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Characterization of Phosphate Binding in the Active Site of Barnase by Site-Directed Mutagenesis and NMR†

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ABSTRACT: Phosphate is a competitive inhibitor of transesterification of GpC by the ribonuclease barnase. Barnase is significantly stabilized in the presence of phosphate against urea denaturation. The data are consistent with the existence of a single phosphate binding site in barnase with a dissociation constant, K_d, of 1.3 mM. The 2D ^{1}H NMR spectrum of wild-type barnase with bound phosphate is assigned. Changes in chemical shifts and NOEs for wild type with bound phosphate compared with free wild type indicate that phosphate binds in the active site and that only small conformational changes occur on binding. Site-directed mutagenesis of the active site residues His-102, Lys-27, and Arg-87 to Ala increases the magnitude of K_d for phosphate by more than 20-fold. The 2D ^{1}H NMR spectra of the mutants His-102 → Ala, Lys-27 → Ala, and Arg-87 → Ala are assigned. Comparison with the spectra of wild-type barnase reveals that His-102 → Ala and Lys-27 → Ala have essentially the same structure as wild type, while some structural changes occur in Arg-87 → Ala. It appears that phoshate binding by barnase is effected mainly by positively charged residues including His-102, Lys-27, and Arg-87. This may have applications for the design of phosphate binding sites in other proteins.

About half of all known proteins bind compounds containing phosphoryl groups (Schultz & Schirmer, 1979). The recognition and binding of phosphoryl groups are important in the interaction of proteins with a wide range of molecules including substrates, cofactors, allosteric effectors, and ligands. The structures of phosphoryl binding sites in a number of proteins are known from X-ray crystallography, for example, nucleotide binding proteins such as human c-H-*ras* oncogene protein complexed with GDP (de Vos et al., 1988), triosephosphate isomerase complexed with dihydroxyacetone phosphate (Lolis & Petsko, 1990), and bovine pancreatic ribonuclease A complexed with mono- and dinucleotide inhibitors [for a summary see Wlodawer (1985)]. Electrostatic interactions between the negatively charged phosphate and positive groups on proteins are important for the binding of phosphate by proteins. This includes charge–charge interactions between phosphate and positively charged side chains that are found in virtually all phosphate binding sites, charge–dipole interactions between phosphate and α-helix dipoles (Hol et al., 1978), and hydrogen bonding between phosphate and side-chain or main-chain peptide groups as, for example, in the Gly-X-X-X-X-Gly-Lys phosphate binding loop of purine nucleotide binding proteins (Campbell-Burk, 1989; Redfield & Papastavros, 1990). There is, however, little experimental data on the energetics of interactions between proteins and phosphate, and the specificity of phosphate binding sites is poorly understood. Here, we characterize a phosphate binding site in which phosphate is bound mainly by positively charged side chains in the ribonuclease barnase.

Barnase is a small endoribonuclease of 110 amino acids from *Bacillus amyloliquefaciens*. It is a member of a family of guanine-specific and guanine-preferential microbial endoribonucleases, including enzymes from both bacterial and fungal species (Hill et al., 1983). Hydrolysis of RNA catalyzed by the microbial ribonucleases occurs in a two-step

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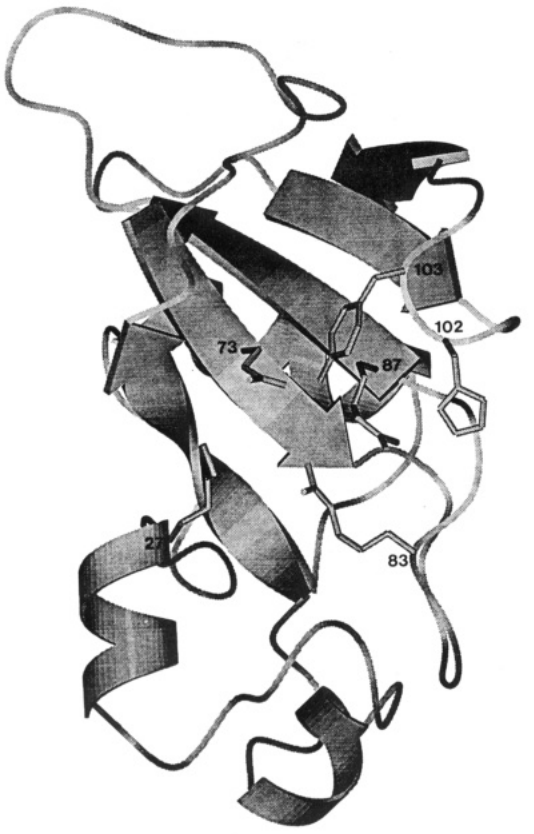

FIGURE 1: Overview of the active site of barnase (from coordinates kindly provided by Professor G. Dodson and Dr. C. Hill). Barnase consists of two N-terminal α-helices (residues 6–18 and 26–34) packed onto a five-stranded antiparallel β-sheet (residues 50–55, 70–75, 85–91, 94–101, and 106–108). The active site is located in a shallow groove on the surface of the enzyme. The side chains of the catalytic residues, His-102 and Glu-73, and of residues Tyr-103, Arg-87, Arg-83, and Lys-27 are shown. Positively charged residues that may interact with phosphate are clustered in the active site.

process via the formation of a 2′,3′-cyclic phosphate intermediate to yield a fragment of RNA ending primarily in G3′p (Uchida & Egami, 1971). Primary sequence homologies, structural comparisons (Hill et al., 1983), chemical modification (Uchida & Egami, 1971; Takahashi & Moore, 1982), and site-directed mutagenesis studies (Nishikawa et al., 1987; Steyaert et al., 1990; Mossakowska et al., 1989) have implicated three strictly conserved residues as essential for activity. His-102 and Glu-73 act as general acid–base groups during catalysis, while Arg-87 has been proposed to be important in phosphate binding and in forming the structure of the active site. Site-directed mutagenesis and kinetic studies of barnase have revealed that Lys-27, a residue found only in barnase and the highly homologous enzyme binase, from *Bacillus intermedius*, is important in catalysis and may interact with phosphate in the transition state of the reaction (Mossakowska et al., 1989). The crystal structure of barnase is known (Mauguen et al., 1982), as is the crystal structure of binase complexed with 3′-GMP (Pavlovsky et al., 1988; Drs. A. G. Pavlovsky and M. Ya. Karpeisky, personal communication). In the crystal structures, His-102, Arg-87, and Glu-73 are located in a shallow groove on the surface of the enzyme that forms the active site. Positively charged residues, including His-102, Arg-87, and Lys-27, are clustered in the active site and could interact with phosphate (Figure 1).

