Enzyme Kinetics above Denaturation Temperature: A Temperature-Jump/Stopped-Flow Apparatus

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ABSTRACT We constructed a ''temperature-jump/stopped-flow'' apparatus that allows us to study fast enzyme reactions at extremely high temperatures. This apparatus is a redesigned stopped-flow which is capable of mixing the reactants on a submillisecond timescale concomitant with a temperature-jump even as large as 60°C. We show that enzyme reactions that are faster than the denaturation process can be investigated above denaturation temperatures. In addition, the temperature-jump/stopped-flow enables us to investigate at physiological temperature the mechanisms of many human enzymes, which was impossible until now because of their heat instability. Furthermore, this technique is extremely useful in studying the progress of heat-induced protein unfolding. The temperature-jump/stopped-flow method combined with the application of structure-specific fluorescence signals provides novel opportunities to study the stability of certain regions of enzymes and identify the unfolding-initiating regions of proteins. The temperature-jump/stopped-flow technique may become a breakthrough in exploring new features of enzymes and the mechanism of unfolding processes.

INTRODUCTION

Characterization of an enzyme mechanism requires the determination of elementary rate constants for which transient kinetics is an especially efficient method. Since most enzymes have low temperature tolerance, until now, to our knowledge, the kinetics and energetics of enzyme reactions could be investigated only in a narrow temperature range. To overcome this fundamental problem we have designed a ''temperature-jump/stopped-flow'' apparatus (1). This equipment allows us to study enzyme reaction steps at extremely high temperatures even above denaturation temperature if the rates of the reactions are faster than that of denaturation. In addition, it provides an opportunity for a more detailed characterization of the enzyme mechanism if the temperature dependences of the reaction steps are different. Fewer temperature-dependent reaction steps that are hidden at low temperatures may become kinetically separable from the preceding or following steps at higher temperatures. Furthermore, this technique is extremely useful for studies of thermodynamics of protein unfolding.

Until now, large rapid temperature-jumps were difficult to achieve (2). Since even a temperature-jump as large as 60°C can be carried out on a submillisecond timescale by the novel rapid mixing temperature-jump/stopped-flow technique, it provides a very efficient way to determine temperature dependences of unfolding processes in wide temperature ranges. This technique is especially useful for studying the unfolding of heat-sensitive proteins which denature rapidly close to the denaturation temperature. In such an experiment the protein can be stored at native temperature and temperature conditions above the denaturation temperature of the protein can be produced rapidly to initiate the heat-induced unfolding reaction. The temperature-jump/stopped-flow technique is also appropriate to investigate thermodynamics and kinetics of heat-induced irreversible unfolding, which is a difficult process to approach by equilibrium calorimetric techniques. The combination of site-directed labeling of proteins (3,4) and temperature-jump/stopped-flow methods is especially useful to investigate the stability of certain regions of proteins.

The temperature dependence of rate constants characterizes the energetics of reaction steps. Due to the relatively low temperature tolerance of enzymes, Arrhenius and van 't Hoff plots can be determined only in narrow temperature ranges. The consequence is that the accuracy and confidence levels of the calculated thermodynamic parameters are very low. In contrast, by using the temperature-jump/stopped-flow technique, Arrhenius and van 't Hoff plots can be determined in a temperature range that is at least three times wider.

Here we describe the principle of the temperature-jump/stopped-flow technique and the design of the equipment along with the characterization and calibration of the setup. We also present two model experiments using this technique.

In the first experiment we studied a recombinant *Dictyostelium* myosin II motor domain (MD) which contains a native single tryptophan in the relay region of myosin (MD-W501+). This region is a central structural element of the myosin MD because the conformational change of the relay region initiates the movement of the lever arm (5). Recently we found by transient kinetic methods detecting tryptophan fluorescence that the large conformational change of the relay region is kinetically coupled to ATP hydrolysis. Nevertheless, the thermodynamic parameters of these reaction

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steps are difficult to determine because the MD is very unstable above 25°C. Consequently, the rate constants can be determined only in a narrow temperature range by conventional transient kinetic techniques. By using the temperature-jump/stopped-flow apparatus the reaction rate constants were determined up to 60°C, which is a significantly higher temperature than the denaturation transition temperature of the MD determined by scanning calorimetry (45°C) (6).

