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Structural characterization of an engineered tandem repeat contrasts the importance of context and sequence in protein folding

(sequence duplication/α-helices/T4 lysozyme)

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ABSTRACT **To test a different approach to understanding the relationship between the sequence of part of a protein and its conformation in the overall folded structure, the amino acid sequence corresponding to an α-helix of T4 lysozyme was duplicated in tandem. The presence of such a sequence repeat provides the protein with "choices" during folding. The mutant protein folds with almost wild-type stability, is active, and crystallizes in two different space groups, one isomorphous with wild type and the other with two molecules in the asymmetric unit. The fold of the mutant is essentially the same in all cases, showing that the inserted segment has a well-defined structure. More than half of the inserted residues are themselves helical and extend the helix present in the wild-type protein. Participation of additional duplicated residues in this helix would have required major disruption of the parent structure. The results clearly show that the residues within the duplicated sequence tend to maintain a helical conformation even though the packing interactions with the remainder of the protein are different from those of the original helix. It supports the hypothesis that the structures of individual α-helices are determined predominantly by the nature of the amino acids within the helix, rather than the structural environment provided by the rest of the protein.**

It is the *sine qua non* of protein folding that the sequence of a protein determines its structure. What is less clear is whether the local sequence determines the local structure. Is the structure of a strand, turn, or helix determined by the sequence of that element, or is it dictated by the fold of the rest of the protein (1–5)? We address this question by duplicating, within the same polypeptide chain, the sequence of a secondary structure element.

The specific context in which the above approach was tested was the amphipathic α-helix that encompasses residues 39–50 of bacteriophage T4 lysozyme. The helix is packed against a small β-sheet region in the amino-terminal domain (Fig. 1*A*) and is flanked by loops that have been shown to permit small amino acid insertions (6, 7). The sequence between residues Asn-40 and Ile-50 was copied and inserted after residue Ser-38 as shown schematically in Fig. 1*E*. We refer to the first half of the tandem repeat as the inserted sequence and the second half as the parent sequence (Fig. 1*E*). The mutant protein will be referred to as the duplication mutant, or, more briefly L20.

The presence of the duplicated sequence could lead to a number of structural alternatives, some of which are illustrated in Fig. 1. In Fig. 1*B*, for example, the parent sequence forms the same α-helix that it does in wild type, and the inserted sequence adopts some structure, either ordered or disordered, at the amino terminus of the parent helix. Conversely, the inserted sequence might take the place of the wild-type α-helix, in which case it would be the parent sequence that would form some structure at the carboxyl terminus of the replaced α-helix (Fig. 1*C*). Because the duplicated helix was designed to be essentially "in phase" with the parent helix, and to retain a common hydrophobic face (Fig. 1*E*), it was also possible that a replacement α-helix might be made up in part of both the parent and the insert (Fig. 1*D*). Of course it was also possible that the modified protein might not fold at all or adopt some radically new structure.

MATERIALS AND METHODS

Cloning and Protein Purification. Mutant L20 was generated by using the Kunkel method on a single-stranded M13 phage DNA T4 lysozyme template plasmid (8). The mutant was cloned with a 72-mer primer of sequence: TTC AGA TTT AGC AGC ATT GAT TGC CTT GTC CAG CTC GGA CTT TGC TGC ATT GAT TGA TGG ACT TTT TGT AAG. The codons of the inserted regions were chosen to mismatch the original (to be copied) sequence by using the GCG high-frequency codon usage listing of *Escherichia coli*. The mutant was constructed in a cysteine-free version of the T4 lysozyme gene designated as WT* (9).

Mutants were screened with an *in vivo* halo assay. Mutants that were confirmed by sequencing subsequently were subcloned and expressed as described (8). The mutant protein remained fully soluble and monomeric as judged by elution profiles from sizing columns (data not shown). *In vivo* halo assays indicated that the mutant proteins remain catalytically active. Direct assay using purified peptidoglycan (10) showed the mutant to have 50% the activity of WT*.