We characterize the binding of phosphate by wild-type barnase using inhibition of GpC transesterification and equilibrium binding studies. Comparison of the 2D ^{1}H NMR spectra of barnase with and without bound phosphate is used to locate the binding site and investigate the structural consequences of the binding of phosphate. The roles of His-102, Arg-87, and Lys-27 in phosphate binding are investigated by mutating these residues to alanine and measuring the binding of phosphate by the mutant enzymes. The NMR spectra of the mutants are compared with those of wild-type barnase in order to determine the structural effects of the mutations.

Materials and Methods

Reagents. SP-Trisacryl was purchased from IBF, and dialysis tubing of M_r cutoff 3500 was obtained from Spectrum Medical Industries, Inc. Ampholine PAGplate gels were from Pharmacia. Enzyme-grade urea was purchased from Bethesda Research Laboratories, and NMR reagents were from Sigma. All other reagents were purchased from either Sigma or BDH Limited. All solutions were made using water purified by Elgastat Spectrum System B.

Protein Purification. Wild-type and mutant barnases were purified from cultures of *Escherichia coli* containing the plasmid pMT410 (Paddon & Hartley, 1987) as described previously (Mossakowska et al., 1989). It has been found that mutant enzymes are often obtained in lower yield than wild-type barnase. Better yields have been obtained by growing cultures at lower temperature. Cultures of His-102 → Ala and Lys-27 → Ala were grown for 24 h at 30 °C, while Arg-87 → Ala was grown for 72 h at 25 °C. The resulting protein is homogeneous as judged by NaDodSO$_4$–polyacrylamide gel electrophoresis and isoelectric focusing. Purified protein was dialyzed extensively against deionized H_2O, then flash frozen, and stored at −70 °C. Protein for NMR experiments was lyophilized after dialysis and stored at −20 °C.

Enzyme Activity Assays. Experiments were carried out at 25 °C. The concentration of enzyme was determined spectophotometrically at 280 nm where $E_{0.1\%} = 2.21$ (Loewenthal et al., 1991).

(*a*) *RNA Hydrolysis.* Barnase activity was assayed using yeast torula RNA as described by Mossakowska et al. (1989). The assay solution contained 2 mg/mL purified yeast RNA in 50 mM Tris-HCl/79 mM Tris, pH 8.5. Wild-type and Arg-87 → Ala activities were measured in parallel, and the activity of the mutant enzyme was calculated as a percentage of the activity of wild type.

(*b*) *GpA Transesterification.* The transesterification step of GpA hydrolysis by barnase was determined from initial velocities by following the increase in absorbance at 280 nm as described by Mossakowska et al. (1989). The assay solution contained 530–640 mM GpA in 7.8 mM acetic acid/100 mM sodium acetate, pH 5.9. Aliquots of stock enzyme solution were added to give a final enzyme concentration of 18 μM for Arg-87 → Ala and 1.2 μM for wild type.

Determination of Inhibition Constant for Phosphate. The inhibition constant, K_i, for phosphate was measured against GpC transesterification. The transesterification of GpC was measured as for GpA, except that the reactions were carried out in 46.2 mM acetic acid/30.0 mM sodium acetate buffer, pH 4.5. Substrate concentrations were 100, 200, and 400 μM GpC. NaH_2PO_4 was added to solutions of substrate before the addition of enzyme. Final concentrations of NaH_2PO_4 were from 1.5 to 9 mM. Phosphate exists only as a monoanion at pH 4.5 and does not significantly affect the final pH of the buffered protein solution. Measurements of initial velocities were determined in duplicate for each reaction condition. The data were analyzed with nonlinear regression analysis using the program Enzfitter (published by Elsevier-Biosoft, Cambridge).

Determination of Dissociation Constants for Ions. (*a*) *Urea Denaturation.* Binding of NaH_2PO_4 and NaCl to barnase was measured from their effects on protein stability as measured by urea denaturation. Solutions containing 9 μM barnase, 416 mM acetic acid/270 mM sodium acetate, pH 4.5, and 0–1.8 M NaH_2PO_4 or NaCl were prepared. Aliquots (100 μL) of this enzyme solution were added to 800 μL of urea solutions as described by Kellis et al. (1989). Complete denaturation curves were obtained at each salt concentration.

(*b*) ^{31}P *NMR.* Solutions of barnase, made from lyophilized protein, contained 3–5 mM protein and 30.3 mM acetic acid/19.7 mM sodium acetate, pH 4.5, in 90% H_2O/10% D_2O. Aliquots of NaH_2PO_4 solutions were added to 500 μL of barnase solution in an NMR tube. Chemical shifts were measured after each addition of phosphate at a field strength of 202.4 MHz using a Bruker AM 500 spectrometer, thermostated at 26.1 ± 0.5 °C. A maximum of 512 transients were collected for the lowest concentration of NaH_2PO_4 (0.1 mM), ranging to a minimum of 16 transients for the highest concentration (250–300 mM). Line broadening of 1 MHz was used in processing the data. The spectral reference was external 80% phosphoric acid in 20% D_2O.

2D ^1H *NMR.* Samples were prepared from lyophilized protein to give a final protein concentration of 3–5 mM in 90% H_2O/10% D_2O or 100% D_2O. The pH or pD of protein solutions was adjusted to 4.5 using DCl and NaOD. Spectra were acquired at 37 °C for wild-type barnase with and without bound phosphate and for the mutants His-102 → Ala and Lys-27 → Ala, and at 27 °C for Arg-87 → Ala and wild type. COSY, DQF-COSY, TOCSY, and NOESY spectra in H_2O and D_2O were recorded for wild type with and without bound phosphate and for Arg-87 → Ala. For His-102 → Ala and Lys-27 → Ala, COSY and NOESY spectra in H_2O and D_2O were recorded. Experimental data were acquired on a Bruker AM 500 or AMX 500 spectrometer and processed as described previously (Bycroft et al., 1990).

Assignment of 2D ^1H *Spectra.* The 2D ^{1}H NMR spectrum of wild-type barnase has recently been assigned and the solution structure of the protein has been determined (Bycroft et al., 1990, 1991). Assignments were obtained for wild-type barnase with bound phosphate and mutants by comparing the $C^{\alpha}H$–NH fingerprint region of the COSY spectra of these proteins with that of free wild type at the same temperature. Peaks at the same positions were initially assumed to originate from corresponding protons in the two proteins. These assignments were confirmed and extended to residues in which protons have different chemical shifts from wild type using standard sequential assignment methods (Wüthrich, 1986) and comparisons with spectra of wild type. Complete side chains were assigned for residues in which the main-chain protons exhibited changes in chemical shift >0.05 ppm.

