Inactivation kinetics of dihydrofolate reductase from Chinese hamster during urea denaturation

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The kinetic theory of substrate reaction during modification of enzyme activity has been applied to the study of inactivation kinetics of Chinese hamster dihydrofolate reductase by urea [Tsou (1988) *Adv. Enzymol. Relat. Areas Mol. Biol.* **61**, 381–436]. On the basis of the kinetic equation of substrate reaction in the presence of urea, all microscopic kinetic constants for the free enzyme and enzyme–substrate binary and ternary complexes

have been determined. The results of the present study indicate that the denaturation of dihydrofolate reductase by urea follows single-phase kinetics, and changes in enzyme activity and tertiary structure proceed simultaneously in the unfolding process. Both substrates, NADPH and 7,8-dihydrofolate, protect dihydrofolate reductase against inactivation, and enzyme–substrate complexes lose their activity less rapidly than the free enzyme.

INTRODUCTION

All the information necessary for the formation of the three-dimensional structure of a protein is contained in its amino acid sequence [1]. However, the mechanism by which this complex conformational change takes place is not known in detail. A variety of equilibrium and kinetic techniques have been used to study the folding/unfolding of a number of globular proteins. Because secondary and tertiary structures can be usefully described by several methods such as CD, fluorescence and NMR, the folding and unfolding processes have been specified mainly on the basis of these structural features. Although the importance of conformational integrity for enzyme activity is generally recognized, in the vast amount of literature on the unfolding of proteins, only a limited number of authors have compared conformational and activity changes during the course of enzyme denaturation [2]. This is probably because of the lack of a suitable method for quantitatively measuring fast rates of enzyme inactivation.

Some years ago, a systematic study on the kinetics of substrate reaction during the irreversible modification of enzyme activity was presented [3–5]. It was shown not only that the apparent rate constant for the irreversible modification of enzyme activity can be obtained in a single experiment, but that the effect of substrate complexing and competition with the modifier can also be ascertained. In the present study, this approach has been extended to study the kinetics of enzyme denaturation. The kinetic equation of substrate reaction in the presence of denaturant has been derived, and, as an example, the kinetics of inactivation of dihydrofolate reductase (DHFR) by urea has been studied.

DHFR (EC 1.5.1.3) catalyses the NADPH-dependent reduction of 7,8-dihydrofolate (DHF) to tetrahydrofolate. The enzyme has been the subject of extensive study not only because of its importance in intermediary metabolism but also because it is the target for a number of chemotherapeutic agents [6]. Furthermore, DHFR from Chinese hamster is a small monomeric protein and has no prosthetic groups or disulphide bonds [7,8]. Therefore, it is an ideal candidate for folding studies. The results of the present study indicate that DHFR denaturation by urea exhibits single-phase kinetics, and changes in enzyme activity and tertiary structure proceed simultaneously during the

unfolding processes. Both substrates, NADPH and DHF, protect DHFR against inactivation, and enzyme–substrate complexes lose their activity less rapidly than the free enzyme.

MATERIALS AND METHODS

Materials

DHF (approx. 90%) and NADPH (approx. 97%) were purchased from Sigma. Methotrexate was obtained from ICN; its concentration was determined spectrophotometrically in 0.1 M NaOH, using a molar absorption coefficient (ϵ) of $22\,100\text{ M}^{-1}\cdot\text{cm}^{-1}$ at 302 nm [9]. The concentrations of DHF and NADPH were determined using $\epsilon_{280} = 28\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$ and $\epsilon_{340} = 6\,200\text{ M}^{-1}\cdot\text{cm}^{-1}$ at pH 7.5 respectively [10]. Urea (ultrapure) was from Boehringer-Mannheim, and solutions were always freshly prepared from a recrystallized sample [11]. Sephadex G-75 was from Pharmacia. Other reagents were local products of analytical grade used without further purification. Double-deionized water was used throughout.

