Tolerance of T4 Lysozyme to Proline Substitutions within the Long Interdomain α-Helix Illustrates the Adaptability of Proteins to Potentially Destabilizing Lesions*

(Received for publication, September 13, 1991)

Uwe H. Sauer‡, Sun Dao-pin§, and Brian W. Matthews¶

From the Institute of Molecular Biology, Howard Hughes Medical Institute and Department of Physics, University of Oregon, Eugene, Oregon 97403

To investigate the ability of a protein to accommodate potentially destabilizing amino acid substitutions, and also to investigate the steric requirements for catalysis, proline was substituted at different sites within the long α-helix that connects the amino-terminal and carboxyl-terminal domains of T4 lysozyme. Of the four substitutions attempted, three yielded folded, functional proteins. The catalytic activities of these three mutant proteins (Q69P, D72P, and A74P) were 60–90% that of wild-type. Their melting temperatures were 7–12 °C less than that of wild-type at pH 6.5. Mutant D72P formed crystals isomorphous with wild-type allowing the structure to be determined at high resolution. In the crystal structure of wild-type lysozyme the interdomain α-helix has an overall bend angle of 8.5°. In the mutant structure the introduction of the proline causes this bend angle to increase to 14° and also causes a corresponding rotation of 5.5° of carboxyl-terminal domain relative to the amino-terminal one. Except for the immediate location of the proline substitution there is very little change in the geometry of the interdomain α-helix. The results support the view that protein structures are adaptable and can compensate for potentially destabilizing amino acid substitutions. The results also suggest that the precise shape of the active site cleft of T4 lysozyme is not critical for catalysis.

Phage T4 lysozyme is a small monomeric protein with its structure divided into two distinct domains (Fig. 1). The active site is located at the junction of the two domains, and it might be expected that the alignment of one domain relative to the other would be critical for catalytic activity (cf. Storm and Koshland, 1970, but see also Jenkins, 1969; Knowles, 1991). On the other hand, the crystal structure of a fully active mutant of T4 lysozyme has recently been described in which there is substantial variability in the "hinge-bending angle" between one domain and the other (Faber and Matthews, 1990). To investigate the ability of T4 lysozyme to compensate for disruptive changes in its structure and to determine the need to conserve the alignment of the active site cleft, a series of proline substitutions was made in the long interdomain α-helix. Studies of proline substitutions and proline replacements in proteins and peptides include Matthews et al. (1987), Alber et al. (1988), O’Neil and DeGrado (1990), Strehlow et al. (1991), and Consler et al. (1991), among others.

The amino acids chosen for substitution with proline, Gin-69, Val-71, Asp-72 and Ala-74, are located in the middle of the long α-helix (residues 60–80) that connects the two domains of T4 lysozyme (Fig. 1). It was expected that the substitution of a proline at any of these sites would tend to significantly distort the α-helix and therefore change the alignment of the "upper" and "lower" domain. By making a series of replacements it was anticipated that the active-site cleft would be distorted in different ways. Also by including different substitutions it was possible to include sites that were both buried and solvent-exposed.

Gln-69 is largely exposed to solvent (Table I, Fig. 1) and its side chain does not obviously participate in stabilizing interactions with other parts of the protein. Val-71 is almost but not entirely buried. Its side chain makes many contacts with the non-polar residues Ile-3, Phe-4, Leu-7, Phe-67, Ala-74, Ile-100, and Phe-104 that contribute to the hydrophobic core both within the carboxyl-terminal domain of T4 lysozyme and connecting one domain with the other. Asp-72 is very solvent exposed and its side chain is located on the "back-side" of the interdomain α-helix relative to the rest of the lysozyme molecule (Fig. 1). Ala-74 is largely, but not entirely, buried, and makes non-polar contacts with residues Val-71, Ile-100, Val-103, and Phe-104 that contribute to the hydrophobic core in the carboxyl-terminal lobe of the molecule. In wild-type lysozyme Asp-70 makes an unusually strong salt bridge with His-31 (Anderson et al., 1990). Asp-70 also accepts a hydrogen bond from the backbone amide of Ala-74. Since the replacement of Ala-74 with proline eliminates any possibility of hydrogen bonding to the amide it was likely that this replacement would perturb the Asp-70...His-31 salt bridge.

