Activation of chicken liver dihydrofolate reductase by urea and guanidine hydrochloride is accompanied by conformational change at the active site

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It has been reported that the activation of dihydrofolate reductase (DHFR) from L1210 mouse leukaemia cells by KCl or thiol modifiers is accompanied by increased digestibility by proteinases [Duffy, Beckman, Peterson, Vitols and Huennekens (1987) J. Biol. Chem. **262**, 7028–7033], suggesting a loosening up of the general compact structure of the enzyme. In the present study, the peptide fragments liberated from the chicken liver enzyme by digestion with trypsin in dilute solutions of urea or guanidine hydrochloride (GuHCl) have been separated by FPLC and sequenced. The sequences obtained are unique when compared with the known sequence of DHFR and thus allow the points of proteolytic cleavage identified for the urea- and GuHCl-activated enzyme to be at or near the active site. It was also indicated by the enhanced fluorescence of 2-*p*-toluidinylnaphthalene 6-sulphonate that conformational changes at the active site in dilute GuHCl parallel GuHCl activation. The above results indicate that the activation of DHFR in dilute denaturants is accompanied by a loosening up of its compact structure especially at or near the active site, suggesting that the flexibility at its active site is essential for the full expression of its catalytic activity.

INTRODUCTION

Dihydrofolate reductase (DHFR; EC 1.5.1.3) in eukaryotic cells can be activated by a diverse group of agents [1], including inorganic salts [2,3], thiol modifiers [3,4] and chaotropes, especially urea and guanidine hydrochloride (GuHCl) [3,5]. Urea and GuHCl, commonly employed as protein denaturants, generally bring about inactivation and unfolding of proteins by disruption of the secondary and tertiary structures. In most cases inactivation occurs at lower denaturant concentrations than required to bring about unfolding of the molecules as a whole [6–8]. However, DHFR can be markedly activated by low concentrations of either urea or GuHCl. Although there have been many reports on the activation of DHFR, the mechanism remains obscure. In a previous paper [9] the urea activation and the subsequent inactivation of chicken liver DHFR was compared with its conformational changes and a scheme for the sequential activation and inactivation of DHFR accompanying its unfolding by increasing concentrations of urea or GuHCl was proposed. Duffy [3] reported that activation of DHFR from L1210 mouse leukaemia cells by KCl and thiol modifiers involved conformational changes in the enzyme molecule as indicated by increased proteolysis and fluorescence of 2-*p*-toluidinylnaphthalene-6-sulphonate (TNS). Here we show, by sequence analysis of the peptide fragments liberated by trypsin digestion, that the increased susceptibility of DHFR is primarily due to conformational changes at or near the active site.

MATERIALS AND METHODS

Materials

DHFR from chicken liver was purified as previously described [9]. Trypsin [type XIII, tosylphenylalanylchloromethane ('TPCK')-treated], dihydrofolate (approx. 90%), NADPH (approx. 97%) were Sigma products. GuHCl (ultrapure) was obtained from ICN Biochemicals. Urea (ultrapure) was from Boehringer Mannheim and solutions were always freshly prepared from a recrystallized sample [10]. N^{α}-Benzoyl-L-arginine ethyl ester (BAEE) was from Dongfeng Biochemicals, Shanghai, China. Other reagents were local products of analytical grade; twice-deionized water was used throughout.

The concentrations of DHFR and trypsin were all determined by measuring the absorbance at 280 nm, taking 28970 $M^{-1} \cdot cm^{-1}$ as the molar absorption coefficient for DHFR [11] and $A^{1\%}_{1\,cm}$ 14.4 for trypsin [12].

Digestion of DHFR by trypsin in various concentrations of urea or GuHCl

Digestion of DHFR with trypsin was performed in 50 mM Tris/HCl buffer, pH 7.4, and 0.01 M $CaCl_2$ at 20 °C. The reaction system contained 0.18 mg/ml DHFR and 0.02 mg/ml trypsin with a molar ratio of about 10:1 at the urea or GuHCl concentrations indicated. To stop the proteolysis, 0.03 M PMSF in methanol as an inhibitor of trypsin was used at a molar ratio of 1200:1.