Construction of Chinese hamster DHFR expression plasmid

The plasmid pSVA75, kindly provided by Dr. P. W. Melera (University of Maryland School of Medicine, Baltimore, MD, U.S.A.), contained the full-length cDNA of Chinese hamster DHFR and was used as a PCR template to amplify the DHFR gene. To facilitate synthesis of DHFR in *Escherichia coli* DH5 α , the DHFR gene was isolated from the PCR product as a *Nco*I–*Sal*I fragment and cloned into *Nco*I and *Sal*I sites in the expression vector pBV221. The resulting plasmid, pDHFR, positions the DHFR gene under the transcriptional control of both the thermoinducible P_R and P_L promoters and the temperature-sensitive cI857 repressor.

Purification of Chinese hamster DHFR

Luria–Bertani medium (4 litres) containing 50 μg of ampicillin/ml was inoculated with overnight culture at a dilution of 1:50. The culture was grown at 30 °C until A_{600} was approx.

Abbreviations used: DHFR, dihydrofolate reductase; DHF, 7,8-dihydrofolate.

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0.6, and then induced at 42 °C for about 6 h. The cells were collected by centrifugation at 3300 *g* (4000 rev./min) for 10 min at 4 °C. To release recombinant Chinese hamster DHFR from the bacterial cytoplasm, the freeze/thaw procedure described by Johnson and Hecht [12] was used. The suspension was centrifuged at 16300 *g* (13000 rev./min) for 10 min at 4 °C, and the supernatant was concentrated by dialysis against solid poly(ethylene glycol)-20000. The concentrated sample was applied to a column (3.5 cm × 110 cm) of Sephadex G-75 equilibrated with 10 mM potassium phosphate, pH 6.8, containing 2 mM 2-mercaptoethanol, and eluted with the same buffer. The combined active fractions were concentrated and stored in small aliquots at −80 °C. The final preparation showed one band on SDS/PAGE and a single peak on reverse-phase HPLC on an Ultrapore C8 column. The concentration of DHFR was determined by methotrexate titration. The specific activity of the purified enzyme was 5.48 μmol/min per mg.

Enzyme assays

The activity of DHFR was determined at 20 °C by following the decrease in NADPH and DHF by A_{340} measurements using a Shimadzu UV-250 spectrophotometer thermostatically controlled at 20 °C. Experiments were performed at pH 7.5 in a buffer mixture containing 50 mM potassium phosphate and 10 mM 2-mercaptoethanol. The concentrations of enzyme, NADPH and DHF are given in the text or in the legends to the Figures. The standard unit of enzyme activity is defined as the amount of enzyme converting 1 μmol of DHF and NADPH to tetrahydrofolate and NADP⁺ respectively per min and is calculated from the change in A_{340} using $\epsilon = 12300 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 20 °C and pH 7.5, which is equal to the sum of the molar absorbance difference between DHF and tetrahydrofolate and that between NADPH and NADP⁺ [13].

For progress-curve experiments, the two-substrate reaction of DHFR was reduced to a one-substrate irreversible reaction by setting one of the substrates at saturating concentration. The kinetics of the inactivation reaction was followed by measuring the substrate reaction in the presence of the denaturant as described previously [3]. All measurements were carried out in 0.05 M potassium phosphate buffer, pH 7.5, at 20 °C.

Fluorescence measurements

Equilibrium unfolding as a function of urea concentration was monitored by fluorescence spectroscopy by exciting the tryptophans at 280 nm on a Hitachi F-4010 fluorimeter. All samples were allowed to equilibrate fully at the appropriate final urea concentration at 20 °C before spectra were recorded. Kinetics of unfolding was also followed by fluorescence spectroscopy using manual mixing methods that have a dead time of 5–8 s. Fluorescence measurements were made by exciting the enzyme at 280 nm. Emission intensity was monitored at a wavelength of 325 nm.

KINETIC ANALYSIS

In the presence of substrate, DHFR inactivation by urea can be written as shown in Scheme 1. [14,15]. If one of the substrates is saturating at all stages, the reaction can then be treated as a one-substrate system (Scheme 2).

