Stevens, R. L., & Ritz, J. (1985) *Nature 318*, 289–291.
Simon, M. M., Hoschützky, H., Fruth, U., Simon, H.-G., & Kramer, M. D. (1986) *EMBO J. 5*, 3267–3274.
Sitkovsky, M. V. (1988) *Immunol. Rev. 103*, 127–160.
Sitkovsky, M. V., & Paul, W. E. (1988) *Nature 332*, 306–307.
Sneath, P. H. A., & Sokal, R. R. (1973) in *Numerical Taxonomy*, Freeman, San Francisco, CA.
Trapani, J. A., Klein, J. L., White, P. C., & Dupont, B. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 6924–6928.
Tschopp, J., & Jongeneel, C. V. (1988) *Biochemistry 27*, 2641–2646.
Von Heijne, G. (1986) *Nucleic Acids Res. 14*, 4683–4690.
Young, J. D.-E., & Cohn, Z. A. (1987) *Adv. Immunol. 41*, 269–332.
Young, J. D.-E., Hengartner, H., Podack, E. R., & Cohn, Z. A. (1986a) *Cell 44*, 849–859.
Young, J. D.-E., Leong, L. G., Liu, C.-C., Damiano, A., Wall, D. A., & Cohn, Z. A. (1986b) *Cell 47*, 183–194.
Zagury, D. (1982) *Adv. Exp. Med. Biol. 146*, 149–163.
Zalman, L. S., Brothers, M. A., Chiu, F. J., & Müller-Eberhard, H. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5262–5266.

Long-Range Electrostatic Interactions Can Influence the Folding, Stability, and Cooperativity of Dihydrofolate Reductase†

Kathleen M. Perry,‡ James J. Onuffer,§ Mitchell S. Gittelman, Lawrence Barmat,‖ and C. Robert Matthews*

Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802
Received March 13, 1989; Revised Manuscript Received June 9, 1989

ABSTRACT: To test the possibility that long-range interactions might influence the folding and stability of dihydrofolate reductase, a series of single and double mutations at positions 28 and 139 were constructed and their urea-induced unfolding reactions studied by absorbance and circular dichroism spectroscopy. The α carbons of the two side chains are separated by 15 Å in the native conformation. The replacement of Leu 28 by Arg and of Glu 139 by Gln resulted in additive effects on both kinetic and equilibrium properties of the reversible unfolding transition; no evidence for interaction was obtained. In contrast, the Arg 28/Lys 139 double replacement changed the equilibrium folding model from two state to multistate and showed evidence for interaction in one of the two kinetic phases detected in both unfolding and refolding reactions. The results can be explained in terms of a long-range, repulsive electrostatic interaction between the cationic side chains at these two positions.

The complex three-dimensional structures of proteins are stabilized by a large number of noncovalent interactions such as hydrogen bonds, van der Waals interactions, and electrostatic interactions and by the hydrophobic effect (Kauzmann, 1959). Although the individual interactions are relatively weak, their propensity to act in a cooperative fashion results in the folding of newly synthesized polypeptides to unique, stable conformations. The cooperative nature of the forces that stabilize proteins raises the possibility that an amino acid at one site may interact in an energetic sense with amino acids at distant sites. Such long-range interactions play an important role in a variety of biological phenomena including allosteric regulation of enzyme function and in the cooperative binding of substrates by multisubunit enzymes.

One approach toward probing the possibility of long-range interactions in proteins is to examine the effects of amino acid replacements on the reversible unfolding transition. Specifically, a set of mutant proteins can be constructed that includes single amino acid replacements at the two sites of interest and the double mutant with simultaneous replacements at the two sites. Comparison of the sum of the effects of the single replacements on the stability and folding kinetics with the effect of the double replacement provides a test for interaction. Additivity of the free energy of folding or the activation free energies of unfolding and refolding rate constants would mean that the replacements act independently. Nonadditivity would provide direct evidence that the residues at these positions interact (Ackers & Smith, 1985).

Previous application of this approach to the α subunit of tryptophan synthase showed that the residues at positions 175 and 211 interact with each other in the native conformation (Hurle et al., 1986). Examination of the preliminary X-ray structure provides a simple explanation for this interaction in that residues 175 and 211 are adjacent in the three-dimensional structure (Hyde et al., 1988). Although similar studies on the lysozyme from phage T4 (Becktel et al., 1987) and staphylococcal nuclease (Shortle et al., 1988; Shortle & Meeker, 1986) have generally found that double replacements have an additive effect on stability, the Gly 46 → Ala/Gly → 48 Ala double mutant in the N-terminal domain of the λ repressor protein was reported to have a nonadditive effect (Hecht et al., 1986).

Because a consistent pattern for such interactions has not yet emerged, we felt that it was important to further test this

† This work was supported by the National Science Foundation, Grant DMB-8705673.
‡ Present address: Department of Biochemistry and Biophysics, University of California Medical Center, San Francisco, CA 94143.
§ Present address: Department of Biochemistry, University of California, Berkeley, CA 94720.
‖ Present address: Temple University Medical School, Philadelphia, PA 19122.

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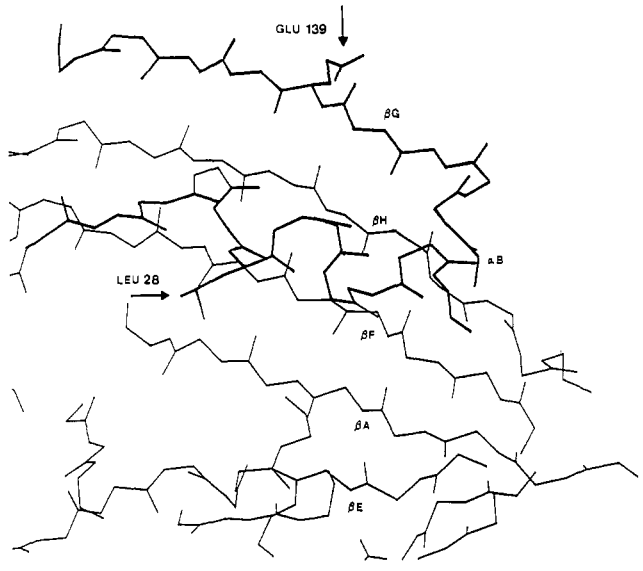

FIGURE 1: Region of *E. coli* DHFR containing strands βE, βA, βF, βH, and βG and helix αB [from Bolin et al. (1982)]. Strand βG and helix αB are shown in bold, as are Leu 28 and Glu 139 side chains; these side chains are also labeled.

approach on a protein with a high-resolution, refined X-ray structure. Accordingly, two sets of single and double mutants were constructed in dihydrofolate reductase (DHFR)[1] from *Escherichia coli*. DHFR contains 159 amino acids and is a member of the α/β class of supersecondary structures (Richardson, 1981). The highly refined X-ray structure shows that an eight-stranded β sheet forms the central hydrophobic core of the protein; four amphipathic α helices are arrayed on its surface (Bolin et al., 1982).

