Circular Dichroism Studies of Barnase and Its Mutants: Characterization of the Contribution of Aromatic Side Chains

Stéphane Vuilleumier,† Javier Sancho,‡ Ron Loewenthal,§ and Alan R. Fersht*

Cambridge Centre for Protein Engineering, Hills Road, Cambridge CB2 2QH, England, U.K.

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ABSTRACT: The circular dichroism spectrum of barnase has been analyzed by examining the spectra of a series of mutants in which every single aromatic residue has been replaced. The spectrum of wild-type barnase is quite atypical for a protein of the $\alpha + \beta$ class, with very low intensities and a minimum in the far-UV at 231 nm. The minimum at 231 nm is associated with the presence of Trp-94. Many other mutations involving aromatic residues have an effect on the spectral features in the far-UV. The major features in the near-UV spectra arise from essentially additive contributions of the three tryptophan residues Trp-35, Trp-71, and Trp-94. Tyrosine contributions are less prominent, with Tyr-78 and Tyr-97 contributing the most to the CD spectrum. The close charge–aromatic interaction between Trp-94 and His-18, which is important for the fluorescence properties of the protein, contributes little to the CD spectrum, as does the close aromatic–aromatic interaction between Tyr-13 and Tyr-17. However, the observed near-UV spectrum of wild-type barnase could not be simulated by the sum of the contributions of aromatic residues defined by difference spectra of protein variants carrying aromatic residues. Aromatic residues play an important role in determining the circular dichroism spectrum of proteins not only in the near-UV but also in the far-UV region.

Barnase has proved to be a successful model for the study of interactions involved in the stability and the folding of proteins (Kellis *et al.*, 1988; Fersht *et al.*, 1991; Horovitz & Fersht, 1992; Loewenthal *et al.*, 1992; Matouschek *et al.*, 1992; Serrano *et al.*, 1992a,b). Its fluorescence properties have been used to monitor protein stability and folding kinetics and have been characterized in detail. The fluorescence spectrum of wild-type barnase is dominated by the contribution from Trp-35 (Loewenthal *et al.*, 1991). Interaction of His-18 with Trp-94 causes a pH-dependent quenching of its fluorescence (Loewenthal *et al.*, 1992). In addition, energy transfer occurs between Trp-71 and Trp-94, as determined by multifrequency phase fluorometry (Willaert *et al.*, 1992).

Circular dichroism (CD)[1] spectroscopy is an important method complementary to fluorescence spectroscopy in studies of protein stability and folding (Kuwajima *et al.*, 1991; Sugawara *et al.*, 1991; Chaffotte *et al.*, 1992; Elove *et al.*, 1992; Kiefhaber *et al.*, 1992; Radford *et al.*, 1992). CD spectra of proteins are usually quite complex, as they represent the sum of positive and negative sign contributions of backbone peptide groups in different conformations, aromatic side chains, and coupling of electronic transitions arising from interactions involving aromatic residues, peptide bonds, and side-chain amide and charged groups in an asymmetric environment (Sears & Beychok, 1973; Strickland, 1974; Johnson, 1985; Woody, 1985).

Many attempts have been made at resolving and understanding individual contributions of amino acids and the backbone conformation to the circular dichroism spectra of proteins, through the comparison of protein spectra, selective chemical modification, model derivatives of aromatic amino acids and peptides (Sears & Beychok, 1973; Strickland, 1974; Woody, 1985), and theoretical calculations of the electronic transitions involved (Strickland, 1972, 1974; Sears & Beychok, 1973; Woody, 1978, 1985; Kahn, 1979; Manning *et al.*, 1988; Manning & Woody, 1989).

The application of protein engineering methods provides new possibilities for defining the contribution of specific residues to the circular dichroism spectra of a protein (Craig *et al.*, 1989; Kanaya *et al.*, 1991; Shire *et al.*, 1991; Muraki *et al.*, 1992). We report here on the circular dichroism features of wild-type barnase and of mutants involving amino acid replacements in every single aromatic residue of the enzyme.

MATERIALS AND METHODS

Materials. Urea (ultrapure enzyme grade) was obtained from Bethesda Research Laboratories, (Bethesda, MD). SP-Trisacryl was from IBF (Villeneuve-La-Garenne, France). Radiochemicals were obtained from Amersham International. 2-(*N*-Morpholino)ethanesulfonic acid (MES) and all other reagents were of analytical grade and were purchased from Sigma.

Mutagenesis. Previously described barnase mutants used in this study are W35F, W71Y, W94F, W94L, H18G (Loewenthal *et al.*, 1991); H18G/W94L (Loewenthal *et al.*, 1992); Y13F, Y17F, Y13F/Y17F (Serrano *et al.*, 1991); F7L (Kellis *et al.*, 1988); Y78F (Matouschek *et al.*, 1989); H102A (Mossakowska *et al.*, 1989), Y24F, Y103F (Serrano *et al.*, 1992a); and W35F/W71Y, W35F/W94F, W71Y/W94F (Loewenthal, 1992). The other mutants (W71F, Y90F, Y97F, F56M, F82L, T105M) were obtained by oligonucleotide-

* Author to whom correspondence should be addressed.
† Swiss National Research Foundation Postdoctoral Fellow, Grant 823A-031993. Present address: Institut für Mikrobiologie, ETH Zürich, Switzerland.
‡ EEC Postdoctoral Fellow. Present address: Dept. Bioquímica y Biología Celular, Fac. Ciencias, Universidàd de Zaragoza, 50009 Zaragoza, Spain.
§ Present address: Division of Pediatrics, Chaim Sheba Medical Centre, Tel-Hashomer 52621, Israel.
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[1] Abbreviations: CD, circular dichroism; CNBr, cyanogen bromide; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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directed site-specific mutagenesis as described previously (Serrano *et al.*, 1990) and checked by direct sequencing of the entire barnase gene.

Expression and purification of mutants was achieved as described previously (Serrano *et al.*, 1990), except in the cases of the W71F and W35F/W71Y mutants, where the propensity of these mutants to stick to the FPLC cation-exchange column used for purification required the addition of urea (7 M) in the buffers used for chromatography. Expression yields of the mutants were between 1 and 10 mg/L of culture. Cyanogen bromide cleavage of the T105M mutant to yield the 1–105 fragment of barnase lacking the sequence Thr-106 to Arg-110 was performed as described previously (Sancho & Fersht, 1992). The purified proteins were dialyzed against water and flash-frozen in aliquots for storage.

Extinction coefficients were determined according to the method of Gill and von Hippel (1989). The values for wild-type (ϵ_{280} = 27 411), W35F (ϵ_{280} = 21 547), W71Y (ϵ_{280} = 23 339), W94F (ϵ_{280} = 22 481), and W94L (ϵ_{280} = 22 277) have been determined previously (Loewenthal *et al.*, 1991). The value of ϵ_{280} = 26 000 ± 300 obtained for both Y78F and Y90F mutants was used for all other Tyr→Phe mutants. His and Phe residues were assumed to make negligible contributions to the extinction coefficient. The value for W71F (ϵ_{280} = 22 100) was estimated from the average extinction coefficient of 5310 per Trp residue observed in single Trp mutants. Estimates for Trp double mutants W35F/W71Y (ϵ_{280} = 17 500), W35F/W94F (ϵ_{280} = 16 620), and W71Y/W94F (ϵ_{280} = 18 400) were obtained from the values determined for the single mutants.

Circular Dichroism Spectroscopy. CD spectra were recorded on a Jasco J720 spectropolarimeter fitted with a thermostated cell holder and interfaced with a Neslab RTE-110 water bath. The instrument was calibrated with (+)-10-camphorsulfonic acid, assuming ϵ_{285} = 34.5 L/mol·cm (Johnson, 1985), $\Delta\epsilon_{290.5}$ = 2.36 L/mol·cm, and $\Delta\epsilon_{192.5}$ = –4.9 L/mol·cm (Toumadje *et al.*, 1992). Cell pathlengths of 0.02, 0.05, 0.1, 0.5, and 1 cm were used. The proteins were dialyzed twice against the buffer used in the experiment prior to measurement. The combined absorbance of sample, cell, and solvent was kept below 1 over the measured range. Spectra were usually recorded at 25 ± 0.5 °C in 5 mM MES buffer, pH 5.8. Spectra at high pH were recorded in 5 mM glycine buffer, pH 9.0. Spectra were acquired at a scan speed of 20 nm/min with a 1-nm slit and 1-s response time, averaging 12 scans, and corrected by subtraction of the solvent spectrum obtained under identical conditions. Units of $\Delta\epsilon$ are L/mol·cm per backbone amide in the far-UV and L/mol·cm per mole of protein in the near-UV. Spectra were measured several times for each protein using different preparations. The spectrum of wild-type protein was measured in each series of measurements as a control. The curves resulting from the subtraction of mutant from wild-type CD spectra represent the contribution of the deleted residue to the CD spectrum and are termed difference CD spectra. They are denoted with Δ preceding the name of the mutant; for example, ΔW94F represents the contribution of Trp-94 as defined by the difference between the spectra of the wild-type protein and the W94F mutant.