In the other model experiment we studied the temperature-induced heat denaturation of the MD-W501+ construct by monitoring tryptophan fluorescence. We directly followed the stability of the protein structure around the W501 side chain located in the relay region. We found that the heat-induced collapse of this structure is a multistep process. Thermodynamic parameters were determined, and interestingly we found that there is a transition temperature (56°C) at which the energetics of unfolding steps changes drastically.

These model experiments clearly demonstrate that the novel temperature-jump/stopped-flow technique can provide important information about enzyme kinetics and the mechanism of protein unfolding. In this work we also discuss further possibilities for the application of the temperature-jump/stopped-flow method.

MATERIALS AND METHODS

All reagents were purchased from Sigma Chemie (St. Louis, MO) except for the nucleotides that were obtained from Roche (Indianapolis, IN) and *N*-bromo-succinimide (NBS) from Sigma-Aldrich Chemie (St. Louis, Mo).

Applied protein

The myosin MD was prepared and purified as reported by Manstein et al. (7). The applied MD was a single Trp mutant of the M761 *Dictyostelium discoideum* myosin II (MD-W501+) previously described by Malnasi et al. (4). If not stated otherwise experiments were carried out in assay buffer consisting of 20 mM HEPES, pH 7.2, 40 mM NaCl, 1 mM $MgCl_2$, 3 μM 2-mercaptoethanol, 5 mM benzamidine. Before measurement protein preparations were centrifuged at 14,000 $\times$ *g* rpm at 4°C, yielding a clear solution. Buffers were heated to 80°C and stirred in vacuum to remove solved gases that could generate bubbles during warming and could cause artifacts. If not stated otherwise, all concentration values are postmix concentrations.

Temperature-jump/stopped-flow apparatus

The modified stopped-flow apparatus discussed in detail here is based on a KinTek 2004 Stopped-Flow apparatus equipped with two thermocontrollers (Supertech (Pécs, Hungary) STC05A): one that is responsible for adjusting the temperature of the cuvette and the other adjusts the temperature of the so-called heating loop. The heating loop is a 55-μl Teflon loop built in a heating element (resistor based heating element, max 12 V, 3 A, 36 W) including a high-tech thermosensor (Dallas Semiconductor DS1820) controller. The heating loop is connected to the mixing chamber with a 50-μl Teflon tube. A 100-μl shot volume is sufficient to wash out the total volume of the hot buffer from the heating loop and get into the 25-μL cuvette. The cuvette house is heated by a heating element (resistor-based heating element, maximum 24 V, 4 A, 96 W) including a thermosensor similar to the one cited above, which was tightly fixed to the bottom surface of the cuvette house. The temperature of cuvette was detected directly by a thermosensor attached to the wall of the cuvette, and 280-nm and 297-nm excitation wavelength was applied in the case of *N*-acetyl-L-tryptophanamide (NATA) and MD-W501+, respectively, with a 4-nm slit width. On the emission side a 340-nm interference filter (Corion, Newport, RI, CFS-001999 9L134) was used, which does not have significant fluorescence compared to other filters (3).

Temperature calibration of the heating loop and the cuvette house

Utilizing the temperature dependence of the intensity of tryptophan fluorescence, NATA was used to establish correct temperature settings for both heating elements. A total of 20 μl NATA was mixed with 100 μl heated buffer to generate different experimental temperatures in the temperature-jump/stopped-flow apparatus. A constant fluorescence intensity of NATA indicated that the correct temperature values were adjusted by the thermocontrollers. Temperature pairs to be adjusted were determined for the experimental temperatures in the range of 20–70°C in 2°C increments.

Dead time determination

Dead time determination was based on the NBS-NATA reaction (8,9). In these experiments phosphate buffer (140 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) was applied. Measurements were carried out at 25°C applying an 18 ml/s flow rate, and 42 μM NATA was reacted with increasing concentrations of NBS: 33, 83, 167, 417, 833, and 1667 μM. Single exponentials were fitted to the transients. The delay between the intercept of the fitted exponentials and the first data point that joins the fitted exponentials provides an estimate of the instrumental dead time at the applied flow rate.

