Activation of dihydrofolate reductase following thiol modification involves a conformational change at the active site

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Compared with the activation of dihydrofolate reductase (DHFR) by protein denaturants and inorganic salts, activation of the enzyme by thiol modification is relatively slow. Thus it is an ideal system for kinetic study of the activation mechanism. We describe here a kinetic study of the activation of DHFRs from chicken liver and Chinese hamster ovary by *p*-hydroxymercuribenzoate (*p*-HMB). The conformational changes in the enzyme molecule that result from the modification were monitored by measuring fluorescence enhancement due to the binding of 2-*p*-toluidinylnaphthalene-6-sulphonate (TNS), and by monitoring changes in the intrinsic fluorescence of the enzyme. Both activation and the conformational change probed by TNS followed pseudo-first-order kinetics, and the rate constants

obtained are in good agreement with each other. The change in intrinsic fluorescence is a biphasic process. The rate of the fast phase, which may reflect a change in the microenvironment of Trp-24 at the active site, coincides with the rate of activation and the conformational change probed by TNS. The rate of the slow phase, which reflects a global conformational change, is about one order of magnitude lower than that of activation. The results indicate that the activation of DHFR by *p*-HMB is due to modification-induced conformational changes at its active site, rather than the modification of the thiol group itself, which is almost complete within the dead-time of the experiment. This study provides kinetic evidence for the proposal that flexibility at the active site is essential for full expression of catalytic activity.

INTRODUCTION

Dihydrofolate reductase (DHFR; EC 1.5.1.3) from eukaryotic cells can be activated by a diverse group of agents [1], including inorganic salts [2,3], thiol-group modifiers [3,4], chaotropes, urea and guanidine hydrochloride [3,5]. Although many studies have described the activation of DHFR, the mechanism remains obscure. The activation of chicken liver DHFR by urea and guanidine hydrochloride has been studied in this laboratory by comparison of activity and conformational changes [6–8]. Chicken liver DHFR is activated by low concentrations of urea or guanidine hydrochloride without any significant conformational changes being detectable by intrinsic fluorescence. Subtle conformational changes of the activated enzyme were, however, detected by proteolysis and enhanced fluorescence of 2-*p*-toluidinylnaphthalene-6-sulphonate (TNS). Sequence analysis of the peptide fragments liberated by trypsin digestion showed that the activation of DHFR in dilute denaturants is accompanied by a loosening of its compact structure, particularly at or close to the active site of the enzyme. This suggests that the active site of DHFR is more flexible than the rest of the molecule, and that flexibility at the active site is essential for full expression of the catalytic activity [9]. Duffy et al. [3] reported that activation of DHFR from L1210 mouse leukaemia cells by KCl and by thiol-group modifiers involves conformational changes in the enzyme molecule, as indicated by increased proteolysis and fluorescence of TNS.

Because the activation of DHFR by denaturants is too fast to be followed even using a stopped-flow apparatus, it is impossible to compare the rates of activation and conformational change. However, obtaining direct kinetic evidence for a link between enzyme activation and conformational changes is key to elucidating the mechanism of activation. There is one cysteine residue in the N-terminal region of the enzyme, at position 11 in the avian enzyme and at position 6 in the mammalian enzyme. The

activation of DHFR by thiol modification is much slower than that by denaturants. Thus the former is an ideal system for kinetic analysis of the relationship between enzymic activity and conformational flexibility at the active site. In the present study, the rates of *p*-hydroxymercuribenzoate (*p*-HMB)-induced activation of DHFR from chicken liver and of the recombinant enzyme from Chinese hamster ovary were determined and compared with the rates of conformational changes detected by monitoring intrinsic fluorescence and binding of the fluorescent probe TNS.

MATERIALS AND METHODS

Materials

Purification of DHFR from chicken liver [6], overexpression in *Escherichia coli* and purification of recombinant Chinese hamster ovary DHFR [10] were carried out as previously described. The final preparations used typically had specific activities of 12.6 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ for chicken liver DHFR and 4.7 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ for recombinant DHFR. Dihydrofolate ($\approx 90\%$ pure), NADPH ($\approx 97\%$) and *p*-HMB were from Sigma. TNS was from ICN. Other reagents were local products of analytical grade. Double-deionized water was used throughout. Since β -mercaptoethanol cannot be used in the modification and assay system, precautions were taken to remove oxygen from the samples. The buffer was aerated with pure nitrogen for about 30 min before use.