From Scheme 2, the concentration of the product formed is given by [4]:

$$[P] = \frac{v_0}{A + k_{-0}} \left\{ k_{-0} t + \frac{A}{A + k_{-0}} \left[1 - e^{-(A + k_{-0})t} \right] \right\} \quad (1)$$

Scheme 1 DHFR inactivation by urea in the presence of substrate

E and *D* represent the native and denatured enzyme, and *S* and *R* are two substrates, NADPH and DHF respectively. It is assumed that neither NADPH nor DHF can bind to the denatured enzyme.

where $[P]$ is the concentration of product formed at time t , and v_0 and A are the initial velocity of the substrate reaction and the apparent inactivation rate constant in the presence of urea respectively:

$$v_0 = \frac{k_c[E]_0[S]}{K_s + [S]} \quad A = \frac{k''_{+0}K_s + k'''_{+0}[S]}{K_s + [S]}$$

When reaction time t is sufficiently large, the exponential term in eqn. (1) can be neglected and the progress curve approaches a straight line with a slope of $v_0 k_{-0}/(A + k_{-0})$. Generally speaking, k''_{+0} and k'''_{+0} will increase, and k_{-0} decrease with increasing urea concentration. When k_{-0} becomes very small compared with A , the reaction can be virtually treated as irreversible, and eqn. (1) can be written as:

$$[P] = \frac{v_0}{A} (1 - e^{-At}) = [P]_\infty (1 - e^{-At}) \quad (2)$$

where

$$[P]_\infty = \frac{v_0}{A} = \frac{k_c[E]_0[S]}{k''_{+0}K_s + k'''_{+0}[S]}$$

It should be noted that $k_{-0} = 0$ does not necessarily mean that the protein denaturation is irreversible, by which we mean that simple return to the native environment will not lead to a return to the native conformation of the protein. It can be seen from eqn. (2) that, when reaction time t approaches infinity, $[P]$ approaches constant values, $[P]_\infty$. Plots of $\ln([P]_\infty - [P])$ against t give straight lines with slopes of $-A$. The values of v_0 can be

Scheme 2 DHFR inactivation by urea when one substrate is saturating

R is the saturating substrate, and k_c , K_S , and k''_{+0} , k'''_{+0} , k_{-0} are kinetic parameters for enzymic reaction, unfolding and refolding rate constants in the presence of urea respectively. All these constants are functions of the urea concentration.

determined directly by the normal method of drawing a tangent to the initial rate phase of the reaction progress curve. Alternatively, with the values of $[P]_{\infty}$ and A , v_0 can also be determined according to $v_0 = A[P]_{\infty}$. Note that the values of v_0 so determined are the initial velocities of the native enzyme-catalysed reaction in the presence of urea, which are, in general, not the same as those obtained from separate experiments in the absence of urea, since both the formation of specific complexes between urea and enzyme and the effect of urea on the dielectric constant of the medium may change the kinetic parameters of an enzyme-catalysed reaction [16]. Thus, from the relationships between v_0 , $[P]_{\infty}$ and $[S]$, all the kinetic parameters, k_c , K_s , k''_{+0} , k'''_{+0} , can be determined as described previously [4]. Similarly, when S is saturating at all stages, K_R , k'_{+0} , k'''_{+0} can be obtained by the same procedure.

RESULTS

Steady-state kinetics of Chinese hamster DHFR

The kinetic behaviour of DHFR from different sources has been extensively recorded in the literature [17–20]. In the present

Table 1 Kinetic parameters of DHFR-catalysed reaction

	Steady-state kinetics	Fluorescence titration
$K'_{\text{(DHF)}} (\mu\text{M})$	0.29 ± 0.04	0.25 ± 0.03
$K'_{\text{(NADPH)}} (\mu\text{M})$	0.37 ± 0.05	0.34 ± 0.04
$K_{\text{(DHF)}} (\mu\text{M})$	0.48 ± 0.03	—
$K_{\text{(NADPH)}} (\mu\text{M})$	0.63 ± 0.03	—
$k_{\text{cat}} (\text{min}^{-1})$	92.62 ± 12.06	—

Figure 1 Initial-velocity pattern of DHFR-catalysed reaction at 20 °C in 50 mM potassium phosphate buffer, pH 7.5, containing 10 mM 2-mercaptoethanol

The enzyme concentration was 0.5 $\mu\text{g/ml}$. The concentrations of DHF for lines 1–6 were 0.40, 0.53, 0.79, 1.06, 1.59 and 3.17 μM respectively. The inset shows the secondary plots of the slopes (○) and the ordinate intercepts (●) against $1/[\text{DHF}]$.