RESULTS AND DISCUSSION

Principle and construction of the temperature-jump/stopped-flow

In a conventional stopped-flow apparatus, reactants are rapidly pushed from two syringes (A and B) into a small mixing chamber where they combine. Then, through a short tube, the reaction mixture reaches the cuvette where the progress of the chemical reaction can be detected by an optical signal. Detection starts after the quick stop of the pistons, but the reaction starts immediately when the reactants are mixed. Aging of the reaction mixture on the way from the mixing chamber to the cuvette causes the dead time of the apparatus. Typically, the dead time of a stopped-flow instrument is on the millisecond timescale, which is short enough to investigate most enzymatic reactions. The syringes and the cuvette house are adjusted to the same temperature by a water circulator. The applied temperature is limited by the temperature sensitivity of the reactants because keeping them at a high temperature for a longer time would denature them in the syringe before the reaction starts.

A schematic view of the temperature-jump/stopped-flow instrument is presented in Fig. 1 *A*. In this equipment the enzyme and its substrate are stored in syringes A and B, respectively, at native temperature by using water bath temperature control, and the cuvette is kept at the experimental temperature by means of an inserted heating element and temperature controller. Furthermore, a heating loop is inserted between the substrate syringe (syringe B) and the mixing

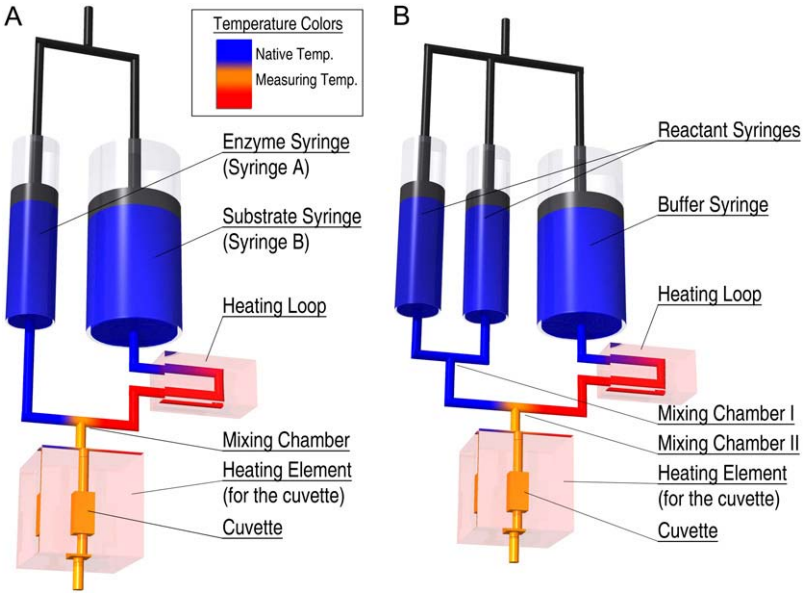

FIGURE 1 Schematic representation of the *temperature-jump/stopped-flow* apparatus in single (*A*) and double (*B*) mixing experiments. (*A*) The heat-sensitive reactant (enzyme) is loaded into syringe A and the non-heat-sensitive reactant (substrate) is loaded into syringe B, both thermostated at 20°C (indicated by *blue*). Upon the stopped-flow push the non-heat-sensitive reactant flows through a heating loop in which it is heated above the reaction temperature (indicated by *red*). The enzyme and the hot reactant combine in the mixing chamber, and they contribute to the new reaction temperature (indicated by *orange*) according to their temperatures and volumes. The process of the reaction at an elevated experimental temperature can be monitored in the cuvette using, e.g., spectroscopic signals. (*B*) Reactants are loaded into syringes A and B, which are both kept at native temperature (indicated by *blue*). Upon the first stopped-flow push the reactants are mixed in Mixing Chamber I thermostated at native temperature (also indicated by *blue*) then flow into a delay line. The motor then stops for a desired delay time, allowing the mixture to reach equilibrium. On the next push the solution is mixed with hot buffer (indicated by *red*) in Mixing Chamber II and forced into the observation cell. The process of reaching the new equilibrium upon the rapid temperature-jump perturbation can be monitored in the cuvette using, e.g., spectroscopic signals.

chamber in which the substrate (the non-heat-sensitive reactant) can be heated to higher temperature than the reaction temperature. The shot volume is adjusted so that after the stopped-flow mix the entire substrate component of the cuvette contents originates from the heating loop segment (see Materials and Methods for more detail). The temperature of the heating loop is controlled by another thermocontroller.

