High-Resolution Structure of the Temperature-Sensitive Mutant of Phage Lysozyme, Arg 96 → His†

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ABSTRACT: The structure of the temperature-sensitive mutant lysozyme of bacteriophage T4 in which arginine 96 is replaced by histidine has been determined crystallographically and refined to a residual of 17.6% at 1.9-Å resolution. Overall, the three-dimensional structure of the mutant protein is extremely similar to that of wild type. There are local distortions in the mutant structure suggesting that the substituted His 96 residue is under strain. This appears to be one of the major reasons for the decreased thermostability. In wild-type lysozyme the guanidinium of Arg 96 is located at the carboxy terminus of α-helix 82–90 and makes a pair of hydrogen bonds to two of the carbonyl groups in the last turn of the helix. The loss of this "helix dipole" interaction also appears to contribute to the destabilization. The pK_a* of His 96 in the mutant lysozyme has been determined by nuclear magnetic resonance and found to be 6.8 at 10 °C. This relatively normal value of the histidine pK_a* suggests that the protonated and unprotonated forms of the imidazole ring are perturbed equally by the protein environment or, what is equivalent, the mutant lysozyme is equally stable with either histidine species.

The lysozyme from bacteriophage T4 is being used as a model system to study the effects of mutations on the structure, folding, stability, dynamics, and spectroscopic properties of proteins [e.g., see Alber et al. (1986, 1987a,b), Matthews (1987), Hawkes et al. (1984), Becktel and Baase (1987), Becktel and Schellman (1987), Griffey et al. (1985), and McIntosh et al. (1987) and references cited therein].

One of the advantages of the lysozyme system is that temperature-sensitive mutants can be selected by a random genetic screen (Streisinger et al., 1961). One such variant is the lysozyme in which Arg 96 is replaced by histidine (Grütter et al., 1979). The mutant was initially identified by its laboratory code name N20. Subsequently, the mutant lysozyme has been given the rational name R96H. Preliminary crystallographic analysis showed that the three-dimensional structure of R96H was very similar to that of wild type, even though it was substantially less thermostable (Grütter et al., 1979). This mutant lysozyme has been the subject of extensive thermodynamic analysis (Grütter et al., 1979; Hawkes et al., 1984; Becktel & Baase, 1987; Becktel & Schellman, 1987; Kitamura & Sturtevant, 1989; W. Becktel and W. Baase, personal communication). In this paper we describe the detailed, high-resolution structure of R96H on the basis of crystallographic refinement at 1.9-Å resolution. Also, the pK_a of His 96 in the mutant lysozyme has been determined by nuclear magnetic resonance.

Materials and Methods

Crystallography. The methods used for the genetic selection, identification, purification, and crystallization of the mutant lysozyme have been outlined by Grütter et al. (1979). Additional details on the selection procedure are given by Grütter et al. (1987) and Alber and Matthews (1987). Prior to X-ray photography, the crystals were equilibrated with a "standard mother liquor" of 1.05 M K_2HPO_4, 1.26 M NaH_2PO_4, 0.23 M NaCl, and 1.4 mM mercaptoethanol, pH 6.7.

The initial crystallographic analysis was to a nominal resolution of 2.4 Å on the basis of data measured by precession photography (Grütter et al., 1979). Data to 1.9-Å resolution were collected by oscillation methods (Schmid et al., 1981). Details of the data-collection statistics are given in Table I. Within experimental error the cell dimensions of the mutant lysozyme are the same as those of wild type.

Initial crystallographic refinement was carried out with the program EREF (Jack & Levitt, 1978) and was subsequently continued with the TNT package of programs developed in this laboratory (Tronrud et al., 1987). The final crystallographic R value was 17.6% for a model with good stereochemistry (Table I). The estimated overall accuracy of the coordinates on the basis of Luzzati plots (not shown) and comparisons with other mutant lysozymes (see below) is about 0.15 Å. The refined wild-type coordinates used for comparison with the R96H structure were similar to those reported by Weaver and Matthews (1987) but subjected to additional refinement with

Table I: Data Processing and Refinement Statistics

data processing	
film packs	27
total reflections measured	45 064
no. of independent reflections	15 106
R_{merge} (on intensities) (%)[a]	8.7
cell dimension	
a, b (Å)	61.2
c (Å)	96.8
refinement statistics	
average deviations from ideal values	
bond lengths (Å)	0.019
bond angles (deg)	3.2
planarity (Å)	0.013
resolution limits (Å)	6.0–1.9
reflections included	14 300
crystallographic residual (%)	17.6

[a] R_{merge} is the agreement between equivalent intensities measured on different films.