Crystallographic Analysis. Crystallization was attempted by using the FastScreenII crystallization kit with 48 different crystallization conditions as available from Hampton Research (Riverside, CA). All proteins were dialyzed against 50 mM Tris·HCl, pH 7.5 with 100 mM NaCl before crystallization.

Mutant L20, at a concentration of 20 mg/ml, crystallized in two forms in 20% polyethylene glycol 6000, 20% isopropanol, and 50 mM Tris·HCl, pH 7.5. The first crystal form, isomorphous with wild type, grew within 6 days in space group $P3_221$ to a size of about $0.3 \times 0.3 \times 0.5$ mm. The second crystal form, in space group $P2_1$, grew within 3–4 days to very thin plates of about $0.4 \times 0.5 \times 0.01$ mm.

Diffraction data for the $P2_1$ crystals were collected on a Rigaku (Tokyo) R-AXISIIc image plate detector. The crystallizing medium permitted flash-freezing without additional cryoprotection allowing data collection at low temperature. For the $P3_221$ crystals, synchrotron diffraction data were

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Data deposition: The coordinates and structures reported in this paper have been deposited in the Protein Data Bank, www.rcsb.org. (PDB ID codes 261L and 262L).

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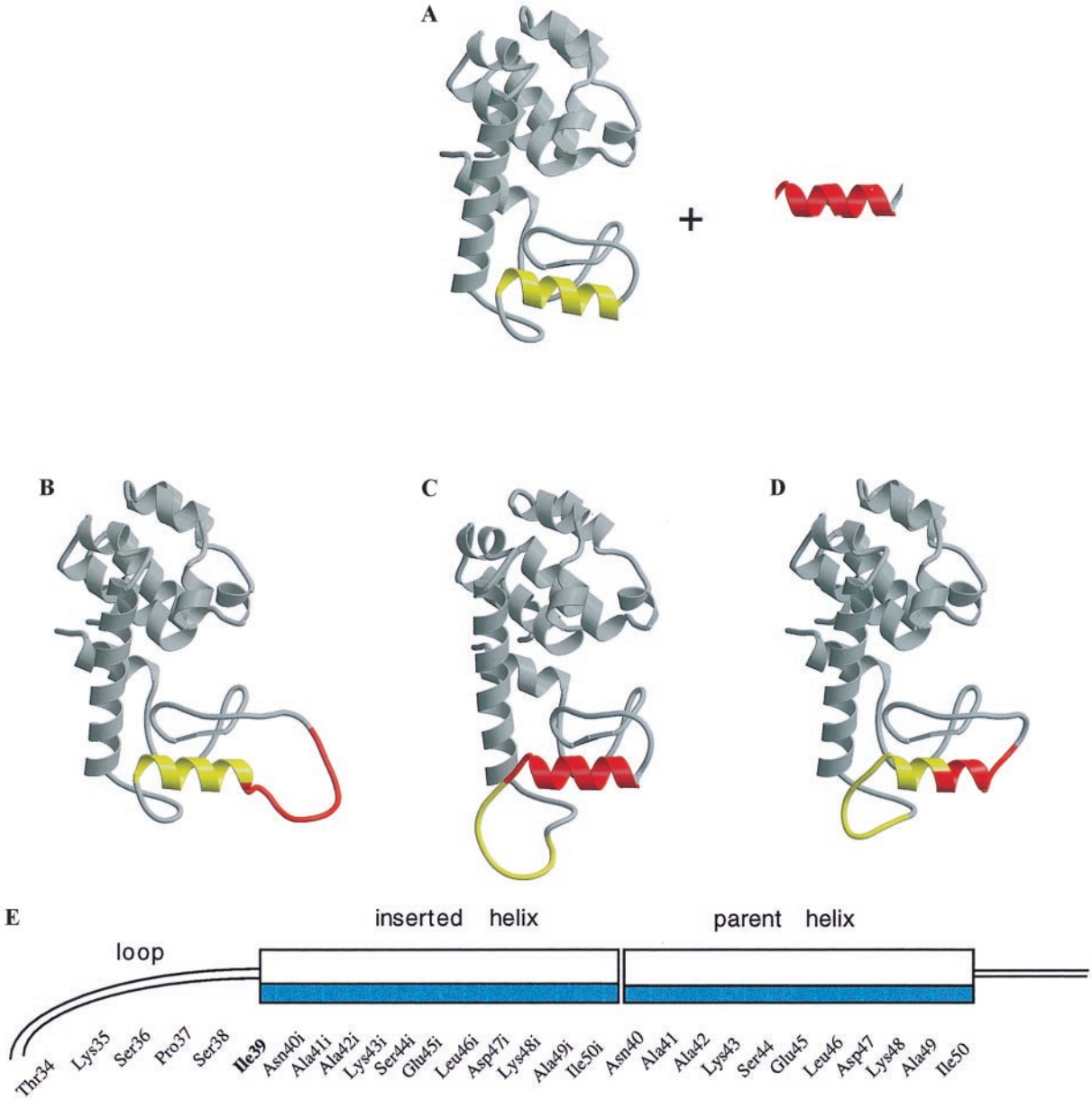