EXPERIMENTAL PROCEDURES

Mutagenesis—The proline mutations were introduced by site-directed mutagenesis according to the uracil template method developed by Kunkel (Kunkel, 1985; Kunkel et al., 1987). The cysteine-free "pseudo wild-type" lysozyme (WT*), in which the 2 cysteine residues present in wild-type had been replaced in order to facilitate thermodynamic measurements (Wetzel et al., 1988; Matsumura and Matthews, 1989; Fjura et al., 1990) was used as the reference protein. The lysozyme ε-gene contained within a 630 base-pair BamHI-HindIII fragment had been previously cloned into phage M13 mp18 yielding the derivative M13 mp18 Tα6 C54T C9TA. It was then transformed into Escherichia coli strain CJ236 (dut, ung, thy1, relA/pCJ105(CM))

* This work was supported in part by National Institutes of Health Grant GM21967 and the Lucille P. Markey Charitable Trust. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ Present address: European Molecular Biology Laboratory, Postfach 10.2209, Meyerhofstrasse 1, W-6900 Heidelberg, Germany.
§ Present address: Laboratory of Molecular Biology, NIDDKD, National Institutes of Health, Bldg. 2, Rm. 316, Bethesda, MD 20892.
¶ To whom correspondence should be addressed.

The abbreviation used is: WT*, pseudo wild-type.
Proline Substitutions in T4 Lysozyme

which was used to prepare uracil-containing single-stranded template DNA. After annealing of the mutagenic primer to WT* template DNA, circularization was accomplished by using the Klenow fragment of E. coli DNA polymerase I and T4 DNA ligase. The double-stranded DNA was subsequently used to transform competent E. coli JM101 cells. Sequencing of the whole lysozyme gene was carried out to confirm that no changes had occurred other than the ones introduced.

Protein Purification—Following subcloning into the plasmid expression vector pPH1403 (Muchmore et al., 1989; Poteete et al., 1991) 100 ml of cells grown overnight in 100 ml of LB broth (10 g of tryptone, 5 g of NaCl. 1 ml of 1 M NaOH/liter) was added to 3 liters of LB broth (12 g of tryptone, 5 g of yeast extract, 10 g of NaCl, 1 g of glucose/liter) and grown at 37 °C under constant agitation (700 rpm) and air flow (12 liters/min) in a 5-liter fermenter until the density reached a value of 1.2. The temperature was then decreased to 26 °C, agitation and aeration were reduced to 200 rpm and 7 liters/min, respectively. Isopropyl-β-thio-galactoside (800 mg) was added to the growth media in order to induce lysozyme expression which was allowed to proceed for 100 min under continued stirring and aeration. The cells were then harvested into a 5-liter Erlenmeyer flask where they lysed. A few grains of DNase I were added to the now thick and viscous lysate which was left stirring at 4 °C for 2.5 h. The lysed cell suspension remained almost the viscosity of LB broth, then was taken out to room temperature, and placed on a magnetic stirrer for 30 min to allow for complete cell lysis. This step almost doubled the yield of proline-containing mutant lysozymes. The lysate became more viscous again and was placed back at 4 °C, stirring for another 1.5 h until it regained the fluidity of LB broth. All the subsequent purification steps were carried out at 4 °C. After centrifugation at 10,000 rpm (17,700 × g) for 2 h, only the supernatant contained mutant lysozyme. It was dialyzed against 20 mM phosphate buffer at pH 6.5 until the conductivity reached a value of 3.8 mS/cm (1 Siemens = 1 Ω). The dialyzed supernatant was loaded onto a 2.5 × 10 cm CM-Sepharose ion-exchange column which had previously been equilibrated with 400 ml of 50 mM Tris buffer at pH 7.3. Mutant lysozyme was eluted using an 800-mI salt gradient in the range from 50 to 300 mM NaCl in 50 mM Tris buffer, pH 7.3. Elution was monitored at a wavelength of λ = 280 nm. Peak fractions (absorbance at a wavelength of 280 nm above 0.4 units) were pooled, dialyzed against 50 mM phosphate buffer at pH 5.8 for 12 h, and concentrated on a 1 × 5 X SP-Sephadex column previously equilibrated with the same buffer. The protein was eluted with SP buffer (100 mM NaF04, pH 6.5, 550 mM NaCl, 0.02% NaN3) and stored in this buffer at 4 °C. Based on sodium dodecyl sulfate and high performance liquid chromatography analysis the purity of the mutant T4 lysozyme was estimated to be over 95%. Final amounts of 60–120 mg of mutant T4 lysozyme proteins were obtained. Wild-type T4 lysozyme typically yields about 150 mg/3-liter preparation.