Assay of DHFR

The activity of DHFR was assayed by the procedure of Mathews [13] with a Shimadzu UV-250 spectrophotometer thermostatically controlled at 20 °C. The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.5, 10 mM 2-mercaptoethanol, 0.1 mM dihydrofolate (DHF), 0.1 mM NADPH and the required concentrations of urea or GuHCl.

Assay of trypsin

Activity measurements of trypsin at pH 7.4 and 20 °C were made with BAEE as substrate [14]. The reaction mixture contained 50 mM Tris/HCl buffer, pH 7.4, 10 mM $CaCl_2$, 30 μM BAEE, 0.02 mg/ml trypsin and required concentrations of urea. Trypsin

Abbreviations used: ANS, 1-anilinonaphthalene-8-sulphonate; BAEE, N^{α}-benzoyl-L-arginine ethyl ester; DHF, dihydrofolate; DHFR, dihydrofolate reductase; GuHCl, guanidine hydrochloride; TNS, 2-*p*-toluidinylnaphthalene-6-sulphonate.

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was first incubated with urea at required concentrations in the same buffer for 5 h.

Fluorescence measurements

The time-dependent change in fluorescence of the hydrophobic probe TNS was measured with a Perkin-Elmer MPF-66 fluorimeter. The excitation and emission wavelengths of TNS were 324 and 450 nm respectively. All measurements were made at 20 °C. Initially, 4.0 μM TNS was present in the quartz cuvette in 50 mM potassium phosphate buffer, pH 7.5, DHFR was added to a final concentration of 0.5 μM, and then 6 M GuHCl was added to make the indicated final concentrations.

FPLC systems

Analysis and separation of trypsin digestion products of DHFR were performed with a ProRPC HR5/10 reverse-phase column on a Phamacia FPLC System and eluted with a linear gradient of water and acetonitrile in 0.1 % trifluoroacetic acid at a flow rate of 0.5 ml/min; the absorbance of the effluent was monitored at 214 nm. Peptide fragments from the digestion products were collected, pooled from several separations when necessary, and freeze-dried before being subjected to N-terminal sequence analysis.

Determination of N-terminal sequence

The N-terminal sequences of the peptide fragments were determined with a MilliGen-6600 Microprotein Sequencer through Edman auto-degradation.

RESULTS

Activation of DHFR in solutions of urea and GuHCl

With increasing concentrations of either urea or GuHCl, DHFR is first activated and then gradually inactivated with a further increase in the concentrations of the denaturants (Figure 1). The enzyme is activated about 2-fold in 0.5 M GuHCl and nearly 5-fold in about 4 M urea. It is nearly completely inactivated when the concentration of GuHCl is higher than 2.0 M, but it is still about twice as active as the native enzyme even in 7 M urea. The urea-activated enzyme is unstable when the urea concentration is higher than 3 M [9].

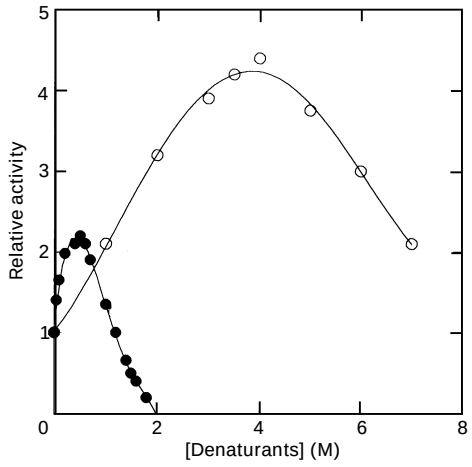

Figure 1 Effects of urea and GuHCl on the activity of DHFR

The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.5, 10 mM 2-mercaptoethanol, 0.1 mM DHF, 0.1 mM NADPH and the indicated concentrations of urea (○) or GuHCl (●). The reaction was initiated by the addition of the enzyme at 1 μg/ml.

