Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 8851–8855, September 1994
Biophysics

The structure of a complex of hexameric insulin and 4′-hydroxyacetanilide

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Communicated by Herbert A. Hauptman, May 31, 1994 (received for review December 22, 1993)

ABSTRACT **X-ray crystallographic studies have been carried out on human insulin crystals grown in the presence of 4′-hydroxyacetanilide (Tylenol) and show that this nontoxic phenolic derivative can induce the T → R transition, producing a T_3R_3 hexamer. Two different crystals, grown under different conditions, are rhombohedral, space group *R*3, with cell constants *a* = 81.11, *c* = 37.97 and *a* = 80.88, *c* = 37.60 Å. The T_3R_3 hexamer is symmetric, resulting from the presence of a crystallographic threefold axis, and the asymmetric unit consists of a TR dimer. Data to a resolution of 1.9 Å were measured on a crystal from each of the two crystallizations and the structures have been refined to residuals of 0.168 and 0.173. The guest molecule is bound by the R-state monomer through the formation of two hydrogen bonds from the hydroxy group of Tylenol to the carbonyl oxygen and the nitrogen of A6 Cys and A11 Cys, respectively. Due to steric constraints of the phenolic binding site, the acetamide group of Tylenol is rotated ≈50° out of the plane of the phenyl group and the methyl group is *cis*; no hydrogen bonds exist between the acetamide group and the hexamer. Although the zinc ion, which is bound to the R-state trimer, has tetrahedral coordination in both structures, the T-state zinc is observed to have octahedral coordination in one structure but tetrahedral coordination in the other. The side chain of A10 Ile in the R-state monomer adopts a high-energy conformation as a result of close contact to a residue in an adjacent dimer and may explain in part the differences between therapeutic preparations of beef insulin, for which A10 is a Val residue, and human insulin.**

The allosteric behavior of insulin has been used unknowingly for many years to produce therapeutic, microcrystalline preparations of insulin, which have a long timing of action for the control of diabetes (1). The results of crystallographic studies on crystals, grown under similar conditions, have been able to explain the reduced rate of dissociation of hexameric insulin to the biologically relevant monomer as due to a change in conformation of eight N-terminal B-chain residues from extended to α-helical in three of the six monomers (2). This crystalline modification of insulin was initially named 4-zinc insulin (3) but, using a newer and less confusing nomenclature, it is now known as the T_3R_3 hexameric form, with T and R referring to an extended or α-helical conformation of the first eight residues of the B-chain (4). While the addition of chloride or thiocyanate is necessary to transform the T_6 hexamer to that of T_3R_3 (5), phenol or phenolic derivatives are necessary to complete the transformation to R_6, a hexameric form that has a much slower exchange of zinc ion than other hexameric forms (4). However, the toxicity of phenol and closely related derivatives precludes their use at the concentrations required to produce R_6 therapeutic preparations.

X-ray crystallographic studies have provided accurate structural models for all three allosteric hexamers (2, 3, 6–9) and the phenolic binding site in the R_6 hexamer has been described in detail. These results have shown that there is sufficient space in the binding site to accommodate phenolic derivatives that possess *m* or *p* substituents containing several atoms. The existence of computer programs to explore the potential of binding a small molecule of known structure within a cavity or active site of a protein (10) and the large number of small molecule entries in the Cambridge Structural Data Base (11) provides a means for identifying and selecting a series of nontoxic phenolic derivatives that might be accommodated in the phenolic binding site. This information can be used to design crystallization experiments with various derivatives, and the subsequent determination of the structure of the putative complex can verify the presence of the guest molecule and the details of its binding.