Positions 28 and 139 in DHFR are particularly attractive candidates for a test of their potential to interact:

(1) Leu 28 is located on the hydrophobic face of helix αB and has direct contacts with several of the residues in the central β sheet. Glu 139 is located in strand βG, the edge strand in the sheet. Although the α carbons of these two position are 15 Å apart, the β sheet could act as a conduit for long-range structural perturbations. The relative positions of residues 28 and 139 and their involvement in secondary structure are shown in Figure 1.

(2) The Glu 139 → Lys mutation (E139K) decreases the enzymatic activity of DHFR from 70 to 51 units mg^{-1} in the standard assay (Perry et al., 1987). Because position 139 is not in direct contact with any of the active-site residues, the decreased activity implies a long-range interaction between the residue at position 139 and the active site. Position 28 is at the active site, adjacent to Asp 27, which is known to play a key role in the reduction of dihydrofolate to tetrahydrofolate (Stone & Morrison, 1984; Howell et al., 1986). The replacement of Leu 28 by Arg (L28R) reduces the activity to 3 units mg^{-1} (Perry et al., 1987).

(3) Previous studies on the stability and folding of the L28R and E139K single amino acid replacements found that both replacements have measurable and distinct effects on the equilibrium and kinetic properties of folding (Perry et al., 1987). Therefore, the potential additivity of changes in the free energy of folding or the activation free energies of unfolding and refolding can be readily tested.

Equilibrium and kinetic folding studies presented in this paper demonstrate that an interaction exists when Leu 28 is replaced by Arg and Glu 139 by Lys. Long-range electrostatic forces play a significant role in this process.

Materials and Methods

Protein Source and Purification. Wild-type DHFR was isolated from *E. coli* strain MC294 containing the plasmid pTY1 whose construction from the original clone by Smith and Calvo (1980) has been described previously (Perry et al., 1987). The L28R mutation appeared spontaneously after continued exposure of cells containing this plasmid to high levels of trimethoprim (T. Yaegashi and J.-T. Chen, unpublished results). A T → G replacement at the second base in the codon corresponding to amino acid 28 accounted for the change. The plasmid containing this mutation has been designated pG93.

The E139K mutation was obtained by oligonucleotide-directed mutagenesis. A single-stranded nick was made in pTY1 at a unique *Eco*RI site in the presence of ethidium bromide. A gap in this region was made by digesting with both exo III exonuclease and DNA polymerase I. An 18-mer containing a change from G to A at the first base in the codon corresponding to residue 139 was annealed to the exposed single strand; the gaps were filled with the Klenow fragment of DNA polymerase I and closed with DNA ligase. This hybrid plasmid was used to transform strain MC294. The successful incorporation of this oligonucleotide results in the loss of the unique *Eco*RI restriction site. Therefore, the resulting plasmids were purified, digested with *Eco*RI endonuclease in the absence of ethidium bromide, and transformed again. Because of the poor transformation efficiency of linear DNA, a higher percentage of mutant plasmids lacking the *Eco*RI site survived. Approximately 60% of the colonies selected from the second transformation carried plasmids that lost the *Eco*RI site. The E139Q Gln single mutant was obtained in an identical manner with an oligonucleotide containing a G to C change coding for Gln. The L28R/E139K and L28R/E139Q double mutants were also isolated from plasmids constructed in this fashion from pG93 described above. DNA sequencing confirmed the desired base changes.

All proteins were purified according to the methods of Baccanari et al. (1975, 1977). Purity was demonstrated by the observation of single bands on both $NaDodSO_4$ and native gels. Protein concentration was measured by the absorbance at 280 nm with a molar extinction coefficient of 3.11×10^4 M^{-1} cm^{-1} (D. Baccanari, personal communication). Enzymatic activity was monitored as described previously (Hillcoat et al., 1967).

Spectroscopic Methods. Equilibrium unfolding as a function of urea was monitored by UV difference spectroscopy, where the change in tryptophan absorbance was measured at 293 nm on a Cary 118 spectrophotometer, and by circular dichroism spectroscopy by following the loss of secondary structure at 222 nm on a Jasco J-20 spectropolarimeter. All samples were allowed to fully equilibrate at the appropriate final urea concentration at 15 °C before spectra were taken. Equilibrium experiments were performed in 10 mM potassium

[1] Abbreviations: DHFR, dihydrofolate reductase; $NaDodSO_4$, sodium dodecyl sulfate; K_2EDTA, ethylenediaminetetraacetic acid dipotassium salt; CD, circular dichroism; L28R, the replacement of Leu 28 by Arg; E139Q, the replacement of Glu 139 by Gln; E139K, the replacement of Glu 139 by Lys; L28R/E139Q, the simultaneous replacement of Leu 28 by Arg and of Glu 139 by Gln; L28R/E139K, the simultaneous replacement of Leu 28 by Arg and of Glu 139 by Lys.

phosphate, 0.2 mM K_2EDTA, and 1 mM 2-mercaptoethanol at pH 7.8. The effect of high salt on protein stability was tested by using this phosphate buffer with the addition of 1 M NaCl. The above phosphate buffer was also employed for the CD experiments, however the K_2EDTA was deleted. Final protein concentrations ranged from 0.15 to 0.6 mg/mL.

Kinetics of folding were followed by UV difference spectroscopy using a manual mixed method that has a dead time of 10–15 s. A Cary 118 spectrophotometer was interfaced to a PDP 11/23 computer to facilitate kinetic measurements. Kinetic experiments were performed in the phosphate buffer described above at pH 7.8, 15 °C.

Data Fitting. Equilibrium unfolding data were fit to a two-state model involving a single native and unfolded species and assuming a linear dependence of the free energy of folding on the urea concentration (Schellman, 1978)

$$\Delta G_{app} = \Delta G_{app}^{H_2O} + A[\text{urea}]$$

where $\Delta G_{app}^{H_2O}$ is the apparent Gibbs free energy change between the native and unfolded species in the absence of denaturant, ΔG_{app} is the apparent change in Gibbs free energy between the native, N, and unfolded, U, states, which varies as a function of final urea concentration, and A is a parameter that reflects the cooperativity of the transition. The midpoint of the transition, Cm, occurs when $\Delta G_{app} = 0$ and can be calculated by $Cm = -\Delta G_{app}^{H_2O}/A$.

