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THERMAL TRANSITION OF A MUTATED DIHYDROFOLATE REDUCTASE

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SUMMARY

A mutated dihydrofolate reductase(DHFR), in which Cys^{152} of wild DHFR was replaced by Glu and three amino acid residues(Ile-Gln-Ile) were added at C terminal, was obtained by a recombinant DNA method. The thermal transition of the mutant was measured by CD and DSC at pH 7.0 and 7.7 and compared with that of wild DHFR. Analysis of the DSC data revealed that the thermal transition was the three-state one and the thermodynamic functions and mole fractions of native, intermediate and denatured states were calculated.

INTRODUCTION

Dihydrofolate reductase (DHFR, EC1.5.1.3) is a pharmacologically interesting enzyme since it is necessary for maintaining intracellular pools of tetrahydrofolate and its derivatives. The structure, function and utilization of DHFR have been studied intensively over the last decade (refs. 1-3). Our previous study revealed that mutated DHFRs from Escherichia coli in which several amino acid residues were added at their carboxyl terminal regions, fused proteins, express DHFR enzyme activity as high as that of the wild type (ref. 3). In order to utilize the multifunctional enzymes created by the protein fusion-system, fundamental knowledge on enzyme stability is required. On the other hand, owing to the development of scanning microcalorimetry (refs. 4,5) and the theory of calorimetry data analysis (refs. 6-10), calorimetry is now one of the effective methods for analyzing the structure and stability of multi-domain proteins which have intermediate states during their thermal transition (ref. 5).

In this paper, the thermal stability and thermodynamic structure of a mutant of DHFR evaluated from the calorimetry data are reported.

MATERIALS AND METHODS

Enzyme

The 3'-coding region of the E. coli DHFR gene was modified and a versatile expression plasmid vector, pTP70-1, was constructed. Using this plasmid, a mutated DHFR(G-DHFR), in which the latter of the two cysteines, Cys^{85} and

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Cys^{152}, was replaced by Glu and Ile-Gln-Ile amino acid residues were added at the carboxyl terminal of wild DHFR, was prepared. G-DHFR from cultured E. coli was purified by DEAE-Toyopal column chromatography and MTX bound affinity chromatography (ref. 3). G-DHFR was dissolved in a potassium phosphate buffer solution(10 mM) containing 0.2 mM EDTA and 1 or 2 mM dithiothreitol(DTT). To avoid the effect of oxidation of enzyme on the CD and DSC measurements, the dialysis was carried out against the above buffer for 24 hours with continuous bubbling of N_2 gas to the outer solution. The concentration of G-DHFR was determined photometrically at 280 nm (ref. 11).

CD and DSC measurements

Thermal transition of G-DHFR was measured with JASCO 40A and 600A spectropolarimeters at a heating rate of 0.25 K/min and a differential adiabatic scanning microcalorimeter DASM-1M (DSC) at a heating rate of 1 K/min. The acquisition of data from the calorimeter was carried out using a computer system with a pre-amplifier, AD converter (Discovery-1,Canopus), isolator and PC9801 NEC microcomputer. The concentrations of the enzyme for CD and DSC measurements were 0.1 - 2.56 and 0.86 - 0.96 mg/ml, respectively.

Deconvolution of DSC data

An attempt to interpret the transition in terms of sequential independent two-state transitions was made by Spink et al.(ref. 6) and their method was applied by Takahashi and Fukada (ref. 7) to DSC data on several proteins. Another method was offered by Freire and Biltonen (ref. 8) based on the partition function of the system calculated from the DSC data. To apply the latter method to the analysis of DSC data, Filimonov et al. (ref. 9) proposed a practical procedure for sequential deconvolution, and Kidokoro and Wada (ref. 10) improved the deconvolution method to make possible the calculation of the thermodynamic properties as functions of temperature.