The activity of the mutant lysozyme was measured at room temperature using the turbidity assay described by Tsagita (Tsagita et al., 1968). Since absolute rates were not reproducible, activities were normalized to a wild-type control. Measurement of Thermal Stability—Thermal denaturations at pH 2.0 and 6.5 were monitored by circular dichroism (CD) at a wavelength of λ = 229 nm on a Jasco J-500C spectropolarimeter as a function of temperature (Elwess and Schellman, 1975; Dao-pin et al., 1990). The temperature was varied from 0 to 75 °C at a rate of 1 °C/min with a Hewlett-Packard 89100A temperature controller interfaced to a Hewlett-Packard 87 XM computer. The protein concentration was adjusted to 0.02 mg/ml by measuring the absorbance at λ = 280 nm using a double beam Varian 2290 spectrophotometer. A probe immersed in the sample solution just above the UV beam recorded the temperature. The solution was continuously stirred with a magnetic stirbar. Buffer solutions (150 mM KCl, 10 mM HCl at pH 2.0 and 150 mM KCl, 10 mM potassium phosphate at pH 6.5) were prepared from doubly desozonized, degassed H2O and were filtered before use through a 22-μm Millipore filter unit.

Thermal denaturations were repeated at least three times. Measurements of the mutants were flanked by WT* thermal denaturations under exactly the same conditions in order to minimize errors (Dao-pin et al., 1990). The data were analyzed using standard van’t Hoff techniques (Becktel and Schellman, 1987; Alber and Matthews, 1987) under conditions similar to those used for wild-type lysozyme. Both methods yielded crystals of D72P isomorphous with WT in the space group P212121.

Refinement was carried out using the TNT package of refinement programs (Tronrud et al., 1987). The positional and the temperature factors were refined simultaneously using the “conjugate directions” option in TNT which improves convergence.1

The starting model for refinement was the refined structure of the cysteine-free wild-type (Weaver and Matthews, 1987; Alber and Matthews, 1987) under conditions similar to those used for wild-type lysozyme. Both methods yielded crystals of D72P isomorphous with WT in the space group P212121.

RESULTS

Expression of Mutant Proteins

Gln-69 → Pro—Mutant Q69P could be expressed and purified in a straightforward manner, yielding up to 100 mg of protein from 4 liters of culture medium. The activity of the protein is close to that of wild-type (Table I), but it is less stable, with melting temperature 12.9 °C less than wild-type at pH 2.2 and 7.6 °C lower at pH 6.5. This behavior corresponds to that of a typical “temperature sensitive” mutant of

1 A. E. Eriksson, W. A. Baase, and B. W. Matthews, manuscript in preparation.

2 D. E. Tronrud, submitted for publication.
Proline Substitutions in T4 Lysozyme

Protein from a 4-liter culture. Its activity is about 60% that purified by the standard procedure, yielding up to 130 mg of crystals were also obtained at 15°C, but have not been examined. 

TABLE I
Sites of proline replacement

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Fraction of side chain accessible to solvent</th>
<th>Mutant</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gln-69</td>
<td>0.75</td>
<td>Q69P</td>
<td>88</td>
</tr>
<tr>
<td>Val-71</td>
<td>0.06</td>
<td>V71P</td>
<td></td>
</tr>
<tr>
<td>Asp-72</td>
<td>0.76</td>
<td>D72P</td>
<td>57</td>
</tr>
<tr>
<td>Ala-74</td>
<td>0.04</td>
<td>A74P</td>
<td>66</td>
</tr>
</tbody>
</table>

* Purified protein was not obtained for V71P.