Nonlinear least-squares fits to kinetic data were obtained by using the Statistical Analysis Systems program NLIN (SAS Institute Inc., Cary, NC) and the equation

$$A(t) = \sum A_i \exp(-t/\tau_i) + A_\infty$$

where $A(t)$ is the total amplitude at time t, A_∞ is the amplitude at infinite time, A_i is the amplitude corresponding to the individual phase, i, at zero time, and τ_i is the associated relaxation time.

Results

The effects of amino acid replacements at positions 28 and 139 on the folding of DHFR were monitored by both equilibrium and kinetic techniques. Equilibrium studies provide a quantitative assessment of the changes in stability and kinetic studies a means of pinpointing when these changes occur during the folding reaction. The equilibrium studies of the reversible urea-induced unfolding reaction were performed with both difference UV and circular dichroism spectroscopies. The difference UV spectrum at 292 nm reflects changes in the exposure of tryptophanyl residues to solvent during unfolding and, thereby, monitors the tertiary structure. The molar ellipticity at 222 nm principally reflects the helical content of DHFR and, thereby, monitors the secondary structure. Comparison of the transition curves obtained from these two techniques provides a means of determining the appropriate equilibrium model. Kinetic studies of folding were performed with difference UV spectroscopy.

As noted above, the choice of the 28–139 pair of residues was dictated by several factors, including the availability of the Arg 28 and Lys 139 single replacements from a prior study. Because we anticipated that long-range electrostatic forces might play a significant role for these particular mutations, a second set of single and double mutants was constructed. The L28R mutant was also combined with the replacement of glutamic acid at position 139 with glutamine (E139Q). Glutamine is similar in size to glutamic acid and is capable of hydrogen bonding. Relevant to the issue of electrostatic interactions, the absence of a formal charge on glutamine provides an opportunity to test the role of an anionic (Glu), a neutral (Gln), and a cationic (Lys) side chain at position 139.

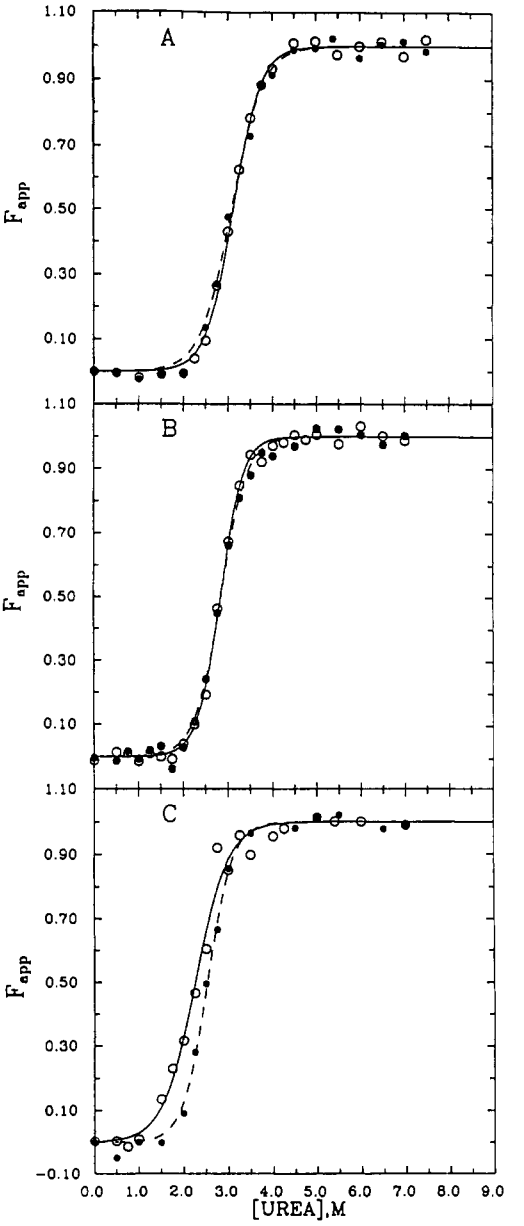

FIGURE 2: (A) Dependence of the apparent fraction of unfolded wild-type protein, F_{app}, on the urea concentration at pH 7.8, 15 °C, as monitored by difference UV (○–○) and CD (●–●) spectroscopies. (B) Dependence of F_{app} for the L28R/E139Q protein on the urea concentration at pH 7.8, 15 °C. (C) Dependence of F_{app} for the L28R/E139K protein on the urea concentration at pH 7.8, 15 °C. The symbols for plots B and C have the same meaning as for plot A.

Equilibrium Studies. Plots of apparent fraction of unfolded protein, F_{app}, as a function of urea concentration for the wild-type DHFR and the L28R/E139K and L28R/E139Q double replacements are shown in Figure 2. As has been previously reported (Touchette et al., 1986), the transition curves obtained from difference UV and circular dichroism spectroscopic studies on the wild-type protein are coincident within experimental error (Figure 2A). These and other data led to the conclusion that the urea-induced unfolding of wild-type DHFR at equilibrium follows a two-state model, N ↔ U, where N and U are the native and unfolded states, respectively.

Similar comparative plots for the L28R, E139K, and E139Q single mutations (data not shown) and the L28R/E139Q

Table I: Thermodynamic Parameters for the Urea-Induced Unfolding Reaction of Wild-Type and Mutant Dihydrofolate Reductase at pH 7.8, 15 °C

protein	$\Delta G_{app}^{H_2O}$ (kcal M^{-1})	A [kcal M^{-1} (M urea)$^{-1}$]	Cm[a] (M urea)	$\Delta\Delta G$[b] (kcal M^{-1})
wild-type DHFR	5.92 ± 0.34	1.90 ± 0.11	3.12 ± 0.02	
L28R	7.64 ± 0.68	2.29 ± 0.20	3.34 ± 0.02	+0.50 ± 0.09
E139K	4.94 ± 1.03	2.34 ± 0.48	2.11 ± 0.06	−2.36 ± 0.51
E139Q	5.50 ± 0.43	2.20 ± 0.17	2.50 ± 0.02	−1.36 ± 0.12
L28R/E139K	nd[c]	nd	2.26 ± 0.07 2.54 ± 0.03[d]	nd
L28R/E139Q	6.38 ± 0.56	2.26 ± 0.20	2.82 ± 0.02	−0.68 ± 0.08

[a] The urea concentration at which $F_{app} = 0.50$, as detected by difference UV spectroscopy. [b] Difference in free energies between the mutant and wild-type DHFR at 3.12 M urea, pH 7.8, 15 °C. $\Delta\Delta G = \Delta G_{mut}$(3.12 M urea) − ΔG_{wt}(3.12 M urea), where ΔG_{wt}(3.12 M urea) = 0. [c] Not determined. [d] The urea concentration at which $F_{app} = 0.50$, as detected by circular dichroism spectroscopy.

