Activation mechanism and modification kinetics of Chinese hamster dihydrofolate reductase by *p*-chloromercuribenzoate

Jia-Wei WU and Zhi-Xin WANG¹

National Laboratory of Biomacromolecules, Institute of Biophysics, Academia Sinica, Beijing 100101, Peoples' Republic of China

Substrate effects on the activation kinetics of Chinese hamster dihydrofolate reductase by *p*-chloromercuribenzoate (pCMB) have been studied. On the basis of the kinetic equation of substrate reaction in the presence of pCMB, all modification 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 modification of Chinese hamster dihydrofolate reductase by pCMB shows single-phase kinetics, and that changes in the enzyme activity and tertiary structure proceed simultaneously during the modification pro-

cess. Both substrates, NADPH and 7,8-dihydrofolate, protect dihydrofolate reductase against modification by pCMB. In the presence of a saturating concentration of NADPH, the value of k_{cat} for 7,8-dihydrofolate in the enzyme-catalysed reaction increased four-fold on modification of Cys-6, accompanied by a two-fold increase in K_m for the modified enzyme. The utilization of the binding energy of a group to increase k_{cat} rather than reduce K_m implies that the full binding energy of the group is not realized in the formation of the enzyme–substrate complex, but is used to stabilize the enzyme–transition-state complex.

INTRODUCTION

Dihydrofolate reductase (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 [1]. Furthermore, DHFR from Chinese hamster is a small monomeric protein and has no prosthetic groups or disulphide bonds [2,3]. Therefore, this protein is an ideal candidate for folding studies.

The protein-sequence information available for vertebrate and bacterial DHFRs [4,5] shows that in all of the vertebrate DHFRs a cysteine residue is located at position 6 or 11. Bacterial DHFRs, by contrast, do not contain a cysteine residue within the first 50 amino acids of the sequence and in some cases contain no cysteine. From these comparisons, it has been concluded tentatively that cysteine residues are not required for direct substrate binding or involved in the catalytic mechanism of the DHFR reaction [6,7]. However, it cannot be stated unequivocally that, in the native DHFR, cysteine plays no role in the binding or catalytic properties of the enzyme. The activity of DHFR from most vertebrates can be stimulated markedly when treated with a diverse group of agents [4]. These include: (i) thiol modifiers [8,9]; (ii) salts such as KCl and NaCl; and (iii) chaotropes such as urea or guanidine hydrochloride. Activation by different agents is not additive, suggesting that a common mechanism may be involved. Although there have been many reports on the activation of DHFR, the possible mechanism to account for these effects is still unclear.

Some years ago, a systematic study on the kinetics of the substrate reaction during the irreversible modification of enzyme activity was presented [10–14]. It has been 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 applied to study the activation kinetics of

Chinese hamster DHFR by *p*-chloromercuribenzoate (pCMB). The role of a unique cysteine residue located at position 6 in the native enzyme is addressed. The results of the present study indicate that the modification of Cys-6 in the enzyme by pCMB shows single-phase kinetics, and changes in the enzyme activity and tertiary structure proceed simultaneously in the modification processes. Both substrates, NADPH and DHF, protect DHFR against the modification reaction, and the enzyme–substrate complexes are activated less rapidly than the free enzyme. In the presence of a saturating concentration of NADPH, the values of k_{cat} for DHF in the enzyme-catalysed reaction increase four-fold on modification of Cys-6, accompanied by a two-fold increase in the value of K_m for the modified enzyme. The value of k_{cat}/K_m was about twice as high for modified DHFR. The utilization of the binding energy of a group to increase k_{cat} rather than reduce K_m implies that the full binding energy of the group is not realized in the enzyme–substrate complex but is used to stabilize the enzyme–transition-state complex. The geometry of the enzyme is such that the small changes in bond lengths and angles during the reaction enable a better fit between the enzyme and the transition state of the substrate rather than between the enzyme and the substrate itself.

MATERIALS AND METHODS

Materials

DHF (approx. 90%) and NADPH (approx. 97%) were purchased from Sigma. Methotrexate was obtained from ICN. The concentration of methotrexate was determined spectrophotometrically in 0.1 M NaOH, using molar extinction coefficients of 23 250 M^{−1}·cm^{−1} at 258 nm and 22 100 M^{−1}·cm^{−1} at 302.5 nm, respectively [15]. The concentrations of DHF and NADPH were determined at pH 7.0 with molar extinction coefficients of $\epsilon_{282} = 28\,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [16] and $\epsilon_{340} = 6220 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [17], respectively. Urea, ultrapure, was from Boehringer Mannheim and solutions were always prepared freshly from a re-crystallized

Abbreviations: DHFR, dihydrofolate reductase; DHF, 7,8-dihydrofolate; pCMB, *p*-chloromercuribenzoate.