FIG. 1. Model structures illustrating some possible structural responses to sequence duplication. The original helix sequence is highlighted in yellow and the sequence of the insert in red. (*A*) Wild-type T4 lysozyme. (*B*) The inserted sequence is looped out at the amino terminus of the parent helix. (*C*) Looping out at the carboxyl terminus of the parent helix. (*D*) A helix similar to that in WT* lysozyme is formed in part by the parent sequence and in part by the inserted sequence. Loop structures would be formed at each end of the helix. (*E*) Sketch showing the sequence of the inserted region (Asn-40i–Ile-50i) relative to the sequence and secondary structure of wild-type lysozyme. If the parent and the insert were to form a single, continuous α-helix, it would be amphipathic with the hydrophobic side shown shaded. To maintain this continuous hydrophobic surface, and also to ensure that the sequence included an exact 11-aa repeat, Leu-39 in the wild-type sequence was replaced by an isoleucine and is designated Ile-39. As a consequence the connection between the two helices is not interrupted by any residues that contribute to loops or other nonhelical structures in the native protein. Figure drawn with BOBSCRIPT (19), MOLSCRIPT (20), and RASTER3D (21).

collected at room temperature at the Cornell High Energy Synchrotron Source (beamline A1, $\lambda = 0.908$ Å) by using a Quantum1 charge-coupled device detector. The data were integrated and scaled with XDS (11, 12) or MOSFLM (13).

The isomorphous crystal was refined (14, 15) by standard methods (16) (Table 1). The nonisomorphous crystal has two molecules per asymmetric unit, and the structure was determined by molecular replacement using the AMORE programs (17). By first using the carboxyl-terminal domain of T4 lysozyme comprising residues 74–162 as a search model, it was possible to locate two such fragments within the asymmetric unit. The amino-terminal domains then could be identified in weighted electron density maps with amplitudes ($3F_o$-$2F_c$) or (F_o-F_c) and phases from the model. The overall orientation and structural changes within these domains were immediately apparent. Further model building was guided by various "omit" and other maps calculated with PROTIN/REFMAC (13) with residues in question deleted. Model building was performed by using the program O (18). Figures were prepared by using BOBSCRIPT, MOLSCRIPT, and RASTER3D (19–21).

The coordinates of the mutant structures have been deposited in the Protein Data Bank and will be released at the time of publication (PDB ID codes 261L and 262L).

RESULTS

Structure of the Mutant Protein. The mutant protein crystallized in two forms under identical conditions. (Both forms were observed in the same crystallization droplets.) One

Table 1. X-ray data collection and refinement statistics for mutant L20

Space group	$P3_221$	$P2_1$
Data collection	CHESS	R-Axis II
Molecules per asymmetric unit	1	2
Cell dimensions		
a	61.5 Å	57.7 Å
b	61.5 Å	55.8 Å
c	98.2 Å	65.2 Å
α	90.0°	90.0°
β	90.0°	111.0°
γ	120.0°	90.0°
Resolution	30.0–2.5 Å	55.0–2.5 Å
R_{sym}	8.4%	7.0%
Completeness of data	86.5%	91.0%
R	17.0%	22.7%
$\Delta_{\text{Bond lengths}}$	0.010 Å	0.012 Å
$\Delta_{\text{Bond angles}}$	2.0°	2.4°