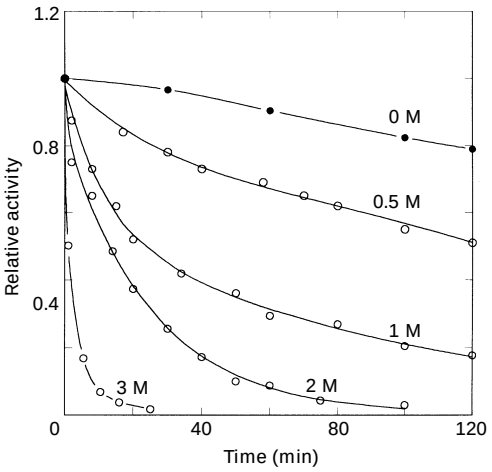

Figure 2 Activity change of DHFR during trypsin digestion in the presence of various concentrations of urea

Digestion mixtures containing 0.18 mg/ml DHFR, 0.02 mg/ml trypsin in 0.05 M Tris/HCl buffer, pH 7.4, containing 0.01 mM $CaCl_2$ and different concentrations of urea as indicated were incubated at 20 °C. Aliquots (10 μl) were taken at intervals and diluted to 1.2 ml of assay mixture containing the same concentrations of urea for activity measurements.

Susceptibility of urea-activated DHFR to trypsin digestion at various concentrations of urea

The activated form is stable and no significant conformational change can be detected by intrinsic fluorescence measurements at urea concentrations lower than 3 M [9]. During the course of tryptic hydrolysis at various concentrations of urea, the decrease in DHFR activity is as shown in Figure 2. The hydrolysis rate of native DHFR by trypsin is very slow; about 80 % activity remained after exposure to trypsin for 120 min. The rate of digestion increases markedly with urea concentrations. In 3 M urea the activated enzyme loses its activity completely after exposure to trypsin for only 25 min. The increase in the rate of hydrolysis is not due to an activation effect of urea on trypsin, as control experiments show that the activity of trypsin towards BAEE decreases slightly in the presence of urea and is about 75 % as active in 5 M urea as in its absence. The difference between the digestion rates of native and activated DHFR can therefore be ascribed to changes in the DHFR molecule only, most probably an opening up of its compact three-dimensional structure. A comparison with previous results shows that in increasing concentrations of urea, the increase in the digestion rate by trypsin accompanies the activation of DHFR and precedes significant conformation changes of the molecule as a whole [9].

Tryptic digestion of DHFR in GuHCl solutions

Chicken liver DHFR is activated by GuHCl at low concentrations, and the activity of the enzyme reaches a maximum at 0.5 M GuHCl. With further increasing of concentration of GuHCl, the activity decreases rapidly, with complete inactivation in 2.0 M GuHCl (Figure 1), whereas no significant conformational change is observed by either intrinsic fluorescence or

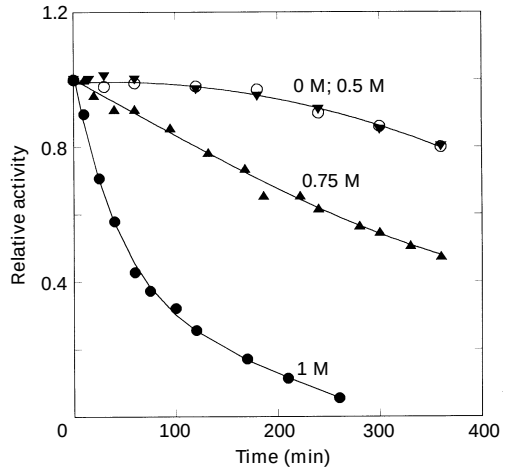

Figure 3 Activity change during trypsin digestion in the presence of various concentrations of GuHCl

Details are as in the legend of Figure 2 with the substitution of GuHCl for urea at the indicated concentrations.

CD up to a GuHCl concentrations of 1.2 M [15]. The time courses of decrease in DHFR activity during trypsin digestion of DHFR in different concentrations of GuHCl are shown in Figure 3. The decrease in activity of 0.5 M GuHCl-activated DHFR is apparently identical with that of the native enzyme, probably owing to the increased susceptibility to proteolysis in GuHCl compensating for the decreased activity of trypsin in this denaturant [16]. However, when the concentration of GuHCl reaches 0.75 M, the rate of digestion increases conspicuously; at a GuHCl concentration of 1 M the enzyme is 1.3-fold activated with a much more marked increase in the rate of tryptic digestion.