According to Kidokoro and Wada (ref. 10), the key relation between mole fraction, f_i, and difference enthalpy function, $\Delta H_i(T)$, for i-th state is given by the equation,

$$d \ln f_i(T)/dT = - \Delta H_i(T)/RT^2 \qquad (1)$$

where $\Delta H_i(T)$, defined as $H(T) - H_i(T)$, can be calculated by integrating the difference heat capacity function, $\Delta C_i(T)$. The latter function is defined by $C(T) - C_i(T)$ and can be calculated directly from the DSC data. Calorimetric enthalpy function, $\Delta H^{cal}(T)$ of the transition between native(N) and denatured(D) state is

$$\Delta H^{cal}(T) = H_D(T) - H_N(T) \qquad (2)$$

If the system can be described by the two-state transition between N and D states, the van't Hoff enthalpy, $\Delta H^{vH}(T)$, is defined by

$$\Delta H^{vH}(T) = RT^2[\Delta C_N(T)/\Delta H_N(T) - \Delta C_D(T)/\Delta H_D(T)] \quad (3)$$

An obtained value of $\Delta H^{cal}/\Delta H^{vH}$ of more than 1 means that at least one intermediate state exists. As the sum of the mole fractions must be equal to one, the calculated values of f_N and f_D by the double deconvolution method using equation (1) allow estimation of the mole fraction of the intermediate state, f_I. To avoid the ambiguities of the thermodynamic parameters obtained by the double deconvolution method and to estimate their errors, an error analysis of the nonlinear least-squares method (Ref. 12) was carried out with a Facom M-780 computer system using the SALS program (Ref. 13).

RESULTS AND DISCUSSION

pH dependence

In the calorimetry measurements of the enzyme, DTT, which was added to protect the enzyme from oxidation reacted with the oxygen in the enzyme solution during the heating. The large exothermic effect caused by the oxidation reaction inhibited observation of the small endothermic effect of thermal denaturation of the enzyme. Consequently, the enzyme solution which had been dialyzed under N_2 gas was set in the calorimeter cell and N_2 gas was blown over it for 1 min before the lid of the cell was closed. The enzyme solution was then heated up to 323 K at a heating rate of 1 K/min to remove the oxygen, which was remained in the solution, by the oxidation reaction of DTT. The calorimeter data at the second heating were used for the data analysis and the reversibility of thermal transition was checked by comparing data of the second and third runs. Although the reversibility was not complete (less than 70%), data were analyzed on the assumption of thermodynamic equilibrium.

The experimental and calculated DSC curves for G-DHFR at pH 7.0 and 7.7 are shown in Figure 1. A shoulder is observed in a DSC peak at pH 7.0 and it becomes clearer at pH 7.7. The values of the ratio of van't Hoff and calorimetric enthalpy of thermal transition of G-DHFR are 2.0 for data at both pH, which indicates that the transition consists of at least three states. Table 1 shows the root mean square deviations(RMSD) of the nonlinear least-squares fitting with different models to the calorimetric data of G-DHFR. The models based on two-state transition clearly deviate from the experimental data. However, the values of RMSD for all of the three-state models are within the experimental error. We therefore adopted the 6th model with least number of parameters as the best one.

Figure 2 shows the mole fraction of native(f_N), intermediate(f_I) and denatured(f_D) states calculated with the 6th model in Table 1. The values of f_D

depend little on pH. On the other hand, the temperature and the value of maximum f_I change from 325 K to 323 K and from 0.67 to 0.73, respectively, which causes the shift of the peak in DSC curve to lower temperature with increasing pH. Table 2 lists the thermodynamic functions for the thermal transition of G-DHFR at pH 7.0 and 7.7. The transition temperature at pH 7.7 is a little lower than at pH 7.0, while the values of ΔH_{Ni} at both pH are almost the same.

At pH 6.0 the observed DSC curve had a single sharp peak at about 313 K. The oscillation was observed in the DSC curve and the transition was completely irreversible. The results suggest that aggregation of the enzyme occurred at pH 6.0.

TABLE 1

Root mean square deviations (RMSD) of the nonlinear least-squares fitting to the data of G-DHFR with different models at pH 7.0 and 7.7.

No.	Model	Number of parameters	RMSD(μW/ml) pH 7.0	pH 7.7
1	N⇌D	6	1.1	0.8
2	N⇌I⇌D	10	0.4	0.2
3	N⇌I⇌D	8	0.4	0.3
4	N⇌I⇌D	7	0.4	0.3
5	N⇌I⇌D	7	0.4	0.4
6	N⇌I⇌D	6	0.4	0.4

Number of data point are 380 - 400. The restrictions on parameters for 3rd - 6th models, are,

3. The temperature dependence of the heat capacity of all states are restrained to be equal.

In addition to the restriction of 3rd model,

4. Heat capacity difference between N and I states, ΔC_{NI}, is restrained to be fixed to zero.
5. Heat capacity difference between I and D states, ΔC_{ID}, is restrained to be fixed to zero.
6. Heat capacity differences, ΔC_{NI} and ΔC_{ND}, are restrained to be fixed to zero.