The structures were refined by using data between the specified resolution limits. R_{sym} gives the agreement between equivalent intensities. *R* is the conventional crystallographic residual following refinement. $\Delta_{\text{Bond lengths}}$ and $\Delta_{\text{Bond angles}}$ give the average discrepancies of bond lengths and bond angles from ideal values. CHESS, Cornell High Energy Synchrotron Source.

crystal form is isomorphous with WT* (22) with one molecule in the asymmetric unit. The second crystal form is in space group $P2_1$ with two molecules in the asymmetric unit. Taken together (Table 1), these provide three independent determinations of the structure of the mutant. As will become apparent, all three structures are quite consistent.

Structure in Space Group $P3_221$. The first electron density map calculated at 3-Å resolution after rigid-body refinement immediately showed that helix 39–50 is substantially extended at the amino terminus (Fig. 2*A*). Even at this early stage of the refinement process the structure of almost the entire inserted sequence was apparent. Further refinement and model building confirmed that the insert had folded into a unique structure, which is illustrated in Fig. 2 *B* and *C*. The bulk of the inserted residues adopt a helical conformation (23) and extend the WT* helix by two additional turns. The remainder of the inserted residues, together with residues 35–38 of the original sequence, connect the end of this helix back to the rest of the protein (Fig. 2*C*). Otherwise, however, the overall structure of the amino-terminal domain and its relationship to the rest of the protein remain almost unchanged. In this crystal form the amino-terminal domain of WT* lysozyme is involved in relatively few crystal contacts (22) and space is available to allow incorporation of the additional structural element.

Structure in Space Group $P2_1$. The mutant protein crystallizes in this space group with two molecules per asymmetric unit. Although the packing environments of these two molecules differ and are unrelated to the $P3_221$ crystal form, all three structures are quite similar. This finding suggests that the additional fold is retained in solution. For both molecules the density showing the extension of the helical region (Fig. 3 *A* and *B*), as well as the overall trace of the chain, is clear, although the details of the structure in the turn region are uncertain. Subsequent refinement to 2.5-Å resolution suggested the apparent conformation for the entire inserted region of molecule B. In molecule A, however, the density in the vicinity of the loop (residues 40i-44i) is very weak and the coordinates are unreliable. It is possible that the conformation in this loop may be the same as in molecule A, but an alternative structure, or multiple conformations, are not excluded. There is one place where the structures of molecules A and B are somewhat different. In molecule B the residues that extend the β-strand (Ser-36–Ile-39) move up to 4 Å relative to molecule A, including a rotation of about 80° in the pyrrolidine ring of Pro-37 (Fig. 3 *A-C*). In association there is a structural rearrangement of up to 3.5 Å in the adjacent loop that includes Tyr-18–Tyr-25 (Fig. 3*C*). The structure of molecule A in these regions, and, indeed, throughout the entire molecule, remains virtually identical with that in the $P3_221$ crystal form.

Properties in Solution. At temperatures below 25°C, the CD per residue at 223 nm is essentially the same for WT* and L20 (not shown), which implies that the amount of α-helix does not differ between the two constructs. Because T4 lysozyme is 60% α-helical (24), for this to be the case, about seven of the 11 additional residues would have to be α-helical, which is consistent with the structures seen by x-ray diffraction.

Thermal unfolding curves for WT* and L20 are both well modeled as two-state transitions. In 0.1 M NaCl, 10 mM sodium acetate, pH 5.4, the duplication decreases the melting temperature by 4.1°C and destabilizes the protein by only 1.6 kcal/mol, a value that might be expected for a typical single site surface mutation (25). The concomitant decrease in van't Hoff enthalpy, from 130 to 110 kcal/mol, is modest and does not significantly exceed that expected on the basis of the decrease in melting temperature (estimating ΔC_p to be 15 cal/mol residue-°K).