The temperature of the enzyme syringe (syringe A) and the heating loop are adjusted so that the mixture of the enzyme solution and the high-temperature substrate solution gives the reaction temperature. The temperature of the cuvette house is adjusted to this temperature to keep the reaction mixture at a constant temperature during the reaction (see next section for calibration procedure). Mixing the reactants yields the new, high temperature of the reaction mixture. Consequently the temperature-jump does not influence the dead time of the measurement, which is determined by the mixing time of the stopped-flow (1 ms) (Fig. 2 *B*). To achieve high temperature-jumps, asymmetric mixing is applied: one volume of heat-sensitive cold reactant (e.g., enzyme in syringe A) is mixed with five volumes of non-heat-sensitive hot reactant (e.g., substrate in syringe B). It should be noted that the temperature dependence of the pH of the applied buffer should be taken into consideration in all experiments involving temperature-jumps. Buffers with low temperature dependence such as phosphate and HEPES are recommended.

In a typical experiment after the first push of the drive syringes, the solution from the substrate syringe (syringe B)

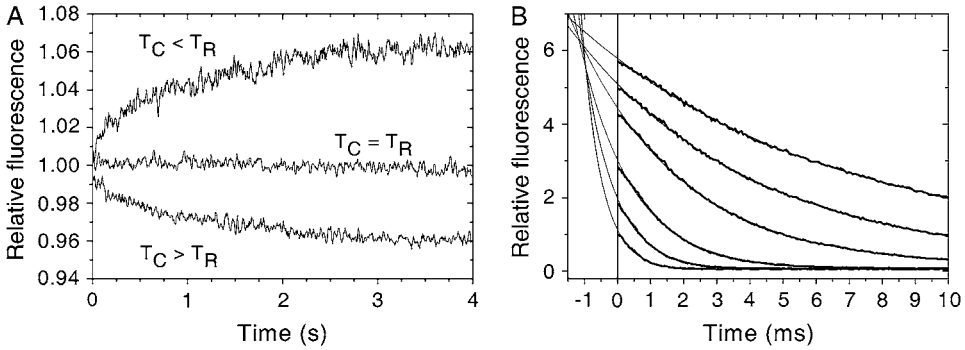

FIGURE 2 Temperature calibration of the heating elements (*A*) and determination of the dead time of the *temperature-jump/stopped-flow* apparatus (*B*). (*A*) *Temperature-jump/stopped-flow* fluorescence records on mixing 50 μM NATA thermostated at 20°C with hot buffer thermostated at 58°C, 60°C, and 62°C and then pushed into a 52°C cuvette house. A fluorescent intensity increase or decrease indicates that the temperature of the reaction mixture (T_R) was higher or lower than the temperature of the cuvette (T_C), respectively. A constant fluorescence intensity indicates that the temperature of the reaction mixture was identical to the temperature of the cuvette. (*B*) Temperature-jump/stopped-flow records of the reaction of NATA with increasing concentrations of NBS at 25°C to determine mixing dead time (for details see Materials and Methods). The instrument dead time was determined to be 0.9 ms.

reaches the heating loop inserted before the mixing chamber, where the substrate solution can be warmed up even to 80–90°C. Due to the second push, the hot substrate solution from the heating loop and the colder enzyme solution (from syringe A) combine in the mixing chamber. The solutions of reactants contribute to the combined temperature according to their volumes, and thus even a 50–60°C temperature-jump of the enzyme solution can be achieved immediately. While the reaction proceeds, the next portion of the substrate solution warms up in the heating loop, so the next shot is prepared for the forthcoming measurement.

Goldmann and Geeves (10) suggested an arrangement called slow temperature-jump, in which the syringes are kept at low temperature and the mixed cold reactants are shot into the hot cuvette. Using this technique, temperature equilibration of the reaction mixture takes 150 ms. Verkman et al. (11) constructed other equipment called stopped-flow temperature-jump which has a 60-ms dead time. The long dead times of these instruments severely limit their applicability. In comparison the dead time of the novel temperature-jump/stopped-flow is much shorter (1 ms) because the reaction temperature is immediately set upon mixing due to the prewarming of substrate.