The structures of wild type with phosphate bound and mutants were investigated by comparing chemical shifts and patterns of sequential and secondary structure NOEs of main-chain protons with those of free wild type (Bycroft et al., 1990). The chemical shifts of protons are very sensitive to changes in magnetic environment due to local conformational changes in a protein, and a lack of change of chemical shifts of protons in related proteins (such as wild type and mutant) in a particular region is good evidence that the structures of the proteins in this region are the same. NOEs are sensitive probes of the distances between protons. Characteristic patterns of NOEs are observed for secondary structural elements in proteins (Wüthrich, 1986). The two α-helices of barnase (residues 6–18 and 26–34) are characterized by intraresidue Nβ, sequential d_{NN} and $d_{\beta N}$, and $d_{\alpha N(i,i+3)}$ and $d_{\alpha N(i,i+4)}$ medium-range NOEs. The five-stranded antiparallel β-sheet (residues 50–55, 70–75, 85–91, 95–101, and 105–108) is characterized by runs of $d_{\alpha N}$ sequential connectivities connected by long-range interstrand NOEs. The patterns of sequential and secondary structure NOEs were used to investigate the conformation of the polypeptide backbone in regions where changes in chemical shifts occurred.

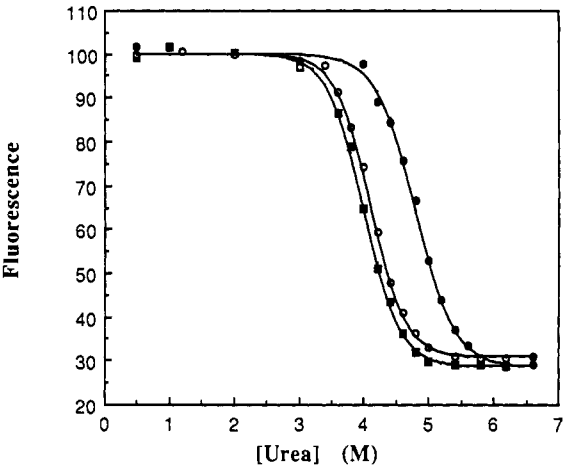

FIGURE 2: Intrinsic fluorescence of barnase as a function of urea concentration. Urea denaturation curves of wild-type barnase (1 μM) in the presence of (■) no added salt, (○) 20 mM NaCl, and (●) 20 mM NaH_2PO_4 in 46.2 mM acetic acid/30 mM sodium acetate, pH 4.5, 25 °C. It can be seen that the protein is significantly and specifically stabilized by H_2PO_4.

RESULTS

Enzyme Purification. Typical yields were 10–20 mg of protein/L of culture for wild-type barnase and His-102 → Ala, and 2–3 mg of protein/L of culture for Lys-27 → Ala. Previous attempts to purify and characterize Arg-87 → Ala were unsuccessful due to extremely low yields of enzyme obtained when cultures were grown at 37 °C (Mossakowska et al., 1989). By growing cultures at 25 °C, however, it was found that 2–3 mg of protein/L of culture could be purified.

Activity of Arg-87 → Ala. The activity of Arg-87 → Ala on RNA and GpA is <0.005% and <0.05%, respectively, of the activity of wild-type barnase.

Determination of the Inhibition Constant for Phosphate. The inhibition constant, K_i, for phosphate for wild-type barnase was determined from the initial velocity of GpC transesterification. The type of inhibition was determined by plotting $[S]/v$ versus [I], where [S] is the substrate concentration, v is the initial velocity, and [I] is the inhibitor concentration, at constant substrate concentration. This gives a family of parallel lines indicative of competitive inhibition by phosphate (Cornish-Bowden, 1979). The inhibition constant was obtained by plotting $1/v$ vs [I] at constant substrate concentration. The linear plots for each substrate concentration intersect at a phosphate concentration equal to $-K_i$, giving a value of 1.4 mM for K_i. It is likely that phosphate is binding to the site where hydrolysis occurs, i.e., at the catalytic site, because this would result in pure competitive inhibition of GpC transesterification. Similar measurements for the mutants are not possible because of their low activity. Since, however, phosphate is a pure competitive inhibitor, K_i is equal to the dissociation constant, K_d, which can be measured by urea denaturation and ^{31}P NMR.

Determination of Dissociation Constant for Phosphate. Preferential binding of a ligand to the native state of an enzyme stabilizes it against denaturation. In this study, a large specific

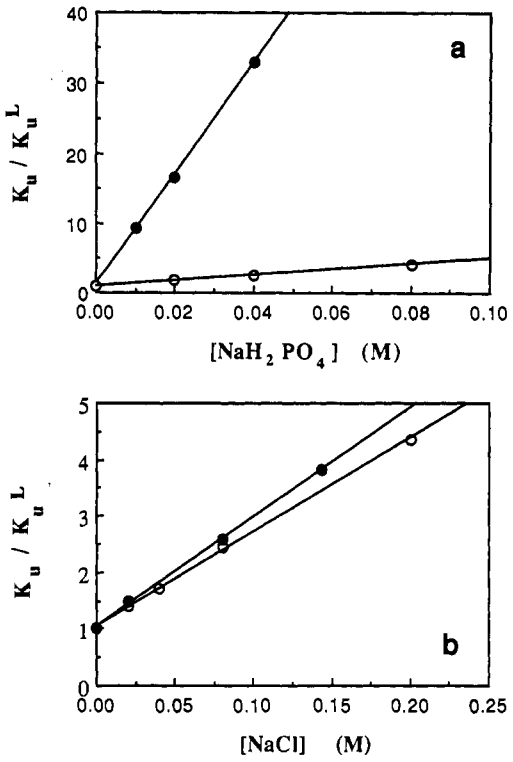

FIGURE 3: Dependence of K_u/K_u^L on ligand concentration for wild type (●) and Arg-87 → Ala (○) for (a) NaH_2PO_4 and (b) NaCl for 1 μM protein in 46.2 mM acetic acid/30 mM sodium acetate, pH 4.5, 25 °C. The plots are linear, indicating that there is one ligand binding site in the native enzyme. The slopes of the plots is $1/K_d$. Comparing (a) and (b), one can see that phosphate binding is specifically decreased in Arg-87 → Ala relative to wild type.

stabilization of wild-type barnase is observed in the presence of phosphate (Figure 2). This stabilization is largest at pH 4.5, and so further experiments were carried out at this pH. Dissociation constants of ligands were measured using urea denaturation as described below.