Equilibrium urea denaturation was done in 50 mM MES, pH 6.3 at 25 °C, the standard conditions used in urea denaturation experiments followed by fluorescence detection, and analyzed in terms of a two-state transition as described previously (Serrano *et al.*, 1992a). The equilibrium constant

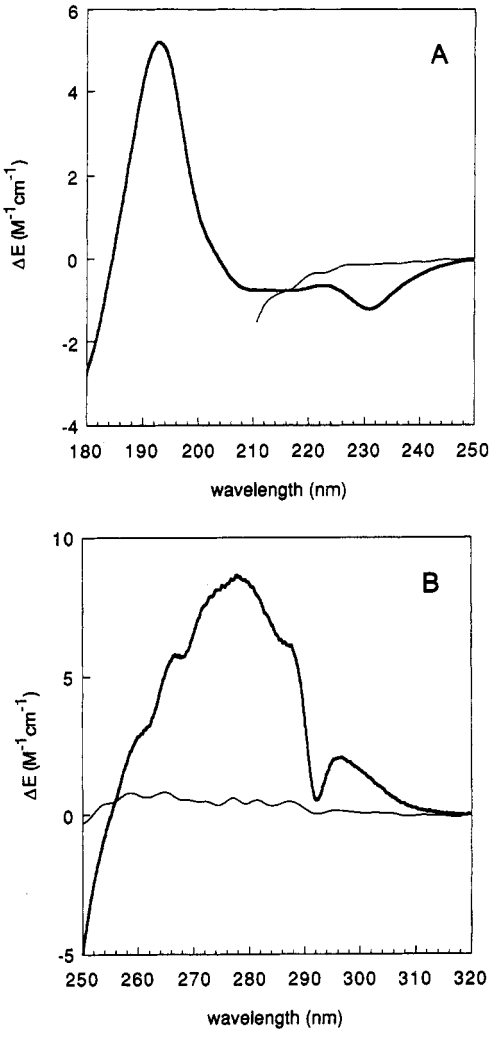

FIGURE 1: CD Spectra of wild-type barnase in 50 mM MES buffer, pH 6.3 (bold line), and 8 M urea in the same buffer (thin line) in the far-UV (A) and the near-UV (B).

K_{F-U} for denaturation at a given urea concentration is defined by the equation

$$K_{U-F} = (I_F - I)/(I - I_U) \quad (1)$$

where I_F is the intensity of the CD signal of the native state, I_U the intensity of the denatured state, and I the intensity at the given urea concentration. The free energy of denaturation is linearly related to the denaturant concentration for many proteins (Pace, 1986), so that

$$\Delta G_{U-F} = \Delta G_{U-F}{}^{H_2O} - m[\text{urea}] \quad (2)$$

In particular, $U_{1/2}$, the urea concentration at the midpoint of transition (ΔG_{U-F} = 0), is given by the following relation:

$$[\text{urea}]_{1/2} = \Delta G_{U-F}{}^{H_2O}/m \quad (3)$$

Using the relation $\Delta G_{U-F}{}^{H_2O} = -RT \ln K_{U-F}$, rearranging eqs 1–3, and assuming that the circular dichroism signal of the folded and unfolded states are dependent upon denaturant concentration in a linear fashion (Santoro & Bolen, 1988) gives the following relation:

$$I = \{I_{F,0}(1 + m_F)\} - \{I_{F,0}(1 + m_F) - I_{U,0}(1 + m_U)\} \exp\{m([\text{urea}] - [\text{urea}]_{1/2})/RT\}/\{1 + \exp(m([\text{urea}] - [\text{urea}]_{1/2})/RT\}\} \quad (4)$$

where $I_{F,0}$ and $I_{U,0}$ are the CD intensitties of the folded state

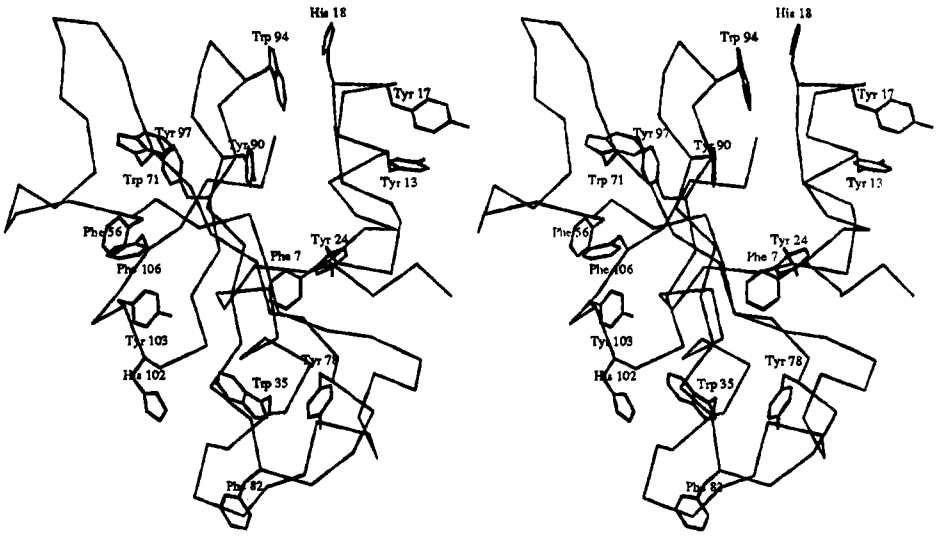

FIGURE 2: Stereoview of the C_α-backbone trace of barnase (pH 6 structure, refined to 2.1 Å; Cameron, 1992) showing the side chains of aromatic residues.

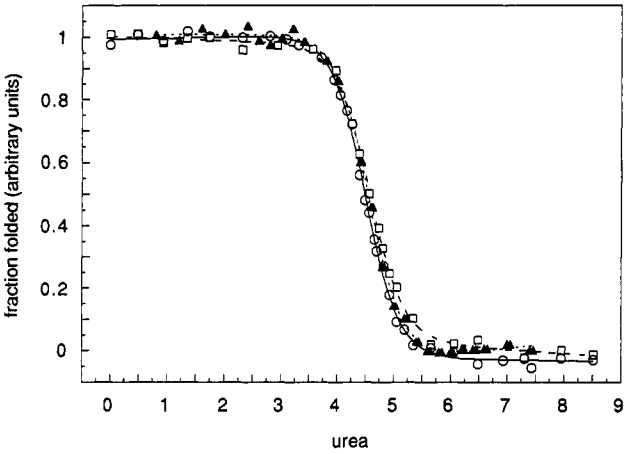

FIGURE 3: Urea denaturation of wild-type barnase in 50 mM MES buffer, pH 6.3, monitored by circular dichroism at 230 (□) and 278 nm (○) and by fluorescence at 315 nm (excitation at 290 nm, ▲). The curves of best fit obtained in the nonlinear least-square analysis are shown.

and the unfolded state in buffer and m_F and m_U are the slopes for the linear dependence with urea of the CD signal of the folded state and the unfolded state, respectively. The CD data were fitted directly to eq 4 by nonlinear least-squares analysis with Kaleidagraph (Abelbeck Software). This yields best-fit values for $U_{1/2}$, m, and hence $\Delta G_{U-F}{}^{H_2O}$.

Secondary Structure Prediction from CD Spectra. The programs Contin (Provencher & Glöckner, 1981), the convex constraint analysis method of Fasman and co-workers (Perczel *et al.*, 1992), and the variable selection method of Johnson and co-workers (Toumadje *et al.*, 1992) were kindly made available by their authors and used according to the instructions provided.

RESULTS AND DISCUSSION

The far-UV circular dichroism spectrum of barnase, a protein of the $\alpha + \beta$ class, does not resemble combinations of spectra of secondary structure elements. It has low overall spectral intensities and a minimum at 231 nm (Figure 1). In the near-UV, the complex spectrum reflects the high proportion of aromatic residues in the protein, which contains three tryptophan (Trp-35, Trp-71, and Trp-94), seven tyrosine (Tyr-13, Tyr-17, Tyr-24, Tyr-78, Tyr-90, Tyr-97, andTyr-103), four phenylalanine (Phe-7, Phe-56, Phe-82, and Phe-106), and two histidine residues (His-18 and His-102) (see Figure 2). The urea-denatured protein has spectral features typical of an unordered polypeptide backbone in the far-UV and of the lack of asymmetric environment in aromatic residues in the near-UV (Figure 1).

Table I: Equilibrium Urea Denaturation of Wild-Type Barnase by Circular Dichroism

wavelength (nm)	$[\text{urea}]_{1/2}$ (M)[a]	m[a]	$\Delta G_{U-F}{}^{H_2O}$ (kcal mol^{-1})[a,b]
230	4.58(0.02)	1.96(0.17)	9.29(0.78)
260	4.52(0.02)	2.00(0.10)	9.13(0.46)
266	4.51(0.01)	2.10(0.07)	9.11(0.32)
278	4.51(0.02)	1.99(0.08)	9.11(0.36)
286	4.52(0.02)	2.07(0.09)	9.27(0.41)
fluorescence[c]	4.58(0.01)	1.92(0.03)	8.79(0.14)

[a] The standard error obtained from the nonlinear least-square fit is given in parentheses. [b] Value obtained using eq 3, using $\langle m \rangle = 2.02 \pm 0.05$, the average of m values obtained at the different wavelengths listed in this table. [c] Average value of six experiments using different preparations of protein.