¹ To whom correspondence should be addressed (e-mail zwxwang@sun5.ibp.ac.cn).

Scheme 1 Modification of DHFR by pCMB in the presence of substrate

E and E^* represent the native and modified enzyme, M is pCMB, S and R are the two substrates, NADPH and DHF, P and Q are the two products, NADP and tetrahydrofolate, respectively. k_{+0} , K_{+0} , K'_{+0} , and K''_{+0} are the modification rate constants for free enzyme, and the enzyme–substrate(s) binary and ternary complexes.

sample [18]. Sephadex G-75 was from Pharmacia. Other reagents were local products of analytical grade 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, Baltimore, MD, U.S.A.), contained the full length of Chinese hamster DHFR and was used as a PCR template for amplifying the DHFR gene. To facilitate synthesis of DHFR in *Escherichia coli* DH5 α , the DHFR gene was isolated from the PCR product as an *Nco*I/*Sal*I fragment and cloned into *Nco*I and *Sal*I sites in the expression vector pBV221. The resulting plasmid, pDHFR, positioned the DHFR gene under the transcriptional control of both the thermoinducible P_R and P_L promoters and the temperature-sensitive cl857 repressor.

Purification of Chinese hamster DHFR

Luria–Bertani medium (4 l) containing 50 μ g 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. 0.6, and then induced at 42 °C for about 6 h. The cells were collected by centrifugation at 3300 g 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 [19] was used. The suspensions was centrifuged at 16300 g for 10 min at 4 °C, and the supernatant was concentrated by dialysis against solid PEG-20000. The concentrated sample was applied on to a column of Sephadex G-75 (3.5×110 cm) equilibrated with 10 mM potassium phosphate, pH 6.0, containing 2 mM β -mercaptoethanol and eluted with the same buffer. Fractions containing DHFR activity were pooled and concentrated. The pooled fractions were applied to a Mono-Q FPLC column pre-equilibrated with 10 mM potassium phosphate buffer (pH 7.2). A gradient of 0.5 M KCl in the same buffer was then applied.

The combined active fractions were concentrated and stored in small aliquots at -80 °C. The final preparation showed one band in SDS/PAGE and a single peak in reversed-phase HPLC by a Ultrapore C₈ column. The concentration of DHFR was determined by methotrexate titration. The specific activity of the purified enzyme was 5.48 μ mol/mg per min.

Enzyme assays

The activity of DHFR was determined by following the decrease in absorbance at 340 nm using a Shimadzu UV-2101PC spectrophotometer held at 25 °C [20]. Experiments were performed at pH 7.0 in a buffer mixture containing 50 mM potassium phosphate. 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 the enzyme converting 1.0 μ mol of DHF and NADPH to 5,6,7,8-tetrahydrofolate and NADP per min, and is calculated from the change in absorbance at 340 nm using an extinction coefficient for the reaction of 12300 $M^{-1} \cdot cm^{-1}$ at 25 °C and pH 7.0, which is the sum of contributions from NADPH oxidation and DHF reduction [21].

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 activation reaction followed by the substrate reaction in the presence of the modifier were as described previously [11]. All measurements were carried out in 0.05 M potassium phosphate buffer (pH 7.0) at 25 °C.

Fluorescence measurements

Equilibrium unfolding as a function of urea was monitored by fluorescence spectroscopy with an excitation wavelength of

Scheme 2 Modification of DHFR by pCMB when one substrate, *R*, is saturating

280 nm on a Hitachi F-4010 fluorimeter. All samples were allowed to equilibrate fully at the appropriate final urea concentration at 25 °C before spectra were taken. Kinetics of conformational change of the enzyme in the presence of pCMB and/or urea were also followed by fluorescence spectroscopy using manual mixing methods that have a dead time of 5–8 s. Fluorescence measurements were made by exciting at 280 nm. The emission intensity was monitored at 325 nm.

Kinetic analysis

In the presence of substrate, the activation mechanism of DHFR by pCMB can be written as shown in Scheme 1 [12,13]. If the concentration of NADPH is saturating at all stages, the reaction can then be treated as a one-substrate system, as shown in Scheme 2.