Calibration of the temperature-jump/stopped-flow

Taking advantage of the temperature dependence of fluorescence emission (12), our system was calibrated to ensure that the temperature of the mixed solution and the cuvette are the same. Since the rates of reactions and the intensity of fluorescence are temperature sensitive, temperature equilibration of the reaction mixture while already in the cuvette during the course of the reaction would cause artifacts. Small-molecule fluorophores like NATA are useful to calibrate the temperatures of the reaction mixture and the cuvette house to the same value. NATA was loaded into the cold syringe A and rapidly mixed with hot buffer pushed through the heating loop inserted between syringe B and the mixing chamber.

Change in fluorescence intensity of NATA in the cuvette indicated that the temperature of the reaction mixture and the cuvette were not identical right after mixing and temperature reequilibration occurred in the cuvette. A fluorescence intensity decrease indicated that the reaction mixture was colder than the cuvette, and the solution warmed up in the cuvette and vice versa (Fig. 2 *A*). A constant fluorescence intensity indicated that the temperature of the cuvette and the reaction mixture was the same. The time constant of temperature equilibration in our system was determined to be $\tau = 1.2$ s. Since the temperature dependence of fluorescence intensity of NATA is sensitive enough to calibrate our system with 0.1°C accuracy, the temperature to be set for the heating loop and the cuvette can be easily determined for each experimental temperature. A set of calibration values of the temperatures of the cuvette, heating loop, and enzyme syringe could be established. This set of values is specific to the instrument and valid as long as dilute aqueous solutions are used.

The main advantage of this setup is that the fast temperature-jump occurs simultaneously with the rapid mixing of the reactants so the dead time of mixing and temperature-jump are identical if the temperatures of the reaction mixture and the cuvette are identical (i.e., the instrument is properly calibrated). We found that modification of the conventional stopped-flow apparatus did not increase the dead time, which was determined with the reaction of NATA and NBS (8,9) to be 0.9–1 ms (Fig. 2 *B*).

Reaction kinetics of the myosin motor domain at extremely high temperatures

We studied the temperature dependence of the kinetics of the ATP-induced fluorescence change in MD-W501+. We chose this reaction as a model experiment to test the novel temperature-jump/stopped-flow technique because it is a well-characterized enzymatic process (4,5,13). The fluorescence change of W501 upon reacting with ATP can be described with a simplified three-step mechanism (Scheme I):

$$\mathrm{M + ATP} \underset{}{\overset{\text{Step 1}}{\rightleftharpoons}} \mathrm{M.ATP} \overset{\text{Step 2}}{\rightleftharpoons} \mathrm{M^*.ATP} \overset{\text{Step 3}}{\rightleftharpoons} \mathrm{M^*.ADP.P_i}$$

SCHEME 1

where M* represents high fluorescent MD-W501+ states, and the lengths of the arrows indicate the relative magnitudes of elementary rate constants. After ATP binding (Step 1) a large conformational change occurs in the relay region (Step 2), which moves the lever arm of myosin and causes an ~50% fluorescence enhancement of W501 as measured by stopped-flow (4). This fast equilibrium reaction step (Step 2) is coupled to the relatively slower hydrolysis step (Step 3), which is shifted to the hydrolyzed product state. The hydrolysis step is not associated with any fluorescence change of W501; therefore a fast fluorescence burst phase precedes a slower phase. Since the equilibrium constant of the large conformational change (Step 2) is highly temperature dependent, the characteristics of the detected stopped-flow records are highly influenced by temperature. The equilibrium (Step 2) is shifted to the M*ATP state by temperature; therefore the higher the temperature the larger the amplitude of the burst phase. Since this well-characterized mechanism is highly temperature dependent (5), it is an appropriate model system to test our novel technique. A recently reported scanning calorimetry study showed that the structure of the MD collapses at 45°C (6). The question arises: Can enzyme reactions be detected above this temperature?