DISCUSSION

The fundamental assumption underlying the experiment described here is that a protein with a duplicated segment of amino acid sequence can provide new information regarding the relation between sequence and structure. The results show, first, that such a mutant, constructed in T4 lysozyme, forms a fully soluble, active protein, somewhat less stable than the wild-type protein, but very amenable to structural characterization. Second, the duplicated region of sequence was found to adopt a well-defined conformation. This finding, of itself, suggests that this region has a propensity to form a distinct structure. The fact that the additional structural element is essentially the same in three independent copies of the molecule strongly indicates that the structure is not induced by crystal packing interactions.

The inclusion of the duplicated sequence results in an additional structured region at the N terminus of helix B. It appears that as many of the duplicated residues as possible adopt a helical conformation, compatible with connecting the end of the extended helix back to the rest of the protein (Figs. 2*C* and 3*C*). It can be asked why the duplicated sequence adopts this particular conformation, rather than one of the other alternatives such as shown in Fig. 1. The entropy change upon closure of a single loop as in Fig. 1 *B* or *C* would be expected to be essentially equal for closure at either end of helix B. This equality is because mostly identical amino acids would be involved depending on exactly where the loops were considered to begin. The situation in Fig. 1*D* would be equivalent to immobilization of an additional point somewhere in the loop and would involve greater reduction of entropy. From an enthalpic viewpoint, structures as in Fig. 1 *B* and *C* would maintain the full and mostly native interface between helix B and the rest of the protein. An out-of-register α-helix as in Fig. 1*D*, however, would have different amino acids substituted throughout the interface and presumably suffer a reduction in favorable interactions.

The choice between a structure with looping out at the amino terminus of the helix rather than at the carboxyl terminus presumably is determined in part by constraints imposed by the local structure of the protein, and in part by the structures that are preferred by, or accessible to, the duplicated sequence. In the present case model building (18, 26) suggested that the helix present in the wild-type protein could, in principle, be extended in either direction without obvious steric interference from the rest of the protein. It is not clear, however, whether extension at the amino terminus is preferred because it results in the preservation of interactions present in the native structure (e.g., those including Asp-47, Arg-52, and Glu-62), or because it allows the insertion to adopt a favorable fold including a hydrophobic core consisting of Pro-37, Leu-46i, and Ala-49i.