Equilibrium denaturation by urea is a cooperative process as measured by CD (Figure 3), confirming the results first obtained by absorbance (Kellis *et al.*, 1988) and fluorescence spectroscopy (Kellis *et al.*, 1989). A clear two-state transition occurs, within error, uniformly at all wavelengths, at a concentration of urea identical, within experimental error, to that determined by fluorescence (Table I). Both probes give the same transition upon urea denaturation (Table I, Figure 3), confirming that the global unfolding of the protein is being monitored by fluorescence and circular dichroism spectroscopy.

Contributions from aromatic residues and disulfide bonds have been shown to account for unusual features in far-UV spectra (Sears & Beychok, 1973; Strickland, 1974; Woody, 1978, 1975; Brahms & Brahms, 1980; Khan *et al.*, 1989; Manning & Woody, 1989; Kuwajima *et al.*, 1991; Arnold *et al.*, 1992; Radford *et al.*, 1992). Barnase has no sulfur-containing amino acids, and so a contribution of disulfides can be excluded. We investigated the contribution of aromatic residues to the CD spectra of barnase, using mutants of all aromatic residues of barnase obtained by site-directed mutagenesis. A number of barnase variants with modifications at positions occupied by aromatic residues had already been obtained to characterize residues involved in enzymatic activity

Table II: Close Contacts between Aromatic Residues in Wild-Type Barnase[a]

interaction type	residue 1	residue 2	distance (Å)[b]	angle (deg)[b]
Trp → His	His-18	Trp-94	4.0	52.3
Trp→Tyr	Trp-71	Tyr-97	6.4	81.1
	Tyr-90	Trp-94	7.5	9.1
Trp→Phe	Phe-56	Trp-71	7.2	94.5
Tyr→Tyr	Tyr-13	Tyr-17	4.1	32.0
Tyr→His	His-102	Tyr-103	5.6	95.0
Tyr→Phe	Phe-56	Tyr-103	5.7	12.8
	Tyr-103	Phe-106	7.3	44.5
	Tyr-97	Phe-106	7.4	74.7
Phe→Phe	Phe-56	Phe-106	6.2	56.9

[a] pH 6.0 structure (C-chain refined to 2.1 Å (Cameron, 1992). [b] Calculated with a program written by Kim Henrick. Distances are between the centroids of the side chains. Angles are between the planes defined by the aromatic rings. A cutoff distance of 8 Å was chosen.

of barnase (Mossakowska *et al.*, 1989), to study residues contributing to the fluorescence of the protein and its pH dependence (Loewenthal *et al.*, 1991), and to characterize the contributions to protein stability and folding of charge–aromatic interactions (Loewenthal *et al.*, 1992), aromatic–aromatic interactions (Serrano *et al.*, 1991), and interactions in the hydrophobic core of the protein (Kellis *et al.*, 1988; Matouschek *et al.*, 1989, 1992; Serrano *et al.*, 1992a,b). Additional mutants were made specifically for this study to investigate the contribution of each individual aromatic residue in barnase to its circular dichroism properties.

Studies with model compounds of aromatic amino acids have revealed that Trp, Tyr, and Phe residues show circular dichroism bands both in the near- and the far-UV [Shiraki, 1969, as reported by Sears and Beychok (1973); Strickland, 1974; Brahms & Brahms, 1980; Woody, 1985]. Changes in the CD spectra of mutant proteins compared with the wild-type protein may, therefore, originate from an altered content in amino acid residues rather than from alterations in the conformation of the polypeptide backbone. Bands arising from Trp, Tyr, and Phe residues in the near-UV are of different intensities and cover different spectral regions, which overlap only partially. In contrast, contributions from the different aromatic amino acids overlap considerably in the far-UV and have comparable intensities. Therefore, based on spectra of model amino acid derivatives, replacement of Trp with Phe residues is expected to reveal essentially the net contributions of Trp residues in the near-UV, while the situation may be less clear-cut in the far-UV region. Similarly, replacement of Tyr by Phe is expected to reveal the Tyr contribution, even if the spectral regions of these residues show some overlap in the near UV, because of the considerable difference in the characteristics of their contributions (Strickland, 1974). In any event, a difference between wild-type and mutant spectra would indicate if and how the mutated residue contributes to the CD spectrum. No changes were observed so far in the far-UV CD spectra of several variants of the protein with point mutations in charged, hydrophobic, or hydrophilic residues; changes in the near-UV were observed so far only when slight disruptions in the tertiary structure of the protein were expected (not shown). We assume that the structurally conservative replacements of Trp and Tyr by Phe and the replacement of Phe by Met or Leu represent the best compromise between maximal retention of physical properties of the side chain and maximal change in circular dichroism upon mutation.

Many interactions involving aromatic residues have the potential to contribute to the CD features of a protein, including coupling between electronic transitions of aromatic residues, aromatic–aromatic interactions, and interactions between π-orbitals of aromatic rings and π-orbitals from other types of functional groups, such as side-chain amides and carboxylate groups, guanidino groups, and main-chain peptide bonds (Holzwarth, 1972; Strickland, 1974). These interactions will depend on the intrinsic dipole moments and rotational strengths of the electronic transitions of the functional groups involved, as well as on the distance and orientation between interacting groups. The closest interactions between aromatic side chains in barnase are reported in Table II, and a summary of some characteristics of aromatic residues in barnase and of their interactions with neighboring residues are given in Table III.

The spectra of the three barnase mutants W35F, W71F, and W94F where one specific Trp residue has been replaced by Phe are shown in Figure 4. Aromatic residues make contributions to the CD spectrum of barnase over essentially the entire spectral range. Removal of Trp-94 is clearly associated with the disappearance of the most distinctive band

Table III: Conformation, Solvent Exposure, and Neighboring Groups of Aromatic Residues in Wild-Type Barnase[a]

			side-chain contacts					
residue	conf[b]	solvent expos[b] (Å²)	mc[c] <8 Å[d]	sc[c] <8 Å[d]	polar <8 Å[d]	polar <6 Å[e]	arom <8 Å[d]	arom <6 Å[e]
Phe-7	helix	27	14	11	5	N77, D86		
Tyr-13	helix	37	13	8	3		1	Y17
Tyr-17	helix	119	9	6	2		1	Y13
His-18	helix	94	11	7	2		3	W94
Tyr-24	extended	19	19	13	2	N23	2	
Trp-35		18	14	12	3	N41, D86	2	Y78
Phe-56		32	11	12	5	N58, E73	3	W71, Y103, F106
Trp-71	extended	8	13	12	5	R69	3	F56, Y97
Tyr-78		81	15	12	5		2	
Phe-82	bend	142	7	5	2		1	
Tyr-90	extended	20	14	12	3	R72	2	W94
Trp-94		80	11	8	4		2	H18
Tyr-97	bend	35	13	11	2		3	W71
His-102	turn	98	7	6	5	R87	1	Y103
Tyr-103	turn	43	11	8	5	E73, R87	2	F56, H102
Phe-106		36	12	14	7		3	F56, Y103

[a] pH 6.0 structure (C-chain), refined to 2.1 Å (Cameron, 1992). [b] Conformation and side-chain solvent exposure according to the DSSP program (Kabsch & Sander, 1983). [c] mc, sc: number of main-chain and side-chain contacts, respectively, from the centroid of the side-chain aromatic ring. [d] Number of contacts from the centroid of the side-chain aromatic ring to polar or aromatic side chains. [e] Close interactions between aromatic side chains and other polar or aromatic side chains involving bonds with π-orbitals.

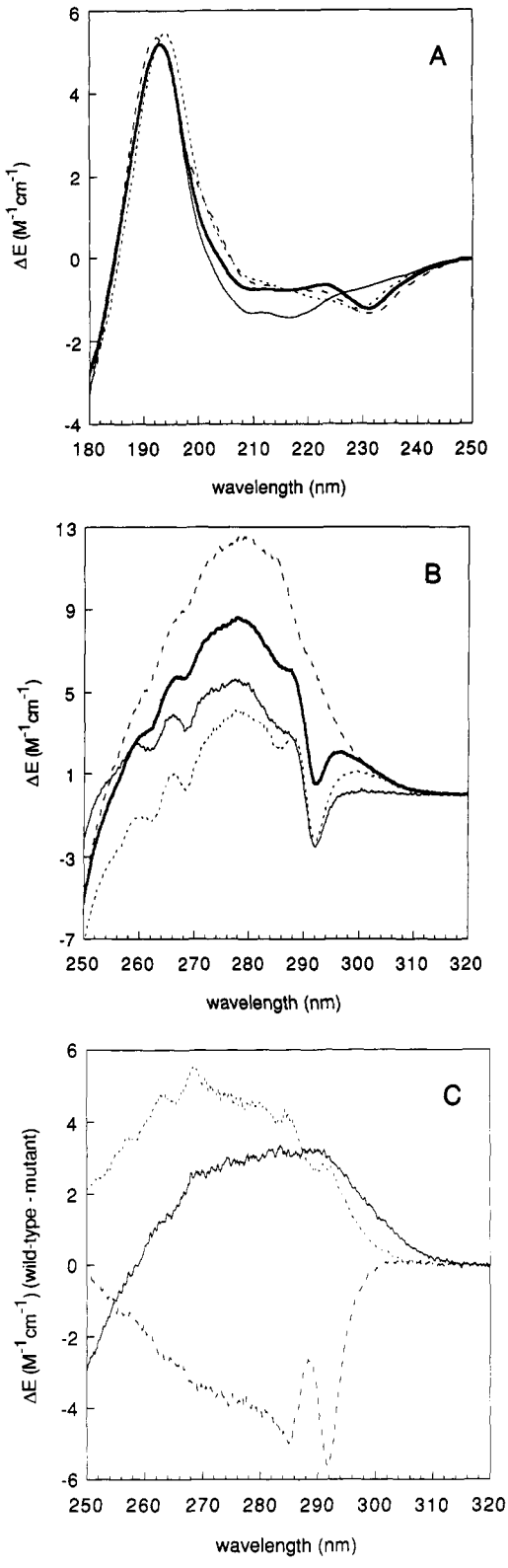

FIGURE 4: CD spectra of mutants W35F (dotted line), W94F (solid line), and W71F (dashed line) in the far-UV (A) and the near-UV-(B). Wild-type CD spectra are shown for comparison (bold line). (C) Difference spectra (wild-type – Trp→Phe mutant: ΔW35F, ΔW71F, ΔW94F) in the near-UV. Conventions as in A and B.