TABLE II
Thermal stability of proline-containing mutants

<table>
<thead>
<tr>
<th>Protein</th>
<th>pH</th>
<th>Tm of mutant (°C)</th>
<th>ΔTm</th>
<th>ΔG (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q69P</td>
<td>2.0</td>
<td>25.6</td>
<td>-12.9</td>
<td>-3.3</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>55.6</td>
<td>-7.6</td>
<td>-2.9</td>
</tr>
<tr>
<td>D72P</td>
<td>2.0</td>
<td>28.3</td>
<td>-10.2</td>
<td>-2.6</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>56.3</td>
<td>-7.1</td>
<td>-2.7</td>
</tr>
<tr>
<td>A74P</td>
<td>3.5</td>
<td>39.3</td>
<td>-18.2</td>
<td>-5.7</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>46.9</td>
<td>-15.3</td>
<td>-5.0</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>53.3</td>
<td>-12.4</td>
<td>-4.5</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>50.8</td>
<td>-12.1</td>
<td>-4.6</td>
</tr>
</tbody>
</table>

T4 lysozyme selected by the random screen of Streisinger et al. (1961) (cf. Gutter et al., 1979, 1987; Hawkes et al., 1984). When stored at 4°C at about 50 mg/ml, Q69P tended to form white opalescent aggregates which dissolved on warming to room temperature. This process was reversible and seemed to have no effect on stability or activity. Small crystals of the protein were obtained, apparently non-isomorphous with wild-type.

Val-71 → Pro—When V71P DNA was transformed into E. coli and the ability of a bacterial extract tested to form a halo on isopropyl-1-thio-β-D-galactopyranoside lysis indicator plates, no halo could be seen at 37°C. After 24 h at 4°C, a small halo was visible but we cannot rule out the possibility that this might be due to a small amount of WT* present as an impurity. Attempts to purify V71P by the method described above, or by using a French press to break open the bacterial cell walls, or by an alternative method described by Dao-pin et al. (1991b) were all unsuccessful. We presume that V71P is very unstable and/or is rapidly degraded by proteolysis.

Asp-72 → Pro—As with Q69P this protein could be readily purified by the standard procedure, yielding up to 130 mg of protein from a 4-liter culture. Its activity is about 60% that of wild-type (Table I) and it is less stable (Table II), again, roughly comparable to a typical temperature-sensitive mutant. Crystals isomorphous with wild-type could be grown at 4°C using both batch and hanging-drop techniques. The latter method gave the largest crystals, 0.5 × 0.65 × 0.3 mm, from 2.0 mM phosphate at pH 7.1. Some apparently non-isomorphous crystals were also obtained at 15°C but have not been examined.

Ala-74 → Pro—As for Q69P and D72P, A74P behaved normally and yielded ~140 mg of protein/preparation from a 4-liter culture. The apparent activity is about two-thirds that of wild-type (Table II). This mutant is less stable than both D72P and Q69P (Table II). Because of the low stability the protein tends to be partially unfolded at low pH, so the stability measurements under these conditions are less reliable than at higher pH. Some crystals of this protein were obtained from 50 mM phosphate, pH 7.9, 16% PEG 8000 and are non-isomorphous with wild-type.

Structure of D72P

The crystals of D72P appeared to be somewhat more sensitive to radiation than wild-type lysozyme and did not diffract as well. For this reason the exposure time per frame on a Xuong-Hamlin area detector system operating with graphite-monochromated CuKα radiation from a Rigaku generator (40 kV, 100 mA) was increased to 60 s, compared to the usual 30 s for WT. Under these conditions the data set to 1.9 Å was 89% complete (Table III).

The map showing the difference in density between D72P and WT* lysozyme is shown in Fig. 2a. The positive density feature confirms the addition of the pyrrolidine ring. Also there is negative density at the site previously occupied by the carboxylate of Asp-72. Positive and negative densities also indicate the movement of the carbonyl oxygen of Asn-68 away from the helix axis (Fig. 2b). A negative feature next to the carbonyl group of Asp-70 indicates a movement of the oxygen toward the helix axis. The side chain of Asp-70 also moves slightly, as does His-31 (not shown) maintaining the strong (Anderson et al., 1990) His-31...Asp-70 salt bridge. The movement of His-31 together with slight adjustments occurring throughout the lower lobe give the impression that the lower part of the long α-helix and the lower domain move as an essentially connected unit.