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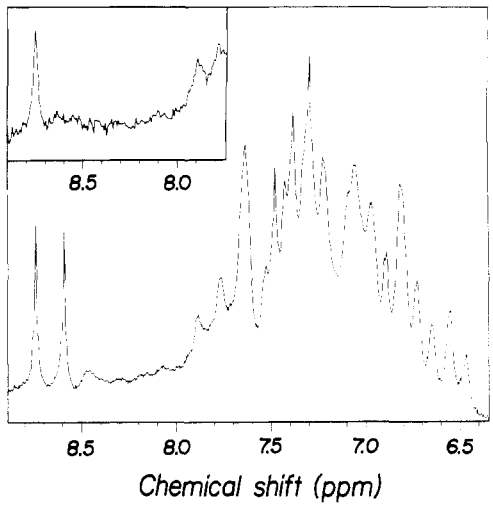

FIGURE 1: Proton magnetic resonance spectrum of the downfield resonances of R96H lysozyme at pH* 4.5 in 95% D_2O. Prior to recording of this spectrum, the protein was incubated at 35 °C and pH 2.0 for 1 h to exchange amide hydrogens. The resonances at 8.75 and 8.60 ppm are due to the C2 protons of His 31 and His 96. The insert shows the corresponding part of the spectrum of wild-type lysozyme which includes the single resonance due to His 31.

recollected native data (unpublished results).

Magnetic Resonance. Proton magnetic resonance spectra were obtained at 500.1 MHz on a GN-500 spectrometer. Protein concentrations ranged from 2 to 10 mg/mL in 100 mM KCl–10 mM phosphate buffer. No changes in chemical shift of line widths were observed over this concentration range. Chemical shifts are reported relative to the most upfield (valine 94) methyl resonance of T4 lysozyme at −0.70 ppm. Spectra were obtained in D_2O containing 1–10% H_2O with an off-resonance jump–return pulse sequence to suppress residual water signals (Plateau & Gueron, 1983). The pH values reported are those obtained by standardization in H_2O with no correction for isotope effects.

Results

Crystallography. The crystallographic refinement confirms the initial finding that the structure of R96H is very similar to that of wild-type lysozyme, except for the replacement of the side chain at position 96. Prior to detailed structure comparison, the mutant lysozyme coordinates were rotated and translated to minimize the root mean square deviation from those of wild type. The transformation matrix is

$$X' = 1.000X + 0.0004Y + 0.000Z + 0.197$$
$$Y' = -0.0004X + 1.0000Y + 0.0012Z - 0.073$$
$$Z' = 0.000X - 0.0012Y + 1.000Z - 0.049$$

(X, Y, Z) are the refined mutant lysozyme coordinates in the standard lysozyme Cartesian system (Weaver & Matthews, 1987). These coordinates will be deposited in the Protein Data Bank concurrent with the publication of this paper.

Following the above transformation the rms discrepancy between all coordinates common to the mutant and wild-type protein structures is 0.16 Å. (This comparison excludes the side chains of Glu 5 and Asn 68 which appear to be mobile and poorly defined.) For the respective main-chain atoms the rms difference is 0.15 Å. These numbers presumably reflect the level of error in the refined coordinates.

Magnetic Resonance. Figure 1 shows the proton magnetic resonance spectrum of R96H at pH* 4.5. Two sharp downfield proton peaks are observed. Comparison with the corresponding spectrum for wild-type lysozyme shows that the resonance at 8.75 ppm corresponds to the C2 proton of the single histidine (His 31) and the resonance at 8.60 ppm is due to His 96.

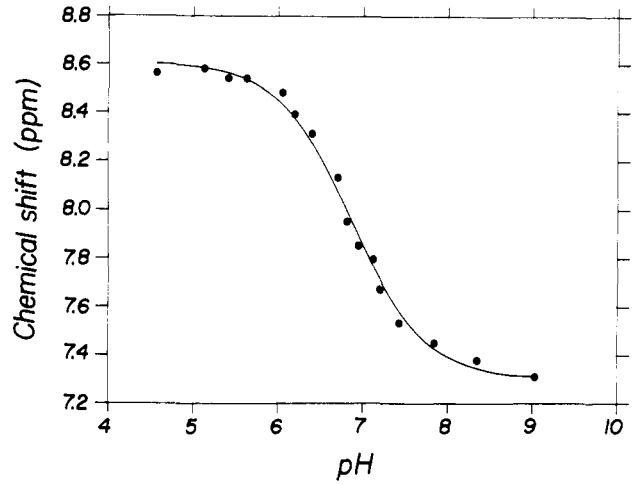

FIGURE 2: pH dependence of the chemical shift of the C2 proton of histidine 96. Experimental data are shown as solid circles. The continuous line shows the theoretical dependence corresponding to a single pK_a* of 6.8.