(a) Urea Denaturation. In the presence of a ligand which binds preferentially to one binding site in the native enzyme, the following equilibria exist in urea solution:

$$NL \rightleftarrows N + L \rightleftarrows D + L \quad (1)$$

where N is the native protein, NL is the native protein–ligand complex, D is the denatured protein, and

$$K_u = [D]/[N] \quad (2)$$

$$K_d = [N][L]/[NL] \quad (3)$$

$$K_u^L = [D]/([N] + [NL]) \quad (4)$$

where K_u is the equilibrium constant of unfolding, K_u^L is the apparent equilibrium constant of unfolding in the presence of ligand, and K_d is the native protein–ligand dissociation constant. From eqs 2–4:

$$K_u/K_u^L = 1 + [L]/K_d \quad (5)$$

There may be various nonspecific, low-affinity binding sites for ligands in both the native and the denatured protein. If so, K_u/K_u^L will vary nonlinearly with [L]. Where there is only one high-affinity binding site in the native protein, however, the effects of binding at the high-affinity site will dominate at low concentrations of ligands, and the effects of nonspecific binding will be insignificant. K_d may be determined by measuring K_u^L as a function of [L] and fitting the data to eq 5. The binding of NaH_2PO_4 and NaCl to wild-type barnase and the mutant Arg-87 → Ala was studied in this way. In order to measure K_u^L accurately, complete urea denaturation curves were determined at each ligand concentration. The fluorescence intensities of both native and denatured barnase are essentially independent of ligand concentration, and so denaturation data were fitted to the following equation using the program Enzfitter as described by Kellis et al. (1989):

$$F = F_N - (F_N - F_D)\{\exp[(m[\text{urea}] - \Delta G_u^\circ)/(RT)]\}/\{1 - \exp[(m[\text{urea}] - \Delta G_u^\circ)/(RT)]\} \quad (6)$$

Here F is the observed fluorescence, F_N is the fluorescence of native barnase, F_D is the fluorescence of denatured barnase, m is a measure of the increase in exposure of buried groups upon unfolding, and ΔG_u° is the free energy of barnase unfolding in water. The values of m and ΔG_u° were used to calculate K_u^L according to

$$K_u^L = \exp[(m[\text{urea}] - \Delta G_u^\circ)/(RT)] \quad (7)$$

The data are plotted in Figure 3, and the values of *Kd* calculated from the slopes of the plots are given in Table I. Figure 3 shows that K_u/K_u^L varies linearly with [L], in agreement with the model of one ligand binding site in the native enzyme. Both phosphate and chloride can be bound at this site, but the site is preferential for phosphate because phosphate binding is greatly weakened while chloride binding is only slightly weakened in Arg-87 → Ala compared with wild type.

(b) ^{31}P NMR. The binding of phosphate to wild-type barnase was also measured using ^{31}P NMR. This method provides a direct probe of phosphate binding compared with urea denaturation which monitors binding indirectly via changes in enzyme stability. A single ^{31}P resonance line is observed for phosphate in the presence of wild-type barnase, indicating that bound and free phosphate are in fast exchange. The observed chemical shift is, therefore, the weighted average of the chemical shift of bound and free phosphate. Solutions of barnase were titrated with phosphate and data fitted to the following single binding site equation using Enzfitter:

$$\delta_{obs} = \delta_f + (\delta_b - \delta_f) \times [\{E_0 + K_d + L - [(E_0 + K_d + L)^2 - 4E_0L]^{1/2}\}/2L] \quad (8)$$

Here δ_{obs} is the observed ^{31}P chemical shift, δ_f is the ^{31}P chemical shift of free phosphate, δ_b is the ^{31}P chemical shift of bound phosphate, E_0 is the total enzyme concentration, and L is the total phosphate concentration. The dissociation constant obtained for wild-type barnase using this method is 1.9 mM. This value is comparable to the those obtained from inhibition of GpC transesterification and urea denaturation since the experimental conditions are slightly different for this determination (see footnotes to Table I). It is not possible to

Table I: Dissociation Constants for Ions for Wild-Type and Mutant Barnases[a]

enzyme	K_d (mM) H_2PO_4	Cl	enzyme	K_d (mM) H_2PO_4	Cl
wild type	1.3	52	Arg-87 → Ala	28	61
	1.4[b]		Lys-27 → Ala	48	41
	1.9[c]		His-102 → Ala	30	88

[a] Dissociation constants measured by urea denaturation for 1 μM protein in 46.2 mM acetic acid/30.0 mM sodium acetate buffer, pH 4.5, at 25 °C. [b] Inhibition constant measured by inhibition of GpC transesterification for 1.9 μM protein in 46.2 mM acetic acid/30.0 mM sodium acetate buffer, pH 4.5, at 25 °C. [c] Dissociation constant measured by ^{31}P NMR for 4.1 mM protein in 30.3 mM acetic acid/19.7 mM sodium acetate buffer, pH 4.5, in 90% H_2O/10% D_2O, at 26.1 °C.