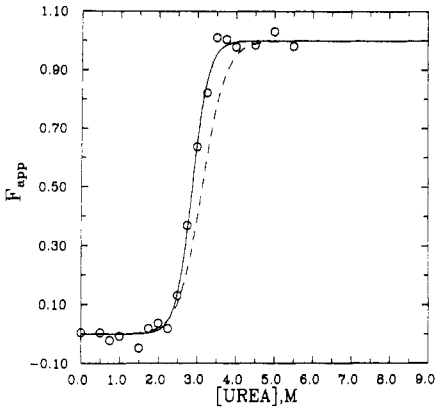

FIGURE 3: Effect of 0.5 M KI on the dependence of F_{app} for the L28R/E139K protein on the urea concentration at pH 7.8, 15 °C, as monitored by difference UV (O) spectroscopy. The curve for wild-type DHFR in the same solvent is shown as a dashed line for reference.

double mutations (Figure 2B) show that the difference UV and circular dichroism transition curves are also coincident within experimental error. Therefore, these proteins, like wild-type DHFR, follow a two-state unfolding model. In striking contrast, the transition curves for the L28R/E139K double mutant are not coincident (Figure 2C). The difference UV transition precedes that for circular dichroism, showing that the tertiary structure is dissipated before the secondary structure. The presence of Arg at position 28 and Lys at 139 results in a breakdown in the two-state folding model and indicates the presence of one or more stable folding intermediates.

For the proteins that obeyed a two-state folding model, i.e., all except the L28R/E139K double mutant, thermodynamic parameters were calculated from the difference UV data by assuming a linear dependence of the free energy change for unfolding on the urea concentration (Schellman, 1978); these parameters are shown in Table I.

The values for $\Delta G_{app}^{H_2O}$ vary from 4.94 kcal mol^{-1} for the E139K mutant to 7.64 kcal mol^{-1} for the L28R mutant (Table I, column 2). With the exception of the L28R mutant, the changes in stability relative to wild-type DHFR are comparable to the estimated errors. Therefore, it is impossible to draw any conclusions regarding interactions on the basis of these data. An alternative method of evaluating the effects of amino acid replacements on stability is to compare the values of ΔG calculated at the urea concentration that corresponds to the midpoint of the unfolding transition for the wild-type protein. This latter procedure avoids the extrapolations required to estimate ΔG in the absence of denaturant and appears to be a more precise way to compare the stabilities of modified proteins (Cupo & Pace, 1983). The calculation is explained in more detail elsewhere (Beasty et al., 1986).

The relative free energy differences, $\Delta\Delta G$, for the three single mutants and the L28R/E139Q double mutant are also shown in Table I, column 5. By use of this method of comparison, at 3.12 M urea the replacement of Leu 28 by Arg increases the stability by 0.50 kcal mol^{-1}, while the replacement of Glu 139 by Gln and Lys decreases the stability by 1.36 and 2.36 kcal mol^{-1}, respectively. The potential for interaction between the L28R and E139Q replacements can be tested by comparing the sum of the individual effects on stability with the effect of the simultaneous replacement at both sites. The observed effect for the double mutant, a *decrease* in stability of 0.68 kcal mol^{-1}, is very close to the value calculated from the sum of the two single mutants, 0.50 − 1.36 = −0.86 kcal mol^{-1}. Therefore, these replacements have independent effects on stability and do not interact.

The multistate unfolding behavior of the L28R/E139K double-mutant protein prohibits a quantitative analysis of the potential interaction between Arg 28 and Lys 139. However, the observation that the wild-type protein and the L28R and the E139K single mutations all obey a two-state model while the L28R/E139K double-mutant protein obeys a multistate model provides convincing qualitative evidence that the presence of Arg at position 28 and of Lys at 139 leads to interaction.

To test the possibility that this long-range interaction has an electrostatic basis, the unfolding of the L28R/E139K double mutant was repeated in the presence of salt. Approximately half of the space between positions 28 and 139 is occupied by solvent, providing an opportunity to vary the screening between the charged side chains. As can be seen in Figure 3, the unfolding transition monitored by difference UV spectroscopy in the presence of 0.5 M KI has a midpoint quite close to that for the wild-type protein (2.88 versus 3.13 M urea) and a slope which is even greater [2.79 versus 1.94 kcal/(mol·mol) (urea)]. The increase in midpoint and slope are consistent with the recovery of a two-state folding model.

A direct comparison of difference UV and CD spectroscopies cannot be made in the presence of iodide because iodide absorbs strongly in the far UV. When such a comparison is made in the presence of 1 M NaCl, the unfolding transition curves are coincident within experimental error (data not shown). The midpoints of the difference UV and CD detected transition curves are 4.64 ± 0.05 and 4.73 ± 0.08 M urea, respectively. The complication with the use of NaCl to alter the ionic strength is that the stability of DHFR increases dramatically; the midpoint of the unfolding transition for wild-type protein in the presence of 1 M NaCl is 5.25 M urea (P. Jennings and C. R. Matthews, unpublished results). This effect appears to be due to chloride ion binding to both native and intermediate forms and is currently under investigation.

Kinetic Studies. Kinetic studies of the single and double replacements at positions 28 and 139 were performed to substantiate the equilibrium results, to pinpoint when the interaction occurs during the folding reaction, and to provide

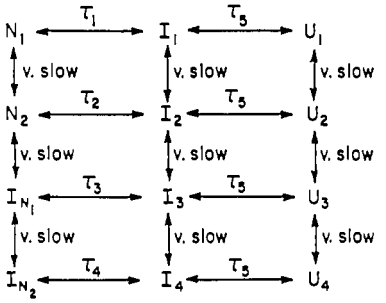

FIGURE 4: Proposed kinetic folding model for dihydrofolate reductase. Definitions of species and relaxation times are provided in the text.

a quantitative estimate of the interaction energy.

Previous studies (Touchette et al., 1986) have led to the proposal of a kinetic model for folding, involving a series of native, intermediate, and unfolded forms (Figure 4). N_1 and N_2 are native conformers that are both present at equilibrium; N_1 and N_2 comprise 15% and 85% of the population, respectively. $^1N_1$ and $^1N_2$ are native-like intermediates in that they are not populated at equilibrium; however, they retain the capacity to bind methotrexate, an inhibitor of DHFR that binds at the active site. I_1–I_4 and U_1–U_4 are a series of transient intermediate and unfolded forms, respectively. Only two relaxation times are detected in unfolding, τ_1 and τ_2, while five are detected in refolding, τ_1–τ_5. The tentative assignment of these phases to specific steps in the model are shown in Figure 4. The vertical steps are proposed to reflect isomerization processes that are slow compared to the folding steps. The protein effectively unfolds and refolds in parallel channels.