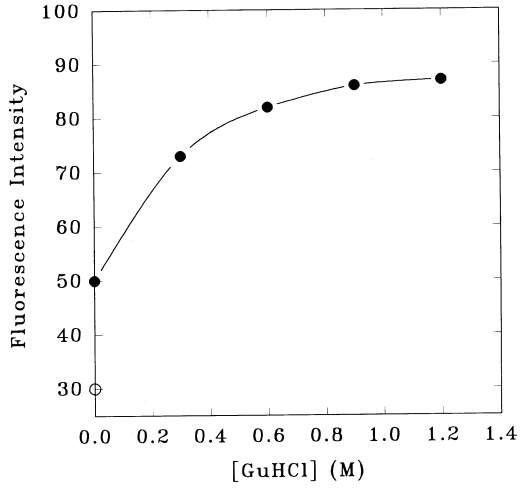

Figure 4 Changes in fluorescence of the DHFR-bound hydrophobic probe TNS with GuHCl concentration

The excitation and emission wavelengths of TNS were 324 and 450 nm respectively and the corresponding slit widths were set at 5 and 7 nm respectively. All measurements were made at 20 °C. Initially, TNS (4.0 μM) was present in 2.7 ml of 50 mM potassium phosphate buffer, pH 7.5; 20 μl of DHFR stock solution was added to a final concentration of 0.5 μM, then 6 M GuHCl was added to the indicated final concentrations. The fluorescence intensity was not corrected for the volume increase.

Conformational change at the active site in dilute GuHCl as monitored by TNS fluorescence

The digestion rate of DHFR is apparently not affected by GuHCl up to 0.5 M. The hydrophobic fluorescence probe TNS, which has been shown to interact with DHFR at the dihydrofolate binding site [17], was employed to investigate the conformation change at the active site in dilute GuHCl, with the result shown in Figure 4. The weak fluorescence of TNS was enhanced immediately on binding to the enzyme. Addition of GuHCl to only 0.3 M caused a further increase in fluorescence, and the fluorescence emission increased with increasing GuHCl concentrations up to 0.9 M. The enhancement of fluorescence of the enzyme-bound TNS was observed immediately after addition of GuHCl, and this was in agreement with the rate of GuHCl activation [15]. The above result suggests that a subtle conformational change of DHFR at the active site occurs concurrently with the activation of the enzyme in dilute GuHCl. 1-Anilinonaphthalene-8-sulphonate (ANS) is an apolar fluorescent probe and its fluorescence is enhanced significantly by GroEL-stabilized DHFR, which has been referred to as a 'molten globule'-like state [18]. However, ANS does not bind to native DHFR and its fluorescence is not enhanced with the addition of the denaturant alone. TNS, a probe similar to ANS, is a linear molecule with an additional methyl group, and is stronger in hydrophobicity for monitoring the slight conformational changes of the protein molecule.

Separation and identification of trypsin digestion products

The tryptic digestion rate of native enzyme is relatively slow, but peptide fragments can be obtained by using a high trypsin concentration (trypsin-to-DHFR molar ratio 1:2) and prolonged digestion for 10 h at 20 °C. Six peaks can be identified by reverse-phase chromatography as shown in Figure 5 (top panel). Control experiments showed that peak VI was derived from trypsin. Four major peaks, I, II, III and IV, were subjected to N-terminal sequence analysis. The sequences obtained are unique and allow the cleavage points to be identified as given in the legend. Peaks II and III have the same N-terminal sequence are probably derived from the same segment but are of different lengths. Peak V has not been sequenced because the amount available was insufficient. By trypsin digestion of DHFR in 2 M urea for 90 min, seven peaks can be identified by the same chromatography system (Figure 5, middle panel). Peak III contains two components (peaks IIIa and IIIb) and was separated in the same FPLC system with a 15 μm PepRPC column. Peak VII was identified as trypsin by a control experiment. The separated peptide fragments of 2 M urea-activated DHFR were subjected to N-terminal sequence analysis and the results are given in the legend. Again, the sequences are unique amd allow the cleavage points to be identified as Arg^{2}–Ser^{3}, Lys^{55}–Thr^{56}, Arg^{70}–Ile^{71}, Arg^{137}–Ile^{138} and Lys^{157}–Leu^{158}. The sequencing of peaks IIIb and VI was not possible because the amount available was insufficient and was contaminated with impurities. The cleavage of the bond Lys^{18}–Asn^{19} identified after digestion of the native enzyme was not detected after digestion of the 2 M urea-activated form. It should be pointed out that for the digestion of the native enzyme, a much higher trypsin-to-DHFR ratio was used and for a much longer time than the digestion in urea. An urea concentration of 2 M was selected because the enzyme is activated more than 2-fold (Figure 1) and is stable, as indicated in a control experiment without trypsin for the same period