There is, however, a movement of the upper domain relative to the lower one. This shift is shown in Figs. 3 and 4. If backbone atoms in the amino-terminal domain of the mutant structure (residues 13-59) are superimposed on the corresponding atoms in WT* lysozyme (Fig. 4a), they have root-mean-square discrepancy of 0.16 Å, which is essentially experimental error. Similarly, the respective backbone structures within the carboxyl-terminal domains are also well conserved (root-mean-square discrepancy of 0.18 Å for residues 81-162, Fig. 4b). This shows that the structures within

TABLE III
Data collection and refinement statistics

<table>
<thead>
<tr>
<th>Data for WT* taken from Eriksson et al.²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>Cell dimensions (Å)</td>
</tr>
<tr>
<td>a, b</td>
</tr>
<tr>
<td>c</td>
</tr>
<tr>
<td>Resolution (Å)</td>
</tr>
<tr>
<td>Completeness of data (%)</td>
</tr>
<tr>
<td>Unique reflections</td>
</tr>
<tr>
<td>Rmerge (on intensities) (%)</td>
</tr>
<tr>
<td>Resolution limits (Å)</td>
</tr>
<tr>
<td>RMS* deviation from ideal values</td>
</tr>
<tr>
<td>Bond length (Å)</td>
</tr>
<tr>
<td>Bond angle (°)</td>
</tr>
</tbody>
</table>

² Root-mean-square.
Proline Substitutions in T4 Lysozyme

the respective NH$_2$-terminal and COOH-terminal domains are conserved. It is the alignment of one domain relative to the other that changes in the mutant structure relative to WT$^*$. As shown in Fig. 4, $a$ and $b$, this movement corresponds to atom shifts up to about 1.5 Å. The pronounced maxima and minima in Fig. 4, $a$ and $b$, are due to different distances of the corresponding atoms from the axis of rotation.

One way to analyze for movements of one part of the structure relative to another is to align the mutant structure with wild-type based on the superposition of relatively short segments of backbone. Two such alignments, based on 6-residue backbone segments on either side of the mutation site, are shown in Fig. 5, $a$ and $b$. In Fig. 5a, in which the alignment is based on residues 60–66, the amino-terminal domain of the mutant structure agrees well with that of WT$^*$. This shows that residues 60–66 and the NH$_2$-terminal domain are connected essentially as a rigid body. In contrast, when the superposition is based on residues 74–80 (Fig. 5b), neither the NH$_2$-terminal domains nor the COOH-terminal domains of the mutant and wild-type structures coincide. This suggests that the residues 74–80 are not connected rigidly to the COOH-terminal domain. The upper part of the 60–80 α-helix appears to be at least in part responsible for the observed flexibility. Small changes in this region can have large effects on the rest of the structure. The regions at the beginning and at the end of the long helix have been previously identified as “hinge-bending” regions in the M61 variant of T4 lysozyme (Faber and Matthews, 1990). The hinge-bending that was observed for the M61 mutant was assumed to be a low energy displacement because different molecules within the same crystal displayed different hinge-bending angles. In contrast, in the present case the long interdomain helix is bent relative to wild-type. Therefore the hinge-bending seen for D72P (Fig. 3) has a very different origin, and presumably very different energetics, relative to that seen for M61.
Proline Substitutions in T4 Lysozyme

2397

Fig. 4. "Shift plots" showing the displacement between corresponding backbone atoms in the mutant D72P and wild-type lysozyme. For each residue the value plotted is the root-mean-square discrepancy between the four backbone atoms in the mutant (N, C, CA, O) and the corresponding atoms in WT*. a, superposition of the structures of D72P and WT* based on residues 13-59 in the amino-terminal domain. The arrowheads indicate those residues that are presumed to contact an extended substrate (Glu-11, Asp-20, Thr-21, Leu-32, Phe-104, Gln-105, Thr-109, Thr-142 and Arg-145) (Anderson et al., 1981). The contacts involving Thr-109 are to sugars in subsites A and B, whereas cleavage is between subsites D and E. b, superposition of D72P and WT* based on residues 81-162.

Another way of detecting differences between two structures is by calculating a difference-distance matrix (Nishikawa et al., 1972). All the intramolecular Cβ-Cβ distances are determined for one protein and then compared to the respective intramolecular distances of the second molecule. An example of such a plot is shown in Fig. 6. Since Cβ-Cβ distances in D72P were subtracted from the corresponding distances in WT*, positive contours (solid lines) reflect shorter distances in the mutant structure; negative contours (dotted lines) represent longer ones. As can be seen, the mutated residue 72 shows the largest shift with respect to residues 95, 122, and 157.