Figure 2 Comparison of activity and conformational changes in DHFR at different concentrations of urea solution at pH 7.5, 20 °C

○, Relative emission intensity at 325 nm, with an excitation wavelength of 280 nm; ●, relative activity. The inset shows the fluorescence emission spectra of DHFR in different concentrations of urea solution. The concentrations of urea for curves 1–6 were 0.0, 1.0, 2.0, 2.5, 2.8 and 3.6 M. The enzyme (14 $\mu\text{g/ml}$) was incubated for 24 h at 20 °C in 50 mM potassium phosphate buffer, pH 7.5, containing 10 mM 2-mercaptoethanol in the presence of the indicated concentration of urea. Samples were removed for assay, with the same concentration of urea present in the assay mixture as in the original incubation. The final concentration of the enzyme in the assay system was 0.7 $\mu\text{g/ml}$. In each case the value corresponding to the enzyme in the absence of urea was 1.0.

study, equilibrium binding and steady-state kinetics of Chinese hamster DHFR in the absence of urea were first studied. The dissociation constants were determined by titration of DHFR with DHF or NADPH. DHF and NADPH were added in small volume to a fixed concentration of enzyme, and changes in fluorescence intensity were recorded. The excitation and emission wavelengths were 280 nm and 325 nm respectively. The intrinsic fluorescence of DHFR is quenched by either of the substrates (results not shown). The values of the dissociation constants for DHF and NADPH were obtained by fitting the experimental titration data to the equation for 1:1 binary complexes [21], and are listed in Table 1. The steady-state velocities obtained by varying NADPH at different fixed concentrations of DHF yielded an intersecting pattern (Figure 1). The lack of deviation from linearity of double-reciprocal plots is consistent with, albeit insufficient evidence for, the conclusions of previous investigators [17,18] that the reaction is a rapid-equilibrium random mechanism. The data were fitted to the equation that describes a rapid-equilibrium random mechanism, and the dissociation constants determined are summarized in Table 1. It can be seen from this Table that the dissociation constants for DHF and NADPH obtained by the two methods are consistent.

Changes in activity and fluorescence of DHFR in different concentrations of urea

Enzyme was incubated in 50 mM phosphate buffer, pH 7.5, in the absence or presence of urea, at 20 °C. When excited at

Figure 3 Time-dependence of unfolding of DHFR in 4.0 M urea at pH 7.5, 20 °C

○, Change in intrinsic fluorescence intensity at 325 nm (excitation at 280 nm). Enzyme concentration was 14 $\mu\text{g/ml}$. The filled symbols refer to changes in enzyme activity. The enzyme (0.7 $\mu\text{g/ml}$) was incubated with the fixed concentration of substrate at 20 °C in 50 mM potassium phosphate buffer, pH 7.5, in the presence of 4 M urea. The assay was started at the indicated times by adding a small volume of substrate. The final concentrations of the substrate in the assay system were NADPH 0.1 mM and DHF 0.1 mM. The substrate concentrations in the incubation mixture were 0 (●), 0.1 mM DHF (▲) and 0.1 mM NADPH (■). The solid lines represent theoretical curves for a single exponential process. The first-order rate constants for generating curves 1–3 were $k_{+0} = 0.0762 \text{ s}^{-1}$, $k'_{+0} = 0.0211 \text{ s}^{-1}$ and $k''_{+0} = 0.0135 \text{ s}^{-1}$ respectively. The inset shows the same data in the corresponding semilogarithmic plots.

280 nm, DHFR exhibited a fluorescence maximum at 325 nm. Changes in emission spectra caused by increasing concentrations of urea are shown in the inset of Figure 2. On addition of urea the emission intensity of the enzyme increased only slightly up to 2 M urea, probably as the result of the change in solvent composition. At 325 nm the emission intensity decreased sharply between 2.0 and 4.0 M urea with a red-shift of the maximum (to 345 nm at 4.0 M urea), indicating that marked structural changes had occurred (Figure 2). On further addition of urea there was a small increase in fluorescence intensity at 325 nm, but no further red-shift of the emission maximum.