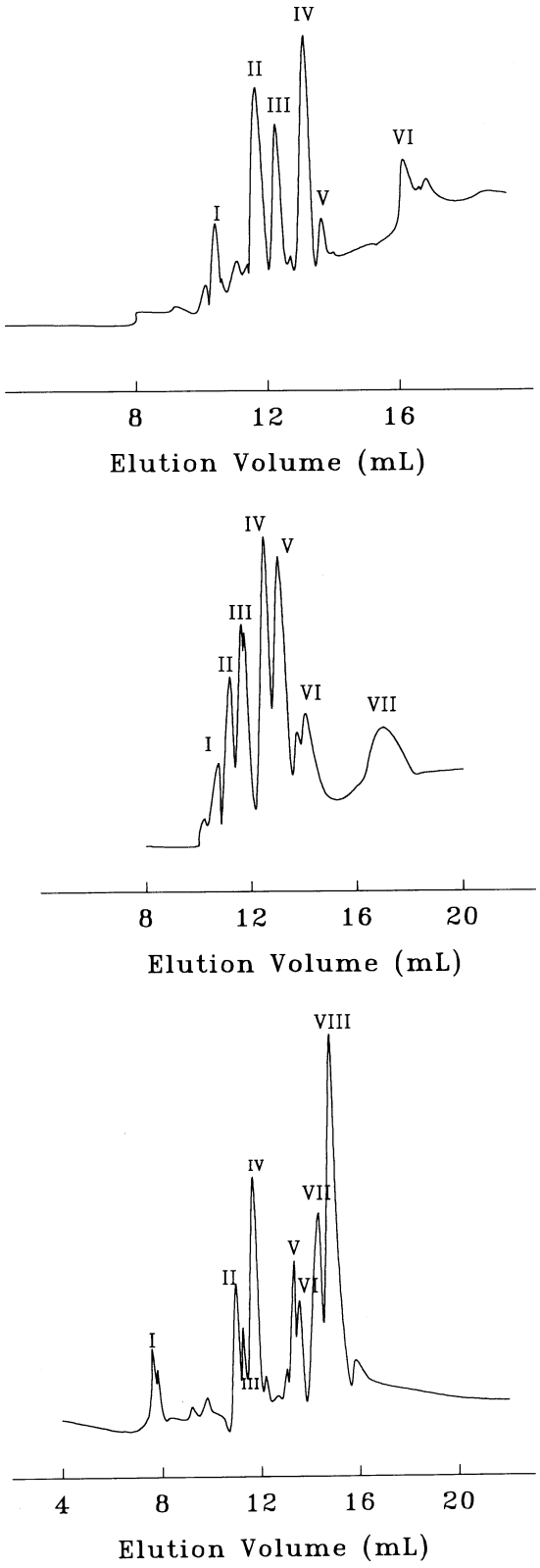

Figure 5 Separation of tryptic digestion products of DHFR

(Top panel) Native enzyme, DHFR (0.18 mg/ml), digested by 0.1 mg/ml trypsin for 10 h at 20 °C; the digests were applied to an FPLC systemas described in the Materials and methods section. The N-terminal sequences of the peaks were: peak I, Asp^{19}-Gly-Asn-Leu; peak II, Leu^{158}-Leu-Thr-Glu; peak III, Leu^{158}-Leu-Thr-Glu; peak IV, Ile^{138}-Leu-His-Gln. (Middle panel) Digestion in 2 M urea; the digestion conditions were as described in the text with a DHFR-to-trypsin molar ratio of 10:1. The digestion was stopped by addition of an excess of PMSF after 90 min and the fragments were applied to the FPLC system. The N-terminal sequences of the peaks were: peak I, Ile^{71}-Asn-Ile-Val; peak II, Thr^{56}-Try-Phe-Ser; peak IIIa, Ser^{3}-Leu-Asn-Ser; peak IV, Leu^{158}-Leu-Thr-Glu; peak V, Ile^{138}-Leu-His-Gln. (Bottom panel) Digestion in 1.0 M GuHCl; other conditions were as above. The N-terminal sequences of the peaks were: peak II, Asp^{19}-Gly-Asn-Leu; peak III, Thr^{56}-Try-Phe-Ser; peak IV, Leu^{158}-Leu-Thr-Glu; peak V, Ile^{138}-Leu-His-Gln; peak VI, Met^{37}-Thr-Ser-His. (mL = ml.)

