Strynadka and James (22) was used as a starting model for 6,127-rcm-lysozyme structure determination. Water molecules and side chains with multiple conformations were deleted from the starting model, as were the first 14 and last 6 amino acids. The 6,127-rcm-lysozyme crystals are similar to those of native lysozyme grown under similar conditions, although the latter are of the tetragonal space group $P4_32_12$ with cell dimensions of $a = b = 79.24$ Å and $c = 37.83$ Å (22). The $P2_12_12_1$ space group of the 6,127-rcm-lysozyme is related to $P4_32_12$ by one two-fold rotation axis. Consideration of this symmetry relationship enabled two copies of the truncated native lysozyme molecule to be placed within the 6,127-rcm-lysozyme asymmetric unit. Rigid-body refinement with XPLOR (23) gave an *R* factor of 26.7% against all 3.0- to 8.0-Å data. This solution was confirmed by rotation function and *R* factor searches using XPLOR. One cycle of simulated annealing refinement using the "slow-cool" protocol was followed by cycles of positional and *B* factor refinement, with several rounds of manual map-fitting using the program FRODO (24) implemented on an Evans and Sutherland ESV graphics station (Salt Lake City). The entire asymmetric unit was inspected in difference maps, water molecules were added, and the protein model was rebuilt where appropriate. Data collection and final refinement statistics are in Table 1.

Ub–Protein Conjugation. Ub-activating enzyme (E1) and the 14-kDa Ub carrier protein ($E2_{14K}$) were purified from calf thymus and rabbit reticulocytes, respectively (25, 26), and ubiquitination assays were done essentially as described (17). Each 10-μl reaction mixture in 50 mM Tris·HCl (pH 7.6) contained 2.5 mM dithiothreitol, 2 mM ATP, 5 mM $MgCl_2$, 5 mM phosphocreatine, 30 mU creatine phosphokinase, 3 mU inorganic pyrophosphatase, and a Ub-depleted rabbit reticulocyte extract (21 μg of protein; see ref. 17) supplemented with 5 μg of bovine Ub (Sigma), 57 nM E1, and 38 nM $E2_{14K}$. After a 5-min preincubation at 37°C with 2 μM Ub–aldehyde to prevent conjugate breakdown by endogenous Ub–protein isopeptidases (16), ^{125}I-labeled substrate (0.4 μg) was added and the incubation was continued for 1 hr. Products were visualized after SDS/polyacrylamide gel electrophoresis and autoradiography (17).

RESULTS AND DISCUSSION

The Folded Form of 6,127-rcm-Lysozyme Is a Ubiquitination Substrate. The 6,127-rcm derivative of lysozyme is thermodynamically less stable than unmodified lysozyme (18, 19), which might suggest that it is the unfolded protein that is recognized by the Ub–protein ligase, E3α. We have found that this is not the case. At 37°C under conditions that mimic a ubiquitination assay, a few percent of 6,127-rcm-lysozyme is in an unfolded state (Fig. 1*A*). As with unmodified lysozyme (28), an oligosaccharide ligand can shift this equilibrium to stabilize the folded form of the 6,127-rcm derivative (Fig. 1*A*), but such stabilization has no effect upon lysozyme ubiquitination (Fig. 1*B*). This same result is obtained over a wide range of lysozyme concentrations, where the substrate is well below the apparent K_m of Ub–protein ligase (unpublished data). Thus, the relatively limited structural differences between the folded conformations of native lysozyme and 6,127-rcm-lysozyme must include features used to distinguish ubiquitination targets from other proteins.

Table 1. Data collection and refinement statistics

Parameter	Value
Resolution range, Å	1.9–10.0
No. of reflections	18,295
Completeness, %	93.8
R_{sym}*	0.042
R_{factor}† (all data)	0.185
No. of water molecules included	133
B factor	
Molecule A average, Å^2	24.7
Molecule B average, Å^2	23.9
Solvent average, Å^2	36.3
rms deviations from ideal geometry	
Bond distances, Å	0.014
Bond angles, degrees	2.8
Dihedral angles, degrees	24.3
rms deviation of main-chain bond *B* values, Å^2	1.7

*$R_{sym} = (\Sigma|I_i - I_{av}|)/\Sigma I_{av}$.
†$R_{factor} = (\Sigma||F_{obs}| - |F_{calc}||)/\Sigma|F_{obs}|$.

