Large Flexibility of Dihydrofolate Reductase as Revealed by Temperature Effects on the Volume and Compressibility

Tadashi Kamiyama, Eiji Ohmae, and Kunihiko Gekko*
Department of Chemistry, Faculty of Science, Hiroshima University, Higashi-Hiroshima, Hiroshima 739-8526

(Received March 11, 1999; CL-990166)

The partial specific volume and adiabatic compressibility of dihydrofolate reductase (DHFR) from *Escherichia coli* remarkably increased as temperature was higher, corresponding to the conformational changes as revealed by spectroscopic measurements. These results demonstrate that this protein has a highly flexible structure at the native state whose tertiary structure or hydrophobic core is easily expanded with temperature to produce the internal cavities.

Protein dynamics, being a basis for understanding the structure-function relationships of enzymes, has been widely investigated by many techniques.[1] The partial specific volume and adiabatic compressibility are important physical quantities directly related to the volume fluctuation or compactness of protein molecule because they involve two contributions of surface hydration and internal cavity.[2-4] DHFR [EC 1.5.1.3] from *Escherichia coli* is a monomeric protein of 159 amino acids with no disulfide bond. It catalyzes reduction of the substrate, dihydrofolate (H_2F) to tetrahydrofolate with the aid of the coenzyme, NADPH. These bulky ligands, comprising 6.6% of DHFR in weight, should require the highly flexible conformation of DHFR for their accommodation. However, there is no direct measurement on the flexibility of DHFR, although this is expected from the temperature-sensitive circular dichroism (CD) and fluorescence spectra[5] as well as the broad thermogram in differential scanning calorimetry.[6] In this paper, we have examined the effects of temperature on the partial specific volume and adiabatic compressibility of DHFR, in order to elucidate the conformational flexibility at the native state.

DHFR was purified as described previously[5] and dialyzed against a solvent (10 mM potassium phosphate buffer, pH 7.0, containing 0.1 mM EDTA and 0.1 mM DTT) at 4 °C, followed by centrifugation at 14000 rpm for 20 min to remove aggregates. Five or six sample solutions of different protein concentrations (0.3-0.8%) were prepared by diluting the dialyzed stock solution with the dialysate.

The partial specific volume, v^o, and partial specific adiabatic compressibility of the protein, β_s, at infinite dilution were calculated with the following equations

$$v^o = \lim_{c\to 0}(1/c)[1-(d-c)/d_o] \quad (1)$$

$$\beta_S = -(1/v^o)(\partial v^o/\partial P) = (\beta_o/v^o)\lim_{c\to 0}(1/c)[(\beta/\beta_o)-(d-c)/d_o] \quad (2)$$

where P is the pressure; d and d_o the densities of the solution and solvent, respectively; β and β_o the adiabatic compressibilities of the solution and solvent, respectively; c the concentration of protein in grams per milliliter of solution. The values of β and β_o were calculated from the sound velocity, u, and the density, d, of the solution or solvent with the Laplace equation, $\beta=1/u^2d$. The sound velocity was measured with an accuracy of 1 cm/sec by means of a "sing-around pulse method" at 5.8 MHz. The density was measured with an accuracy of 10^{-6} g/cm^3 using a precision density meter, DMA-02C (Anton Paar, Gratz). Both measurements were made at 15, 20, 25, 30 and 35±0.01 °C, which were maintained with a thermobath (Neslab RTE-111). Protein concentration was photometrically determined with an extinction coefficient of 1730 cm^3/(g·cm) at 280 nm after sound velocity and density measurements. The apparatus and experimental procedures were essentially the same as those used in previous papers.[3,4,7]