Table 1 A comparison of the trypsin cleavage points of the native and the urea- and GuHCl-activated enzymes

Native	In 2 M urea	In 1 M GuHCl
	Arg^{2}–Ser^{3}	
Lys^{18}–Asp^{19}		Lys^{18}–Asp^{19}
		Arg^{36}–Met^{37}
	Lys^{55}–Thr^{56}	Lys^{55}–Thr^{56}
	Arg^{70}–Ile^{71}	
Arg^{137}–Ile^{138}	Arg^{137}–Ile^{138}	Arg^{137}–Ile^{138}
Lys^{157}–Leu^{158}	Lys^{157}–Leu^{158}	Lys^{157}–Leu^{158}

required for proteolysis, and although it can be further activated by higher concentrations of urea, the activated enzyme is no longer stable for the period required for proteolysis [9].

Figure 5 (bottom panel) shows the FPLC profile for the peptide fragments liberated from DHFR by tryptic digestion in 1 M GuHCl for 60 min at 20 °C. Peaks VII and VIII are trypsin and intact DHFR respectively. The results of N-terminal sequence analyses of peaks II, III, IV, V and VI are given in the legend. The sequences are also unique and allow the cleavage points to be identified as Lys^{18}–Asn^{19}, Arg^{36}–Met^{37}, Lys^{55}–Thr^{56}, Arg^{137}–Ile^{138} and Lys^{157}–Leu^{158}. A concentration of 1 M GuHCl was selected because the digestion rate was too slow in the concentration (0.5 M) for maximal activation, and in 1.0 M GuHCl DHFR was 1.3-fold activated and stable over the digestion period.

The points of cleavage of the DHFR molecule in the absence and presence of denaturants are compared in Table 1 and indicated in Figure 6, together with the known essential residues at the active site [19]. Note in Table 1 that the peptide bonds Arg^{2}–Ser^{3} and Arg^{70}–Ile^{71} are cleaved in 2 M urea but not in 1 M GuHCl, whereas Lys^{18}–Asp^{19} and Arg^{36}–Met^{37} are hydrolysed in GuHCl but not in urea. These results suggest that the unfolding pathways of DHFR are different in urea and GuHCl, with differently structured intermediates. Apart from some bonds susceptible to both denaturants, urea- and GuHCl-susceptible bonds seem to be located in different regions of the enzyme molecule, responsible respectively for NADPH and DHF binding (Figure 6).

DISCUSSION

Activation of DHFR is accompanied by conformational change at the active site

In previous reports from this laboratory, the activation and inactivation of DHFR from chicken liver during denaturation by urea [9] or GuHCl [15] have been shown to precede detectable global conformational changes of the enzyme molecule. The activated enzyme shows a greatly increased V_{max}, which more than compensated for the increase in the dissociation and Michaelis constants compared with that of the native enzyme, suggesting that the activation is due to a more open and flexible