in the spectrum at 231 nm. Hence, whereas Trp-35 is the main contributor to fluorescence of barnase (Loewenthal *et al.*, 1991), Trp-94 is a major determinant of the far-UV CD of the protein. Interestingly, the contribution of Trp-94 revealed by subtraction of the mutant CD spectrum from the wild-type curve (Figure 4C) is of negative sign, whereas

Table IV: Positions and Intensities of Apparent Maxima from Aromatic Contributions to the CD Spectra of Wild-Type Barnase Detected by Difference CD Spectra[a]

residue	replacement	far-UV transitions, ΔE_{max} (λ_{max})	near-UV transitions, ΔE_{max} (λ_{max})
Trp-35	Phe	0.6 (186), –1.0 (197), 0.4 (224)	5.4 (269),[b] 2.7 (291)
Trp-71	Phe	–0.5 (189), –0.7 (202)	–4.9 (285), –5.5 (292)
Trp-94	Phe	0.6 (210–217),[c] –0.5 (231)	3.3 (290)[c]
Tyr-13	Phe	–0.4 (197)	–0.7 (270)
Tyr-17	Phe	–0.4 (190/198)	–0.7 (270)
Tyr-24	Phe	–1.6 (194), –0.3 (232)	–0.3 (264), 0.6 (281/287), –0.3 (297)
Tyr-78	Phe	–0.5 (188)	1.0 (262), 1.3 (269), 1.4 (275), 1.6 (285)
Tyr-90	Phe	–1.3 (194), 0.7 (221)	–0.8 (278)
Tyr-97	Phe	0.4 (187), –0.5 (198), –0.3 (221)	–2.4 (268/275), –1.9 (284), –1.1 (291)
Tyr-103	Phe	–0.6 (192)	–0.5 (279)[c]
Phe-7	Leu	–0.6 (199)	–0.5 (263/269), –0.3 (286), –0.7 (292)
Phe-56	Met	0.8 (194)	–0.9 (262/269), –1.1 (285), –1.3 (292)
Phe-82	Leu	1.7 (196)	0.6 (265,[c] –0.3 (293)
Phe-106	*d*	–1.3 (199)	–0.8 (262/269), –0.8 (292)
His-18	Gly	0.6 (193)	1.0 (280),[c] 1.7 (291)
His-102	Ala	0.5 (193)	1.0 (280)[c]

[a] Only contributions greater in magnitude than $\Delta E \geq 0.3$ M^{-1} cm^{-1} at their maximum are given. The errors in ΔE were estimated from the scatter in all measurements of wild-type spectra: $\Delta E_{193} = 0.3$ M^{-1} cm^{-1}/backbone amide, $\Delta E_{231} = 0.1$ M^{-1} cm^{-1}/backbone amide, $\Delta E_{278} = 0.2$ M^{-1} cm^{-1}, respectively. [b] Very broad maximum (see Figure 4C). [c] Broad band. [d] Measured with the 1–105 cyanogen bromide cleavage fragment; includes the contribution of Phe-106 and of all interactions lost in the truncated form of the protein.

previous calculations indicate that Trp contributions of positive sign are preferred (Sears & Beychok, 1973; Woody, 1978, 1985). However, contributions of tryptophan residues in the far-UV have been difficult to calculate because of the uncertainty in estimates of their transition moments resulting from the low symmetry of the indole side chain.

Tryptophan residues are major determinants of the near-UV spectra as expected (Figure 4B). Difference spectra between wild-type and Trp→Phe mutants reveal the contribution of the deleted tryptophan residue (Figure 4C). As mentioned above, the much smaller contribution of the Phe residue used for replacement can be neglected as a first approximation in the near-UV, where transitions of Phe and Trp do not overlap (Strickland, 1974), and the intensity expected for the contribution of a phenylalanine relative to that of a tryptophan is negligible. Difference spectra reveal the widely varying net contributions of the three tryptophan residues in barnase (Figure 4D). Their intensities (Table IV) are at the higher end of the reported range (Strickland, 1974). The CD contribution of Trp-71 is of opposite sign to that of Trp-35 and Trp-94. The contributions of Trp-94 and Trp-35 appear to be predominantly of the L_a type of tryptophan electronic transition (Strickland, 1974). The contribution of Trp-35 is most intensive at the lower end of the near-UV spectrum, while that of Trp-94 is slightly red-shifted, in line with this residue being more exposed to solvent (Table III). In the case of W94L mutant, comparison of the chemical shifts of the mutant with those of the wild-type show only local alterations in the region of mutation, indicating that no structural rearrangement has occurred in this mutant (Loewenthal *et al.*, 1992). In contrast, the contribution of Trp-71 suggests a very intensive L_b transition (Strickland, 1974) and shows more detailed vibrational structure with two prominent vibrational bands at 285 and 282 nm (Table IV), presumably as a result of Trp-71 being less exposed to solvent (Table III).

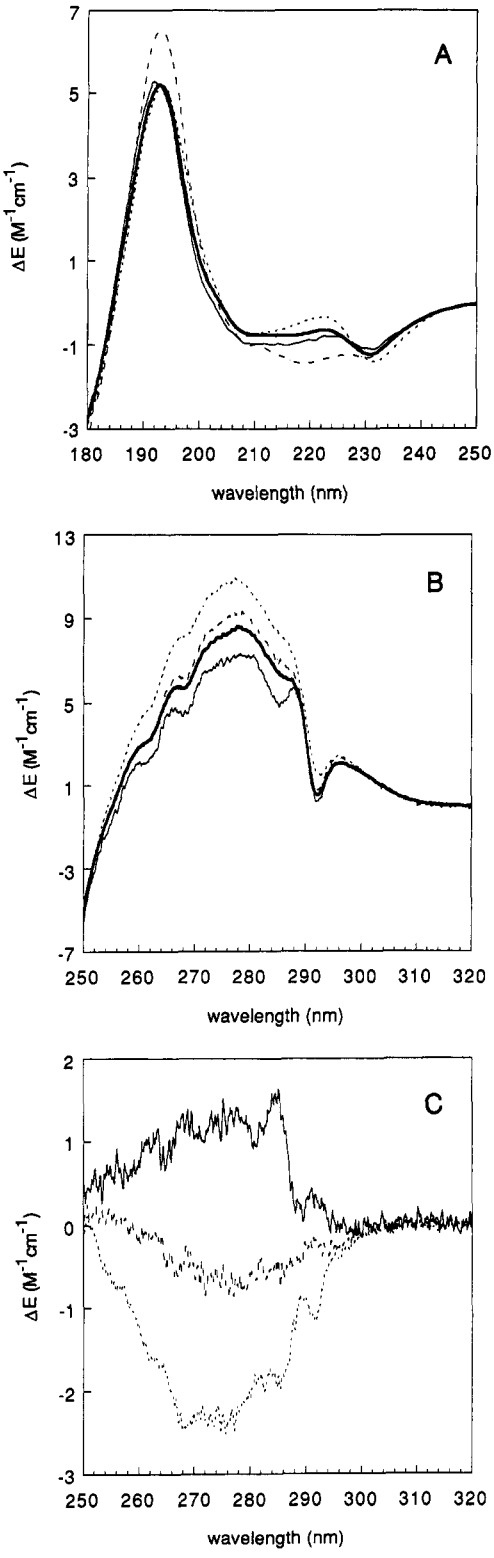

FIGURE 5: Far-UV CD spectra (A) and near-UV CD spectra (B) of selected Tyr→Phe mutants: Y78F (solid line), Y90F (dashed line), and Y97F (dotted line). Wild-type spectra are shown for comparison (bold line). (C) Difference CD spectra in the near-UV showing the contribution of Tyr-78, Tyr-90, and Tyr-97 to the circular dichroism spectrum of barnase.