There were good linear relationships between c and $(1/c)[1-(d-c)/d_o]$ or $(1/c)[(\beta/\beta_o)-(d-c)/d_o]$ at all temperatures, indicating no intermolecular interaction or aggregation. The values of v^o and β_s of DHFR at each temperature were listed in Table 1, together with the sound velocity increment per unit protein concentration, $\Delta u/c$. The root mean square volume fluctuation (δV_{rms}) calculated by statistical thermodynamics[2] was approximately 38 ml/mol at 25 °C, corresponding to 0.29% of the total protein volume. These values of v^o, β_s, and δV_{rms} are comparable with those for other globular proteins at 25 °C,[4] but a characteristic difference of DHFR consists in their large temperature dependence. As shown in Figure 1, the v^o and β_s values extensively increased with increasing temperature. From the slope of these plots, the thermal expansion coefficient, $\alpha=(1/v^o)(\partial v^o/\partial T)$, was roughly estimated to be 13 x 10^{-4} K^{-1} at 25 °C. This value is two or three-fold larger than those of other proteins.[8] This was also the case with β_s. These results suggest that the native structure of DHFR, different from other globular proteins, is highly flexible and easily expanded with temperature to a relaxed conformation.

The experimentally determined expansibility and adiabatic compressibility of a protein would mainly consist of two contributions, hydration and cavity, as follows[3,4]

$$\alpha = (1/v^o)[\partial V_{cav}/\partial T + \partial \Delta V_{sol}/\partial T] \quad (3)$$

$$\beta_S = -(1/v^o)[\partial V_{cav}/\partial P + \partial \Delta V_{sol}/\partial P] \quad (4)$$

Table 1. Partial specific volume and adiabatic compressibility of DHFR at various temperatures (1dyn=10^{-5}N)

Temperature (°C)	v^o (ml/g)	$\Delta u/c$ (m ml/g s)	β_S x 10^{12} (cm^2/dyn)
15	0.723±0.001	312±3	1.7±0.3
20	0.728±0.005	300±6	3.4±0.2
25	0.735±0.002	292±7	4.4±0.7
30	0.740±0.001	258±8	8.0±0.7
35	0.742±0.002	258±4	8.0±0.6

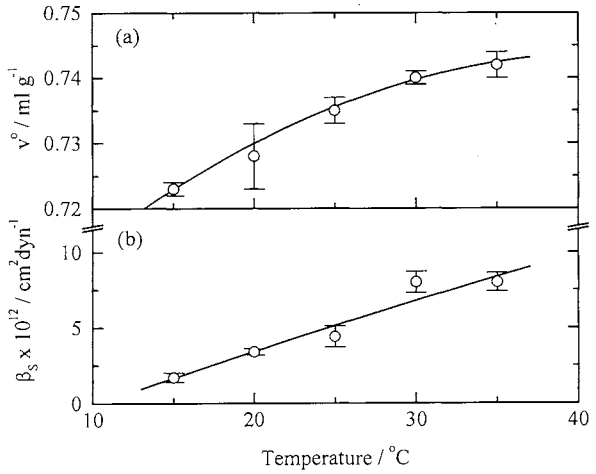

Figure 1. Temperature dependence of the partial specific volume (a) and adiabatic compressibility (b) of DHFR. Solid lines were drawn by means of least-squares quadratic regression.

where V_{cav} is the cavity volume in a protein molecule due to imperfect atomic packing and ΔV_{sol} is the volume change due to solvation or hydration. In general, the cavity and hydration terms contribute positively to α, while the cavity term contributes positively to β_s but the hydration term negatively. At present, it is difficult to estimate separately both contributions of cavity and hydration. However, the large increase in v^o and β_s with temperature would be dominantly ascribed to expansion of cavity because the dehydration effect might be partly compensated by an increase in hydration due to swelling of the structure.