For the purposes of this study, we were principally interested in monitoring the effects of mutations on the τ_1 and τ_2 folding channels. The approach that we have developed to analyze the effects of mutations requires a knowledge of the perturbations on both the unfolding and refolding relaxation times (Beasty et al., 1986). Both relaxation times are available only for the τ_1 and τ_2 channels.

The dependence of the τ_1 and τ_2 relaxation times on the final urea concentration for the E139Q single replacement at pH 7.8, 15 °C, is shown in Figure 5A; the results for wild-type DHFR are shown as a reference. The replacement of Glu 139 by Gln has a selective effect on the relaxation time for unfolding for both the τ_1 and τ_2 phases. At 6 M urea, the relaxation time for the τ_1 phase decreases from 420 to 143 s and that for the τ_2 phase from 95 to 27 s. In contrast, the refolding relaxation times for the mutant are within experimental error of those for wild-type DHFR. These results show that the E139Q replacement selectively increases the free energy of the native conformations, N_1 and N_2, relative to the intermediates, I_1 and I_2, and to the transition states linking these N and I species. This behavior has been classified as that characteristic of an equilibrium mutant (Beasty et al., 1986). Note that a selective increase in the free energy of the native conformers in the E139Q mutant is consistent with the decrease in stability observed in the equilibrium studies (Table I).

The similar effect of the E139Q replacement on the two folding channels contrasts with that previously observed for the E139K replacement [Figure 7B in Perry et al. (1987)]. In the latter case, the relaxation times for both unfolding and refolding in the τ_1 channel are decreased, behavior characteristic of a kinetic mutant[2] (Beasty et al., 1986). However, similar to the effect observed with Gln at 139 (Figure 5A), the E139K replacement selectively decreases the relaxation time for unfolding in the τ_2 channel. The different effects of the E139K replacement on the two folding channels must reflect differences in the interactions of the lysyl side chain with its environment in the different native and intermediate conformations.

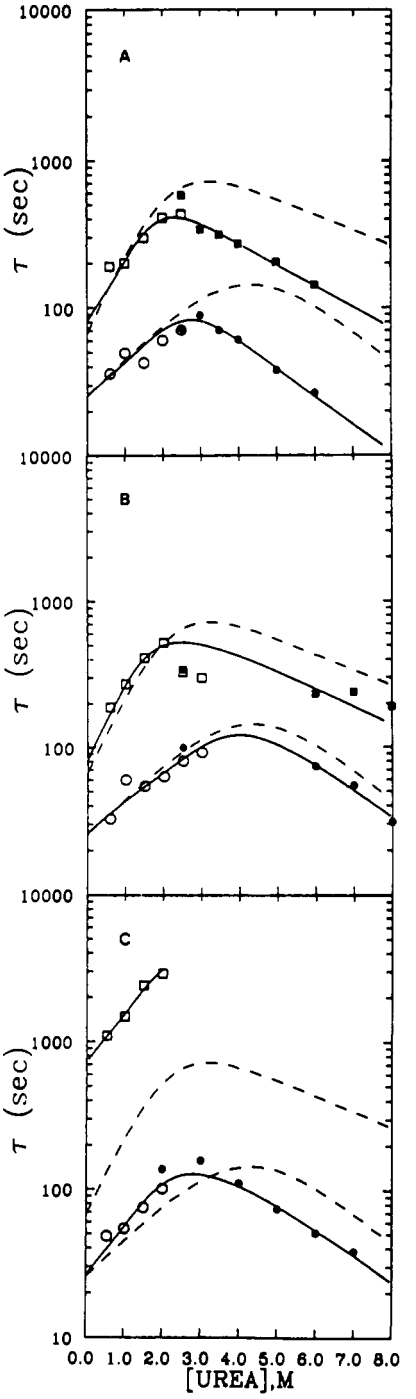

FIGURE 5: (A) Urea dependence of the unfolding (solid symbols) and refolding (open symbols) relaxation times for the τ_1 (■, □) and τ_2 (●, ○) reactions for the E139Q protein at pH 7.8, 15 °C. (B) Urea dependence of the unfolding (solid symbols) and refolding (open symbols) relaxation times for the τ_1 (■, □) and τ_2 (●, ○) reactions for the L28R/E139Q protein at pH 7.8, 15 °C. (C) Urea dependence of the unfolding (solid symbols) and refolding (open symbols) relaxation times for the τ_1 (■, □) and τ_2 (●, ○) reactions for the L28R/E139K protein at pH 7.8, 15 °C. In all cases, the values for the wild-type protein are shown as dashed lines for reference. Lines drawn through the data are to aid the reader.

[2] In a kinetic mutant, the replacement would have a selective effect on the free energy of the transition state linking N and I, relative to the free energies of N and I.

Table II: Activation Free Energies for Unfolding[a] and Refolding[b] of Wild-Type and Mutant Dihydrofolate Reductases

protein	phase	activation free energies[c] (kcal mol^{-1})		relative activation free energies[d] (kcal mol^{-1})			
		$\Delta G^{\ddagger}_{refold}$	$\Delta G^{\ddagger}_{unfold}$	$\Delta\Delta G^{\ddagger obs}_{refold}$	$\Delta\Delta G^{\ddagger cal}_{refold}$	$\Delta\Delta G^{\ddagger obs}_{unfold}$	$\Delta\Delta G^{\ddagger cal}_{unfold}$
wild type	τ_1	19.91	20.31	0		0	
	τ_2	19.15	19.46	0		0	
L28R	τ_1	19.94	20.80	0.03		0.49	
	τ_2	18.95	19.96	−0.20		0.50	
E139Q	τ_1	19.88	19.69	−0.03		−0.62	
	τ_2	19.09	18.74	−0.06		−0.72	
E139K	τ_1	19.59[e] (est)	19.35	−0.32		−0.96	
	τ_2	19.10	18.40[f] (est)	−0.05		−1.06	
L28R/E139Q	τ_1	20.06	19.97	0.15	0.00	−0.34	−0.13
	τ_2	19.19	19.31	0.04	−0.26	−0.15	−0.22
L28R/E139K	τ_1	21.02		1.11	−0.29	n.o.	−0.47
	τ_2	19.13	19.10	−0.02	−0.25	−0.36	−0.56