Figure 2 shows the pH* dependence of the chemical shift of the C2 proton of histidine residue 96. The continuous line represents a best-fit least-squares fit of the data to a single pK_a* of 6.8. The temperature dependence of this pK_a was monitored by observation of a sample at pH 6.6 in the range 10–30 °C. At higher temperatures irreversible aggregation was observed. The ΔH of ionization was estimated to be about 5 kcal/mol over the temperature range 10–30 °C, consistent with values in the literature (Cohn & Edsall, 1965). This extrapolates to a pK_a* value for histidine residue 96 of about 6.2 at 60 °C.

Discussion

The exact reduction in free energy of unfolding of the temperature-sensitive lysozyme R96H relative to that of wild type depends somewhat on salt concentration, pH, and temperature (Grütter et al., 1979; Hawkes et al., 1984; Becktel & Baase, 1987; Becktel & Schellman, 1987; Kitamura & Sturtevant, 1988; W. Becktel, personal communication). A representative value measured in 20 mM KH_2PO_4, 25 mM KCl, and 0.1 mM dithiothreitol is $\Delta\Delta G$ = 3.2 kcal mol^{-1} at pH 3.0 and 40 °C (Kitamura & Sturtevant, 1989).

Arginine 96 in wild-type lysozyme forms part of the surface of the molecule (Figure 3). Its side chain is well packed between the backbone of residues 91–93 and the side chain of Tyr 88. The guanidinium group forms two hydrogen bonds to the backbone carbonyls of Tyr 88 and Asp 89. These multiple interactions are reflected in the fact that the side chain is well ordered, with an average crystallographic thermal factor of 21.6 Å^2. It should be mentioned, however, that nitrogen HN1 of Arg 96 makes contacts with Asp 52 and Arg 76 of an adjacent lysozyme molecule in the crystal. These contacts could also help to inhibit motion of the side chain in the crystal. The average thermal factor for all side chains is 26.5 Å^2.

In the structure of the mutant lysozyme, His 96 is also relatively well ordered with an average thermal factor of 25.2 Å^2 for the side-chain atoms. The histidine variant lacks the intermolecular crystal contacts observed at this site in the wild-type structure, but this does not noticeably interfere with growth of isomorphous crystals.

As can be seen in Figure 4, the side chain of the histidine in several respects mimics that of the arginine. In particular, carbon CD2 of the histidine approximately superimposes on

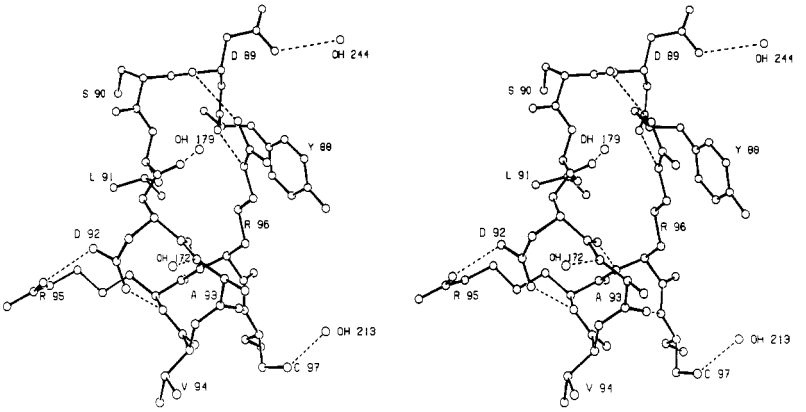

FIGURE 3: Stereo drawing showing the environment of Arg 96 in wild-type lysozyme. Amino acids are identified by the one-letter code. Hydrogen bonds are shown as broken lines.