From the Scheme 2 given above, it can be shown that the concentration of the product formed is given by [11]:

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

where $[P]$ is the concentration of product formed at time t , and v_0 , v_s and A are the initial velocity, the steady-state velocity of the substrate reaction and the apparent activation rate constant in the presence of pCMB, respectively.

$$v_0 = \frac{k_c[E]_0[S]}{K_s + [S]} \quad (2)$$

$$v_s = \frac{k_c^*[E]_0[S]}{K_s^* + [S]} \quad (3)$$

$$A = \frac{k''_{+0}K_s + k'''_{+0}[S]}{K_s + [S]} \quad (4)$$

By fitting the experimental data with Equation 1, the values of v_0 , v_s and A can be obtained. Thus from the relationships between A and $[S]$, the microscopic kinetic parameters, k''_{+0} and k'''_{+0} , can be determined as described previously [11]. Similarly, when S is saturating at all stages, k'_{+0} and k'''_{+0} can be obtained by the same procedure.

RESULTS

Kinetics of modification and activation of DHFR by pCMB

As mentioned above, a well-documented and unusual property of the vertebrate DHFRs is their response to pCMB. This compound reacts specifically with available thiol groups of proteins [22] and, in the case of Chinese hamster DHFR, reacts with its single cysteine at position 6. Activation kinetics of

Figure 1 Comparison of activation and conformational change of DHFR during pCMB modification

(Upper panel) Time course of activation of Chinese hamster DHFR by pCMB. The enzyme (57.5 nM) was incubated with 14 μ M pCMB at 25 °C in 50 mM potassium phosphate buffer, pH 7.0. The assay was started at indicated times by adding a small volume of substrates. ●, Experimental points; solid line, theoretical curve for a single exponential process with a first-order rate constant of $k_a = 7.2 \times 10^{-3} \text{ s}^{-1}$. Inset, the same data in the corresponding semi-logarithmic plot. (Lower panel) Comparison of the enzyme modification rate (4.0 μ M, curve 1) with the rate of enzyme conformational change (1.4 μ M, curve 2) by 14 μ M pCMB under the same conditions as the upper panel. The modification process was monitored directly by absorbance measurements at 255 nm using an extinction coefficient of $\Delta\epsilon_{255} = 6200 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Conformational change of the enzyme was monitored by intrinsic fluorescence intensity at 325 nm (excitation at 280 nm). Solid lines, theoretical curves for a single exponential process. The first-order rate constants for generating curve 1 and 2 were $k_m = 7.4 \times 10^{-3} \text{ s}^{-1}$ and $k_c = 6.7 \times 10^{-3} \text{ s}^{-1}$, respectively.

Chinese hamster DHFR in the presence of pCMB were first studied by the conventional method. The enzyme was incubated with 14 μ M pCMB for a definite time interval and samples were removed for activity assay. Treatment of the enzyme at pH 7.0 and 25 °C with a large excess (240-fold) of pCMB produced a time-dependent increase in catalytic activity that reached its maximal value (about four-fold the initial activity) after about 10 min (Figure 1, upper panel). The corresponding semi-logarithmic plot of $\ln(v_s - v)$ against t gives a straight line, indicating that the activation of DHFR by pCMB is a simple first-order

Figure 2 Plot of the initial rate against substrate concentration

(Upper panel) Plot of the initial velocity of native DHFR (○) and the pCMB-modified enzyme (●) against NADPH concentration in the presence of saturating concentrations of DHF. The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 0.1 mM DHF and 2.95 nM DHFR. Solid curves, the best-fitting results according to the Michaelis–Menten equation with parameters: $V_{\max} = 6.10 \text{ nM} \cdot \text{s}^{-1}$, $K_m = 0.60 \text{ } \mu\text{M}$ for the native enzyme, and $V_{\max} = 22.94 \text{ nM} \cdot \text{s}^{-1}$, $K_m = 1.32 \text{ } \mu\text{M}$ for the modified enzyme. (Lower panel) Plot of the initial velocity of the native (○) and the pCMB-modified enzymes (●) against DHF concentration in the presence of saturating concentration of NADPH. The reaction mixture was as above. Solid curves, the best-fitting results according to the Michaelis–Menten equation with parameters: $V_{\max} = 5.83 \text{ nM} \cdot \text{s}^{-1}$, $K_m = 0.44 \text{ } \mu\text{M}$ for the native enzyme, and $V_{\max} = 23.87 \text{ nM} \cdot \text{s}^{-1}$, $K_m = 0.88 \text{ } \mu\text{M}$ for the modified enzyme.