We report here the identification of a nontoxic phenolic derivative that can be accommodated in the phenol binding site and the subsequent crystallization and structure determination of the complex.¶

EXPERIMENTAL PROCEDURES

Modeling. In preparation for identifying phenolic derivatives that can be accommodated in the phenolic binding site, the rhombohedral R-state dimer (9) was used to construct the threefold symmetric R_6 insulin hexamer. Using a probe radius of 1.3 Å, a Connolly surface (12) was constructed about all atoms of the hexamer within 10 Å of one of the phenol binding sites. A complete search of the Cambridge Structural Data Base (11) for simple *p*- and *m*-substituted phenol derivatives resulted in identifying 52 entries, of which 42 were unique. In Search mode, version 2.0 of DOCK (10) identified 12 derivatives that were too large to be accommodated by the phenolic binding site. Of the remaining 30 structures, 4′-hydroxyacetanilide and ethyl *p*-hydroxybenzoate (Cambridge Structural Data Base REFCODS, HXACAN, and CEBGOF) were ranked as the second and third highest scoring substrates, respectively. Interestingly, phenol ranked last, while *m*-cresol and resorcinol ranked sixth from last. Although an examination of the collection of ligands in the binding site showed that none of them adopted the expected orientation with the formation of a pair of hydrogen bonds to A6 Cys and A11 Cys, there was clearly sufficient space in the binding site to accommodate any of the 30 ligands. Since the primary goal of this research was to identify a nontoxic phenolic ligand that can induce the T → R transition in insulin, further studies were focused on 4′-hydroxyacetanilide, a common analgesic that is sold under the tradename of Tylenol.

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¶The atomic coordinates have been deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973 (reference 1TYL, 1TYM).

Crystallography. Biosynthetic human insulin complexed with zinc was provided by Lilly Research Laboratories (Indianapolis). Buffer, salts, and other reagents were purchased and used without further purification. An initial crystallization was carried out in 0.05 M sodium citrate/0.007 M zinc acetate/0.07 M 4'-hydroxyacetanilide/0.75 M sodium chloride, pH 6.4. The crystals belong to space group $R3$ and were indexed in a hexagonal cell. Intensity data were measured from an insulin crystal with dimensions of approximately 0.5 × 0.5 × 0.5 mm using a Rigaku R-Axis II image plate system and RU-200 rotating anode generator with graphite monochromated CuKα radiation (λ = 1.54178 Å) at 290 K. Information regarding crystallization conditions and data processing is given in Table 1.

The unit cell constants are nearly identical to that of T_3R_3 insulin crystals, which were known in the earlier literature as 4-zinc insulin (2, 3). The initial model of 747 atoms consisted of the entire A-chains and residues B2–B28 of both monomers of the T_3R_3 insulin dimer (7); side chains that had been refined in two alternative conformations were excluded. Using this model in a structure factor calculation using PROFFT (13, 14) produced a residual of 0.282 and the largest peaks from the $2|F_o - F_c|$ and $|F_o - F_c|$ maps corresponded to a pair of zinc ions and a chloride ion located on the threefold axis. Continuous electron density corresponding to a Tylenol molecule was present in the phenolic binding site and was considerably different from the discrete water peaks observed in the T_3R_3 insulin hexamer (7). As a precaution against overinterpreting the maps, restrained least-squares refinement using PROFFT (13, 14) was carried out on a minimal initial model consisting of only the protein atoms of the TR dimer; individual temperature factors were also refined. As the refinement progressed, necessary adjustments were made to the model using FRODO (15), and zinc and chloride ions were added along with well-defined water molecules extraneous to the phenolic binding site. After multiple cycles of refinement and model building, which reduced the residual to 0.212 for 6313 data with $F > 2.0\sigma(F)$ in the resolution range 8.0–1.9 Å, a Tylenol molecule was finally added on the basis of the $2|F_o - F_c|$ electron density map shown in Fig. 1. The continuous electron density in the binding site could be interpreted unambiguously and clearly revealed the orientation of the acetamide and the position of the phenolic hydroxy group, which were modeled accordingly. The refinement was then continued and appropriate adjustments were made to main- and side-chain atoms of the dimer and water molecules were added in accord with the criteria of good electron density and acceptable hydrogen bonds to other atoms. Throughout the refinement, the geometry was continually monitored by Procheck (16). Unlike the T_3R_3 insulin hexamer in which the first eight residues of the R-state B-chain were well defined, the electron density maps provided no indication of the positions of B1.2 Phe and B2.2 Val[‖] of the R-state monomer, suggesting that these residues are probably disordered. The quality of the electron density in the vicinity of the C termini of both B-chains was poor and as a result it was not possible to locate the side chain of B30.1 Thr or the side chain of B29.2 Lys and all but the nitrogen of B30.2 Thr. The side chains of B13.1 Glu, A13.2 Leu, B9.2 Ser, and B21.2 Glu were found to exist in two discrete orientations. The refinement converged at a residual of 0.168 for 6316 data between 8.0 and 1.9 Å resolution. Illustrated in Fig. 2 is the final electron density map in the vicinity of the Tylenol molecule.