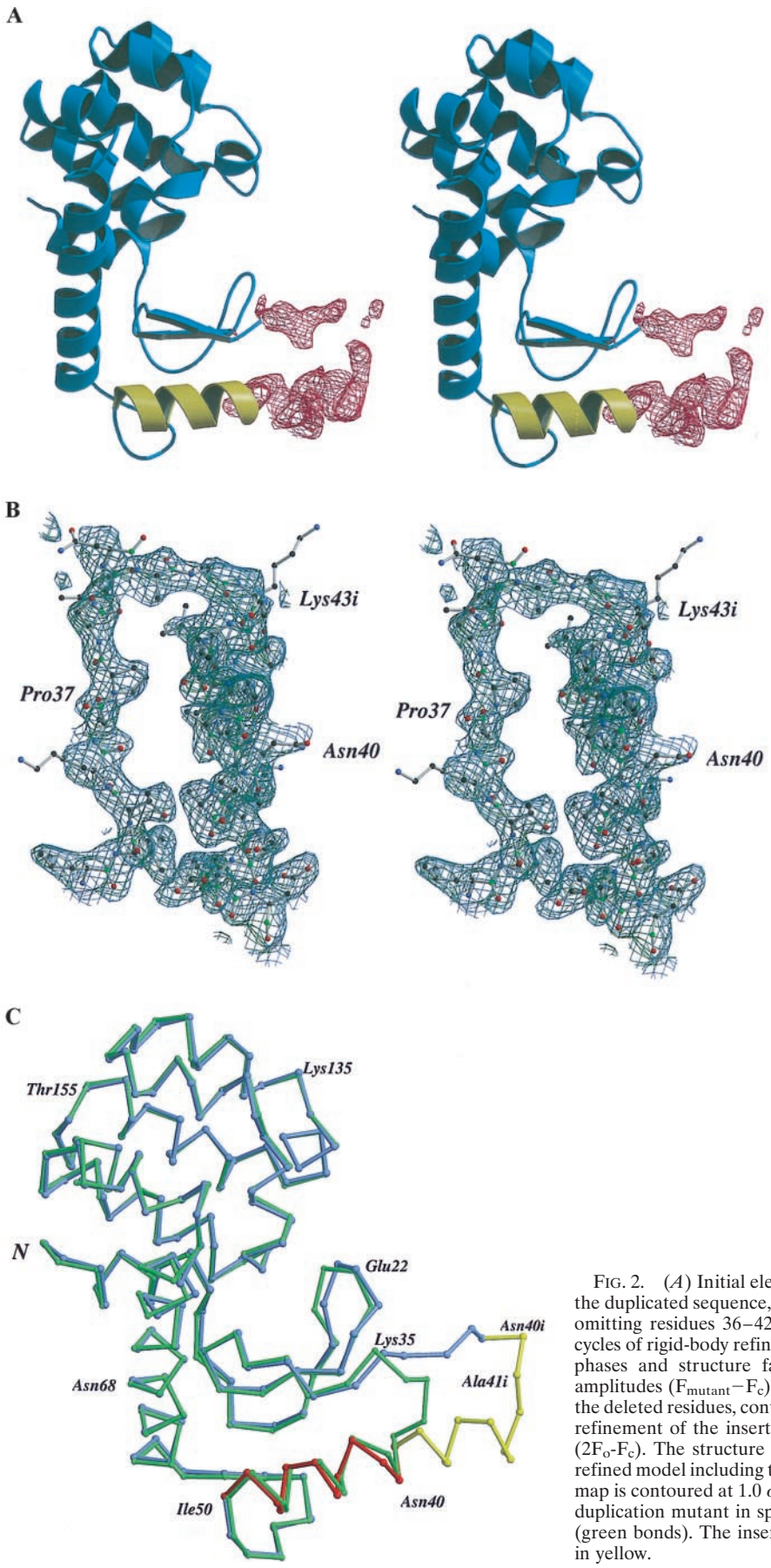

FIG. 2. (*A*) Initial electron density showing the overall conformation of the duplicated sequence, as seen in space group $P3_221$. The WT* structure, omitting residues 36–42 (shown as a ribbon drawing) was subject to 10 cycles of rigid-body refinement in the mutant lysozyme cell. The calculated phases and structure factors, F_c, were used to calculate a map with amplitudes ($F_{mutant} - F_c$) at 3.0-Å resolution. The density in the vicinity of the deleted residues, contoured at 2.5 σ, is shown. (*B*) Electron density after refinement of the inserted region in space group $P3_221$. Coefficients are ($2F_o$-F_c). The structure factors, F_c, and phases were calculated from the refined model including the inserted region. The resolution is 2.5 Å, and the map is contoured at 1.0 σ. (*C*) Superposition of the overall structure of the duplication mutant in space group $P3_221$ (blue bonds) on WT* lysozyme (green bonds). The inserted region in the mutant structure is highlighted in yellow.

The most striking overall result, however, is that more than half of the duplicated sequence (residues 43i-50i) is itself helical (Figs. 2*C* and 3*C*). This finding strongly suggests that the duplicated region has a tendency to form an α-helix, even though its