reaction (Figure 1, upper panel inset). The rate constant of activation in the presence of $14 \text{ } \mu\text{M}$ pCMB is $k_a = 7.2 \pm 0.8 \times 10^{-3} \text{ s}^{-1}$. In order to elucidate the mechanism of mercurial activation, the modification process was monitored directly by absorbance measurements at 255 nm using an extinction coefficient of $\Delta\epsilon_{255} = 6200 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Figure 1, lower panel) [23]. For comparison, conformational change during modification was also followed by changes in intrinsic fluorescence of the enzyme. Treatment of the enzyme with pCMB produced a time-dependent decrease in fluorescence emission at 325 nm (Figure 1, lower panel). Both the processes of Cys-6 modification and conformational change of DHFR can be well described by pseudo-first-order kinetics. By fitting the exper-

Table 1 Kinetic parameters of native and pCMB-modified DHFR-catalysed reaction

	Native DHFR	Modified DHFR
$K_{\text{DHF}} (\text{ } \mu\text{M})$	0.44 ± 0.05	0.88 ± 0.15
$K_{\text{NADPH}} (\text{ } \mu\text{M})$	0.60 ± 0.08	1.32 ± 0.19
$k_{\text{cat}} (\text{s}^{-1})$	2.02 ± 0.08	7.94 ± 0.37

imental data to first-order kinetic equations, the rate constants for the modification and conformational change reactions were determined to be $7.4 \pm 0.1 \times 10^{-3} \text{ s}^{-1}$ and $6.7 \pm 0.1 \times 10^{-3} \text{ s}^{-1}$, respectively, both in agreement with the rate constant of enzyme activation. A similar study has been reported by Duffy et al. [9] for the chemical modification of the mouse L1210 DHFR with pCMB. However, these authors report that the rate of Cys-6 modification by pCMB exceeded the rates of activation and conformational change, and proposed that mercurial activation occurs in a stepwise manner in which pCMB first attaches to the thiol group and subsequently induces a conformational change in the enzyme, which in turn produces the activation of the enzyme. It can be seen from Figure 1 that the modification of DHFR by pCMB shows a single-phase kinetics, and changes in the enzyme activity and tertiary structure proceed simultaneously during the modification processes. However, it is also possible that the mechanism of mercurial activation for Chinese hamster DHFR may be different from that for the mouse L1210 enzyme, since these conflicting studies were performed under identical experimental conditions for the modification reaction and the assay.

Kinetic studies of the native and modified enzymes

To compare the kinetic behaviour of the pCMB-modified enzyme and the native enzyme, two groups of experiments were carried out. The first group of experiments involved varying the concentration of NADPH at saturating concentrations of DHF. Figure 2 (upper panel) shows the dependence of the velocities of the enzyme-catalysed reaction on the NADPH concentration. Both the initial velocities for the native and modified enzymes give rise to the hyperbolic dependence on the NADPH concentration. The experimental data fit the Michaelis–Menten equation, with a K_m of $0.60 \pm 0.08 \text{ } \mu\text{M}$ and $V_{\max}$ of $6.10 \pm 0.28 \text{ nM} \cdot \text{s}^{-1}$ for the native DHFR, and a K_m of $1.32 \pm 0.19 \text{ } \mu\text{M}$ and $V_{\max}$ of $22.94 \pm 1.04 \text{ nM} \cdot \text{s}^{-1}$ for the pCMB-modified enzyme. In the second group of experiments, the initial velocities of enzyme-catalysed reaction were measured by varying the concentration of DHF at saturating concentrations of NADPH (Figure 2, lower panel). By fitting the experimental data to the Michaelis–Menten equation, the values of K_m and $V_{\max}$ for NADPH were determined to be $K_m = 0.44 \pm 0.05 \text{ } \mu\text{M}$, $V_{\max} = 5.83 \pm 0.21 \text{ nM} \cdot \text{s}^{-1}$ for the native enzyme, and $K_m = 0.88 \pm 0.15 \text{ } \mu\text{M}$, $V_{\max} = 23.87 \pm 1.16 \text{ nM} \cdot \text{s}^{-1}$ for the pCMB-modified enzyme. These results are summarized in Table 1.

Effect of substrates on the modification reaction of DHFR with pCMB

Modification kinetics of the free enzyme and enzyme–NADPH binary complexes were first studied by absorbance measurements at 255 nm. Figure 3 shows the time courses of the modification reaction in the presence of different pCMB concentrations. The pseudo-first-order rate constants were observed to be pro-

Figure 3 Time courses of modification of Chinese hamster DHFR

(Upper panel) Time courses with different concentrations of pCMB at pH 7.0, 25 °C. The enzyme concentration was 4.0 μM . The concentrations of pCMB for the curves, from bottom to top, were 6.2, 10.3, 14.4, 20.6 and 41.2 μM , respectively. Inset, the dependence of the first-order rate constant on the concentration of pCMB. (Lower panel) Time courses in the presence of 100 μM NADPH and different concentrations of pCMB at pH 7.0, 25 °C. The enzyme concentration was 3.5 μM . The concentrations of pCMB for the curves, from bottom to top, were 39.6, 59.4, 79.2, 118.8 and 277.3 μM , respectively. Inset, the dependence of the first-order rate constant on the concentration of pCMB.