[a] Unfolding reactions were initiated at 0 M urea and ended at 6.0 M urea, pH 7.8, 15 °C. [b] Refolding reactions were initiated at 5.4 M urea and ended at 1.0 M urea, pH 7.8, 15 °C. [c] The estimated errors in the activation free energies are ±0.20 kcal mol^{-1}. By propagation of errors analysis, the errors in the relative activation free energies are 0.28 kcal mol^{-1}. [d] Observed relative activation energies, $\Delta\Delta G^{\ddagger obs}$, determined by $\Delta\Delta G^{\ddagger obs} = \Delta G^{\ddagger obs}$(mutant) − $\Delta G^{\ddagger obs}$(wild type). Calculated relative activation energies, $\Delta\Delta G^{\ddagger cal}$, for the double mutants were determined from the sum of the observed relative activation energies of each of the single mutants, $\Delta\Delta G^{\ddagger cal} = \Delta\Delta G^{\ddagger obs}$(mutant 1) + $\Delta\Delta G^{\ddagger obs}$(mutant 2). [e] Estimated by linear extrapolation of ln τ_{refold} versus the urea concentration to 1.0 M urea [from Figure 7B in Perry et al. (1987)]. [f] Estimated by linear extrapolation of ln τ_{unfold} versus the urea concentration to 6.0 M urea [from Figure 7B in Perry et al. (1987)].

Previous studies on the L28R replacement observed a selective increase in the relaxation time for unfolding in both the τ_1 and τ_2 phases [Figure 3B in Perry et al. (1987)]. The decrease in the free energies of the two native conformations relative to those for the transition states and the intermediates is also reflected in the increased stability of the L28R mutant (Table I).

Comparison of the unfolding and refolding relaxation times of the L28R/E139Q double mutant with those for wild-type DHFR (Figure 5B) shows that only the unfolding relaxation time for the τ_1 channel is altered; the other values are within experimental error of those for wild-type protein.

For the L28R/E139K double mutant (Figure 5C), the dependence of the τ_2 relaxation time on the urea concentration is very similar to that of wild-type protein; there is a small acceleration in the unfolding reaction. However, the τ_1 relaxation time differs from that of wild-type DHFR in two significant respects: (1) the amplitude for the unfolding reaction has decreased to the point of being undetectable; (2) the refolding relaxation time has increased by a factor of 7 at 1 M urea.

To examine the potential role of long-range electrostatic interactions on the folding kinetics of the L28R/E139K double mutant, unfolding and refolding were repeated in the presence of 0.5 M KI. In unfolding, the τ_1 phase is recovered and the relaxation time at 5.0 M urea (405 s) is within experimental error of that of wild-type DHFR in the presence of salt (414 s); the τ_2 phase is unaffected. When the final concentration of urea for refolding was 0.54 M urea, the relaxation time of the τ_1 phase decreases from 1092 to 247 s, very close to the value for the wild-type DHFR in the presence of salt (300 s). The τ_2 phase in refolding is unaffected by salt. Thus, the dramatic changes in the τ_1 unfolding/refolding channel in the L28R/E139K double mutant are eliminated in the presence of 0.5 M KI.

To test for potential interactions by use of the kinetic data, the relaxation times were first converted to rate constants and then to activation free energies via the Eyring formalism. Under strongly unfolding conditions it can be shown that the observed relaxation time for a first-order process is inversely proportional to the unfolding rate constant, $k_{unfolding} = \tau^{-1}$, and under strongly refolding conditions, $k_{refolding} = \tau^{-1}$ (Matthews, 1987). The activation free energy, $\Delta G^{\ddagger}$, is then computed from

$$\Delta G^{\ddagger} = -RT \ln (kh/k_B T)$$

where k is the rate constant, T is the absolute temperature, and R, h, and k_B are the gas, Planck, and Boltzmann constants, respectively.

The activation free energies for both the τ_1 and τ_2 phases in unfolding, $\Delta G^{\ddagger}_{unfold}$, to 6 M urea and refolding, $\Delta G^{\ddagger}_{refold}$, to 1 M urea for the single and double mutants under discussion are shown in Table II, columns 3 and 4. The values range from 18.40 to 21.02 kcal mol^{-1}. The activation free energies relative to wild-type DHFR, $\Delta\Delta G^{\ddagger}$, reflect the changes caused by the replacements and are shown in Table II (columns 5 and 7). The additivity can be tested by comparing the sum of the $\Delta\Delta G^{\ddagger}$ values observed for each of the single mutants ($\Delta\Delta G^{\ddagger cal}$, columns 6 and 8) with the value of $\Delta\Delta G^{\ddagger}$ observed for the double mutants.

For the L28R/E139Q double mutant, the close agreement between the observed and calculated relative activation free energies for both unfolding and refolding in both channels shows that these replacements independently influence the relaxation times for unfolding and refolding. There is no evidence for interaction. In contrast, the effects on the refolding relaxation times in the τ_1 channel for the L28R/E139K double mutant are distinctly nonadditive: $\Delta\Delta G^{\ddagger cal}_{refold}$ is −0.29 kcal mol^{-1}, while $\Delta\Delta G^{\ddagger obs}_{refold}$ is +1.11 kcal mol^{-1}. The interaction energy, $\Delta\Delta G_I$, which can be defined to be the difference between the observed and calculated values (Hurle et al., 1986), is 1.40 kcal mol^{-1} for the τ_1 phase in refolding. The effects of these two replacements on the τ_2 folding channel are additive, emphasizing once again the differences between the two folding channels.

Discussion

The noncoincidence in the equilibrium unfolding transitions and the nonadditivity in the kinetic studies of folding of the L28R/E139K double-mutant protein very likely reflect a long-range electrostatic interaction between the two cationic side chains at positions 28 and 139 in DHFR. Three lines of evidence support this conclusion: (1) The increase in midpoint and slope of the difference UV transition curve in the presence of 0.5 M KI. (2) The coincidence of the UV and CD curves in 1 M NaCl. (3) The restoration of the properties of the τ_1 phase in unfolding and refolding kinetics in the presence of 0.5 M KI.

The NaCl results must be viewed with some caution because of the significant increase in stability of DHFR. Preferential binding of the salt to the native conformation, relative to the

putative intermediate in the double mutant, could decrease the relative population of the intermediate and lead to a two-state model for folding. Pace and Grimsley (1988) have recently reported that ribonuclease τ_1 is stabilized by cation and anion binding to the native conformation. A detailed study of the effect of salt on the equilibrium and kinetic properties of folding of DHFR is in progress.