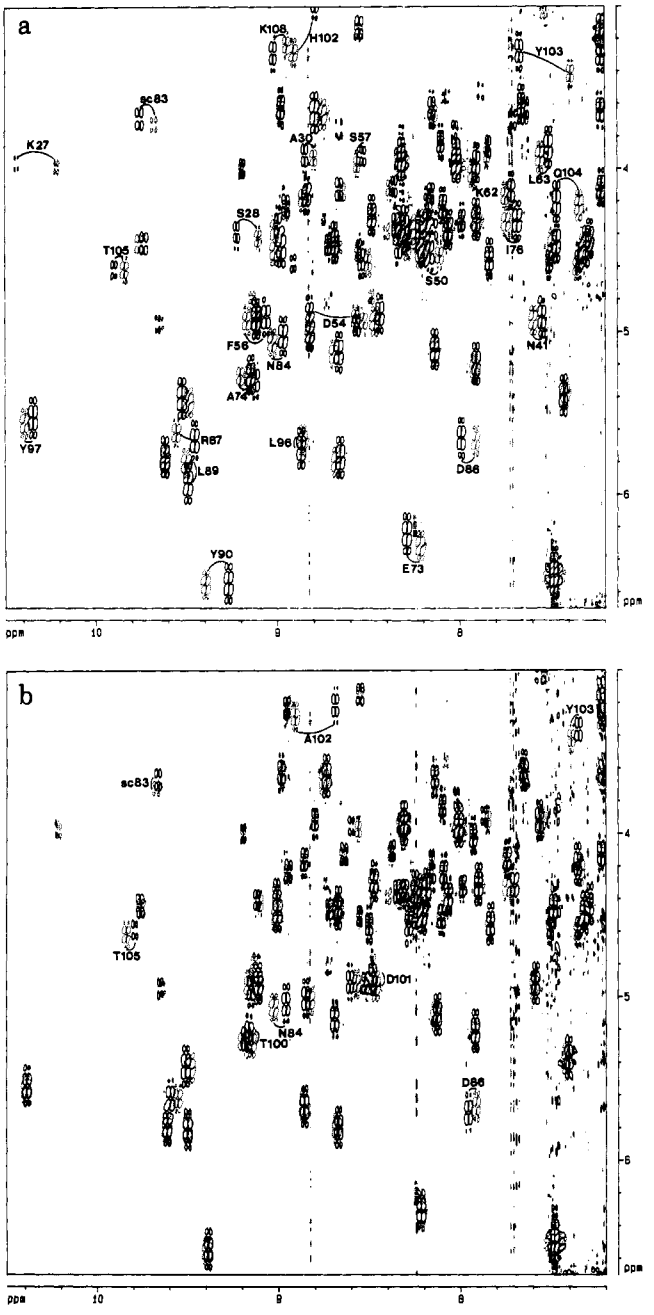

FIGURE 4: Overlay of COSY fingerprint region of (a) wild-type barnase with bound phosphate (solid cross-peaks) and without bound phosphate (dotted cross-peaks). The concentration of barnase is approximately 4 mM and the concentration of NaH_2PO_4 is 20 mM for wild type with bound phosphate. Spectra were obtained in 90% H_2O/10% D_2O, pH 4.5, 37 °C. (b) His-102 → Ala (solid cross-peaks) and wild-type barnase (dotted cross-peaks). Experimental conditions are as in (a). Cross-peaks that are at different positions in the overlayed spectra are marked with arrows. The cross-peak labeled SC83 is a cross-peak between the $C^{\delta}H$ and $N^{\epsilon}H$ of the side chain of Arg-83.

quantitate phosphate binding to mutants using this method because the dissociation constants are approximately 60–80 mM (as estimated from urea denaturation) and the upper limit on the solubility of the enzymes is 4–5 mM. Titration of His-102 → Ala and Lys-27 → Ala with phosphate causes changes in ^{31}P chemical shift of 0.01 and 0.02 ppm at enzyme concentrations of 3.76 and 3.98 mM, respectively. This is consistent with weaker binding of phosphate to the mutants compared with wild-type barnase.

2D ^1H NMR of Wild-Type Barnase with Bound Phosphate

Table II: Protons with Changes in Chemical Shift at 37 °C in Wild-Type Barnase with Bound Phosphate, His-102 → Ala, and Lys-27 → Ala Compared with Free Wild-Type Barnase[a]

		chemical shift			
protein residue	proton	wild type	wild type with bound phosphate	His-102 → Ala	Lys-27 → Ala
Lys-27	NH	10.20	**10.43**		**10.09**
	$C^{\alpha}H$	3.98			4.15
	$C^{\beta}H$	2.14, 2.06			1.73[b]
	$C^{\gamma}H$	1.63	1.53		
Ser-28	NH	9.09	**9.21**		
Ala-30	NH	8.78	8.83		
Gly-40	$C^{\alpha}H$	3.79	3.69		
Asn-41	NH	7.59	7.54		
Leu-42	NH	8.21	8.16		8.06
Ser-50	NH	8.10	8.15		
Gly-52	NH	9.24			9.17
	$C^{\alpha}H$	4.12	4.07		4.03
Asp-54	NH	8.57	**8.79**		**8.82**
Phe-56	NH	9.16	**9.04**		9.11
	$C^{\delta}H$	6.88	6.80		
	$C^{\epsilon}H$	6.77	**6.47**		
	$C^{\zeta}H$	7.37	**7.07**		
Ser-57	$C^{\alpha}H$	3.96	3.91		
Asn-58	NH	6.43	**6.18**	**6.30**	**6.30**
	$C^{\alpha}H$	4.06	**3.94**		
Lys-62	C^{H}	4.01	3.96		
	$C^{\beta}H$	1.91, 1.76	1.83, 1.69		
Leu-63	NH	7.56	7.50		
	$C^{\alpha}H$	3.92	3.86		
	$C^{\gamma}H_3$	−1.00	−1.06		
Glu-73	NH	8.21	8.27		
	$C^{\alpha}H$	6.29	6.23		
Ala-74	NH	9.19	9.10		
Ile-76	NH	7.74	7.67		
Arg-83	NH	8.39	**8.12**	**8.22**	**8.23**
	$C^{\beta}H$	2.04	1.94		
	$N^{\epsilon}H$	9.67	9.73		**9.56**
	$N^{\eta}H$	6.76	**6.53**		
Asn-84	NH	9.02	8.94	8.94	
Ser-85	$C^{\alpha}H$	4.36		**4.55**	
Asp-86	NH	7.90	7.97		
	$C^{\alpha}H$	5.65		5.71	
	$C^{\beta}H$	3.37		3.43	
Arg-87	NH	9.54	9.44		
	$C^{\alpha}H$	5.61	5.67		
	$C^{\beta}H$	1.63	1.69		
	$N^{\epsilon}H$	7.02	**7.85**		
Leu-89	$C^{\alpha}H$	5.81	**5.93**		
	$C^{\delta}H_3$	0.71, 0.48	**0.86**, 0.53		
Tyr-90	NH	9.39	**9.26**		
Ile-96	$C^{\alpha}H$	5.65	5.72		
Tyr-97	NH	10.38	10.33		
Thr-100	$C^{\alpha}H$	5.28		5.21	
Asp-101	$C^{\alpha}H$	4.93	4.88	4.87	
His-102	NH	8.87	8.77	**8.69**	
	$C^{\alpha}H$	3.26	**2.96**	3.21	
	$C^{\beta}H$	3.88, 2.97	**2.61**	**1.40**[b]	
	$C^{2}H$	7.15	**6.64**		
	$C^{4}H$	8.82	8.77		
Tyr-103	NH	7.39	**7.66**		
	$C^{\alpha}H$	3.39	**3.25**		
	$C^{\beta}H$	3.59	3.53	3.51	
	$C^{\delta}H$	6.76	6.70	**6.95**	
	$C^{\epsilon}H$	6.54	6.46	**6.75**	
Gln-104	NH	7.33	**7.45**		
Thr-105	NH	9.84	9.91	9.78	
Lys-108	NH	8.94	8.99		
Arg-110	NH	8.23	8.28		

[a] Protons that have chemical shift changes of 0.05 ppm or greater relative to the corresponding proton in wild-type barnase are listed. The chemical shifts of protons with changes greater than 0.10 ppm are in bold type. [b] Chemical shift of methyl group of alanine in the mutated residue that is a methylene group in wild-type barnase.

and Mutants. (a) Wild-Type Barnase with Bound Phosphate. The spectra of wild-type barnase with and without bound phosphate have many similarities. This can be seen in Figure 4a which is an overlay of the $C^{\alpha}H$–NH fingerprint region of the COSY spectra of wild-type barnase with and without bound phosphate, shown by solid and dotted cross-peaks, respectively. Main-chain protons from all 110 residues were assigned for wild type with bound phosphate. Protons with changes in chemical shift larger than 0.05 ppm are listed in Table II. The majority of protons in wild type with bound phoshate have the same chemical shift as in free wild type. Also, the sequential and secondary structure NOEs in wild type with bound phosphate are essentially identical to those observed in free wild type. Thus, there is no large change in the fold of the polypeptide backbone due to phosphate binding.