portional to pCMB concentrations, indicating that there is no prior complexing with the enzyme before the irreversible modification step. From the slopes of straight lines given in the insets of Figure 3, the second-order rate constants for the modification of the free enzyme and enzyme–NADPH binary complexes were determined to be $k_{+0} = 568 \pm 6.5 \text{ s}^{-1} \cdot \text{M}^{-1}$ and $k'_{+0} = 19.0 \pm 0.38 \text{ s}^{-1} \cdot \text{M}^{-1}$, respectively. The ratio between the modification rate constants for the free enzyme and the enzyme–NADPH complex indicates strong protection against pCMB modification by NADPH.

To determine the modification rate constants of the enzyme–substrate binary complex and enzyme–substrate–NADPH ternary complex, modification kinetics were also studied by using the progress curve method. The time courses of substrate reaction using the saturating DHF and various concentrations of NADPH in the presence of 286 μM pCMB are shown in Figure 4. The general shapes of the curves are as predicted by Equation 1. When t was sufficiently large the curves approached straight

Figure 4 Courses of substrate reaction at different concentrations of NADPH in the presence of 286 μM pCMB and saturating DHF at 25 °C

The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 0.1 mM DHF and 0.046 nM DHFR. Concentrations of NADPH for curves 1–4 were 0.35, 1.06, 2.82 and 10.58 μM , respectively. The enzyme (5 μl) was added to the reaction mixture (1.0 ml) to start the reaction. The inset shows a plot of the apparent activation rate constant against the concentration of NADPH. Solid line, the best-fitting curve using Equation 4 with $K_m = 0.60 \mu\text{M}$, $k'_{+0} = 13.12 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k''_{+0} = 4.31 \text{ M}^{-1} \cdot \text{s}^{-1}$.

Figure 5 Courses of substrate reaction at different concentrations of DHF in the presence of 286 μM pCMB and saturating NADPH at 25 °C

The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 0.1 mM NADPH and 0.046 nM DHFR. Concentrations of DHF for curves 1–4 were 0.36, 0.71, 1.78 and 10.67 μM , respectively. The enzyme (5 μl) was added to the reaction mixture (1.0 ml) to start the reaction. Inset, a plot of the apparent activation rate constant against the concentration of DHF. Solid line, the best-fitting curve using Equation 4 with $K_m = 0.44 \mu\text{M}$, $k'_{+0} = 20.68 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k''_{+0} = 4.38 \text{ M}^{-1} \cdot \text{s}^{-1}$.

lines; the slopes of these lines represent activities of the activated enzyme. By fitting the experimental data to Equation 1, the apparent activation rate constant, A , can be obtained. The inset of Figure 4 shows a plot of A against NADPH concentration. With the known values of K_m for NADPH, the values of k'_{+0} and k''_{+0} were determined to be $13.1 \pm 0.9 \text{ s}^{-1} \cdot \text{M}^{-1}$ and

Table 2 Activation and modification rate constants of Chinese hamster DHFR by pCMB

Modification rate constant	Method of calculation		
	Conventional method	Progress-curve method	
		Saturation with DHF	Saturation with NADPH
k_{+0} ($\text{s}^{-1} \cdot \text{M}^{-1}$)	525 ± 60	—	—
k'_{+0} ($\text{s}^{-1} \cdot \text{M}^{-1}$)	—	13.1 ± 0.9	—
k''_{+0} ($\text{s}^{-1} \cdot \text{M}^{-1}$)	19.0 ± 0.4	—	20.7 ± 1.2
k'''_{+0} ($\text{s}^{-1} \cdot \text{M}^{-1}$)	—	4.31 ± 0.57	4.38 ± 0.34

$4.31 \pm 0.34 \text{ s}^{-1} \cdot \text{M}^{-1}$, respectively. The time courses of activation of DHFR with saturating NADPH but varying concentrations of DHF in the presence of 286 μM pCMB are shown in Figure 5. The inset shows the dependence of the apparent activation rate constant, A , on the concentration of DHF. By the same procedure mentioned above, the values of k'_{+0} and k''_{+0} were determined, and the results are listed in Table 2. It can be seen from Table 2 that the modification and activation rate constants of the enzyme–NADPH binary complex determined by the two different methods are the same within experimental error, suggesting again that the processes of Cys-6 modification and enzyme activation proceed simultaneously. The modification rate constant for free enzyme was about 30-fold greater than that for the enzyme–NADPH complex, and 45-fold greater than that for the enzyme–DHF complex, indicating that both the substrates have strong protection against the modification by pCMB.

