdn·HCl. Furthermore, the relatively high concentration of enzyme used in their study could favor a more rapid association. Our attempts to utilize dimethylsuberimidate as a cross-linkage agent to monitor reassociation were discontinued because we observed the following: (a) the cross-linking yield with native enzymes was usually less than 50%, varied with protein concentration, and required 30 min for completion; (b) experiments with sodium dodecyl sulfate gel electrophoresis of cross-linked CK indicated at least two distinct cross-linked dimers were formed as well as higher molecular weight multimers.

In accordance with the data presented, we propose the following sequence of reactions for the reactivation, renatu-

ration, and reassociation of denatured CK:



D′, D″, and D‴ represent denatured states of CK resulting from unfolding in different denaturants. The respective rates for renaturation, k', k'', k''', are fast and lead to a common polypeptide conformation (D*) which is still significantly unfolded and devoid of enzymic activity but committed to renaturation to the native structure. This intermediate, unfolded state may represent a polypeptide chain containing the "nucleation centers". In a slower step ($k_1 = 3.1 \times 10^{-3}$ s^{-1} for reactivation and $k_1 = 4.7 \times 10^{-3}$ s^{-1} for renaturation from urea), D* becomes the catalytically functional monomer receptive to dimerization ($k_2 = 1.8 \times 10^4$ M^{-1} s^{-1}). Clearly, other kinetic schemes would be compatible with our observations, and more structural information is required, but it is clear that enzyme activity does not increase as a result of association. If reactivation were dependent upon association, a major change in structure, as detected, for example, by fluorescence, should be observed subsequent to dimerization. No such changes were found. Indeed, fluorescence changes were complete before full dimerization. This suggests that the monomeric subunits of CK are enzymatically active and that association proceeds rapidly after formation of native subunits.

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