Inspection of the kinetic data for this L28R/E139K double mutant shows a striking difference from the wild-type DHFR: the τ_1 phase in unfolding is absent. The loss of this phase implies that the corresponding N_1 conformer is destabilized, relative to N_2, and that only the N_2 conformer is populated under equilibrium conditions. If electrostatic repulsion is responsible for the selective destabilization of N_1 in the L28R/E139K double mutant, either the Lys 139 side chain is closer to Arg 28 in this conformer or the dielectric constant of the intervening protein matrix is lower.

It is not known which if either of the N_1 or N_2 conformers corresponds to the X-ray structure of *E. coli* DHFR that was cocrystallized with methotrexate as a binary complex. Therefore, it is not possible to draw any quantitative conclusions relating the observed interaction energy for the τ_1 channel in the L28R/E139K double mutant and the spatial arrangement of the side chains. A semiquantitative estimate of the separation distance between the cationic side chains of Arg 28 and Lys 139 in the N_1 conformation can be obtained from the observed interaction energy and Coulomb's law

$$W = N_0 Z_A Z_B e^2 / 4\pi\epsilon_0 \epsilon d$$

where W is the work required to bring a mole each, N_0, of charged species Z_A and Z_B from infinite separation to distance d in a medium of dielectric constant ϵ; e is the unit charge, 1.60×10^{-19} C; and ϵ_0 is the permittivity of vacuum, 8.85×10^{-12} C^2 N^{-1} m^{-2}. If the interaction energy, $\Delta\Delta G_I$, is principally due to electrostatic repulsion between Arg 28 and Lys 139, then $\Delta\Delta G_I = W = 1.40$ kcal mol^{-1}.

The estimate of distance obviously depends upon the choice of a dielectric constant. Fersht and his colleagues (Russell et al., 1987) have found in a study of subtilisin that the effective dielectric constant for two charges separated by proteinaceous material is 51, very similar to the value for charges separated by aqueous medium, 44. These values apply to ionic strengths in the range from 0.005 to 0.025 M, comparable to that used in the present experiment, 10 mM potassium phosphate. Because the X-ray structure shows that the intervening space between positions 28 and 139 in DHFR is approximately equally divided between water and protein, a value of 48 for the effective dielectric constant seems appropriate. This value results in a predicted distance of 5 Å. Using a value of 27, reported by Rogers et al. (1985) in their study on cytochrome c_{551}, predicts the distance to be 8.8 Å.

Inspection of the X-ray structure shows that if Leu is replaced by Arg at position 28 and Glu by Lys at position 139, the shortest possible distance between the two cationic groups with the backbone unaltered is about 9 Å. Thus, it appears possible that, with some rearrangement of the backbone and/or side chains at positions 28 and 139 in the N_1 conformer, the two positive charges at these positions can approach the estimated distance.

A qualitative argument also supports a long-range, i.e., not nearest-neighbor, interaction. Methotrexate has been observed to bind to both N_1 and N_2 (Touchette et al., 1986), showing that the active sites in both conformers are accessible to this inhibitor. Given that Leu 28 is at the active site, this result means that N_1 has *not* rearranged to such an extent that positions 28 and 139 are adjacent in the three-dimensional structure. Therefore, the presumed electrostatic interactions cannot be from adjacent side chains.

It should also be noted that the presence of the τ_1 phase in the refolding of the L28R/E139K double mutant demonstrates that these replacements do not alter the distribution of the putative unfolded conformers that give rise to the multiple folding channels. Rather, the absence of the τ_1 phase in unfolding shows that the effect of the interaction is clearly on one of the native conformations, N_1.

Although these results support the hypothesis of a significant role for a repulsive electrostatic interaction between Arg 28 and Lys 139 in the N_1 conformer, the strict additivity observed for the L28R/E139Q system would appear to be contradictory. That is, the electrostatic attraction between Arg 28 and Glu 139 would also be expected to give rise to nonadditivity in the L28R/E139Q system. A possible explanation for the absence of an observable interaction in this system resides in the involvement of Glu 139 in a salt bridge with His 141 in the native conformation (Bolin et al., 1982). At a distance of 15 Å, the attraction and repulsion forces of this pair of ions for Arg 28 would nearly cancel. When Glu is replaced by Gln, His 141 will presumably deprotonate and assume a neutral charge at pH 7.8. The Gln/His pair of neutral side chains would obviously not be capable of long-range electrostatic interactions with Arg 28. Thus, additivity, as measured by changes in stability or kinetics of folding in this system of single and double mutants, would be expected.

Arguing along a similar line, the replacement of Glu 139 by Lys would result in the replacement of the Glu 139–His 141 ion pair with a cationic Lys 139 and a neutral His 141 at pH 7.8. This change in net charge of the pair of side chains could be responsible, in part, for the observed interaction in L28R/E139K. The fact that the interaction only appears in the τ_1 channel clearly shows that other factors, such as the placement of the Lys 139 side chain, are also important.

The likelihood that the interaction between Arg 28 and Lys 139 is due to electrostatics and not to a concerted structural change which is propagated through the noncovalent network of bonds that stabilize the native conformation is supported by the additivity of the L28R and E139Q replacements. Both single mutants have measurably altered equilibrium and kinetic properties of folding; however, these effects are not transmitted between the two sites.

Another significant conclusion to draw from these data is that amino acid replacements can be used to break down the cooperativity of the protein folding reaction and populate stable intermediates. The lower midpoint for the difference UV transition compared to that for the CD transition in the L28R/E139K double mutant shows that the tertiary structure is disrupted before the secondary structure. This observation is similar to those for other multistate unfolding transitions (Wong & Tanford, 1973; Robson & Pain, 1976) and is consistent with the framework model for folding (Kim & Baldwin, 1982). This conclusion is also supported by the results of Meeker and Shortle (1986) on a triple mutant in staphylococcal nuclease. Noncoincident fluorescence and CD transition curves for the V66L/G79S/G88V mutant protein show that this process must also involve intermediates, unlike the wild-type protein. Therefore, it appears that there may be some generality in the use of amino acid replacements to break down the cooperativity of the folding reaction and, perhaps, the identification of elementary steps in complex folding reactions.

ACKNOWLEDGMENTS

We thank Patricia A. Jennings for a critical review of the manuscript and for providing the wild-type DHFR equilibrium

and kinetic results in the presence of various salts. We also thank Gail Feldman for typing the manuscript. We also deeply appreciate the assistance of George Rose in estimating the separation distance of side chains in dihydrofolate reductase.

Registry No. DHFR, 9002-03-3; Leu, 61-90-5; Glu, 56-86-0; Arg, 74-79-3; Gln, 56-85-9; Lys, 56-87-1.