Assay of DHFR

The activity of DHFR was assayed by the procedure of Matthews [11], using a Shimadzu UV-250 and Cary 219 UV/visible spectrophotometer thermostatically maintained at 20 °C. The reaction system contained 50 mM potassium phosphate buffer,

Abbreviations used: DHFR, dihydrofolate reductase; *p*-HMB, *p*-hydroxymercuribenzoate; TNS, 2-*p*-toluidinylnaphthalene-6-sulphonate.

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pH 7.5, 0.1 mM dihydrofolate and 0.1 mM NADPH (0.2 mM for *p*-HMB-modified chicken liver DHFR) in a volume of 1.2 ml. Activation of the enzymes, monitored by the time-dependent increase in catalytic activity, was measured in the following manner. Enzyme was preincubated at 20 °C with the indicated concentration of *p*-HMB in 50 mM potassium phosphate buffer, pH 7.5. At various time intervals, aliquots were removed and assayed for catalytic activity as described above.

Modification of the enzymes by *p*-HMB

The modification reaction was monitored as described by Boyer [12], using a Shimadzu UV-250 spectrophotometer at 20 °C. Both the reference and sample cells contained 200 μ M *p*-HMB in 50 mM potassium phosphate buffer, pH 7.5, with 4 μ M enzyme added to the sample. The increase in absorbance at 250 nm was recorded as a function of time.

Fluorescence measurements

Fluorescence measurements were performed at 20 °C using a Hitachi F-4500 spectrofluorimeter. The time-dependent change in intrinsic fluorescence was measured at an emission wavelength of 326 nm with an excitation wavelength of 280 nm. Both excitation and emission slits were set at 5 nm. For measurement of the fluorescence of TNS and enzyme-bound TNS, the excitation and emission wavelengths were 323 and 450 nm respectively, and both slit widths were set at 5 nm. Initially, 4.0 μ M TNS was present in the quartz cuvette in 50 mM potassium phosphate buffer, pH 7.5. The enzyme was added to a final concentration of 0.5 μ M, and then *p*-HMB was added to give the indicated final concentrations.

The kinetic constants of activation and conformational changes were obtained by non-linear least-squares fitting to the equation:

$$A_t = \sum_i A_i \exp(-k_i t) + A_\infty$$

where A_t is the activity or fluorescence intensity at time t , A_i is the amplitude corresponding to the individual phase at zero time, k_i is the associated rate constant and A_∞ is the amplitude at infinite time.

RESULTS

Modification of chicken liver DHFR by *p*-HMB

It has been reported that chicken liver DHFR can be activated approx. 6-fold by modification with a stoichiometric amount of *p*-HMB [4]. The chemical reaction of the enzyme with *p*-HMB was detected directly by measuring the increase in absorbance at 250 nm [12]. As shown in Figure 1 (curve 2), the modification is very fast, and is almost completed within the manual mixing time at a 50-fold excess of *p*-HMB. The K_m values for NADPH and dihydrofolate of the *p*-HMB-modified enzyme are shown in Table 1. It is conceivable that the K_m values for both NADPH and dihydrofolate were increased, indicating that activation of the modified enzyme is not due to an increase in the affinity of the enzyme for the substrates.

Treatment of the enzyme with a 100-fold excess of *p*-HMB produced a time-dependent increase in catalytic activity, which is a much slower process (Figure 1, curve 1). It is obvious that modification of the enzyme is considerably faster than the process of enzyme activation. Because the modified enzyme is not activated immediately, it seems likely that conformational changes following modification are the actual reason for enzyme activation. The increase in activity fits well to a single exponential

Figure 1 Chemical modification and activation of chicken liver DHFR by *p*-HMB in 50 mM potassium phosphate buffer, pH 7.5

Curve 1: activity change (right ordinate); the open circles represent the experimental values and the solid line represents the theoretical values obtained by fitting to a single exponential. Curve 2: chemical modification monitored by the increase in absorbance at 250 nm (left ordinate). Details are as described in the text.

Table 1 K_m values of chicken liver DHFR and Chinese hamster DHFR for substrates dihydrofolate and NADPH

Enzyme	K_m (μ M)	
	Dihydrofolate	NADPH
Chicken liver DHFR		
Native	0.17	2.7
Modified	8.9	22
Chinese hamster DHFR		
Native	1.7	2.6
Modified	8.7	12

function, indicating that the activation is a pseudo-first-order process. The activation rate constant obtained by non-linear least-squares curve fitting is 0.02 s⁻¹ (see Table 2).