Of the other aromatic residues in barnase, Tyr-78 and Tyr-97 appear to contribute the most to the CD spectra of the protein in both the far-UV and the near-UV, as judged from the spectra of point mutants in which these residues were replaced by phenylalanine (Figure 5, Table IV). As with Trp residues, CD contributions of both positive and negative sign are observed. The contribution of Tyr-90 as defined by the Y90F mutant is more prominent in the far-UV region of the spectrum. These three tyrosine residues appear to affect the far-UV spectrum of barnase as much as tryptophan residues. In the case of Tyr-78, a perturbation of the CD spectra by structural rearrangement upon mutation can be clearly excluded. In the wild-type protein, Tyr-78 is H-bonded to both the main-chain amide proton and the carbonyl oxygen of Gly-81. The X-ray structure of the Y78F mutant is essentially superimposable on that of the wild-type, with a mean rms distance between coordinates of the Y78F mutant and wild-type (pH 7.5 structure; Hill, 1986) of 0.17 Å in the main chain and 0.33 Å in the side chains respectively (W. Chen, K. Henrick, and A. R. Fersht, unpublished results). Much smaller perturbations of the CD spectra are observed in other mutants where either tyrosine residue 13 or 17 has been replaced (Table IV; see below, Figure 10). Also, mutation of either Tyr-103 or the adjacent His-102 has little effect (Table IV). These residues are, therefore, expected to make little contributions to the CD spectra of barnase. The contribution of Tyr-24 (Table IV) is uncertain. It is possible that structural rearrangements have occurred in this mutant that could obscure the contribution of Tyr-24 to the CD spectrum of the wild-type protein. Tyr-24 has the largest number of contacting side chains in all aromatic residues of barnase (Table III). Y24F is unique among mutants with replacements in tyrosine and phenylalanine residues reported here in that its second-derivative absorbance spectrum is significantly altered compared with that of the wild-type (data not shown), although its stability is essentially identical to that of wild-type (Serrano *et al.*, 1992a).

Mutations involving phenylalanine residues also affect the CD spectrum of barnase, more predominantly so in the far-UV (Figure 6). Phenylalanine residues were mutated to Leu or Met, except for Phe-106, the contribution of which was probed using a truncated form of barnase lacking the five C-terminal residues 106–110 obtained after CNBr cleavage of the T105M mutant. Significant changes below 210 nm in the far-UV spectra are observed for all proteins where Phe residues have been removed. Surprisingly, the mutation of Phe-82, the aromatic residue in barnase that is most exposed to solvent and has the fewest interresidue contacts of all aromatics in the protein (Table III), causes the largest changes in the far-UV. This suggests that interactions between the aromatic ring of Phe-82 with spatially adjacent peptide bonds in the 37–40 loop 4–5 Å away contribute to the far-UV CD of barnase. No significant changes are observed in the near-UV spectrum of this protein. The contributions of other Phe residues in the near-UV are of modest intensity. The 1–105 fragment used to probe the contribution of Phe-106 has native-like structure, as evidenced by its CD spectra and confirmed independently by ^{1}H NMR and the cooperative two-state transition with urea observed with this fragment (S. Vuilleumier, unpublished work). Phe-56 and Phe-106 are in close interaction in barnase (Table II), but only small perturbations are observed in spectra of mutants where either one of these residues is absent. The minor changes observed in the spectra of F7L are confirmed by the X-ray analysis of the F7L mutant (W. Chen and K. Henrick, unpublished results), which shows only local changes compared with wild-type, with a mean RMS distance between coordinates of mutant and wild-type (pH 7.5 structure; Mauguen *et al.*, 1982; Hill, 1986) of 0.26 Å in the main chain and 0.83 Å in the side chains. In addition, no significant changes in chemical shifts have been observed in NMR spectra of this mutant (D. Sali and M. Bycroft,

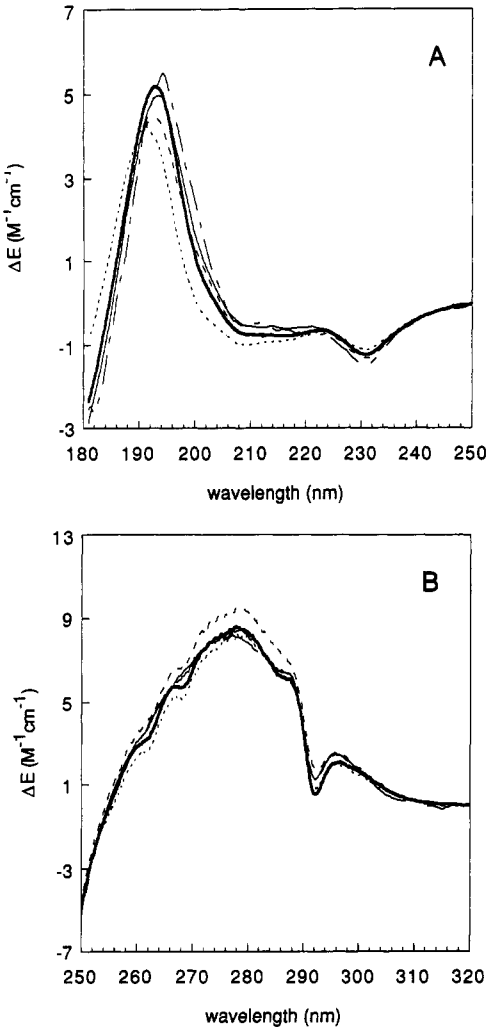

FIGURE 6: Far-UV CD spectra (A) and near-UV CD spectra (B) of selected mutants where phenylalanine has been replaced: F7L (solid line), F56M (short dash), F82L (dotted line), and the fragment 1–105 lacking Phe-106 (long dash). Wild-type spectra are shown for comparison (bold line).

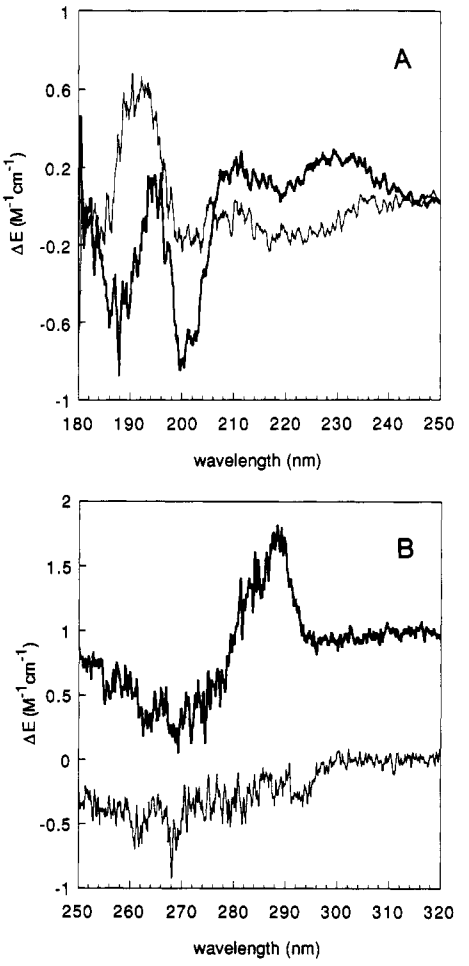

FIGURE 7: Difference CD spectra between W71Y and W71F mutants (bold line) and between W94F and W94L mutants (thin line) in the far-UV (A) and the near-UV (B), revealing the individual contributions to the circular dichroism spectra of extraneous tyrosine and phenylalanine residues introduced at these positions.

personal communication).

In general, replacements of tryptophan or tyrosine residues by phenylalanine are not observed to give rise to well-resolved bands in the 255–270-nm region where such bands are expected. Such bands are more prominent when the orientation of the Phe aromatic ring is fixed in space. In the far-UV, however, some strong contributions are observed at 190 and 200 nm, in line with studies with model compounds (Sears & Beychok, 1973; Woody, 1985). Comparison of the CD spectra of W94F and W94L and of W71Y and W71F (Figure 7) show that the contributions of both Tyr and Phe residues can be quite large in the far-UV region between 185 and 200 nm ($\Delta E \sim 0.6$ M^{-1} cm^{-1}/ backbone amide) and suggest that differences in one single aromatic amino acid residue can cause detectable changes in the position and the intensity of the CD maximum around 190 nm. In the near-UV, the contribution of introduced tyrosine residues is larger than that of phenylalanine as expected (Strickland, 1974). Further, only small differences are observed when using Leu, Phe, or even Tyr residues as replacements for tryptophan residues, indicating that such replacements only marginally affect the determination of the much larger contribution of tryptophan residues to the CD spectrum of the protein.

One main concern in this study, as in studies of the stability and the folding pathway of the protein, was to avoid mutations that could lead to significant structural rearrangements which would complicate the study of interactions of interest. Double mutant cycles (Horovitz & Fersht, 1990) yield further information about the interaction term between two particular residues. Consider two side chains X and Y in a protein. The effect observed upon removal of X from a protein in which both X and Y side chains are present will be the same as upon removal of X from a protein in which Y is missing when there is no interaction, direct or indirect, between X and Y. The double mutant cycle thus reveals that the contributions of side chains X and Y are additive. In the opposite situation, a difference between the two cases indicates that the side chains X and Y interact in some way. When no interaction between X and Y is expected and an interaction term is found, double mutant cycles provide evidence that structural rearrangement has occurred upon mutation.