A matter of concern is why the volumetric properties of DHFR are largely dependent on temperature. A possible answer for this problem may be derived by comparing v^o and β_s with the temperature dependence of the CD and fluorescence spectra.[5] Figure 2 shows plots of the molar ellipticities at 222 and 290 nm, and fluorescence intensity at 344 nm against β_s at the corresponding temperature. The observed good linear relationships confirm that the change in β_s with temperature is mainly brought about by the enhanced fluctuation of the structure. The v^o value also showed good linear correlation with $[\theta]_{222}$, $[\theta]_{290}$, and fluorescence intensity (correlation coefficient, r=0.968, -0.917, and -0.995, respectively). It is known that unusually there is an exciton pair of tryptophan-47 and tryptophan-74 in the structure of DHFR and the decreased intensity of $[\theta]_{222}$ with temperature is not due to the unfolding of the secondary structure but to the breakdown of this exciton pair.[5,9] The changes in $[\theta]_{290}$ and fluorescence intensity also reflect the modification of hydrophobic cores around tryptophan residues. Therefore, it is highly possible that some hydrophobic cores are loosened by breaking the exciton pair with temperature to increase the cavity and β_s. In this relation, it is interesting that a single amino acid substitution on the flexible loops of DHFR has influence on the compressibility, stability, and enzyme activity.[6,10] That such a small perturbation in thermal energy and

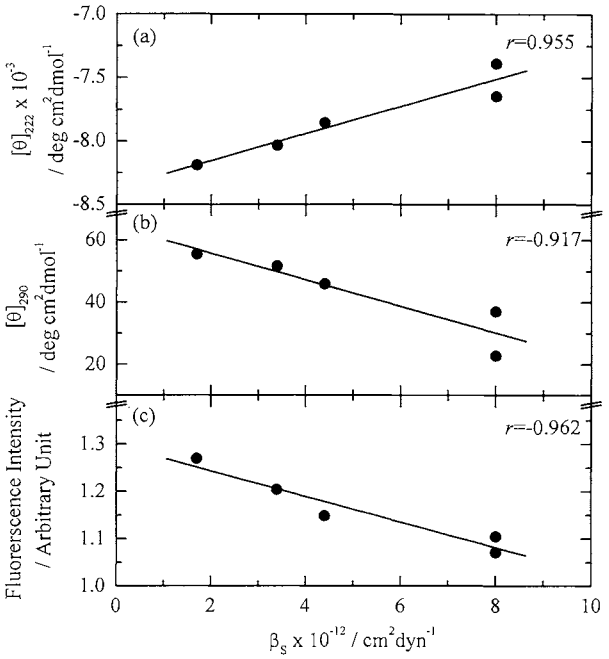

Figure 2. Plots of the molar ellipticity at 222 nm (a) and 290 nm (b), and fluorescence intensity at 344 nm (c) as a function of the adiabatic compressibility. Solid lines were drawn by means of least-squares method with all data. The correlation coefficient, r, is indicated in each figure.

local structure dramatically influences the overall dynamics of the protein molecule is an evidence that apo DHFR takes a largely fluctuating structure at the native state. This is reasonable for the function of DHFR to bind cooperatively bulky ligands, NADPH and H_2F, as appeared in the movie of Sawaya and Kraut.[11]

Reference

1 M. Karplus and J. A. McCammon, *CRC Crit. Rev. Biochem.*, **9**, 293 (1981).
2 A. Cooper, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 2740 (1976).
3 K. Gekko and H. Noguchi, *J. Phys. Chem.*, **83**, 2706 (1979).
4 K. Gekko and Y. Hasegawa, *Biochemistry*, **25**, 6563 (1986).
5 E. Ohmae, T. Kurumiya, S. Makino, and K.Gekko, *J. Biochem.*, **120**, 946 (1996).
6 K. Gekko, Y. Kunori, H. Takeuchi, S. Ichihara, and M. Kodama, *J. Biochem.*, **116**, 34 (1994).
7 K. Gekko, Y. Tamura, E. Ohmae, H. Hayashi, H. Kagamiyama, and H. Ueno, *Protein Sci.*, **5**, 542 (1996).
8 T. V. Chalikian, M. Totrov, R. Abagyan, and K. J. Breslauer, *J. Mol. Biol.*, **260**, 588 (1996).
9 K. Kuwajima, E. P. Garvey, B. E. Finn, C. R. Matthews, and S. Sugai, *Biochemistry*, **30**, 7693 (1991).
10 E. Ohmae, K. Iriyama, S. Ichihara, and K. Gekko, *J. Biochem.*, **123**, 33 (1998).
11 M. R. Sawaya and J. Kraut, *Biochemistry*, **36**, 586 (1997).