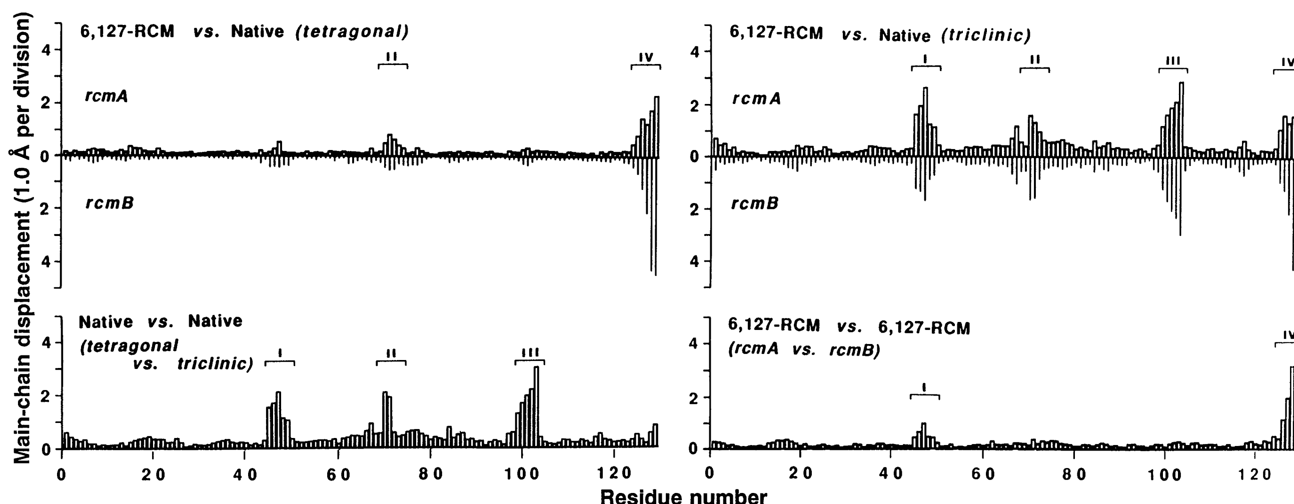

FIG. 3. 6,127-rcm-lysozyme conformation differs uniquely from native lysozyme at its C terminus. Plots of the main-chain rms deviations per residue are shown for all pairwise comparisons of the two 6,127-rcm-lysozyme forms (rcmA and rcmB) and two crystal forms of native lysozyme [tetragonal (22) and triclinic (29, 30)]. For comparisons of 6,127-rcm vs. native forms (*Upper Left* and *Upper Right*), deviations for rcmA are plotted above the horizontal axis and, for rcmB, below the axis. Regions of significant displacement are labeled I–IV. Deviations between the 6,127-rcm and native lysozymes at sites I, II, and III are also seen between the two native forms alone (*Lower Left*) and reflect conformational flexibility within the native protein. However, at the C-terminal five residues (region IV), both 6,127-rcm forms adopt conformations unavailable to native lysozyme.

(18). The crystal structure resolves this issue and points to displacement of the C-terminal segment from its conformation in the native protein as the key alteration that renders three-disulfide lysozyme a ubiquitination target. Substrate recognition by the Ub–protein ligase involves at least two determinants, a permissive N-terminal amino acid and an internal site that may include the ubiquitinatable Lys residue (9–12, 15, 31); these correspond to the E3 "head" and "body" sites postulated by Reiss *et al.* (10, 11). Because accessibility of lysozyme's N terminus (Lys-1) is identical in the 6,127-rcm and native forms, we propose that Cys-6 Cys-127 disulfide cleavage generates a ubiquitination substrate by exposing elements of the second, body site determinant. This site is unlikely to be within the conformationally altered C-terminal region because neither carboxymethylation of Cys-127 nor deletion of Arg-128 and Leu-129 decreases ubiquitination of three-disulfide lysozyme (unpublished work). Instead, as a consequence of the C-terminal rearrangement, there is enhanced exposure of the α-helix delineated by residues 5–15 and which contains Lys-13 as a potential ubiquitination site. Evidence that Lys-13 is the major ubiquitination site has come from experiments with a Lys-13 → Arg mutant form of lysozyme. Unlike the wild-type protein, the Arg-13 variant is ubiquitinated only poorly *in vitro* (unpublished).

Ubiquitination Determinants on Three-Disulfide Lysozyme. A view of the three-disulfide lysozyme surface that presents the N-terminal and putative internal recognition determinants is shown in Fig. 5. The model was constructed from native lysozyme by removal of the Cys-6 Cys-127 disulfide bond and rotation about the N—C_α bond of Gly-126 to swing the C-terminal tetrapeptide into the solvent, as suggested by the 6,127-rcm structures. We propose that this conformational change gives E3α or an E3α·E2 complex access to Lys-13 and allows simultaneous binding to the determinant at the N terminus, Lys-1. This model, in which key binding determinants are accessible only in the locally unfolded molecule, can explain why native lysozyme was unable to compete with the 6,127-rcm derivative in Ub-dependent degradation assays (17). Displacement of the C-terminal residues increases the accessibility of Lys-13 and adjacent residues, particularly Cys-6, Ala-9, and Ala-10, as well as Asp-18 and Leu-25; any or all of these might contribute to a surface for recognition by E3α or an E3α·E2 complex.

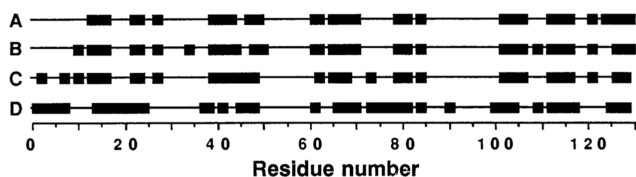

FIG. 4. Intermolecular contacts in the crystal lattice for native and 6,127-rcm lysozymes. Intermolecular contact distances were measured for four lysozyme structures, 6,127-rcm [rcmA (*A*) and rcmB (*B*)] and native [tetragonal (*C*) and triclinic (*D*)]. Superimposed upon lines representing the lysozyme primary sequence, the bars indicate residues ≤4.0 Å away from a symmetry-related protein molecule in the crystal lattice.

In yeast, a flexible polypeptide spacer between the N terminus and Ub attachment site was required for Ub-dependent degradation of modified forms of dihydrofolate reductase and β-galactosidase (15). The need for a minimum spacer length of >5 residues suggested that both determinants may be bound simultaneously to distinct sites on the Ub–protein ligase complex (15). Three-disulfide lysozyme differs from these other model substrates in that the recognition determinants are within a comparatively rigid structure. Indeed, the crystallographic results presented here together with NMR spectroscopy as a probe of solution structure (18) show that in three-disulfide lysozyme the main-chain atom positions are relatiyely fixed from residue 1 to residue 124. Thus, having demonstrated that E3α recognizes the folded form of three-disulfide lysozyme rather than a transiently unfolded species, we can infer that a distance of ≈20 Å separates the N-terminal and internal sites (i.e., head and body sites) on the mammalian E3α Ub–protein ligase.¶ Other constraints upon substrate recognition are likely to involve residues in the vicinity of Lys-13, and E2 bound to E3α also may participate in these interactions. The 6,127-

¶This distance applies only to the "Type I" N-terminal site that accommodates the basic amino acid Lys, Arg, or His (10–12). Results from the *in vivo* experiments of Bachmair and Varshavsky (15) may reflect proximity of the ubiquitination site to a "Type II" N-terminal site (bulky hydrophobic amino acid) on the yeast enzyme.