The location of the bound phosphate cannot be determined directly from NOE data because the phosphate has no observable protons. There is, however, a clustering of protons in the active site of barnase that exhibits relatively large changes in chemical shift when phosphate binds. This includes the main-chain NH of Lys-27, Ser-28, Asp-54, Phe-56, Asn-58, Arg-83, Tyr-90, Tyr-103, and Gln-104, the main-chain $C^{\alpha}H$ of Asn-58, Leu-89, His-102, and Tyr-103, and the side-chain protons of Phe-56, Arg-83, Arg-87, Leu-89, and His-102. The changes in chemical shift are consistent with phosphate binding in the vicinity of these protons and changing their magnetic environment. Such perturbations of chemical shift have also been used to identify a phosphate binding site in cytochrome *c* (Feng & Englander, 1990).

The chemical shifts of other protons located throughout the protein are also significantly perturbed. This may be due in part to small conformational changes. Some changes in tertiary NOEs are observed for protons in residues 54–56 and 101–103 (data not shown). Further studies are in progress to determine the solution structure of barnase complexed with phosphate and other nucleotide inhibitors.

(b) His-102 → Ala and Lys-27 → Ala. The spectra of these two mutants are almost identical to those of wild type, as can be seen in the overlay of the fingerprint region of the COSY spectra of His-102 → Ala and wild type, shown by solid and dotted cross-peaks, respectively (Figure 4b). Main-chain protons were assigned for all residues except Ser-38. It is difficult to obtain assignments for this residue because its protons resonate in crowded spectral regions and have no sequential connectivities, and the main-chain NH is not protected against exchange with solvent. Protons that have changes in chemical shift >0.05 ppm are listed in Table II. The sequential and secondary structure NOEs are the same in His-102 → Ala and Lys-27 → Ala as in wild type. Thus, these mutants have very similar structures to wild-type barnase.

The protons with changes in chemical shift are mainly in residues that are close to the site of mutation in these two proteins. In Lys-27 → Ala, the perturbed protons in residues Lys-27, Gly-52, Asp-54, Phe-56, and Arg-83 are spatially close together. The N terminus of the α_2-helix (residues 25–28) packs onto the β_1-strand (residues 50–55). The side chain of Lys-27 interacts electrostatically with the side chain of Asp-54 which in turn hydrogen bonds to the main-chain NH of Lys-27. Also, the amino group of the side chain of Lys-27 is within 4–5 Å of the guanidinium group of Arg-83. The main-chain NH's of Leu-42, Phe-56, and Asn-58 are more distant from the site of mutation, but no other protons in these residues are perturbed. No NOE changes are observed for the residues in which protons have different chemical shifts from wild type. Thus, very little structural change results from the mutation of Lys-27 to Ala, and the perturbations in

Table III: Protons with Changes in Chemical Shift at 27 °C in Arg-87 → Ala Compared with Wild-Type Barnase[a]

protein residue	proton	chemical shift: wild type	chemical shift: Arg-87 → Ala
Phe-7	NH	8.94	**8.82**
Val-10	NH	8.92	8.86
Ala-11	NH	8.31	8.26
Leu-14	NH	9.13	9.07
Asn-23	NH	8.43	8.33
Lys-27	NH	10.07	10.13
Gln-31	NH	9.26	9.32
Gly-40	$C^{\alpha}H$	2.35	2.42
Lys-49	NH	7.85	7.80
Ile-51	$C^{\beta}H$	1.40	1.46
Gly-52	NH	9.18	**9.07**
	$C^{\alpha}H$	3.98	3.92
Gly-53	NH	9.70	**9.47**
Asp-54	NH	8.52	**8.72**
Phe-56	NH	9.12	9.03
Asn-58	NH	6.32	6.42
Thr-70	NH	8.16	8.11
Trp-71	NH	9.13	9.06
	$N^{\epsilon}H$	8.83	8.76
Arg-72	NH	8.60	8.67
	$C^{\alpha}H$	5.02	5.12
	$C^{\beta}H$	0.25	0.34
Glu-73	NH	8.12	**7.97**
	$C^{\alpha}H$	6.22	**5.95**
	$C^{\beta}H$	2.20	2.13
Ala-74	NH	9.12	**9.31**
	$C^{\alpha}H$	5.18	**4.91**
	$C^{\beta}H$	1.21	1.11
Asp-75	NH	9.60	**9.37**
	$C^{\beta}H$	3.34, 2.46	**3.23, 2.67**
Ile-76	NH	7.70	**8.03**
Asn-77	NH	8.78	8.68
Gly-81	NH	8.77	8.84
Asn-84	NH	8.97	**9.11**
	$C^{\beta}H$	3.72, 3.20	**3.43**, 3.10
Ser-85	NH	8.29	8.35
	$C^{\alpha}H$	4.27	**4.38**
Asp-86	NH	7.77	7.86
	$C^{\beta}H$	3.27, 2.28	**3.08**, 2.34
Arg-87	NH	9.49	**9.34**
	$C^{\alpha}H$	5.51	**5.11**
	$C^{\beta}H$	1.54	**1.34**[b]
Leu-89	NH	9.42	9.32
	$C^{\delta}H_3$	0.66, 0.37	0.73, 0.31
Ser-92	NH	8.03	7.98
Ile-96	$C^{\alpha}H$	5.56	**5.44**
Lys-98	$C^{\alpha}H$	5.71	5.76
	$C^{\beta}H$	1.63	1.72
Thr-99	NH	8.75	**8.97**
Thr-100	NH	9.08	**8.96**
	$C^{\alpha}H$	5.20	5.26
His-102	NH	8.85	**9.23**
	$C^{\alpha}H$	3.16	**3.59**
	$C^{\beta}H$	2.87	2.82
	$C^{2}H$	7.08	**7.25**
	$C^{4}H$	8.74	**8.63**
Tyr-103	NH	7.29	**7.50**
	$C^{\delta}H$	6.67	**6.51**
Gln-104	NH	7.23	7.28
	$C^{\beta}H$	1.95	1.90
Thr-105	NH	9.77	9.72
Phe-106	NH	8.66	**8.54**
Lys-109	NH	8.96	8.91

[a] Assignments were obtained for all main-chain protons except for the $C^{\alpha}H$ of Gly-52 and the $C^{\alpha}H$ of Asp-75, which are probably obscured by the H_2O resonance in both wild type and Arg-87 → Ala. Protons that have chemical shift changes of 0.05 ppm or greater relative to the corresponding proton in wild-type barnase are listed. The chemical shifts of protons with changes greater than 0.10 ppm are in bold type. [b] Chemical shift of methyl group of alanine in the mutated residue that is a methylene group in wild-type barnase.

chemical shift are probably primarily due to changes in the local magnetic environment due to the removal of the positively charged side chain.