The enzyme was incubated in urea solutions in 50 mM phosphate buffer, pH 7.5, at 20 °C for 24 h; samples were removed for assay, with the same concentration of urea present in the assay mixture as in the original incubation. As shown in Figure 2, the enzyme was first activated by low concentrations of urea, but the activity gradually decreased with further increases in the concentration of the denaturant. Enzyme activity increased to about twice that of the native enzyme at 1.5 M urea, and then fell with increased urea concentration until complete inactivation occurred at 4.0 M.

Kinetics of unfolding and inactivation of DHFR

The time course of DHFR unfolding in 4.0 M urea, as measured by the decrease in fluorescence intensity at 325 nm, is shown in Figure 3. The corresponding semilogarithmic plot of $\ln\{(F-F_0)/(F-F_\infty)\}$ against t gave a straight line, indicating that

Figure 4 Time courses of substrate reaction at different concentrations of urea at 20 °C

The reaction mixture contained 50 mM potassium phosphate, pH 7.5, 10 mM 2-mercaptoethanol, 0.1 mM DHF, 0.1 mM NADPH and 0.21 $\mu\text{g/ml}$ DHFR. Urea concentrations for curves 1–3 were 3.3, 3.6 and 4.0 M respectively. The enzyme (10 μl) was added to the reaction mixture (1.2 ml) to start the reaction.

the unfolding of DHFR by urea is a simple first-order reaction (inset of Figure 3). The rate constant of denaturation at 4.0 M urea (k_u) is 0.0765 s^{-1} , as determined by the non-linear least-squares curve-fitting method.

Inactivation kinetics of the free enzyme and enzyme–substrate binary complexes in urea solution was first studied by the conventional method. The enzyme was incubated with 4.0 M urea in the absence or presence of one of the substrates for a defined time interval, and the assay was started by the addition of a small volume of the concentrated substrates, NADPH and/or DHF, to the incubation system. It can be seen from Figure 3 that the DHFR inactivation process in 4.0 M urea follows single-exponential kinetics. By fitting the experimental data to the equation $v_t = v_0 \exp(-k_{+0} t)$, the first-order rate constant of enzyme inactivation (k_{+0}) was determined to be 0.0762 s^{-1} , which is in agreement with the rate constant for conformational change. This suggests that the overall changes are simultaneous with those that occur at the active site. When one of the substrates was included in the incubation mixture, there was clear evidence of protection against loss of enzyme activity. In the presence of saturating NADPH or DHF (100 μM), the inactivation rate constants were $k'_{+0} = 0.0211 \text{ s}^{-1}$ and $k''_{+0} = 0.0135 \text{ s}^{-1}$ respectively.

Kinetics of substrate reaction during the inactivation of DHFR

As pointed out by Tsou [2,22], no substrate is usually present during measurements of protein conformational changes whereas, by necessity, substrate has to be present in an activity assay. The possibility of either substrate protection or partial reactivation leading to an underestimation of the rates and/or extents of inactivation has to be considered. Therefore the experimental observation of parallel changes in activity and

Figure 5 Time courses of substrate reaction at different concentrations of NADPH in the presence of 4.0 M urea and saturating DHF at 20 °C

The reaction mixture contained 50 mM potassium phosphate, pH 7.5, 10 mM 2-mercaptoethanol, 0.1 mM DHF and 0.14 $\mu\text{g/ml}$ DHFR. NADPH concentrations for curves 1–3 were 6.7, 13.3 and 23.3 μM respectively. The enzyme (10 μl) was added to the reaction mixture (1.2 ml) to start the reaction. The inset shows semilogarithmic plots of curves 1–3 plotted according to eqn. (2).

Figure 6 Double-reciprocal plots of (a) $1/v_0$ versus $1/[\text{NADPH}]$ and (b) $1/[\text{P}]_\infty$ versus $1/[\text{NADPH}]$ in the presence of 4.0 M urea and saturating concentration of DHF

The values of v_0 (a) and $[\text{P}]_\infty$ (b) were calculated from the time courses of DHFR inactivation shown in Figure 5. The straight line in (a) represents the best fit with parameters: $V_{\text{max}} = 0.0172 \mu\text{M} \cdot \text{s}^{-1}$, $K_S = 9.440 \mu\text{M}$. The straight line in (b) represents the best fit with parameters: $k'_{+0} = 0.0149 \text{ s}^{-1}$, $k''_{+0} = 0.0060 \text{ s}^{-1}$.

conformation was examined further by using the progress-curve method.