The hydrophobic fluorescence probe TNS, which has been shown to interact with DHFR at the dihydrofolate binding site [13], was used to investigate conformational changes at the active site of the enzyme following modification (Figure 2, curve 1). The weak fluorescence of TNS was enhanced about 1.5-fold immediately upon binding to the enzyme. Addition of *p*-HMB yielded a slow further increase in fluorescence. Like the change in

Table 2 Rate constants for activation of and conformational changes in DHFR on modification by *p*-HMB

Enzyme	Rate constant (s ⁻¹)			
	Activation	TNS fluorescence	Conformational changes	
			Intrinsic fluorescence	
			Fast phase	Slow phase
Chicken liver DHFR	0.02	0.05	0.057	0.005
Chinese hamster DHFR	0.004	0.0043	0.02	0.0004

Figure 2 Conformational changes following modification of chicken liver DHFR by *p*-HMB, monitored by TNS and intrinsic fluorescence

Curve 1: enhancement of TNS fluorescence. Emission values are relative to the fluorescence intensity of free TNS. Curve 2: decrease in intrinsic fluorescence. Emission values are relative to the fluorescence intensity of the unmodified enzyme at the same concentration. The thick continuous lines represent the theoretical curves for fitting single or double exponentials respectively. Details are as described in the text.

Figure 3 Chemical modification and activation of Chinese hamster DHFR by *p*-HMB in 50 mM potassium phosphate buffer, pH 7.5

Curve 1, activity change (right ordinate); curve 2, chemical modification monitored by increase in absorbance at 250 nm (left ordinate). Details are as in the legend to Figure 1.

activity, the conformational change detected by TNS binding is a pseudo-first-order reaction, with a rate constant of 0.05 s^{-1} .

Modification of the enzyme with *p*-HMB also caused a small, but measurable, time-dependent decrease in the intrinsic fluorescence (Figure 2, curve 2). There are three tryptophan residues located in different parts of the chicken liver DHFR molecule, one of which, Trp-24, is located at the active site. The observed fluorescence change may represent both global conformational changes and conformational changes localized to the active site. As shown in Figure 2 (curve 2), the change in intrinsic fluorescence is a biphasic process. Since Trp-24 and the modified Cys-11 are located at the active site, the fast phase may be due to a conformational change at the active site, caused by the change in the microenvironment of Trp-24, while the slow phase may represent a global conformational change. The rate constants of the conformational changes, detected by both TNS and intrinsic fluorescence, were obtained by non-linear least-squares curve

Figure 4 Conformational changes following modification of Chinese hamster DHFR by *p*-HMB, monitored by TNS and intrinsic fluorescence

Curve 1, enhancement of TNS fluorescence; curve 2, decrease in intrinsic fluorescence. Details are as in the legend to Figure 2.

fitting and are compared in Table 2. Although the rates for enzyme activation, the conformational changes probed by TNS fluorescence and the fast phase of the change in intrinsic fluorescence are not identical, they are of the same order of magnitude, and are much faster than the rate of the overall conformational change, as represented by the slow phase of the change in intrinsic fluorescence.

Modification of recombinant Chinese hamster DHFR by *p*-HMB

Chinese hamster DHFR can be activated approx. 2.4-fold by stoichiometric amounts of *p*-HMB [14]. As shown in Figure 3 (curve 2), the chemical reaction of Chinese hamster DHFR with *p*-HMB is slower than that of chicken liver DHFR (Figure 1, curve 2). With a 50-fold excess of *p*-HMB, about 60 % of the enzyme had incorporated *p*-HMB at the time of the first measurement. When a > 300 -fold excess of *p*-HMB is used, the reaction is pseudo-first-order and the rate constant does not increase with further increases of the *p*-HMB concentration, indicating that the chemical reaction is not the rate-limiting step at high *p*-HMB concentrations. The activation caused by modification of the enzyme with a 400-fold excess of *p*-HMB is also a slow process, which follows pseudo-first-order kinetics (Figure 3, curve 1) with a rate constant of 0.004 s^{-1} (Table 2).

The weak fluorescence of TNS was enhanced about 5.4-fold upon binding to Chinese hamster DHFR. The fluorescence intensity of TNS bound to Chinese hamster DHFR is much greater than that of TNS bound to chicken liver DHFR, indicating that the former exhibits a more exposed hydrophobic surface area. Modification of the enzyme by *p*-HMB produces a small, but measurable, further increase in fluorescence intensity, which is also a pseudo-first-order process (Figure 4, curve 1).