REFERENCES

Ackers, G. K., & Smith, F. R. (1985) *Annu. Rev. Biochem. 54*, 597.
Amdur, I., & Hammes, G. G. (1966) *Chemical Kinetics*, McGraw-Hill, New York.
Baccanari, D., Phillips, A., Smith, S., Sinski, D., & Burchall, J. (1975) *Biochemistry 14*, 5267.
Baccanari, D., Averett, D., Briggs, C., & Burchall, J. (1977) *Biochemistry 16*, 3566.
Beasty, A. M., Hurle, M. R., Manz, J. T., Stackhouse, T., Onuffer, J. J., & Matthews, C. R. (1986) *Biochemistry 25*, 2965.
Becktel, W. J., Schellman, J. A., & Baase, W. A. (1987) *Biophys. J. 51*, 11a.
Bolin, J. T., Filman, D. J., Matthews, D. A., Hamlin, R. C., & Kraut, J. (1982) *J. Biol. Chem. 257*, 13650.
Cupo, J. F., & Pace, C. N. (1983) *Biochemistry 22*, 2654.
Hecht, M. H., Sturtevant, J. M., & Sauer, R. T. (1986) *Proteins: Struct., Funct., Genet. 1*, 43.
Hillcoat, B. L., Nixon, D. F., & Blakely, R. L. (1967) *Anal. Biochem. 21*, 178.
Howell, E. E., Villafranca, J. E., Warren, M. S., Oatley, S. J., & Kraut, J. (1986) *Science (Washington, D.C.) 231*, 1123.
Hurle, M. R., Tweedy, N. B., & Matthews, C. R. (1986) *Biochemistry 25*, 6356.
Hyde, C. C., Ahmed, S. A., Padlem, E. A., Miles, E. W., & Davies, D. R. (1988) *J. Biol. Chem. 263*, 17857.
Kauzmann, W. (1959) *Adv. Protein Chem. 14*, 1.
Kim, P. S., & Baldwin, R. L. (1982) *Annu. Rev. Biochem. 51*, 459.
Matthews, C. R. (1987) *Methods Enzymol. 154*, 498.
Pace, C. N., & Grimsley, G. R. (1988) *Biochemistry 27*, 3242.
Perry, K. M., Onuffer, J. J., Touchette, N. A., Herndon, C. S., Gittelman, M. S., Matthews, C. R., Chen, J. T., Mayer, R. J., Taira, K., Benkovic, S. J., Howell, E. E., & Kraut, J. (1987) *Biochemistry 26*, 2674.
Richardson, J. S. (1981) *Adv. Protein Chem. 34*, 168.
Robson, B., & Pain, R. H. (1976) *Biochem. J. 155*, 331.
Rogers, N. K., Moore, G. R., & Sternberg, M. J. E. (1985) *J. Mol. Biol. 182*, 613.
Russell, A. J., Thomas, P. G., & Fersht, A. R. (1987) *J. Mol. Biol. 193*, 803.
Schellman, J. A. (1978) *Biopolymers 17*, 1305.
Shortle, D., & Meeker, A. K. (1986) *Proteins: Struct., Funct., Genet. 1*, 81.
Shortle, D., Meeker, A. K., & Frieire, E. (1988) *Biochemistry 27*, 4761.
Smith, D. R., & Calvo, J. M. (1980) *Nucleic Acids Res. 8*, 2255.
Statistical Analysis Systems Institute Inc. (1985) *SAS User's Guide: Statistics*, Version 5 ed., p 575, Cary, NC.
Stone, S. R., & Morrison, J. F. (1984) *Biochemistry 23*, 2753.
Touchette, N. A., Perry, K. M., & Matthews, C. R. (1986) *Biochemistry 25*, 5445.
Wong, K.-P., & Tanford, C. (1973) *J. Biol. Chem. 248*, 8518.

Vanadium K-Edge X-ray Absorption Spectroscopy of Bromoperoxidase from *Ascophyllum nodosum*†

J. M. Arber,‡,§ E. de Boer,‖ C. D. Garner,‡ S. S. Hasnain,*,§ and R. Wever‖

Department of Chemistry, University of Manchester, Manchester M13 9PL, U.K., Daresbury Laboratory, Warrington WA4 4AD, U.K., and Laboratory of Biochemistry, University of Amsterdam, 1000 HD Amsterdam, The Netherlands

Received August 26, 1988; Revised Manuscript Received June 2, 1989

ABSTRACT: Bromoperoxidase from *Ascophyllum nodusum* was the first vanadium-containing enzyme to be isolated. X-ray absorption spectra have now been collected in order to investigate the coordination of vanadium in the native, native plus bromide, native plus hydrogen peroxide, and dithionite-reduced forms of the enzyme. The edge and X-ray absorption near-edge structures show that, in the four samples studied, it is only on reduction of the native enzyme that the metal site is substantially altered. In addition, these data are consistent with the presence of vanadium(IV) in the reduced enzyme and vanadium(V) in the other samples. Extended X-ray absorption fine structure data confirm that there are structural changes at the metal site on reduction of the native enzyme, notably a lengthening of the average inner-shell distance, and the presence of terminal oxygen together with histidine and oxygen-donating residues.

Bromoperoxidase from the marine brown alga *Ascophyllum nodosum* was recently shown to be a vanadium-containing enzyme (de Boer et al., 1986a), in contrast to the more commonly recognized hemoproteins which are found in most haloperoxidases (Morrison & Schonbaum, 1976). This was the first demonstration of the existence of an enzyme with vanadium at the active site. Subsequently, vanadium-containing bromperoxidases have been isolated from other marine algal species (de Boer et al., 1986b) and vanadium nitrogenases identified (Hales et al., 1986; Robson et al., 1986). Steady-state kinetic studies on vanadium-containing bromoperoxidases have suggested the existence of a bromoperoxidase–bromide inhibitory complex and a bromoperoxidase–hydrogen peroxide intermediate (de Boer et al., submitted for publication). The

† R.W. and E.d.B. gratefully acknowledge the support of DSM NV, Geleen, The Netherlands. Their research is supported by the Netherlands Foundation for Chemical Research (SON) and was made possible by financial support from the Netherlands Technology Foundation and NWO (68-39). C.D.G. and S.S.H. are grateful to the U.K. Science and Engineering Research Council for the funding of their research and providing support to J.M.A. We also acknowledge the Director of Daresbury Laboratory for the provision of facilities.

‡ University of Manchester.
§ Daresbury Laboratory.
‖ University of Amsterdam.

0006-2960/89/0428-7968$01.50/0