Figure 4 shows the time courses of substrate reaction in the presence of different urea concentrations. At each urea concentration, the rate of product formation decreased with increasing time until a straight line was approached. As expected, the slope of the straight line decreased with increasing urea concentration. At 4.0 M urea, the enzyme was inactivated completely, indicating that the denaturation reaction becomes irreversible under these conditions.

The time courses of substrate reaction at saturating DHF and

Figure 7 Time courses of substrate reaction at different concentrations of DHF in the presence of 4.0 M urea and saturating concentration of NADPH at 20 °C

The reaction mixture contained 50 mM potassium phosphate, pH 7.5, 10 mM 2-mercaptoethanol, 0.1 mM NADPH and 0.14 $\mu\text{g/ml}$ DHFR. DHF concentrations for curves 1–3 were 3.3, 6.7 and 13.3 μM respectively. The enzyme (10 μl) was added to the reaction mixture (1.2 ml) to start the reaction. The inset shows semilogarithmic plots of curves 1–3 plotted according to eqn. (2).

various concentrations of NADPH in the presence of 4.0 M urea are shown in Figure 5. With increasing reaction time, the concentration of product formed approached constant final values, $[\text{P}]_\infty$, which increased with increasing NADPH concentration. The corresponding semilogarithmic plots gave straight lines, indicating that inactivation of DHFR in the presence of substrate is a first-order reaction. The apparent inactivation rate constants, A , can be determined from the slopes of the straight lines. With these values, the initial velocities of the native enzyme-catalysed reactions in the presence of urea can be determined from $v_0 = A[\text{P}]_\infty$. Figure 6(a) shows a double-

Figure 8 Double-reciprocal plots of (a) $1/v_0$ versus $1/[\text{DHF}]$ and (b) $1/[\text{P}]_\infty$ versus $1/[\text{DHF}]$ in the presence of 4.0 M urea and saturating concentration of NADPH

The values of v_0 (a) and $[\text{P}]_\infty$ (b) were calculated from the time courses of DHFR inactivation shown in Figure 7. The straight line in (a) represents the best fit with parameters: $V_{\text{max}} = 0.0166 \mu\text{M} \cdot \text{s}^{-1}$, $K_R = 2.216 \mu\text{M}$. The straight line in (b) represents the best fit with parameters: $k'_{+0} = 0.0219 \text{ s}^{-1}$, $k''_{+0} = 0.0062 \text{ s}^{-1}$.

Table 2 Inactivation rate constants of DHFR by 4 M urea

	Inactivation rate constant (s^{-1})		
	Conventional method	Progress curve method	
		Saturation with DHF	Saturation with NADPH
k_{+0}	0.076 ± 0.005	—	—
k'_{+0}	0.021 ± 0.002	—	0.022 ± 0.003
k''_{+0}	0.014 ± 0.002	0.015 ± 0.002	—
k'''_{+0}	—	0.006 ± 0.001	0.006 ± 0.001

Table 3 Comparison of dissociation constants for enzyme–substrate interactions in the presence of different urea concentrations

[Urea] (M)	K_R (μ M)	K_S (μ M)
0.0	0.48 ± 0.03	0.63 ± 0.03
1.0	0.77 ± 0.12	1.46 ± 0.24
2.0	1.07 ± 0.25	2.42 ± 0.34
4.0	2.22 ± 0.30	9.44 ± 0.67