As shown in Figure 4 (curve 2), the change in intrinsic fluorescence of Chinese hamster DHFR due to *p*-HMB modification is also biphasic. The rate constants for activation and for conformational changes probed by TNS fluorescence and intrinsic fluorescence were obtained by non-linear least-squares curve fitting (Table 2). As in the case of chicken liver DHFR, the rates of activation and of conformational changes probed by TNS are very similar, but the fast phase of change in intrinsic fluorescence is about 5-fold faster than the rate of activation. The binding of substrates or TNS may slow down the rate of conformational change at the active site. The slow phase of the

intrinsic fluorescence change is much slower than both activation and the conformational change probed by TNS. Thus the extent and rate of activation of Chinese hamster DHFR by *p*-HMB are different from those with chicken liver DHFR.

DISCUSSION

In the present investigation, the rates of activation of chicken liver and Chinese hamster DHFR by *p*-HMB modification have been studied and compared with the rates of the associated conformational changes. The results show that, compared with activation by urea and guanidine hydrochloride [6–8], activation of DHFR by *p*-HMB is relatively slow, and thus provides an ideal system for kinetic study. The rates of activation of both chicken liver and Chinese hamster DHFR are similar to the rates of conformational changes at the active site, which can be monitored by TNS fluorescence and the fast phase of intrinsic fluorescence change. The global conformational change is much slower, and is represented by the slow phase of the intrinsic fluorescence change (Table 2). The results suggest that activation of DHFR by *p*-HMB is accompanied by a conformational change around the active site of the enzyme.

It has been shown by sequence analysis of the peptide fragments liberated by trypsin digestion that the activation of chicken liver DHFR by urea or guanidine hydrochloride is due to an increase in flexibility at the active site [8]. Activation of DHFR by different agents is not additive, suggesting that a common mechanism may be involved [3]. Covalent incorporation of the derivatizing agent into the enzyme may break some secondary bonds in the molecule and increase the flexibility of its structure, particularly at the active site. The present study provides kinetic evidence for the proposition that flexibility at the active site of DHFR is essential for full expression of its catalytic activity. It is also possible that modification of Cys-11, which is in loop I of the chicken liver enzyme, might affect the motion of this loop, so that the rate of enzyme turnover is greater [15].

As each intermediate step during the entire catalytic process may require the enzyme to be in a particular conformation, rapid interconversion between the different conformational states may well be involved in the catalytic process. Rapid cycling of different conformational states at the active site may be essential for full expression of the catalytic activity of the enzyme [16]. It has been reported that ligand binding induces conformational changes in DHFR, and these changes may be important for activity (reviewed in [1]). The crystal structures of chicken liver DHFR complexed with binary (NADP⁺ and biopterin) and ternary (thio-NADP⁺–biopterin) complexes show clearly that the steric orientation and substrate conformation affect the rate of enzyme turnover [17,18]. Recently, the conformational changes that occur in *E. coli* DHFR during catalysis have been depicted as a movie, which was constructed using six isomorphous crystallographic structures [19]. It is conceivable that covalent modification of the enzyme and chaotropes may have similar effects on the binding of the products, and may accelerate the dissociation of products from the ternary complex, DHFR–NADP–tetrahydrofolate, which has been suggested to be the limiting step in the catalytic mechanism of this enzyme [20–22].

Although the sequences of DHFRs from animal tissues are highly similar, and the three-dimensional structures are nearly superimposable, the degree of activation varies depending upon the source of the enzyme and the modifying agent used [1]. The results in the present paper show that chicken liver and Chinese hamster DHFRs are both activated by *p*-HMB modification, but

that the degrees and rates of activation are significantly different. It has been suggested, from a comparison of the activation of bovine and chicken liver DHFRs and their corresponding N-terminal sequences, that the differences in the activation of the two enzymes by mercurials may reside in the location of and microenvironment surrounding their respective cysteine residues. There is a proline at position 3, two residues away from Cys-6, in the mammalian sequence, whereas in the avian enzyme this helix-breaking residue is absent, and the cysteine is at position 11 rather than position 6 [23]. However, DHFRs from Chinese hamster, bovine liver [19] and mouse L1220 lymphoma cells [3] have identical N-terminal sequences, but exhibit different activation properties. The significant difference between TNS fluorescence when bound to chicken liver and Chinese hamster DHFRs indicates that the conformations of the active sites of the two enzymes are different. The conformational differences are small but significant. It is likely that a slight difference in the overall conformational state of the enzyme, in particular at its active site, influences catalytic efficiency and activation. It is also tentatively concluded that the activation of these enzymes by thiol modification, as well by other agents, might involve a unique control site on the enzyme molecule. This may be located at the active site. It is difficult to define in detail from the present data how the enzyme would be activated upon modification of its single thiol group. However, crystallization of *p*-HMB-modified DHFR and analysis of the structure of the modified enzyme should provide the full picture of how this enzyme is activated upon SH modification.

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