Activation and stability studies of the native and modified enzymes

The changes of activity of the native and pCMB-modified enzymes with increasing concentrations of urea are shown in Figure 6 (upper panel). The modified enzyme was only slightly activated but the native enzyme was markedly activated at low concentrations of urea, suggesting that a common activation mechanism by pCMB and by urea may be involved. Results of the preceding experiments suggested that activation of Chinese hamster DHFR by pCMB involved an opening up of the conformation, as also indicated by previous reports of increased proteolysis [9,24]. In this study, experiments were also conducted to estimate the conformational stability of the native and modified enzymes. The urea-induced denaturation of both the native and modified enzymes under the same experimental conditions is shown in Figure 6 (lower panel). From these measurements, values of the free energy of unfolding, $\Delta G_{\text{N-U}}$, for a two-state process were determined using the relation:

$$\Delta G_{\text{N-U}} = -RT \ln \left(\frac{y - y_{\text{N}}}{y_{\text{D}} - y} \right) \quad (5)$$

where y is the measured optical property in a particular denaturing condition and y_{N} and y_{D} are the properties of the protein in the fully folded and unfolded states under the same conditions, respectively [25]. The values of the Gibbs free-energy change are plotted in the inset of Figure 6 (lower panel). Both the plots of $\Delta G_{\text{N-U}}$ (where N and U represent the folded and unfolded enzymes, respectively) versus urea concentration were analysed using the linear free-energy model:

$$\Delta G_{\text{N-U}} = \Delta G_{\text{N-U}}^0 - m[\text{urea}] \quad (6)$$

where m is the slope of the $\Delta G_{\text{N-U}}$ versus [urea] plot and $\Delta G_{\text{N-U}}^0$ is the value of $\Delta G_{\text{N-U}}$ in the absence of urea. A least-squares

analysis of the urea-induced denaturation results gave values of $\Delta G_{\text{N-U}}^0 = 3.67 \pm 0.33 \text{ kcal} \cdot \text{mol}^{-1}$ and $m = 1.53 \pm 0.13 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{M}^{-1}$ for the native enzyme, and $\Delta G_{\text{N-U}}^0 = 2.99 \pm 0.12 \text{ kcal} \cdot \text{mol}^{-1}$ and $m = 1.96 \pm 0.07 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{M}^{-1}$ for the pCMB-modified enzyme.

Figure 7 shows the unfolding courses of the native and modified enzymes in 3.5 M urea, as measured by the decrease in fluorescence intensity at 325 nm. The corresponding plots of $\ln[(F - F_{\infty})/(F_0 - F_{\infty})]$ against t gave straight lines (Figure 7, inset). The rate constants of denaturation for the native and modified enzymes were determined to be $0.0118 \pm 0.006 \text{ s}^{-1}$ and $0.0357 \pm 0.0014 \text{ s}^{-1}$, respectively. Therefore, both the equilibrium and kinetic results suggest that the native enzyme has a more stable conformation than the pCMB-modified enzyme.

DISCUSSION

The conventional method for determining the rate constant of enzyme modification is to take aliquots from an enzyme-modifier incubation mixture at definite time intervals and assay for the enzyme activity. This method is laborious and not easily applied to fast reactions with a half-life of less than 1 min. In comparison with the conventional method, the advantage of the progress-curve method is not only its usefulness in the study of fast modification reaction but its convenience in the study of substrate effects on enzyme modification. Several years ago, a systematic study of modification kinetics was presented for several two-substrate kinetic mechanisms [12,13]. In order to obtain a reasonable estimation of the kinetic parameters, the substrate concentrations used in experiments should be chosen at values close to the corresponding Michaelis constants [26]. However, this requirement may become impractical for some two-substrate enzyme systems. For the two-substrate systems with very low dissociation constants, when both substrate concentrations are below one-third to one-half of the corresponding dissociation constants, the amount of product formed during activation is often too low to be detected accurately if the change of 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 to those of a one-substrate system can be obtained in the non-saturating substrate. This would enable k'_{+0} , k''_{+0} and k'''_{+0} to be obtained from experiments where each substrate in turn was saturating. Thus, as illustrated in the present study, all activation rate constants can be obtained by using the conventional and the progress-curve methods.