Crystal Structure of 6,127-rcm-Lysozyme. The 6,127-rcm-lysozyme was crystallized into space group $P2_12_12_1$, different from that of native lysozyme crystals from which high-resolution structure coordinates are available. We were able to solve the structure by molecular replacement with a model

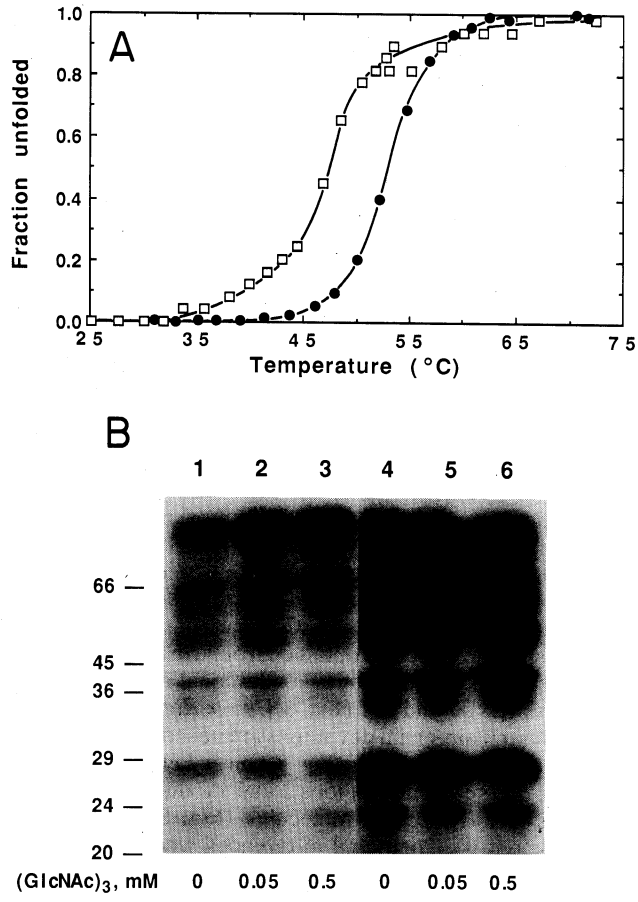

FIG. 1. Ubiquitination of lysozyme does not depend upon global unfolding. (*A*) Thermal denaturation of 6,127-rcm-lysozyme in 50 mM sodium 3-(*N*-morpholino)propanesulfonate buffer (pH 7.0) was monitored by the absorbance change at 280 nm (19), and the data are presented as the fraction of protein in the unfolded state without (□) or with (●) 1.0 mM *N,N′,N″*-triacetylchitotriose [$(GlcNAc)_3$]. The ligand stabilizes the protein, increasing its melting transition temperature from 47.6°C to 52.7°C. (*B*) Ub conjugation assays employed ^{125}I-labeled lysozyme (1×10^5 cpm, lanes 1–3) or 6,127-rcm-lysozyme (1.4×10^5 cpm, lanes 4–6) with 0, 0.05, and 0.5 mM $(GlcNAc)_3$ as indicated; native lysozyme and 6,127-rcm-lysozyme under these conditions are saturated by ≥0.05 mM $(GlcNAc)_3$ (ref. 27; unpublished data). Note that by inclusion of 2.5 mM dithiothreitol in these reactions, 1–2% of the native lysozyme is in the three-disulfide form (17). Conjugates (>20 kDa) were visualized after SDS/polyacrylamide gel electrophoresis and autoradiography. Positions of molecular mass markers (kDa) are to the left of the gel; bands corresponding to the ^{125}I-labeled substrates (14 kDa) were overexposed and are not shown.