Analysis of the CD spectra of double mutants of barnase where two tryptophan residues have been removed, with only the third tryptophan residue remaining, provides evidence for the additivity of the major contributions of the three tryptophans of barnase (Figure 8). Tryptophan residues in barnase are far apart, the closest interaction being between Trp-71 and Trp-94, some 11 Å away (Figure 3). Although energy transfer between these two residues was detected by multifrequency phase fluorometry (Willaert *et al.*, 1992), such a distance is probably too large for it to give rise to a significant CD signal. Other distances between tryptophan residues in barnase are much greater (25 Å between Trp-35 and Trp-94

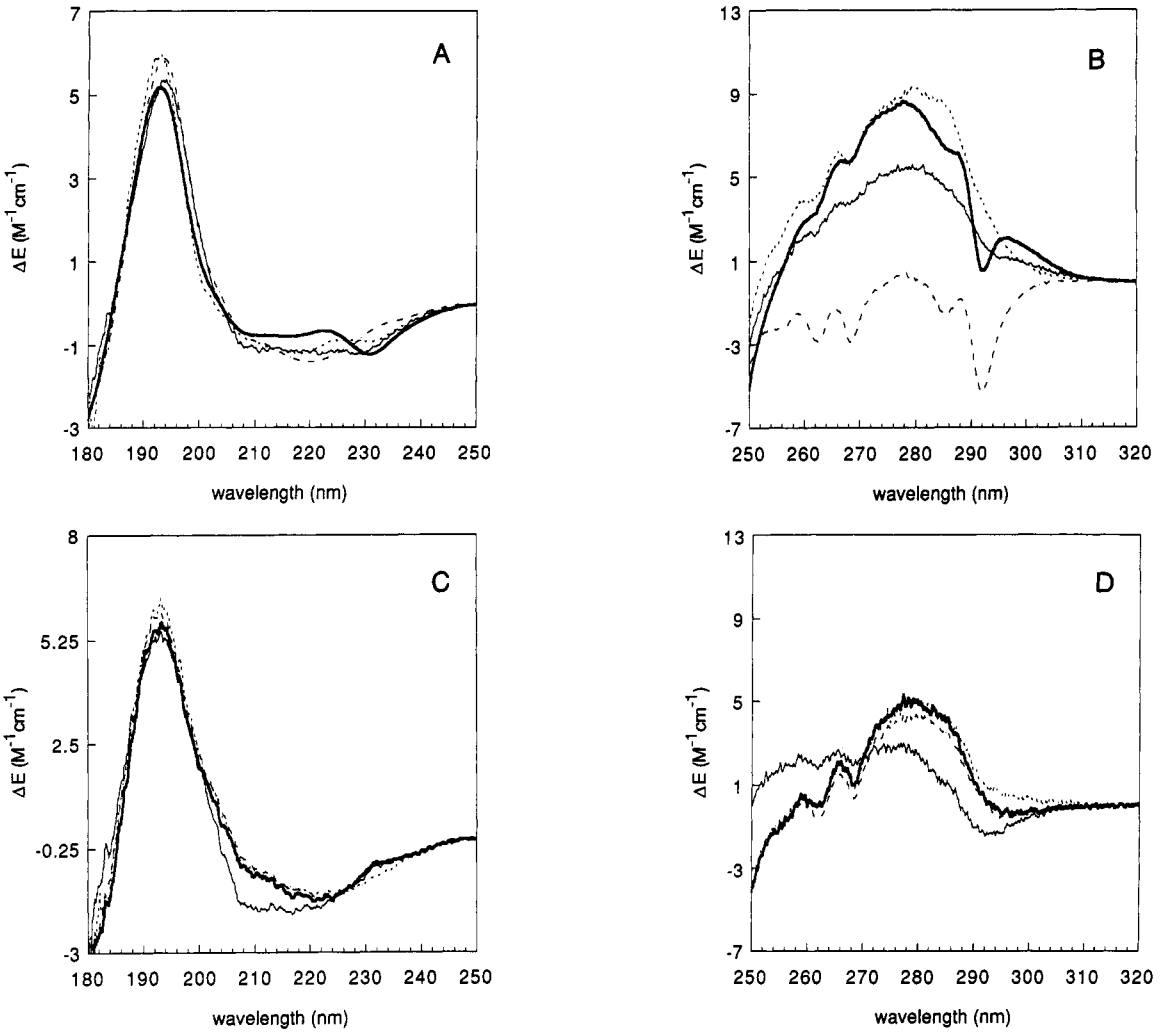

FIGURE 8: CD spectra of mutants W71Y/W94F (dotted line), W35F/W71Y (solid line), and W35F/W94F (dashed line) in the far-UV (A) and the near-UV (B). Wild-type spectra are shown for comparison (bold line). Hypothetical CD curves of barnase in the far-UV (Figure 8C) and the near-UV (Figure 8D), in which all tryptophan contributions are absent, were computed by linear combination of experimental wild-type barnase and single and double tryptophan mutant CD spectra. The different curves should be identical in the case of perfect additivity of the effected mutations (see text). The curves were generated as follows: wild-type – ΔW35F – ΔW71F – ΔW94F (bold line); W71Y/W94F – (W71Y – W71F) – ΔW35F (dotted line); W35F/W71Y – (W71Y – W71F) – ΔW94F (solid line); and W35F/W94F – ΔW71F (dashed line). The (W71Y – W71F) term arises from the need to remove the contribution of tyrosine at position 71 introduced as a replacement for tryptophan in the double mutants W35F/W71Y and W71Y/W94F.

and 23 Å between Trp-35 and Trp-71). Subtracting the contributions of each tryptophan obtained from difference spectra (i.e., wild-type – ΔW35F – ΔW71F – ΔW94F) yields essentially identical results as subtracting the contribution of the third tryptophan from the spectra of the double mutants, in the cases of both mutants W35F/W94F and W71Y/W94F. This holds for both the far-UV (Figure 8C) and the near-UV (Figure 8D) regions of the CD spectrum. However, the spectrum obtained by removing the contribution of Trp-94 from the spectra of W35F/W71Y double mutant is qualitatively different. It is very unlikely that this difference corresponds to a true interaction between Trp-35 and Trp-71 since these two residues are so far apart in the barnase structure (Figure 2). Rather, it is probable that some structural rearrangement has occurred in the W35F/W71Y mutant, a fact corroborated by the low protein yield obtained in the expression of this mutant and by its altered properties during purification. The additivity of all other linear combinations of wild-type and single and double mutants of all three tryptophans suggests that all other single and double mutations involving tryptophan residues have not caused significant structural rearrangement to occur.

The closest side-chain interactions involving aromatic residues in barnase are between Trp-94 and His-18 (4.0 Å) and between Tyr-13 and Tyr-17 (4.1 Å, Figure 2, Table II). It was shown previously that the fluorescence of Trp-94 is quenched by His-18 in a pH-dependent way, the effect being largest at low pH (Loewenthal *et al.*, 1991). Using double mutant cycles, the interaction energy between His-18 and Trp-94 was shown to contribute as much as 1.4 kcal mol^{-1} to protein stability at pH 5.8 (Loewenthal *et al.*, 1992). Difference spectra for H18G and the double mutant W94L/H18G indicate that the band at 231 nm arises mainly from the presence of Trp-94 (Figure 9A). His-18 affects the spectrum little more than the catalytic His-102 (Table IV). The use of a double mutant cycle reveals only a small contribution from the His-18/Trp-94 interaction to the CD of barnase of about $\Delta E = -0.8 \pm 0.5$ M^{-1} cm^{-1} at 192 nm and -2.1 ± 0.3 M^{-1} cm^{-1} at 291 nm (Figure 9B). Incidentally, the pH dependence of fluorescence arising from the quenching of Trp-94 by protonated His-18 is not observed by circular dichroism. Within experimental error, changing the pH from 5.8 to 9.0 has no effect on the CD spectra for wild-type barnase, the single mutants H18G or W94L, or the double mutant

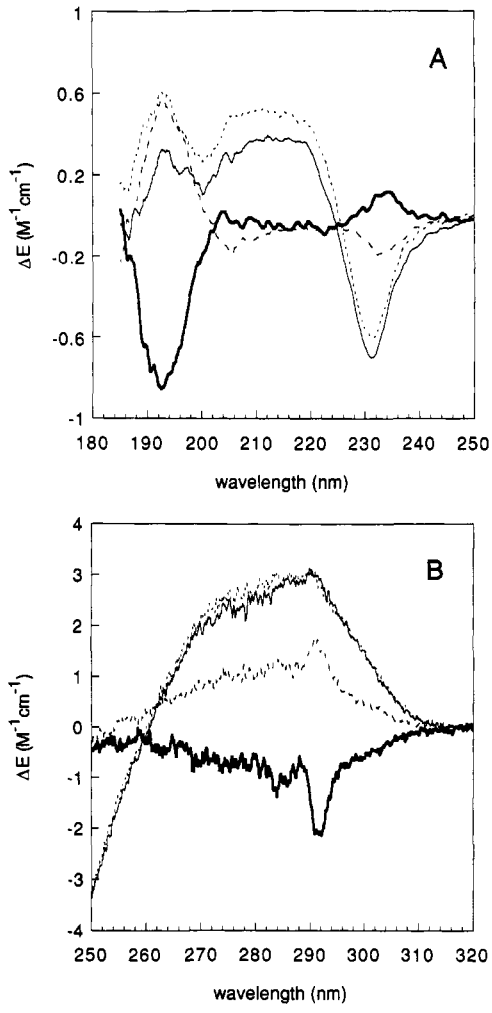

FIGURE 9: Contribution of the His-18/Trp-94 interaction to CD spectra in the far-UV (A) and the near-UV (B). Difference curves are for ΔW94L (dotted line), ΔH18G (dashed line), ΔH18G/W94L (solid line), and the interaction contribution between His-18 and Trp-94 (bold line) calculated from the double-mutant cycle: ΔCD_{int} (H18/W94) = ΔH18G/W94L − (ΔW94L + ΔH18G).