reciprocal plot of $1/v_0$ against $1/[NADPH]$. In the presence of 4.0 M urea and saturating DHF, the kinetic parameters of the native enzyme–DHF binary complex for NADPH are $V_{\max} = 0.0172 \mu\text{M} \cdot \text{s}^{-1}$ and $K_S = 9.440 \mu\text{M}$. Knowing the values of $V_{\max}$ and K_S , the microscopic rate constants for the inactivation of the enzyme–NADPH binary and enzyme–DHF–NADPH ternary complexes can be determined from the relationships between $[P]_{\infty}$ and $[NADPH]$ as described previously. Figure 6(b) shows a plot of $1/[P]_{\infty}$ against $1/[NADPH]$. From the intercept and slope of the straight line, the corresponding inactivation rate constants, $k'_{+0} = 0.0149 \text{ s}^{-1}$ and $k'''_{+0} = 0.0060 \text{ s}^{-1}$, were calculated. Figure 7 shows time courses of DHFR inactivation at saturating NADPH and various concentrations of DHF. The corresponding double-reciprocal plots are shown in Figure 8. By the same procedure as above, the values of K_R , k'_{+0} and k'''_{+0} were determined, and the results are listed in Tables 2 and 3.

For the sake of comparison, the Michaelis constants for DHF and NADPH at various concentrations of urea were also determined by the traditional initial-velocity method (Table 3). In the absence of urea, the Michaelis constants of the enzyme–substrate binary complexes for DHF and NADPH are 0.48 and 0.63 μM respectively. Therefore the Michaelis constant for DHF increased approx. 5-fold and that for NADPH increased about 14-fold in the presence of 4.0 M urea. It can be seen from Table 2 that the values of k'_{+0} and k'''_{+0} determined by the two different methods are the same within experimental error, suggesting that the inactivation rate constant for the free enzyme, k_{+0} , determined by the conventional method is reliable.

DISCUSSION

The conventional method for determining the rate constant of enzyme inactivation in the presence of denaturant is to take aliquots from an enzyme–denaturant incubation mixture at defined time intervals and assay for enzyme activity. This method is laborious and not easily applied to fast reactions with a half-life of less than 1 min, and, for a multistep unfolding mechanism, the initial fast phase of the inactivation process can easily be

missed. Moreover, since substrate has to be present in an activity assay mixture, either substrate protection or substrate-induced partial re-activation could lead to an underestimation of the rates and/or extents of inactivation [2,22]. In comparison with the conventional method, the advantage of the progress-curve method is not only its usefulness in the study of fast inactivation reactions but its convenience in the study of substrate effects on enzyme denaturation. In the case of DHFR, the inactivation rate constants, k'_{+0} and k'''_{+0} , determined by the two methods are the same within experimental error. This suggests that the possibility of substrate-induced re-activation can be ruled out.

A knowledge of the origins of the stability of proteins is essential to the understanding of their structure and function. The stabilization free energy depends on the usual thermodynamic variables of temperature, pressure and composition. Usually, the important composition variables for protein stability are pH, ionic strength, the concentration of denaturants and the concentration of other substances that bind preferentially to the native or unfolded states. A detailed theoretical consideration of the effect of ligand binding or other perturbation on a protein that undergoes a two-state transition has been presented by Becktel and Schellman [23,24]. The present kinetic method provides an alternative approach to the study of effects of substrate on the stability of proteins.

Several years ago, a systematic study of inactivation kinetics was presented for several two-substrate kinetic mechanisms [14,15]. Despite the attractions of this method, it has not been used a great deal in practice. The reason for this is probably that use of the progress-curve method requires that the consumption of substrates is negligible during the course of enzyme inactivation. However, this requirement may become impractical for some two-substrate enzyme systems. As pointed out previously, in order to obtain a reasonable estimation of the kinetic parameters, the substrate concentration used in experiments should be chosen to be as close as possible to the value of the Michaelis constant [25]. For two-substrate systems with very low dissociation constants, when both substrate concentrations are less than one-third to one-half of the corresponding dissociation constants, the amount of product formed during inactivation is often too low to be detected accurately if the change in substrate(s) concentration is within 10% of the initial value. In these cases, however, if one of the substrates is saturating at all stages, kinetic equations identical with those of a one-substrate system can be obtained in the non-saturating substrate. This enables k'_{+0} , k''_{+0} and k'''_{+0} to be obtained from experiments in which each substrate in turn is saturating. Thus, as illustrated in the present study, all inactivation rate constants can be obtained by combining the use of the conventional and progress-curve methods.

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