Fig. 2b illustrates the effect of the Asp-72 → Pro mutation on the long α-helix itself. In this figure residues 60-66 have been superimposed so as to make the structural change more obvious. The helix containing the proline has an overall bend of about 14°. A similar analysis of the structure of wild-type lysozyme, however, reveals that the same helix already has a bend of about 8.5°. Therefore the effect of the Asp-72 → Pro replacement is to increase the bending by about 5.5°.

Fig. 7 compares the hydrogen-bond distances within the interdomain helix in wild-type lysozyme and in the mutant structure. The first α-helical hydrogen bond is from the carbonyl oxygen of Thr-59 to the backbone amide of Ala-63. Not surprisingly, the introduction of the pyrrolidine ring at residue 72 results in a substantial increase in the distance between the nitrogen of residue 72 and the carbonyl oxygen of Asn-68. The nitrogen- oxygen distances for the successive residues are lengthened somewhat, but not excessively so (maximum 3.3 Å), suggesting that the hydrogen bonds within

Fig. 5. a, superposition of D72P on WT* based on the alignment of the amino-terminal part of the interdomain α-helix, residues 60-66. b, superposition of D72P on WT* based on the alignment of the carboxyl-terminal part of the interdomain α-helix, residues 74-80. Note that a is similar to Fig. 4a indicating that residues 60-66 and the amino-terminal domain do not move very much relative to each other upon the proline replacement. b, however, is not very similar to Fig. 4b, showing that the carboxyl-terminal part of the interdomain α-helix does move somewhat relative to the carboxyl-terminal domain of the mutant protein.

Fig. 6. Difference distance matrix comparing distances between all pairs of α-carbon atoms in the mutant structure with the corresponding distances in WT*. The quantity plotted is $\Delta r_{ij} = r_{ij}^{WT} - r_{ij}^{D72P}$ where $r_{ij}^{WT}$ is the distance between the i-th and j-th α-carbon atoms in the structure of wild-type lysozyme, and $r_{ij}^{D72P}$ is the distance between the i-th and j-th α-carbon atoms in the D72P mutant structure. Solid contours are drawn at 0.3 Å, 0.6 Å, 0.9 Å, . . . and indicate pairs of α-carbon atoms that are closer together in the mutant structure than in wild-type. Broken contours, drawn at -0.3 Å, -0.6 Å, -0.9 Å, . . . indicate pairs of α-carbon atoms that are further apart in the mutant than in wild-type. Featureless regions indicate domains within which the structure in the mutant is essentially identical with that in wild-type.
the remainder of the α-helix are maintained. In the mutant, D72P, indicated by the solid line; values for WT are indicated by the broken line. The lower plot shows the difference in the distance between the mutant and wild-type structures.

**DISCUSSION**

The most striking result of the present study is the finding that proline residues can be substituted at several positions within the long interdomain α-helix of T4 lysozyme with only modest effects on catalytic activity. The proteins are destabilized relative to wild-type, but still fold and behave essentially normally. Attempts were made to substitute prolines at four sites and in three cases a functional protein was obtained. It is not as if there is one particular site at which a proline can be accepted. Rather, the data suggest that it may be possible to introduce prolines at additional sites within the interdomain helix, if not at many other sites in the protein as well.

The decrease in stability observed for the two mutants D72P and Q69P is very comparable with that found for temperature-sensitive mutants of T4 lysozyme such as R66H, T157I, and A98V identified by the random genetic screen of Streisinger et al., 1961; Grutter et al., 1969, 1987; Weaver et al., 1989; Dao-pin et al., 1991a). The mutant A74P is, however, less stable than any of these previously described variants. The decrease in stability associated with the proline substitutions seems to be associated to some extent with the inaccessibility of the residue to solvent but the correlation is not perfect. Val-71 is largely buried and a proline replacement at this site did not yield a functional protein. Ala-74 is also largely solvent inaccessible. In addition the proline substitution disrupts the α-helical hydrogen bond between residues 70 and 74 which, in turn, is likely to misalign and perhaps weaken the very strong salt bridge between Asp-70 and His-31 (Anderson et al., 1990). In this case protein was obtained although with substantially reduced stability. O'Neil and DeGrado (1990) found that the energy cost of an alanine to proline replacement within a dimeric α-helical model peptide was 3.4 kcal/mol. Also Yun et al. (1991) found, by free energy simulations, exactly the same value for an alanine to proline replacement within a short polyalanine helix. These values are roughly comparable with those found here (average values of 3.2, 2.7, and 5.2 kcal/mol) but there is no reason to expect close agreement since the context is different in every case.