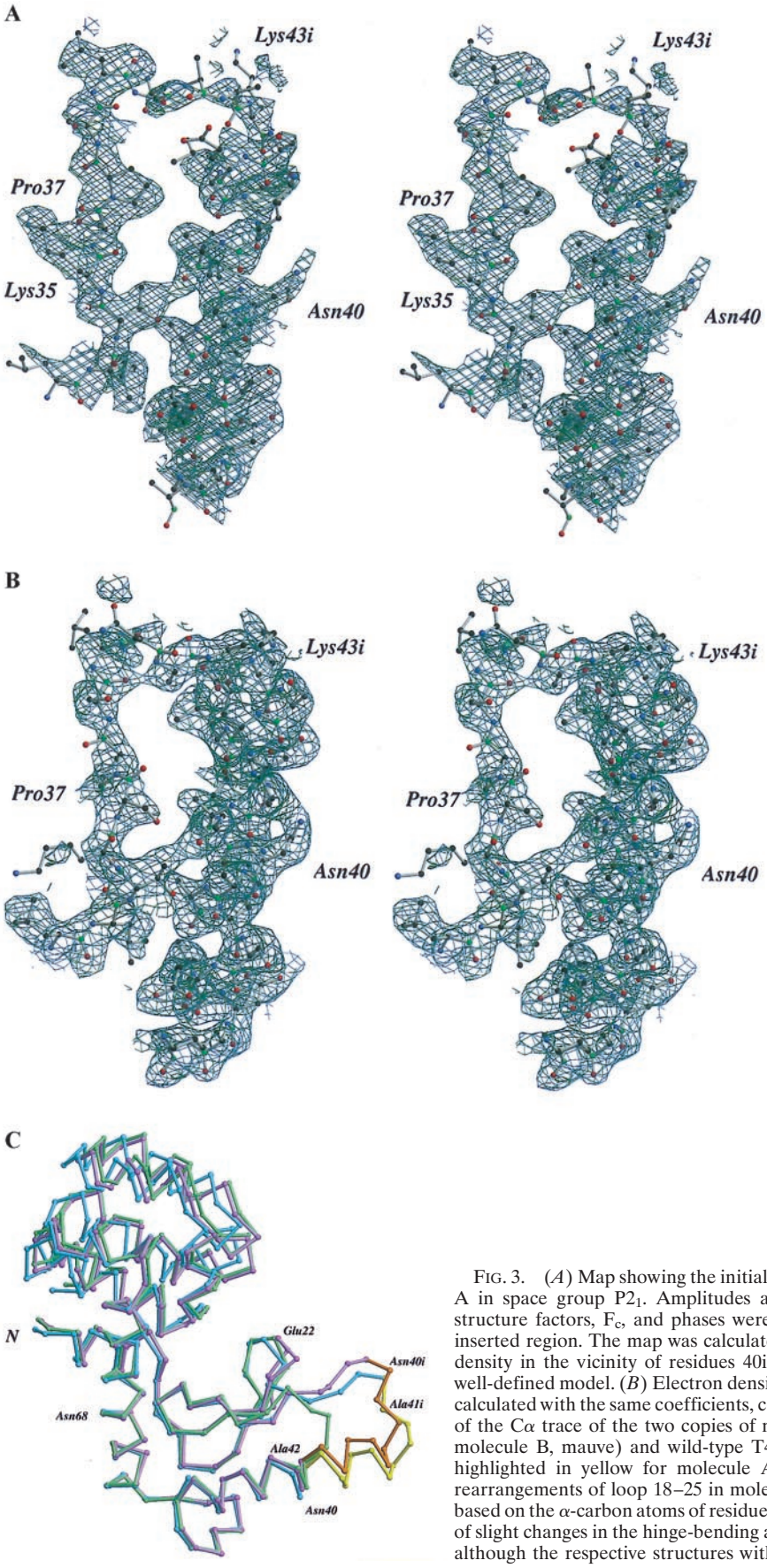

FIG. 3. (*A*) Map showing the initial electron density for the inserted region of molecule A in space group $P2_1$. Amplitudes are $(2F_o\text{-}F_c)$ weighted by REFMAC (15) where the structure factors, F_c, and phases were calculated from the refined model including the inserted region. The map was calculated at 2.5-Å resolution and contoured at 1.0 σ. The density in the vicinity of residues 40i-43i is not well defined and could not be fit by a well-defined model. (*B*) Electron density for molecule B of crystal form $P2_1$. This map was calculated with the same coefficients, contouring, and resolution as in *A*. (*C*) Superposition of the Cα trace of the two copies of mutant L20 in crystal form $P2_1$ (molecule A, blue; molecule B, mauve) and wild-type T4 lysozyme (green). The sequence of the insert is highlighted in yellow for molecule A and in orange for molecule B. The structural rearrangements of loop 18–25 in molecule B are clearly visible. The superpositions were based on the α-carbon atoms of residues 51–80 within the amino-terminal domain. Because of slight changes in the hinge-bending angle the C-terminal domains appear out of register although the respective structures within these regions are very similar.

structural environment is very different from that of the parent helix. In other words, in terms of the question asked in the *Introduction*, it would seem that, at least for this α-helix of T4 lysozyme, sequence is more important than structural context.