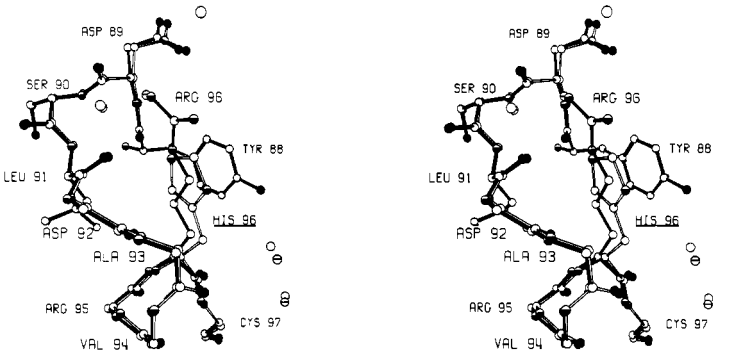

FIGURE 4: Superposition of the structure of the mutant lysozyme Arg 96 → His (open bonds) on that of wild type (solid bonds). For clarity, a number of the side chains have been omitted. Oxygen atoms are drawn solid, nitrogen are drawn half-solid, and carbon atoms are drawn as open circles. Water molecules present in the wild-type structure are drawn as open circles; those present in the mutant structure are indicated by a horizontal line.

CG of the arginine, and the NE2 and NE nitrogens are also close in space. Nitrogen NH2 of the arginine has no direct counterpart in the histidine but is replaced by a bound water molecule in the R96H structure (Figure 4). Furthermore, the histidine also retains the positive charge of the arginine, at least under acidic conditions. Why, then, is the mutant structure less stable than wild type? The following factors appear to contribute.

(*i*) *Introduction of Strain.* The refined crystal structure of R96H suggests that the destablization of the mutant structure may, in part, be due to the introduction of strain. As can be seen in Figure 4, the His 96 side chain is "wedged" between the side chain of Tyr 88 and the backbone of residues 91–93. Nitrogen NE2 of the imidazole is 2.73 Å from the carbonyl oxygen of Tyr 88. Inspection of the crystal structures (Figure 4) suggests that this contact is actually an unfavorable one and results in the His 96 side chain being forced "outward" toward the surface of the molecule. In the structure of R96H (Figure 4) the β-carbon of His 96 is displaced 0.60 Å relative to that of Arg 96, and the γ-carbon is displaced 0.89 Å. This structural change is localized to the side-chain atoms; the respective main chains of residue 96 in the mutant and wild-type structures have practically identical conformations. Inspection of the refined structures shows that the geometry of the α-carbon of Arg 96 in wild-type lysozyme is very close to tetrahedral (Table II). In contrast, the angle C–CA–CB in the mutant structure is 11.1° less than its "ideal" value (Table II). The average deviation of all bond angles in the mutant structure from their ideal values is 3.2° (Table I), which provides an upper estimate for the probable error in the bond angles. On this basis, the apparent strain in the His 96 C–CA–CB angle is statistically significant (3.5σ). Furthermore,

Table II: Comparison of the Geometry of Residue 96 in Wild-Type and R96H Lysozymes

		wild type, Arg 96		mutant, His 96	
bond angle	ideal value (deg)	obsd angle (deg)	deviation from ideal (deg)	obsd angle (deg)	deviation from ideal (deg)
N–CA–C	112.0	109.5	−2.5	107.8	−4.2
N–CA–CB	112.0	111.2	−0.8	110.9	−1.1
C–CA–CB	111.0	110.8	−0.2	99.9	−11.1
CA–CB–CG	113.0	111.7	−1.3	110.8	−1.2

the 11.1° deviation of this bond angle from ideal is actually the largest observed for any bond angle in the entire refined mutant structure [excepting two residues at the extreme C-terminus whose coordinates are known to be unreliable (Weaver & Matthews, 1987)]. The apparent distortion of the C–CA–CB bond angle in the mutant structure corresponds to an estimated strain energy of 2.0 kcal mol^{-1} (Brooks et al., 1983). This corresponds to more than half of the observed loss of stability of R96H relative to that of wild-type lysozyme.

(*ii*) *Changes in Helix Dipole Interactions.* The guanidinium of Arg 96 is perfectly placed to make favorable interactions with the carboxyl terminus of the α-helix that includes residues 82–90. Two of the guanidinium nitrogens make hydrogen bonds to two of the peptide carbonyls within the last turn of the helix. In addition, the position occupied by the positively charged guanidinium moiety is close to the helix axis (Figure 3). Whether one thinks of a helix dipole (Blagdon & Goodman, 1975; Wada, 1976; Hol et al., 1978) as a macrodipole with effective positive and negative changes at the ends of the helix or as localized hydrogen-bond donors and acceptors at

the ends of the helix, it is clear that Arg 96 is optimally positioned to interact favorably with the C-terminus of α-helix 82–90.