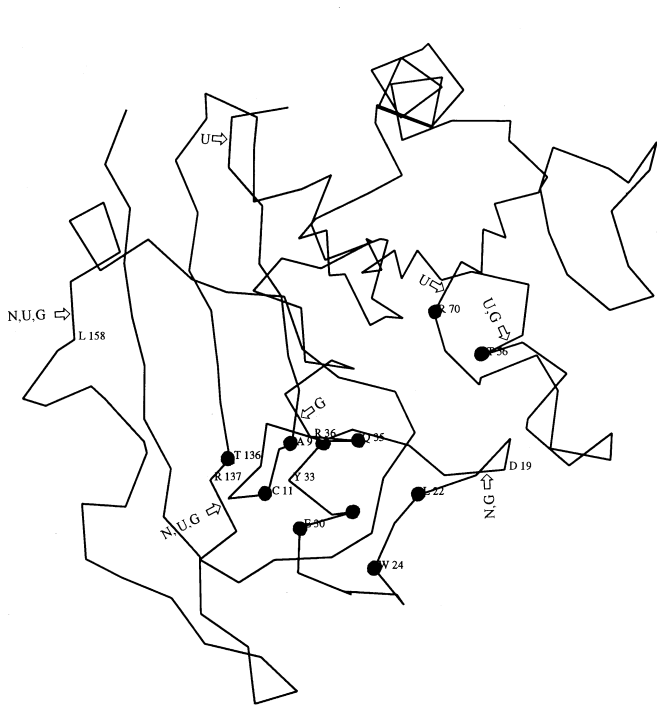

Figure 6 Cleavage positions in the DHFR molecule by proteolysis with trypsin under various conditions

N indicates the peptide bonds in native DHFR hydrolysed by trypsin; U the hydrolysed peptide bonds in the 2 M urea-activated form and G the hydrolysed peptide bonds in 1 M GuHCl. The essential residues at the active site, Ala[9], Cys[11], Leu[22], Trp[24], Glu[30], Try[31], Glu[34], Gln[35], Thr[56], Arg[70] and Thr[136], are marked ●.

conformation of the activated enzymes. A similar suggestion has been made by Duffy et al. [3] for the activation of DHFR from L1210 mouse leukaemia cells by KCl and modification of the thiol group.

Proteolysis has been shown to be a sensitive method of detecting subtle conformational changes in protein molecules [16,20–22]. Native proteins are generally resistant to proteolysis, and their susceptibility increases conspicuously even with a slight unfolding of the compact folded structure [23]. It is now shown that, concurrent with activation in urea and GuHCl solution, conformational change at the active site of DHFR does occur, as indicated by increased susceptibility to digestion by trypsin and to increased TNS binding as indicated by increased fluorescence emission. Although 0.5 M GuHCl does not affect the digestion rate, the susceptibility to digestion by trypsin increases significantly at higher concentrations of GuHCl without noticeable conformational change, as shown by measurements by conventional spectral methods [15].

There are 26 possible tryptic hydrolysable bonds in the DHFR molecule. Fragment separation and N-terminal sequence determinations reveal that hydrolysis of the native enzyme is at the Lys[18]–Asn[19], Arg[137]–Ile[138] and Lys[157]–Leu[158] bonds. The residues Lys[18]–Asn[19] and Lys[157]–Leu[158] are situated in the surface loop of the molecule and are probably therefore prone to hydrolysis; the residue Thr[136] is essential for substrate binding, so the residues Arg[137]–Ile[138] are near the active site. The hydrolysis of bonds after trypsin digestion of the 2 M urea-activated enzyme are identified (Figure 5) as Arg[2]–Ser[3], Lys[55]–Thr[56], Arg[70]–Ile[71], Arg[137]–Ile[138] and Lys[157]–Leu[158], and those after digestion in 1 M GuHCl are Lys[18]–Asn[19], Arg[36]–Met[37], Lys[55]–Thr[56], Arg[137]–Ile[138] and Lys[157]–Leu[158]. The locations of these sites are indicated in Figure 6; comparison with the cleavage positions in the native enzyme (Table 1) shows that, with the exception of Arg[2]–Ser[3] in the N-terminus, all the additional bonds cleaved in 2 M urea or 1 M GuHCl are situated close to the active site. The above results clearly show that although gross conformational change cannot be detected by conventional methods, a subtle change in conformation, mainly at the active site, occurs during activation by denaturants.

The initial stage of denaturation of some proteins at low denaturant concentrations involves limited conformational changes and can be compared with the 'molten globule' state that was proposed for the partly denatured state of α-lactalbumin [24]. The consensus on the concept of the molten globule state has been defined as a high content of secondary structure, considerable molecular compactness, non-specific tertiary structure and significant flexibility [25–27]. The molecule of DHFR denatured in dilute GuHCl or urea is partly loosened especially in the active site region and is thus open to attack by proteolytic hydrolysis without noticeable changes in its secondary structure. The above is consistent with the concept of the molten globule state. However, the initial state of denaturation involves changes mostly in the active site region only and for the molten globule-like intermediate during the refolding of DHFR, global molecular changes are considered [18].