The amino acid sequence of the α-helix in question does not appear to have an unusually high helical propensity. McLeish and coworkers (27) showed that a peptide containing residues 38–51 of T4 lysozyme is unstructured in aqueous solution. Also, the helix content of the 12 residues 39–50 (Fig. 1*E*) theoretically estimated by using the program AGADIR (28) is 1.0%. For the overall duplicated region of 23 residues (Ile-39i–Ile-50, Fig. 1*E*) the maximum estimated helix content rises to 14%, which is still low. Nevertheless, the synergistic advantage in extending an existing α-helix, rather than nucleating a new one, may play a role in determining the observed structure of the duplication mutant.

If insertions (6, 7, 29, 30) of a small number of amino acids (e.g., 1–3) are made within α-helices, the polypeptide chain often is translocated, thereby preserving the overall helical region (6, 7, 31). Sometimes the length of the α-helix remains the same (6, 7). In other cases it can be lengthened, corresponding to the added amino acids (31). These observations suggest that the structures of helical segments within proteins are determined by a compromise between the sequence of amino acids within the helix and the structural context provided by the rest of the protein. At the same time, experimental measurement of the intrinsic α-helix-forming propensities of the 20 different amino acids give similar ranking whether based on α-helices within folded proteins or on isolated helical peptides (32–37). This finding strongly suggests that the amino acids within a given α-helix contribute in a direct and significant way to the formation of that helix. The extended α-helical conformation observed here for the duplicated segment of T4 lysozyme is supportive of this hypothesis.

Some experiments also have suggested that, in contrast to α-helices, the conformations of β-sheet regions of proteins tend to be determined not so much by the amino acid sequence within the β-strands themselves, but by nonlocal interactions, i.e., by the tertiary context provided by the rest of the protein (4, 38, 39). Sequence duplication of individual β-strands may provide a way to test this idea.

It may be instructive to contrast the present experiment, in which a peptide segment is duplicated within an intact protein, with studies of isolated peptides. It is well known that certain polypeptides tend to form α-helices or other structures in solution (40–42). The propensity for such structure formation is, however, always weak, in part because the ends are free and the interactions that stabilize the helix are barely sufficient to compensate for the entropic cost of organizing the polymer. In contrast, in the sequence duplication experiment each end of the inserted segment has to connect to the rest of the protein, which has two consequences that may tend to offset each other. On one hand, fixing the ends may help the inserted segment adopt a well-defined structure. On the other hand, the conformation that the inserted segment prefers to adopt may necessitate moving one or both of the ends to new positions. Indeed, this situation is observed here. At its carboxyl terminus, residue Ile-50i of the inserted segment connects directly to Asn-40, which retains the same position as in the wild-type enzyme. At the amino terminus, however, where Ile-39 connects to Asn-40i, the backbone has moved 13 Å from its position in the wild-type structure (Fig. 2). Thus, in the present case, the constraints imposed by the positions of the ends of the inserted segment seem to be modest and do not prevent the segment from displaying a rather clear preference for helical structure.

In summary, we have duplicated the sequence corresponding to an α-helix in T4 lysozyme. Because the ends of the insertion are connected to the rest of the protein, the entropic cost of forming a well-defined structure will be less than for the equivalent free peptide in solution. At the same time, the ends of the inserted sequence are not rigidly constrained and allow some freedom in the structure that is adopted. In the present case one end of the insertion continues the α-helix that is present in the wild-type structure and it is possible that the pre-existing helix acts as a nucleus for the extension. At the other end of the insertion the conformation of the wild-type protein is altered substantially. The largely helical structure that is adopted by the duplicated sequence, in an environment that is different from that of the parent sequence, suggests that it is the sequence of the helix rather than its structural context that is more important in defining its fold.

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