Fig. 3. shows temperature-jump/stopped-flow records of the ATP-induced intrinsic fluorescence change of MD-W501+ at different temperatures. At 20°C (data not shown) and 26°C a fast burst phase ($k = 161\ \mathrm{s}^{-1}$) followed by a slower phase ($k = 18\ \mathrm{s}^{-1}$) was detected as was expected based on our earlier

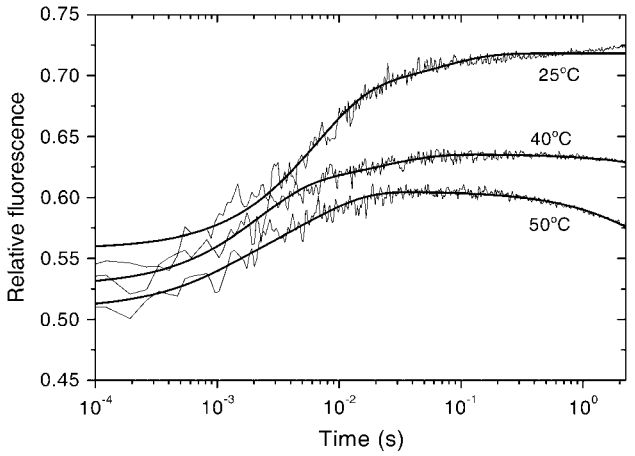

FIGURE 3 Temperature-jump/stopped-flow records of the reaction of myosin MD with ATP; 3 μM MD-W501+ was mixed with 1 mM ATP at different temperatures and the change in tryptophan fluorescence intensity was monitored. At 26°C double exponentials could be fitted to the traces corresponding to ATP binding and the following hydrolysis steps. Above 40°C a third phase with fluorescence intensity decrease appeared indicating that denaturation of myosin occurred.

results (4). The traces had similar characteristics at 40°C; however, after the two phases of the fluorescence increase ($k = 480\ s^{-1}$ and $44\ s^{-1}$) representing the enzyme reaction, a slow fluorescence decrease ($k = 0.016\ s^{-1}$) was observed. Above the denaturation temperature (determined by scanning calorimetry) the enzyme reaction could still be detected (14). The two phases of the fluorescence increase ($k = 950\ s^{-1}$ and $160\ s^{-1}$) were followed by a dominant slow signal decrease ($k = 0.26\ s^{-1}$), indicating the denaturation process of the enzyme (see below). The equilibrium constant of the second reaction step (Step 2) determines the ratio of the relative amplitudes of the two phases as it was characterized recently.

The equilibrium constant of this step increases with temperature (5). We found the same temperature effect up to 40°C: the equilibrium constant increased from $K_2 = 4.5$ at 26°C to $K_2 = 9.5$ at 45°C. Interestingly, above 45°C the ratio of the relative amplitudes did not increase further, in contrast, it dropped to $K_2 = 1.3$ at 50°C. There are three possible explanations for this phenomenon: 1), The temperature dependence of the equilibrium constant of the second reaction step changes drastically at extremely high temperatures; 2), the after hydrolysis step (Step 3) becomes more reversible at extremely high temperatures; and 3), a ''hidden'' reaction step appears above 40°C. Irrespective of which assumption is valid, we can conclude that the temperature-jump/stopped-flow method allows us: A), to detect all the reaction steps that are faster than the events of heat denaturation of the enzyme, and B), to reveal unexpected transitions during enzyme reactions at extremely high temperatures.

We have also investigated other enzyme reactions of the myosin MD. As an example of a first order reaction, ADP off rates were determined at different temperatures using an ATP chasing method (15). We found that even at 65°C enzyme reactions can be detected; however, there is a break in the Arrhenius plot at around 45°C (data not shown) (16).

Site-specific kinetics of the heat-induced unfolding of the myosin motor domain

The novel temperature-jump/stopped-flow gives an opportunity to follow rapid structural changes in enzymes during heat-induced unfolding. By introducing site-specific signals into proteins, a further prospect is provided as local structural rearrangements can be investigated.

To test the novel temperature-jump/stopped-flow method for the investigations of heat-induced protein unfolding, we used the single tryptophan mutant myosin motor domain MD-W501+. In this experiment MD-W501+ was loaded into syringe A, kept at 20°C, and then rapidly mixed with hot buffer yielding different experimental temperatures. The fluorescence intensity change of W501 was followed, which reflects directly the stability of the relay region where the fluorophore is located. Fig. 4 *A* shows temperature-jump/stopped-flow records of the heat denaturation of MD-W501+ at 52°C. The records had