TABLE 2

Thermodynamic functions for the three-state transition of G-DHFR

Thermodynamic functions	Estimated values pH 7.0 N⇌I	N⇌D	pH 7.7 N⇌I	N⇌D
T_{Ni} (K)	318.75 ± 0.06	324.92 ± 0.07	314.9 ± 0.1	322.2 ± 0.2
ΔH_{Ni} (kJ/mol)	195.0 ± 0.9	365.9 ± 1.6	194.4 ± 1.8	346.0 ± 3.4
ΔS_{Ni} (J/K/mol)	611.7 ± 2.9	1126.2 ± 4.7	617.4 ± 5.6	1074 ± 10

i = I or D

Comparison with wild DHFR

The measuring condition for reversible thermal transition of wild DHFR by DSC has not yet been found. CD data of wild DHFR for native state and renatured state(cooled after heat treatment) were not perfectly equal. The calculated values of T_d and ΔH^{vH} from the data of CD at pH 7.0 based on two-state revers-

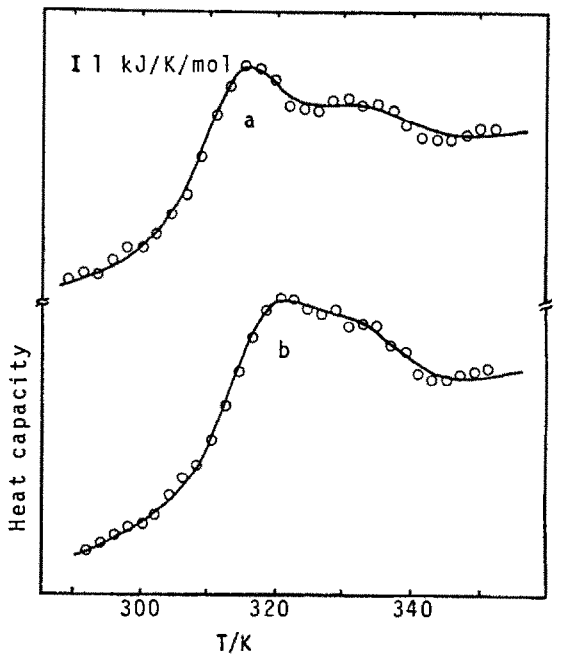

Fig. 1. Molar heat capacity of G-DHFR at pH 7.7(a) and 7.0(b). Circles denote experimental data and solid lines denote the calculated values with the parameters of the 6th model in Table 1.

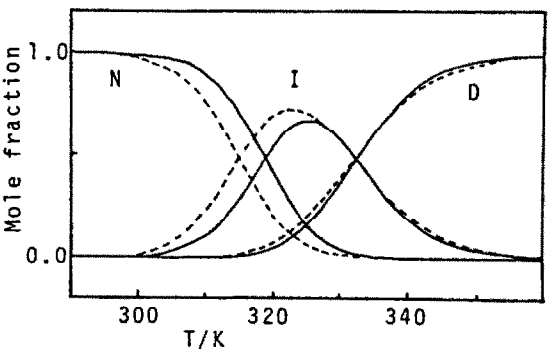

Fig. 2. Mole fraction of native(N), intermediate(I) and denatured(D) states calculated with the parameter of 6th model in Table 1. Solid lines and dotted lines correspond to the values at pH 7.0 and 7.7, respectively.

ible transition depended on the wavelength: 321-325 K at 205-260 nm, and 326-331 K at 291 nm for T_d; 155-160 kJ/mol at 205 and 220 nm and 273-360 kJ/mol at 255-291 nm for ΔH^{vH}. Results suggest that the thermal transition of wild DHFR also is not a two-state one. The agreement of the values of T_d for wild and mutated DHFR suggests that the replacement of Cys^{152} with Glu and the addition of three amino acid residues at the C terminal of DHFR has no major effect on the thermal transition temperature.

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