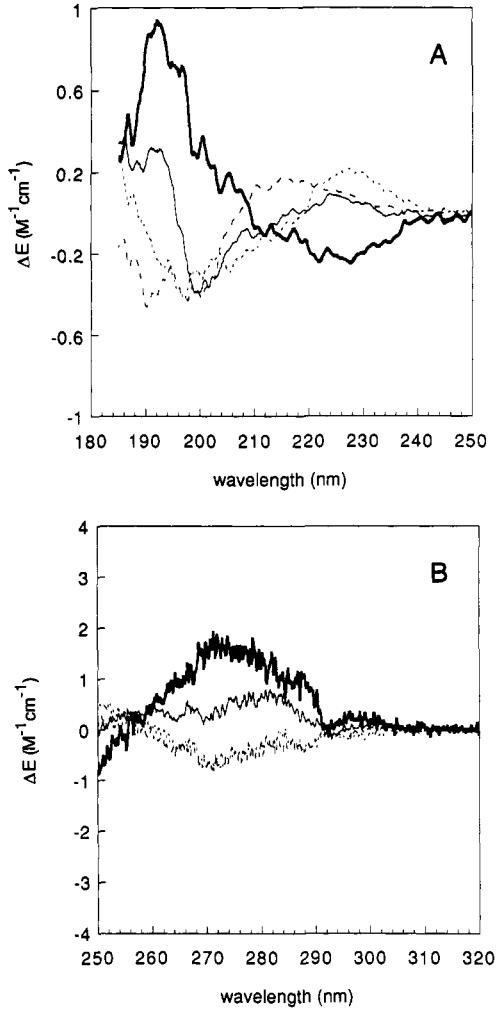

FIGURE 10: Contribution of the Tyr-13/Tyr-17 interaction to CD spectra in the far-UV (A) and the near-UV (B). Curves are for ΔY13F (dotted line), ΔY17F (dashed line), ΔY13F/Y17F (solid line), and the interaction contribution between Tyr-13 and Tyr-17 (bold line) calculated from the double-mutant cycle: ΔCD_{int} (Y13/Y17) = ΔY13F/Y17F − (ΔY13F + ΔY17F).

W94L/H18G (data not shown). The other close interaction between Tyr-13 and Tyr-17 has been studied previously (Serrano *et al.*, 1991) and shown to contribute about 1.3 kcal mol^{-1} to protein stability. Replacement of these residues by phenylalanine leads to very similar small changes in the CD spectra (ΔE = 0.4 ± 0.3 M^{-1} cm^{-1} in the far-UV at 197 nm, and −0.7 ± 0.2 M^{-1} cm^{-1} at 270 nm in the near-UV (Table IV). The changes observed with the double mutant (ΔE = −0.4 ± 0.3 M^{-1} cm^{-1} at 200 nm and 0.7 ± 0.2 M^{-1} cm^{-1} at 281 nm) are also quite small (Figure 10). These small terms add up in the calculation of the interaction between Tyr-13 and Tyr-17 to intensity maxima of 0.9 ± 0.5 M^{-1} cm^{-1} at 192 nm and 1.7 ± 0.3 M^{-1} cm^{-1} at 272 nm. Incidentally, the red shift observed for the contribution of Trp-94 relative to the other two tryptophan residues does not seem to be caused by the interaction with His-18 since essentially the same difference spectrum as for W94L is observed for the W94L/H18G double mutant, in which His-18 is absent.

Do all the contributions of aromatic residues defined here using protein variants with mutations involving all aromatic residues of the protein account for the near-UV spectrum of wild-type barnase? We have shown above that the contributions of tryptophan residues are the major determinants of the CD features of the protein, and the spectra of double mutants with only one Trp remaining show that these are essentially additive (Figure 8). However, adding together all other contributions from Tyr and Phe residues defined by difference spectra between wild-type and aromatic mutants does not yield the curve expected from the non-tryptophan contributions (Figure 11). This is in contrast to previously reported studies, both theoretical (Strickland, 1972) and experimental (Craig *et al.*, 1989). Possible reasons for this are twofold. First, although the effect of a mutation has been tested for each aromatic residue in the protein, interactions conducive to the appearance of CD bands have not yet all been investigated (Tables II and III). Second, replacement of tryptophan and tyrosine residues by phenylalanine will, as a rule, not only remove the electronic interactions responsible for the CD signal of the replaced residue but also introduce an extraneous contribution from the introduced Phe residue, especially in the far-UV region (see Figure 7). However, this has negligible consequences in the case of Trp-94 in barnase in the near-UV region (Figure 7B), where the curve corresponding to the contribution of this residue to the CD spectrum of barnase is found to be essentially identical, irrespective of whether it was defined by the subtraction of the CD spectrum of mutant W94L or of mutant W94F from the wild-type spectrum.

The large contributions of aromatics in the far-UV described above and the small overall intensities of the CD spectrum of

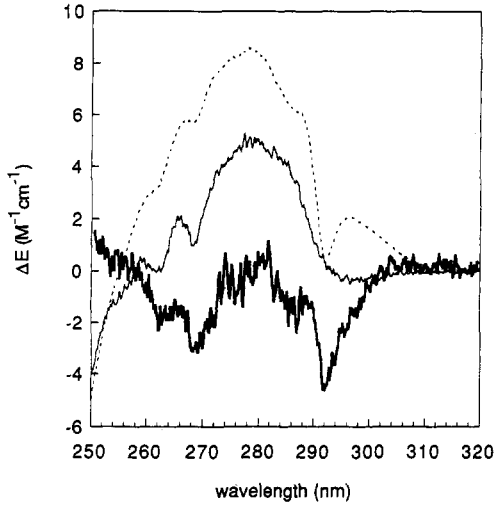

FIGURE 11: Comparison of the computed sum of all non-tryptophan aromatic contributions to the near-CD obtained from the subtraction of mutant from wild-type spectra (bold line) with the computed spectrum of tryptophan-less barnase (thin line, see Figure 8D). The experimental CD spectrum of wild-type barnase is given for comparison (dotted line).

barnase in this region represent a difficult task for algorithms using CD information to predict the secondary structure content of the protein. We tested Contin (Provencher & Glöckner, 1981), the variable selection method of Johnson and coworkers (Toumadje *et al.*, 1992), and the convex constraint deconvolution algorithm (Perczel *et al.*, 1992) (Table V). We used both the far-UV curve of wild-type barnase or a hypothetical "tryptophan-less" spectrum of the protein (see Figure 8C), generated by subtracting the contributions of tryptophan residues defined by the spectra of Trp→Phe mutants of barnase as input data. The Contin program misinterpreted the low CD intensities in barnase spectra. Although the generated curves of best fit matched the input data well, the program predicted 100% and 87% β-sheet content for the wild-type spectrum and the simulated tryptophan-less spectrum, respectively, whereas analysis of the X-ray structure using the program DSSP (Kabsch & Sander, 1983) gives a β-sheet content of 22% for barnase. The curve of best fit corresponding to the prediction of the variable selection method matched the input spectra only poorly. The prediction gave a too-low helix content and a high value for parallel β-sheet (of which barnase has none) but appeared to predict a correct amount for antiparallel β-sheet and turn. The convex constraint deconvolution method fitted the experimental spectrum best when both wild-type and tryptophan-less spectra were included in the reference set of protein spectra to generate the five "pure" component curves required in the analysis, although no combination of five pure component curves could be found which would describe wild-type barnase or its simulated tryptophan-less spectrum. Only one curve, that corresponding to a helix-like component, was reminiscent of spectra typical of elements of sheet, turn, or coil. Unfortunately, the currently available implementation of the program does not perform an automatic search for subsets of the reference data set best suited for the analysis of the protein of interest, as is the case with the variable selection method.

The low-intensity spectra in the far-UV displayed in all barnase mutants described here suggest extensive canceling out of positive and negative CD bands. Further possible CD contributions arising from interactions between aromatic residues and neighboring residues with electronic transitions involving π-orbitals may be defined in the future as more

Table V: Prediction of Secondary Structure Content from CD Spectra of Barnase[a]

method	protein	helix	β (‖)	β (ap)	turn	other
X-ray[b]	wild-type	22		22	14	52
Contin[c]	wild-type		100			
	Trp-free[a]		87		13	
variable selection[d]	wild-type	5	14	23	22	30
	Trp-free	12	23	23	18	24
CCA[e]	wild-type	33	*23*	*35*	*9*	
	Trp-free	33	*23*	*31*		*12*

[a] The spectrum of wild-type barnase (Figure 1A) and a simulated Trp-free spectrum of barnase (Figure 8C) obtained by removing the contributions of all three tryptophans defined by difference spectra between wild-type and single Trp→Phe mutants were used as input. [b] As determined from the X-ray coordinates of barnase (Cameron, 1992) using the DSSP program (Kabsch & Sander, 1983). This program and the different prediction methods tested define the content in each secondary structure type in different ways. The categories used here are helix, α-helix and 3_{10} helix; ‖ β, parallel β-sheet; ap β, antiparallel β-sheet, turn; and other. [c] Three-state prediction method of Provencher & Glöckner (1981), using the 16 reference proteins in the data set for data between 190 and 240 nm at 0.5-nm intervals. The standard error for the β-sheet content given by the program was 1.4% for the wild-type spectrum and 2.3% for the tryptophan-free spectrum. [d] Toumadje *et al.* (1992). The reference data set was the best-fit combination of 19 proteins from the total 22 proteins included in the data set, for data between 180 and 260 nm at 0.5-nm intervals. The rms error given in the program was ±30% for the wild-type spectrum and ±18% for the tryptophan-free simulated spectrum. [e] Convex constraint analysis (Perczel *et al.*, 1992). The reference data set used to generate the five pure component curves included both the wild-type spectrum and the simulated "tryptophan-free" spectrum in addition to the 25 proteins in the basic data set, with data between 195 and 240 nm at 1-nm intervals. The final square error given by the program was 4.1 for the wild-type spectrum and 30.3 for the tryptophan-free simulated spectrum. The percentages given in italics are for the four pure curves which could not be interpreted to correspond to a type of secondary structure.