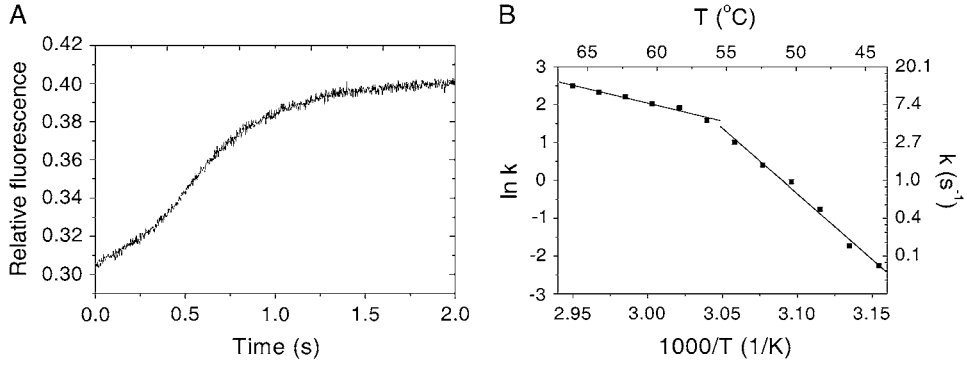

FIGURE 4 Heat-induced denaturation of the myosin MD. (*A*) 6 μM MD-W501+ was mixed with hot buffer, and the progress of denaturation was monitored at 52°C by detecting the tryptophan fluorescence intensity change. A lag phase is followed by a single exponential increase of the fluorescence intensity, indicating that the denaturation of MD-W501+ is a multistep process. (*B*) Arrhenius plot (in k versus $1/T$) of the heat-induced denaturation. There is a break in the plot at 56°C; thus two linear functions could be fitted to the sections above and below this break point ($\Delta H^{\ddagger}_{40\text{-}55°C} = 4.2$ kJ/mol, $\Delta H^{\ddagger}_{55\text{-}70°C} = 1.2$ kJ/mol). This phenomenon suggests that another reaction step becomes the rate-limiting step above 56°C.

intriguing characteristics. The fluorescence intensity increase is preceded by a lag phase, which suggests that the mechanism of unfolding of MD-W501+ consists of at least two steps. Since the signal is structure specific and in the absence of nucleotide, the myosin MD adopts a single state (5), the lag phase indicates that initiation of heat-induced unfolding occurs in another region of MD different from the relay region.

The observed rate constants of the phase after the lag were plotted against temperature (Fig. 4 *B*). We found that there is a transition in the Arrhenius plot of heat-induced denaturation at 56°C, which indicates a further reaction step. Further investigation is required to resolve the mechanism of this unfolding process; however it is clearly demonstrated by this model experiment that heat-induced unfolding is a structurally, kinetically, and thermodynamically complex process which can be studied efficiently by the novel temperature-jump/stopped-flow technique. The combination of the temperature-jump/stopped-flow technique with structure-specific signals opens a new prospect in the investigation of protein-unfolding mechanisms.

Further applications of the temperature-jump/stopped-flow

We show that temperature-jump/stopped-flow is a useful technique to study enzyme reactions at extremely high temperatures, and it is also a new approach to investigate the mechanism of protein unfolding/folding. In addition, there are further opportunities for the application of the equipment. For instance, a breakthrough can be achieved in the characterization of human enzymes in vitro at physiological temperature. Since most purified human enzymes are unstable at 37°C, kinetic measurements have not been possible at the most relevant (in vivo) temperature. This difficulty can be overcome by the application of the temperature-jump/stopped-flow because a rapid temperature-jump can be carried out from the storage temperature (e.g., 20°C) to 37°C with concomitant initiation of the enzyme reaction.

Furthermore, equilibria of transiently appearing reaction populations can be studied by temperature perturbation of the equilibrium if the temperature-jump/stopped-flow is applied in the double-push mode of the equipment. At lower temperatures the enzyme can be rapidly mixed with substrate, and after a certain delay time when the molecule populations of interest are most abundant, hot buffer can be mixed with the reaction solution in the second stopped-flow push to rapidly increase temperature (see Fig. 1 *B*). In this experiment the short-lived preequilibrium is perturbed by temperature which can be a small temperature-jump (~5°C) as it is in the traditional T-jump experiments, or even a very high temperature-jump can be achieved (5–50°C). These equilibrium reactions of intermediates are mostly first order reactions, and thus no additional relaxation occurs due to the dilution of reactants with the hot buffer. This novel technique is a new and straightforward method to characterize equilibria of transiently appearing intermediate populations which were not detectable before.

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