Replacement of Arg 96 with a histidine alters these interactions in two obvious ways. First, the two hydrogen bonds of the arginine are replaced by one from the histidine (and, as discussed below, the geometry of this H-bond is very poor). Second, the histidine either has no charge or, in the protonated form, has the charge located about 5 Å off the axis of the helix.

The Arg 96 → His substitution therefore apparently weakens the helix dipole interactions of helix 82–90. Mitchison and Baldwin (1986) have shown that changes in the charge at the end of the C-peptide of ribonuclease A can alter the T_m by up to 6 °C and the stability of the protein by an estimated 1–2 kcal mol^{-1}. Theoretical estimates (Hol et al., 1978) suggest that the energy of interaction of a helix dipole with a charged group may be substantially higher. Therefore, a substantial fraction of the reduction in free energy of unfolding could come from the loss of helix dipole stabilization in the mutant structure.

It can be asked whether the disruption of the helix dipole interaction causes changes in the helix. There is no indication of significant structural adjustments of this sort. The two hydrogen-bonding carbonyl groups at the end of the helix (Tyr 88 and Asp 89) do, however, become slightly more mobile in the mutant structure with crystallographic thermal factors increasing from (14.9 Å^2, 18.5 Å^2) to (20.1 Å^2, 25.1 Å^2). There is an interesting situation near the other end of the helix where residues 75–82 move bodily by about 0.3 Å. (Maximum movements in backbone atoms are 0.41 Å for the carbonyl oxygens of Leu 79 and Asn 81.) No other segment of the backbone shows a comparable shift. It is tempting to speculate that this small but apparently significant alteration in the backbone conformation might be due to some signal being transmitted via the helix, but a more plausible reason involves changes in crystal contacts. As mentioned above, the Arg 96 → His substitution causes changes in crystal contacts with Arg 76. The residue is within the long α-helix 60–80, and it is possible that this altered contact could cause the changes seen for the 75–82 backbone segments.

As was noted above, the imidazole of His 96 is in the vicinity of the C-terminus of α-helix 82–90 and is about 5 Å off-axis. The histidine is also located in the first turn (i.e., toward the amino terminus) of α-helix 93–106 and in this case is about 5.5 Å from the helix axis (Figure 4). In addition, the imidazole of His 96 is about 6.5 Å from the carboxylate of Asp 92, a residue that stabilizes the helix dipole of helix 93–106 (Figure 3). The observation that the pK_a of His 96 is essentially unperturbed shows that the effects of these neighborhood α-helix dipoles and charged groups must essentially cancel, so far as His 96 is concerned.

(*iii*) *Hydrogen-Bonding Geometry.* It was noted in the previous section that nitrogen NE2 of His 96 is 2.73 Å from the carbonyl oxygen of Tyr 88. At first glance (Figure 4) this appears to suggest that there is a strong hydrogen bond between these two atoms, analogous to one of the hydrogen bonds formed by the guanidinium of Arg 96. More detailed analysis, however, shows that the geometry for a hydrogen bond is poor. The angle His 96 NE2—H···O(=C) Tyr 88 is 131°. Both in small molecule structures and in proteins the N–H···O angle is rarely less than 140° (Olovsson & Jönsson, 1976; Baker & Hubbard, 1984). Therefore rather than having an energetically favorable hydrogen bond between NE2 and the carbonyl oxygen of Tyr 88, the actual geometry suggests that the hydrogen bond is weak at best.

This analysis assumes that NE2 is protonated. Because the X-ray analysis does not reveal the locations of the proton(s), one also has to consider the possibility that NE2 might not be protonated, at least at higher pH values. This, however, seems unlikely since deprotonation of NE2 would leave both the NE2 nitrogen and the carbonyl oxygen of Tyr 88 as buried, nonsatisfied, hydrogen-bond acceptors (Figure 4). Rather, it seems more reasonable to assume that NE2, the buried nitrogen of His 96 (Figure 4), remains protonated at all pH values, while ND1, the solvent-exposed nitrogen, changes its protonation state as the imidazole is titrated. This is also supported by the NMR results showing the histidine pK_a* to be 6.8, a relatively normal value, suggesting that the protonated and the unprotonated forms of His 96 are stabilized (or destabilized) essentially equally by the native environment. The crystal structure analysis was carried out at pH 6.7 and so presumably represents an average of the mutant structures in which His 96 is charged and neutral. Because His 96 is seen to be well ordered in the R96H crystal structure, it suggests that the imidazole undergoes little if any conformational change on titration (est. $\lesssim$0.2 Å). This is also consistent with nitrogen NE2 remaining protonated at low and high pH values.