interactions in the barnase structure are probed by protein engineering. Other localized conformations in the protein backbone, such as β-turns, which can give rise to a wide variety of spectra, may also co-determine the observed features (Woody, 1985). It is also known that small variations in band position or bandwidth in one of the far-UV peptide or aromatic transitions may result in large changes in the overall appearance of the spectrum (Woody, 1978; Manning *et al.*, 1988; Manning & Woody, 1989). As for the near-UV region, there is the possibility that some of the interactions involving aromatic residues responsible for large compensating positive and negative features are retained in phenyalanine mutants, since all aromatic residues have electronic transitions in the far-UV region which could give rise to a CD signal (Woody, 1985). On the other hand, less conservative mutations of aromatic residues may involve such large destabilization of the protein backbone that the resulting alteration of the spectral features would prevent the characterization of this interaction.

In conclusion, we have attempted to characterize experimentally the contributions of all aromatic residues in barnase to the circular dichroism spectra of barnase in both the far-UV and the near-UV regions. Using mutants of the protein obtained by protein enginering in which aromatic residues have been replaced, we could obtain estimates of the relative contribution of different amino acid residues to the CD spectrum of the wild-type protein (Table IV). Tryptophan contributions in barnase were found to be additive and to contribute the major part of the CD intensity in the near-UV, the other main contribution arising from tyrosine residues 78 and 97. While all tryptophan and several tyrosine and phenylalanine residues in the protein were observed to make significant contributions to the far-UV CD of the protein, Trp-94 was shown to be associated with the observed minimum

of the spectrum at 231 nm. Thus, Trp-94 is the main structural probe in CD studies of barnase, whereas Trp-35 is the main contributor to the fluorescence of the protein (Loewenthal et al., 1991). Since the cooperative two-state denaturation of barnase with urea is identical with both fluorescence and circular dichroism detections, the global nature of the denaturation process detected with these two relatively localized probes is confirmed. The information gained in this study will be of value in the characterization of the structural integrity and the stability of barnase mutants, in investigations of partially folded states of the protein, and in stopped-flow kinetic studies of the folding pathway of the protein using circular dichroism detection. It is also hoped that the findings reported here will prompt theoretical investigations on the structural basis for the circular dichroism features of barnase in both the near-UV and the far-UV.

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REFERENCES

Arnold, G. E., Day, L. A., & Dunker, A. K. (1992) *Biochemistry 31*, 7948–7956.
Brahms, S., & Brahms, J. (1980) *J. Mol. Biol. 138*, 149–178.
Cameron, A. D. (1992) Ph.D. Thesis, University of York, U.K.
Chaffotte, A. F., Guillou, Y., & Goldberg, M. E. (1992) *Biochemistry 31*, 9694–9702.
Craig, S., Pain, R. H., Schmeissner, U., Virden, R., & Wingfield, P. T. (1989) *Int. J. Pept. Protein Res. 33*, 256–262.
Elove, G. A., Chaffotte, A. F., Roder, H., & Goldberg, M. E. (1992) *Biochemistry 31*, 6876–6883.
Fersht, A. R., Matouschek, A., Bycroft, M., Kellis, J. T., Jr., & Serrano, L. (1991) *Pure Appl. Chem. 63*, 187–194.
Gill, S. C., & von Hippel, P. H. (1989) *Anal. Biochem. 182*, 319–326.
Hill, C. (1986) Ph.D. Thesis, University of York, U.K.
Holzwarth, G. (1972) in *Membrane Molecular Biology* (Fox, C. F., & Keith, A. D., Eds.), pp228–286, Sinauer Associates Inc., Stamford, CT.
Horovitz, A., & Fersht, A. R. (1990) *J. Mol. Biol. 214*, 613–617.
Horovitz, A., & Fersht, A. R. (1992) *J. Mol. Biol. 224*, 733–740.
Johnson, W. C., Jr. (1985) *Methods Biochem. Anal. 31*, 61–163.
Kabsch, W., & Sander, C. (1983) *Biopolymers 22*, 2577–2637.
Kahn, P. C. (1979) *Methods Enzymol. 61*, 339–378.
Kanaya, S., Katsuda-Nakai, C., & Ikehara, M. (1991) *J. Biol. Chem. 266*, 11621–11627.
Kellis, J. T. J., Nyberg, K., Sali, D., & Fersht, A. R. (1988) *Nature 333*, 784–786.
Kellis, J. T. J., Nyberg, K., & Fersht, A. R. (1989) *Biochemistry 28*, 4914–4922.
Khan, M. Y., Villanueva, G., & Newman, S. A. (1989) *J. Biol. Chem. 264*, 2139–2142.
Kiefhaber, T., Schmid, F. X., Willaert, K., Engelborghs, Y., & Chaffotte, A. (1992) *Protein Sci. 1*, 1162–1172.
Kraulis, P. (1991) *J. Appl. Crystallogr. 24*, 946–950.
Kuwajima, K., Garvey, E. P., Finn, B. E., Matthews, C. R., & Sugai, S. (1991) *Biochemistry 30*, 7693–7703.
Loewenthal, R. (1992) Ph.D. Thesis, University of Cambridge, U.K.
Loewenthal, R., Sancho, J., & Fersht, A. R. (1991) *Biochemistry 30*, 6775–6779.
Loewenthal, R., Sancho, J., & Fersht, A. R. (1992) *J. Mol. Biol. 224*, 759–770.
Manning, M. C., & Woody, R. W. (1989) *Biochemistry 28*, 8609–8613.
Manning, M. C., Illangasekare, M., & Woody, R. W. (1988) *Biophys. Chem. 31*, 77–86.
Matouschek, A., Kellis, J. T., Jr., Serrano, L., & Fersht, A. R. (1989) *Nature 342*, 122–126.
Matouschek, A., Serrano, L., & Fersht, A. R. (1992) *J. Mol. Biol. 224*, 819–835.
Mauguen, Y., Hartley, R. W., Dodson, E. J., Dodson, G. G., Bricogne, G., Chothia, C., & Jack, A. (1982) *Nature 29*, 162–164.
Mossakowska, D. E., Nyberg, K., & Fersht, A. R. (1989) *Biochemistry 28*, 3843–3850.
Muraki, M., Harata, K., & Jigami, Y. (1992) *Biochemistry 31*, 9212–9219.
Pace, C. N. (1986) *Methods Enzymol. 131*, 266–279.
Perczel, A., Park, K., & Fasman, G. D. (1992) *Anal. Biochem. 203*, 83–93.
Provencher, S. W., & Glöckner, J. (1981) *Biochemistry 20*, 33–38.
Radford, S. E., Dobson, C. M., & Evans, P. A. (1992) *Nature 358*, 302–307.
Sancho, J., & Fersht, A. R. (1992) *J. Mol. Biol. 224*, 741–747.
Santoro, M. M., & Bolen, D. W. (1988) *Biochemistry 27*, 8063–8068.
Sears, D. W., & Beychok, S. (1973) in *Physical Principles and Techniques of Protein Chemistry* (Leach, S. J., Ed.), pp445–593, Academic Press, New York.
Serrano, L., Horovitz, A., Avron, B., Bycroft, M., & Fersht, A. R. (1990) *Biochemistry 29*, 9343–9352.
Serrano, L., Bycroft, M., & Fersht, A. R. (1991) *J. Mol. Biol. 218*, 465–475.
Serrano, L., Kellis, J. T., Jr., Cann, P., Matouschek, A., & Fersht, A. R. (1992a) *J. Mol. Biol. 224*, 783–804.
Serrano, L., Matouschek, A., & Fersht, A. R. (1992b) *J. Mol. Biol. 224*, 805–818.
Shiraki, M. (1969) *Sci. Pap. Coll. Gen. Educ., Univ. Tokyo 19*, 151.
Shire, S. J., Holladay, L. A., & Rinderknecht, E. (1991) *Biochemistry 30*, 7703–7711.
Strickland, E. H. (1972) *Biochemistry 11*, 3465–3474.
Strickland, E. H. (1974) *CRC Crit. Rev. Biochem. 3*, 113–175.
Sugawara, T., Kuwajima, K., & Sugai, S. (1991) *Biochemistry 30*, 2698–2706.
Toumadje, A., Alcorn, S. W., & Johnson, W. C., Jr. (1992) *Anal. Biochem. 200*, 321–331.
Willaert, K., Loewenthal, R., Sancho, J., Froeyen, M., Fersht, A. R., & Engelborghs, Y. (1992) *Biochemistry 31*, 711–716.
Woody, R. W. (1978) *Biopolymers 17*, 1451–1467.
Woody, R. W. (1985) in *Conformation in Biology and Drug Design* (Hruby, V. J., Ed.), pp15–114, Academic Press, Inc., New York.