The availability of three-dimensional structures of DHFR from X-ray crystallography has provided the means to integrate kinetic investigations with structural ones. The Brookhaven protein database currently contains about 30 entries of various structures of DHFR and its ligand complexes. Despite limited

Figure 6 Comparison of enzyme activity and conformational change at different concentrations of urea

(Upper panel) Comparison of activity changes of the native DHFR (○) and pCMB-modified DHFR (●) at different concentrations of urea at pH 7.0, 25 °C, with the value for the native enzyme in the absence of urea as 1.0. The enzyme (6.5 μM) was incubated for 6 h at 25 °C in 50 mM potassium phosphate buffer, pH 7.0, in presence of the indicated concentration of urea; samples were removed for assay, with the same concentration of urea present in the assay mixture as during incubation. The final concentration of the enzyme in the assay system was 23 nM. (Lower panel) Urea-induced denaturation of the native DHFR (○) and pCMB-modified DHFR (●) at pH 7.0 and 25 °C. The enzyme concentration was 6.5 μM. The fluorescence intensity at 325 nm was measured after excitation at 280 nm. Inset: ΔG_{N-U} as a function of urea molarity for the native DHFR (○) and the modified DHFR (●). Solid lines, calculated according to Equation 6 with the following parameters: $\Delta G_{N-U}^0 = 3.67 \pm 0.33 \text{ kcal} \cdot \text{mol}^{-1}$, $m = 1.53 \pm 0.13 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{M}^{-1}$ for the native enzyme, and $\Delta G_{N-U}^0 = 2.99 \pm 0.12 \text{ kcal} \cdot \text{mol}^{-1}$, $m = 1.96 \pm 0.07 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{M}^{-1}$ for the pCMB-modified enzyme.

sequence similarity between bacterial and vertebrate DHFRs, all of them display highly conserved α -carbon backbone geometries, i.e. four major α -helices and seven-eight β -sheets in corresponding positions of an 18–22 kDa monomer. Alignment of DHFR primary sequences shows the high sequence similarity of 95 % between Chinese hamster and mouse L1210 DHFR. It can be concluded, therefore, that the Cys-6 in Chinese hamster DHFR may also belong to the first β -sheet segment (βA) near the substrate- and cofactor-binding sites [27–30]. This is in agreement

Figure 7 Time-dependence of unfolding of the native DHFR (○) and pCMB-modified DHFR (●) in 3.5 M urea at pH 7.0, 25 °C

The symbols refer to the change in the intrinsic fluorescence intensity at 325 nm (excitation at 280 nm). Concentrations of the native enzyme and the modified enzyme were 3.2 and 2.4 μM, respectively. Solid lines, theoretical curves for a single exponential process. The first-order rate constants for curves 1 and 2 were 0.0118 and 0.0357 s⁻¹, respectively. The inset shows the same data in the corresponding semi-logarithmic plots.

with the experimental observations that both the substrate and cofactor strongly protect against the modification by pCMB.

It is now generally recognized that the function of protein molecules is related to their dynamic properties. The well-known ‘induced fit’ model of enzyme action postulates that enzymes exist in many different conformational states at equilibrium with one another, and that this equilibrium can be perturbed by the presence of substrates or other ligands [31]. Full expression of catalytic activity may require rapid shifts in conformational states at the enzyme active site [32]. Compared with the native DHFR, the modified enzyme shows a four-fold increased k_{cat} and a decreased stabilization free energy, suggesting that the activation is due to a more open and flexible conformation of the enzyme induced by attachment of the mercurial to the thiol moiety on Cys-6. As pointed out by Prendergast et al. [7], a possibility is that Cys-6 in the native enzyme may ionize and form a salt bridge with another residue and, in doing so, stabilize and protect the enzyme against urea denaturation. In contrast, the pCMB-modified cysteine residue would not be expected to ionize and thus could explain the altered properties of this modified enzyme.

For the simple Michaelis–Menten mechanism (where $K_m = K_s$), we have:

As pointed out by Fersht [33], the Michaelis–Menten equation may be rewritten as the useful form:

$$v = \frac{k_{\text{cat}}[E][S]}{K_m} \quad (9)$$

the enzyme–transition-state complex, i.e. the transition state of the substrate binds to the modified enzyme more strongly than the native enzyme.

This work was supported in part by Grant 39421003 from the China Natural Science Foundation and the Pandeng Project of the National Commission for Science and Technology.