In His-102 → Ala the protons with perturbed chemical shifts are also close together. In the crystal structure of wild type, the ring of His-102 lies above the main-chain atoms of residues 83–86. In particular, the N and the α- and β-carbons of residue 85 are within 3–4 Å of the ring atoms. No NOE changes are observed for any of the residues in which protons have different chemical shifts from wild type. Thus, again, the changes in chemical shift of protons in residues 83–86, 101–103, and 105 are probably mainly due to changes in the magnetic environment due to removal of the histidine side chain.

(*c*) *Arg-87 → Ala.* The reduced stability of this mutant and aggregation of the protein with increased sample temperature required that NMR data be acquired at a lower temperature (27 °C). This decreased the spectral resolution because of increased line broadening and caused more peaks to be overlapped or obscured by the H_2O resonance. Main-chain assignments were, however, obtained for all residues. Although more protons have different chemical shifts from wild type in Arg-87 → Ala than in Lys-27 → Ala and His-102 → Ala, the majority of protons have the same chemical shift (Table III) and sequential NOEs. The same secondary structure NOEs are observed in the α-helices. A number of interstrand NOEs in the β-sheet (NH 53 to $C^{\alpha}H$ 74, $C^{\alpha}H$ 52 to $C^{\alpha}H$ 74, $C^{\alpha}H$ 52 to NH 75, $C^{\alpha}H$ 75 to $C^{\alpha}H$ 87) could not be compared because peaks were overlapped or obscured by the H_2O resonance; however, the majority of the β-sheet NOEs were resolved and present as in wild type. Thus, the structure of most of the polypeptide backbone is maintained in Arg-87 → Ala.

A large number of protons in the β-sheet and some in the α-helical portion of the protein have changes in chemical shift (Table III). These protons are not all located near the site of mutation. Arg-87 is in the central strand of the β-sheet and makes van der Waals contacts to many active site residues in the crystal structure of barnase. The guanidinium group hydrogen bonds to the main-chain carbonyl of Asn-84 and Asp-86, to the side-chain carbonyl of Asn-84, and to the hydroxyl of Tyr-103. It also makes electrostatic interactions with the carboxyl groups of Asp-73 and Asp-75. Many of the protons that have large changes in chemical shift are close to the side chain of Arg-87 in wild-type barnase. This includes protons in residues 73–76, 84–86, 99, 100, 102, and 103. However, a number of protons that have large changes in chemical shift, including those in residues 52–54 and 96, or smaller changes in chemical shift are distant from the site of mutation. The only marked changes in sequential NOEs, however, are between residues 84–86. Strong $d_{\beta N}$ connectivities between Asn-84 and Ser-85 and a strong d_{NN} connectivity between Ser-85 and Ser-86, which are present in wild type, are not present in this mutant, though a $d_{\alpha N}$ connectivity between Asn-84 and Ser-85 is maintained. The structure of Arg-87 → Ala is clearly disrupted around Ser-85 due to the deletion of the hydrogen bonds from the arginine side chain. It is possible that some other conformational changes occur which contribute to the changes in chemical shift of protons more distant from the site of mutation. Some changes in NOEs that are not detected due to overlap of peaks with the H_2O resonance may occur for residues 52–54.

Discussion

We have characterized a phosphate binding site in the ribonuclease barnase. The following evidence suggests that the binding site is in the active site and is the site at which hydrolysis occurs. The binding of phosphate inhibits the transesterification of GpC in a competitive manner. Site-directed mutagenesis of His-102, Arg-87, and Lys-27 to alanine greatly weakens phosphate binding in addition to greatly decreasing enzyme activity (Mossakowska et al., 1989). Protons that experience relatively large changes in chemical shift when phosphate binds are clustered in the active site, indicating that phosphate is binding in this area.

It is not possible, in general, to rationalize the chemical shifts of protons in proteins in terms of structural parameters. However, it is noteworthy that the $N^{\epsilon}H$ of Arg-87 experiences a large downfield change in chemical shift of approximately 0.8 ppm when phosphate binds. This suggests that the end of the side chain is close to the bound phosphate and may make a hydrogen bond with it. Similar downfield shifts have been observed for protons in the guanidinium groups of arginines that are involved in salt bridges (Hyberts & Wagner, 1990; Bycroft et al., 1991) and main-chain NH protons in *ras* complexed with GDP that are proposed to hydrogen bond to a phosphate group (Redfield & Papastavros, 1990). One might expect that the chemical shift of the C^2H of His-102 would also be shifted downfield if it interacts with phosphate. This has been observed for histidines in bovine pancreatic RNase A when they interact with phosphate-containing ligands (Jardetsky & Roberts, 1981). In barnase, the C^2H of His-102 experiences a large upfield change in chemical shift of approximately 0.5 ppm when phosphate is bound. This may be because the ring of His-102 moves closer to the aromatic ring of Tyr-103.

The global fold of the polypeptide backbone seems to be unaffected by phosphate binding because the sequential and secondary structure NOEs are qualitatively the same in free wild type and wild type with bound phosphate. It is, however, possible that small changes in the main-chain conformation occur that have not been detected by the qualitative comparison of NOEs. This, together with the conformational changes of side chains, may be part of the reason why many protons in the β-sheet have relatively large changes in chemical shift. Changes in tertiary NOEs for protons in residues 54–56 and 101–103 suggest that small conformational changes may occur in these residues when phosphate binds. The solution structures of wild-type barnase complexed with phosphate and other inhibitors are in progress to define these changes and will be reported elsewhere.