In the case of A72P, for which the crystal structure is available, the pyrrolidine ring is seen to introduce three unfavorable contacts with neighboring atoms (2.93 Å between Cα and the peptide nitrogen of Val-71; 3.04 Å between Cα and the peptide nitrogen of Val-71; 3.00 Å between Cα and the carbonyl oxygen of Asn-68). Each of these contacts corresponds to an unfavorable van der Waals interaction energy in the range 1-2 kcal/mol (Levitt, 1974). These steric clashes are, presumably, a major factor in the destabilization of the mutant structure relative to wild-type.

The results provide further evidence that protein structures are adaptable and can compensate for amino acid substitutions at many sites (Matthews, 1987; Sondek and Shortle, 1990). It also illustrates the redundancy that is present in the amino acid sequence of a protein. Not every amino acid in the linear sequence is necessary for folding (Reidhaar-Olson et al., 1988; Zhang et al., 1991). Amino acid substitutions that are expected to distort and destabilize the folded structure of the protein, and disrupt a major α-helix that might be a key folding intermediate, do not prevent the formation of a folded functional protein.

Functional proteins were obtained with prolines substituted at positions 69, 72, and 74. Residues 69 and 72 are located in successive turns on the same side of the helix. Residue 74, however, is on the opposite side of the α-helix. Therefore one cannot argue that prolines are only accommodated on one side of the α-helix such that each substitution bends or distorts the α-helix in the same direction.

The substitution of a proline in an α-helix that is part of a protein is not the same as a proline substitution of an isolated...
α-helix. In the absence of any constraints, a substituted proline is likely to completely disrupt the helix (Strehlow et al., 1991) or to introduce a bend of up to 45° (Barlow and Thornton, 1988; Karle et al., 1991). The structural consequences of a proline introduced into an α-helix in a protein, however, will be modulated by interactions between the α-helix and the rest of the protein. Prolines in α-helices in known protein structures are associated with bend angles that average 26 ± 5° (Barlow and Thornton, 1988), but this is not to say that an introduced proline will cause a change of this magnitude. The determination of the structure of D72P provides an example of the structural compromise that can result. The presence of the proline increases the bend angle of the α-helix, but only by 5.5°. Thus, it is not to be expected that each individual proline substitution will cause a major re-arrangement of the two domains in T4 lysozyme. Nevertheless it can be anticipated that the different substitutions will cause distinct changes in the alignment of one domain relative to the other. This suggests that the precise shape of the active-site cleft in the resting enzyme is not critical for catalysis. At the same time it should be noted that the key catalytic residues are at the base of the active site cleft, and the structural changes in this region may therefore be relatively small (≤0.5 Å). The residues in T4 lysozyme that are presumed to contact an extended oligosaccharide substrate are indicated in Fig. 4a.

It should be noted that the crystals that were obtained for the replacement Asp-72 → Pro are isomorphous with wild-type. It is possible that the formation of such isomorphous crystals constrains the structure of D72P to be more similar to WT* than is the case in solution. The differences between the structures of D72P and WT* in solution may therefore be larger than those seen in Figs. 3 and 4. The mutants Q69P and A74P give crystal forms that are not isomorphous with wild-type. Hopefully, the structure analysis of these crystals will give a better overall impression of the structural changes that are induced by the proline substitutions.

The first determination of the structure of a temperature-sensitive mutant lysozyme (Grütter et al., 1979) showed that relatively large changes in stability were accompanied by minimal changes in structure, these being localized to the immediate vicinity of the substituted amino acid. The ability of T4 lysozyme to accommodate destabilizing mutations in this manner has subsequently been seen on a number of occasions (Matthews, 1987) and is further exemplified by the present study.

Acknowledgments—We thank Drs. Walt Baase, Larry Weaver, Eric Anderson, and Elisabeth Eriksson for advice and help with, respectively, thermodynamic measurements, data collection, NMR measurements, and crystallographic refinement. Joan Wozniak and Sheila Pepiot also provided advice in protein purification, and D. W. Heinz and X.-J. "Cai" Zhang helped with data analysis. We also thank Dr. J. A. Schellman for facilities.

REFERENCES