REFERENCES

- 1 Blakley, R. L. (1969) *The Biochemistry of Folic Acid and Related Pteridines*, Elsevier, New York
- 2 Melera, P. W., Lewis, J. A., Biedler, J. L. and Hession, C. (1980) *J. Biol. Chem.* **255**, 7024–7028
- 3 Melera, P. W., Davide, J. P. and Oen, H. (1988) *J. Biol. Chem.* **263**, 1978–1990
- 4 Blakley, R. L. (1984) in *Folates and Pterins*, vol. 1 (Blakley, R. L. and Benkovic, S. J., eds.), pp. 191–255, Wiley Interscience, New York
- 5 Freisheim, J. H. and Matthews, D. A. (1984) in *Folate Antagonists as Therapeutic Agents*, vol. 1 (Sirotnak, F. M., Burchall, J. J., Ensminger, W. D. and Montgomery, J. A., eds.), pp. 69–131, Academic Press, Orlando
- 6 Kaufman, B. T., Kumar, A. A., Blankenship, D. T. and Freisheim, J. H. (1980) *J. Biol. Chem.* **255**, 6542–6545
- 7 Prendergast, N. J., Delcamp, T. J., Smith, P. L. and Freisheim, J. H. (1988) *Biochemistry* **27**, 3664–3671
- 8 Duffy, T. H., Sato, J. K., Vitols, K. S. and Huennekens, F. M. (1986) *Adv. Enzymol. Regul.* **24**, 13–25
- 9 Duffy, T. H., Beckman, S. B., Peterson, S. M., Vitols, K. S. and Huennekens, F. M. (1987) *J. Biol. Chem.* **262**, 7028–7033
- 10 Tian, W. X. and Tsou, C. L. (1982) *Biochemistry* **21**, 1028–1032
- 11 Tsou, C. L. (1988) *Adv. Enzymol.* **61**, 381–436
- 12 Wang, Z. X. and Tsou, C. L. (1987) *J. Theor. Biol.* **127**, 253–270
- 13 Wang, Z. X., Preiss, B. and Tsou, C. L. (1988) *Biochemistry* **27**, 5095–5100
- 14 Wang, Z. X., Wang, H. R. and Zhou, H. M. (1995) *Biochemistry* **34**, 6863–6868
- 15 Seeger, D. R., Cosulich, D. B., Smith, J. M. and Hultquist, M. E. (1949) *J. Am. Chem. Soc.* **71**, 1753–1758
- 16 Dawson, R. M. C., Elliot, D. C., Elliot, W. M. and Jones, K. M. (1969) in *Data for Biochemical Research*, p. 206, Oxford University Press, Oxford
- 17 Kornberg, A. and Horecker, R. L. (1953) in *Biochemical Preparations*, vol. 3 (Snell, E. C., ed.), p. 27, Wiley, New York
- 18 Maragos, P. J. and Constantinides, S. M. (1974) *Biochemistry* **13**, 904–910
- 19 Johnson, H. and Hecht, M. H. (1994) *BioTechnology* **12**, 1357–1360
- 20 Mathews, C. K., Scrimgeour, K. G. and Huennekens, F. M. (1963) *Methods Enzymol.* **6**, 364–368
- 21 Hillcoat, B. L., Nixon, P. F. and Blakley, R. L. (1967) *Anal. Biochem.* **21**, 178–189
- 22 Means, G. E. and Feeney, R. E. (1971) in *Chemical Modification of Proteins*, p. 19, Holden-Day, San Francisco
- 23 Boyer, P. H. (1954) *J. Am. Chem. Soc.* **76**, 4331–4337
- 24 Fan, Y. X., Ju, M., Zhou, J. M. and Tsou, C. L. (1996) *Biochem. J.* **315**, 97–102
- 25 Pace, C. N. (1986) *Methods Enzymol.* **131**, 266–280
- 26 Wang, Z. X. (1990) *J. Theor. Biol.* **142**, 551–563
- 27 Stammers, D. K., Champness, J. N., Beddell, C. R., Dann, J. G., Eliopoulos, E., Geddes, A. J., Ogg, D. and North, A. C. T. (1987) *FEBS Lett.* **218**, 178–184
- 28 Oefner, C., D'Arcy, A. and Winkler, F. K. (1988) *Eur. J. Biochem.* **174**, 377–385
- 29 Davies, J. F., Delcamp, T. J., Prendergast, N. T., Ashford, V. A., Freisheim, J. H. and Kraut, J. (1990) *Biochemistry* **29**, 9467–9479
- 30 Cody, V., Luft, J. R., Ciszak, E., Klamann, T. I. and Freisheim, J. H. (1992) *Anti-Cancer Drug Des.* **7**, 483–491
- 31 Koshl and D. E. Jr. (1976) *FEBS Lett.* **62**, E47–E52
- 32 Tsou, C. L. (1995) *Biochim. Biophys. Acta* **1253**, 151–162
- 33 Fersht, A. R. (1985) in *Enzyme Structure and Mechanism*, pp. 311–346, W. H. Freeman, New York