Pancreatic ribonuclease A is inactivated in GuHCl at low concentrations before the unfolding of the molecule as a whole as can be detected by either intrinsic fluorescence or ultraviolet absorbance [28]. It has also been shown that, concurrently with the inactivation of ribonuclease in dilute GuHCl, conformational change in the molecule does occur, as indicated by increased susceptibility to proteolysis by both trypsin and proteinase K [16]. Fragment separation and N-terminal sequence determination revealed that the bonds were at the active site of the ribonuclease molecule.

The proteolysis studies of DHFR clearly confirm the proposition that the conformation of active sites is more flexible and more sensitive to denaturation than the molecule as a whole [7,8] and that the activation of DHFR is due to the subtle conformational change at the active site of the molecule, suggesting that flexibility at the active site is important for DHFR activity. It would be interesting to study whether the activation of other enzymes is accompanied by subtle conformation change at the active sites without perturbation of the conformation of the molecules as a whole.

Requirement for flexibility at the active site for the full expression of the catalytic power of DHFR

The dynamic nature of protein structure is now generally recognized [29]; however, the flexibility at the active site has been only occasionally emphasized [30]. The well-known 'induced fit' hypothesis by Koshland [31] implies the existence of multi-conformational states of enzymes in equilibrium with one another, which can be perturbed by the presence of substrates or other ligands [32]. As each intermediary step during the entire catalytic process may require the molecule to be in a particular conformation, rapid interconversion between the different conformational states may well be involved in the catalytic process. As a relatively fragile and consequently flexible active site has now been envisaged [8], it seems that for the full expression of the catalytic activities of enzymes, a rapid cycling of the different conformational states of the active site is essential. For example, the stabilization of lactate dehydrogenase by either ammonium

sulphate or cross-linking with glutaraldehyde decreases its activity; the original activity can be restored by low concentrations of GuHCl, which probably restores the conformational flexibility required for the full expression of the activity of lactate dehydrogenase [33]. Our previous studies also showed that ammonium sulphate stabilizes DHFR against denaturation by urea or GuHCl and greatly reduces the extent of activation of DHFR by denaturant [9,17].

Sequential conformational change during unfolding of DHFR

It has been suggested that the denaturation of DHFR followed the two-state model [34]. However, the activation, inactivation and intrinsic fluorescence changes occur at different urea or GuHCl concentrations. In addition, the activated state at low concentrations of the denaturants can in no way be envisaged as a mixture of the native and the unfolded state and is incompatible with the two-state model. Consequently a sequential scheme for the stepwise unfolding accompanying the denaturation of DHFR in increasing concentrations of denaturants has been proposed [9]. A subtle conformational change at the active site has now been shown to accompany activation of this enzyme before any global conformation change can be detected by conventional spectral methods. The gradual increase in the rates of proteolysis by trypsin with the increase in denaturant concentration also suggests the gradual opening up of the compact enzyme molecule. GuHCl at concentrations lower than 0.5 M apparently does not affect the rates of digestion by trypsin; however, in view of the inhibition of the proteolytic enzymes by GuHCl this could be a composite effect of increased proteolysis owing to the opening up of the compact enzyme molecule and a decrease in proteolysis by GuHCl inhibition of trypsin. A subtle conformational change accompanying activation can also be detected by the fluorescence of TNS, a hydrophobic probe that has been shown to interact with DHFR on the dihydrofolate binding site of the molecule [17]. The enzyme seems to open up sequentially with increasing concentrations of denaturants until a critical concentration is reached beyond which further disruption of the weak bonds holding together the overall conformation of the molecule may occur co-operatively, leading to a collapse of the native structure. The two-state model probably holds only under specific conditions where the breaking of a few of the non-covalent bonds leads to a collapse of the native conformation in a highly co-operative manner. Similarly, it has also been proposed that the refolding of urea-denaured DHFR is a sequential process [35].

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