(*iv*) *Changes in Solvent Structure.* The mutant lysozyme has a bound water molecule (Figure 4) not present in the native structure. Consideration of other mutant lysozyme structures suggests that the localization of water molecules is common and need have little to do with changes in the free energy of folding of a mutant structure relative to that of wild type. The mutant structures Thr 157 → Ile (Grütter et al., 1987), Thr 157 → Gly (Alber et al., 1987), Gly 156 → Asp (Gray & Matthews, 1987), and Ile 3 → Tyr (Masazumi et al., 1988) all have a localized solvent molecule not seen in wild-type lysozyme and are less stable. In addition the variant Ala 82 → Pro lacks a bound solvent present in wild-type lysozyme and is more thermostable (Matthews et al., 1987). Thus, in all four cases the "bound-water" variant is the least stable. On the other hand there are other factors that are thought to explain the changes in thermostability observed in each of the above cases.

The most direct information available from mutant lysozyme structures on the energetic consequences of bound solvent is provided by the two variants with serine and glycine at position 157 (Alber et al., 1987b). In this case the glycine structure has a bound water molecule that mimics the hydrogen-bond network formed by the serine. The hydrogen-bonding geometry in the two structures is very similar, and the free energy of stabilization of the Gly variant is only 0.45 kcal mol^{-1} less than that of the Ser 157 lysozyme (Alber et al., 1987b). This relatively small difference could, in turn, be attributed to the difference in backbone entropy of folding for serine and glycine (Nemethy et al., 1966; Matthews et al., 1987). Thus, the Gly/Ser 157 comparison suggests that the hydrogen-bond energy of the bound water molecule is essentially equal to the entropy cost of its immobilization. In the present case the new water molecule bound to the R96H structure also makes several hydrogen bonds, to the carbonyl oxygens of Asp 89 and Leu 91 and to another solvent molecule (Figure 2). Taking the Gly/Ser 157 lysozyme pair as a model, it would appear that the localization of the additional water molecule seen in the R96H structure contributes little, if any, to the destabilization of the mutant structure relative to wild type.

Summary

The observed temperature sensitivity of the mutant lysozyme R96H appears to be due primarily to two factors. First, the replacement with histidine introduces strain into the folded

mutant structure, which is manifested by distortion in the bond angles at the α-carbon of the substituted residue. Second, the loss of Arg 96 eliminates favorable helix dipole interactions with the C-terminus of helix 82–90.

Consistent with other mutant lysozymes (Alber et al., 1987a), the consequences of the Arg 96 → His substitution can be explained in terms of alterations to the rigid parts of the folded (as opposed to the unfolded) protein.

The results suggest that the use of site-directed mutagenesis to introduce favorable helix dipole interactions or to eliminate strain in the folded structure of a protein may lead to increased thermostability. In fact, some success along both these lines has already been obtained (Nicholson et al., 1988; unpublished observations).

The results also highlight the need for sophistication in the calculation of protein energetics. For example, an appropriate algorithm needs to be developed to model helix dipole interactions. Also, procedures that assume rigid bond angles would be incapable of modeling the type of structural distortion seen in this particular mutant lysozyme structure.

It should be noted that the availability of the refined wild-type and R96H mutant coordinates supports some but not all of the conclusions reached in the initial report of this mutant (Grütter et al., 1979). The main finding, namely, that the structure of the temperature-sensitive lysozyme is virtually identical with that of native lysozyme except for the replacement of Arg 96 by histidine, is confirmed by the present work. It was argued previously that the replacement of the hydrocarbon portion of the Arg 96 side chain with an imidazole might destabilize the hydrophobic core of the molecule, but the correspondence between the Arg and His side chains seen in the refined structures (Figure 4) suggests that this is probably not the case. It was also argued that the introduction of the histidine might destabilize α-helix 82–90. This now appears to be the case but is rationalized in terms of the loss of interactions with the helix dipole rather than the reasons given initially.

Understanding the properties of mutant proteins is seen to require not only their crystal structures but the structures refined to high resolution. In addition, the pH-dependent properties of ionizable centers are required to fully understand their energetic role in the folded structure.

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