The phosphate binding site at the catalytic site is specific for phosphate. Phosphate stabilizes the enzyem to urea denaturation far more than does chloride. The residues that are involved in phosphate binding originate in different parts of the structure of barnase and point into the binding site from various directions (Figure 1). The ϵ-amino group of Lys-27 is approximately 9–11 and 12–14 Å from the guanidinium portion of Arg-87 and the ring of His-102, respectively. The guanidinium portion of Arg-87 is within 4–6 Å of the ring of His-102. This positioning of the residues allows sufficient space for a phosphate group to be bound, and all the residues can simultaneously interact with phosphate. The positioning of the residues may account for the specificity of the binding site for phosphate because a smaller anion such as chloride could not interact strongly with all the binding residues at the same time.

The binding of phosphate to barnase measured by inhibition of transesterification, stabilization to urea denaturation, and ^{31}P NMR fits a single binding site model with a moderate K_d of approximately 1.3 mM. This value of K_d is comparable to the values of approximately 4 mM and 3.5 mM obtained for

phosphate binding to RNase A and RNase T1, respectively (Anderson et al., 1968; Pace & Grimsley, 1988). Barnase may have other binding sites for the phosphate portions of its RNA substrate. Kinetic studies of transesterification of dinucleotides, trinucleotides, and tetranucleotides (A. Day, unpublished data) support the existence of such sites at positions other than the catalytic site. The binding of phosphate to these subsites is not detected here, and so they must be relatively low-affinity binding sites compared with the major phosphate binding site at the catalytic site.

Functional and Structural Roles of His-102, Lys-27, and Arg-87. The mutation of His-102, Lys-27, and Arg-87 to alanine specifically decreases the binding of phosphate as measured by urea denaturation and ^{31}P NMR. The decrease in binding could be due to the removal of a positively charged side chain that interacts electrostatically with phosphate and/or to structural changes due to the mutation. We have investigated the structural consequences of the mutations using ^1H 2D NMR.

In the ^1H 2D NMR spectra of His-102 → Ala and Lys-27 → Ala, very few protons have different chemical shifts from wild type, and the sequential and secondary structure NOEs of the mutants are essentially identical to those in wild type. This is strong evidence that the structures of His-102 → Ala and Lys-27 → Ala are very similar to that of wild-type barnase. Some protons in residues close to the site of mutation in the primary sequence and in the tertiary structure do experience small changes in chemical shift. This may be related to minor local conformational changes. The structure of most of the protein, including the region of the active site and phosphate binding site, is unaffected by the mutations; therefore, His-102 → Ala and Lys-27 → Ala are nondisruptive deletions and the decreased binding of phosphate by these mutants is probably due to the removal of positively charged side chains that interact electrostatically with it in wild-type barnase.

For nondisruptive deletions, the apparent binding energy of a side chain and a ligand can be calculated from the ratio of the dissociation constants of wild-type and mutant enzyme (Fersht, 1988). The apparent binding energy of His-102 and Lys-27 with phosphate is approximately 1.9 and 2.1 kcal/mol, respectively. This binding energy may approximate the incremental binding energy for these side chains and phosphate because the mutations His-102 → Ala and Lys-27 → Ala probably allow the access of water to the site of mutation and the ligand (Fersht, 1988).

The NMR data indicate that structural changes occur in Arg-87 → Ala. The global fold of the polypeptide backbone is maintained because many protons have the same chemical shift and sequential and secondary structure NOEs as in wild-type barnase. However, structural changes occur in the polypeptide backbone of residues 84–86 since large changes in chemical shift and sequential NOEs are observed for protons in these residues. Many other protons throughout the β-sheet also have perturbed chemical shifts perhaps due to other small conformational changes. Folkers et al. (1989) have found that the mutation of an active site Lys to Glu in hirudin also causes a small structural change in the vicinity of the mutation and that a number or protons, particularly NH and $C^{\alpha}H$ protons, that are distant from the site of mutation have perturbed chemical shifts. Clearly, large changes in chemical shift must not be overinterpreted as evidence for large structural changes in the absence of NOE data; however, they can be sensitive indicators of potential regions of structural change.

As Arg-87 makes many contacts in the crystal structure of barnase and some structural changes occur in Arg-87 → Ala, this residue plays a role in forming the structure of the active site. Arg-87 → Ala is a disruptive deletion, and so the energetic contribution of this side chain to phosphate binding cannot be determined using this mutant. It is likely, however, that Arg-87 contributes to phosphate binding by electrostatic interactions because the chemical shift of the $N^{\epsilon}H$ of 87 is greatly affected when phosphate binds to wild-type barnase.

Relation of Phosphate Binding to Enzyme Activity. The decrease in the binding of phosphate due to mutating His-102, Lys-27, and Arg-87 to Ala is correlated with a dramatic decrease in activity of the mutant enzymes (Mossakowska et al., 1989). This suggests that these residues have important structural and/or functional roles in the binding of phosphate during catalysis. In particular, Lys-27 is probably involved in the binding of phosphate in the transition state rather than the ground state of GpA transesterification because mutation of this residue affects mainly k_{cat} rather than K_m (Mossakowska et al., 1989).

Application of Phosphate Binding Studies for de Novo Design of Phosphate Binding Sites. Phosphate binding by barnase is effected mainly by a group of positively charged side chains. Electrostatic interactions between the negatively charged phosphate and positively charged side chains are relatively strong (e.g., an individual side chain may contribute up to 2 kcal/mol to the binding of phosphate by barnase) and are less sensitive to distance and geometry than other noncovalent interactions. Minimal conformational changes accompany the binding of phosphate by barnase. This suggests that it may be feasible to engineer phosphate binding sites onto the surface of a protein by using the protein backbone as a scaffold for positively charged residues. The requirements for a phosphate binding site of moderate affinity may be less stringent than, for example, a high-affinity metal binding site. Electrostatic interactions in proteins are becoming better understood (Sternberg et al., 1987; Åqvist et al., 1991; Sharp & Honig, 1990), and the binding of phosphate to a binding site in the active site of RNase A has been successfully modeled (Matthew & Richards, 1982). It may now be possible to engineer phosphate binding sites in proteins de novo.

Phosphate binding sites could be engineered for a variety of applications. They could, for example, be used to stabilize proteins (Pace & Grimsley, 1988). Barnase is stabilized by approximately 1.3 kcal/mol in 10 mM H_2PO_4 and 2.6 kcal/mol in 100 mM H_2PO_4, due to the binding of phosphate. Clearly, it is possible to significantly stabilize a protein at moderate concentrations of ligand by engineering a relatively moderate-affinity binding site. Phosphate binding sites may also be designed to bind the phosphoryl portions of a wide variety of biological molecules such as substrates, cofactors, or allosteric effectors.

Registry No. His, 71-00-1; Lys, 56-87-1; Arg, 74-79-3; barnase, 37300-74-6; phosphate, 14265-44-2.

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