Table 1. Crystallization conditions and data analysis

	Crystal 1	Crystal 2
Tylenol, M	0.07	0.07
NaCl, M	0.75	1.00
pH	6.4	5.6
a, Å	81.11	80.88
c, Å	37.97	37.60
Resolution, Å	1.9	1.8
Total data	28940	44075
Independent data	6783	7866
R_{merge}	0.034	0.061
Resolution range, Å	% reflections with $F_o \geq 2\sigma(F_o)$	
∞–3.0	100	99
3.0–2.5	99	99
2.5–2.2	96	96
2.2–2.0	82	90
2.0–1.9	46	67
1.9–1.8		37

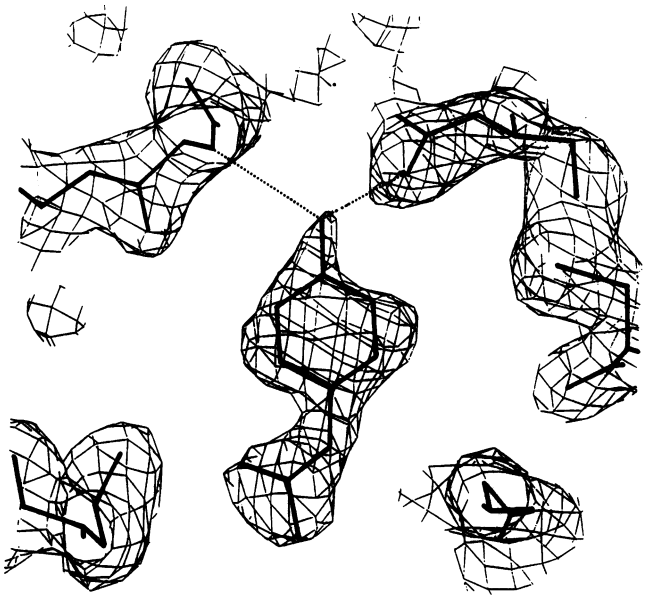

FIG. 1. $2|F_o - F_c|$ electron density (1.0σ) in the phenolic binding site prior to inclusion of the Tylenol molecule in the refinement. The crystallographic R-factor was 21.2% at the time this map was calculated. Coordinates for the Tylenol molecule were extracted from the Cambridge Structural Data Base and were manually adjusted to fit the observed electron density; hydrogen bonds to the oxygen and nitrogen atoms of A6 and A11 Cys, respectively, are illustrated as dotted lines.

To further verify the presence of the Tylenol molecule in the phenolic binding site, data were measured from a second crystal grown under similar conditions from the first but with 1.0 M NaCl and at pH 5.6; data were measured and processed as described (Table 1). A comparison of the two data sets using NORMAN (17) resulted in a residual of 0.174 for those data between 8.0 and 1.9 Å resolution and with $F < 2\sigma(F)$; the largest weighted differences existed in the lower resolution shells and the normal probability plot was nonlinear. A structure factor calculation using an intermediate set of coordinates, which included Tylenol, zinc, and chloride atoms from the refinement of the first set of data (876 atoms) and with a single overall temperature factor, produced a residual of 0.289. Several cycles of restrained least-squares refinement allowing individual temperature factors to refine reduced the residual to 0.204. The Tylenol and adjacent water molecules were excluded from the next few cycles of refinement and the resulting $|F_o - F_c|$ electron density map again verified the presence of Tylenol. The

[‖]The 1 or 2 in the decimal portion of the chain name and residue number refers to monomer 1 or 2, respectively.

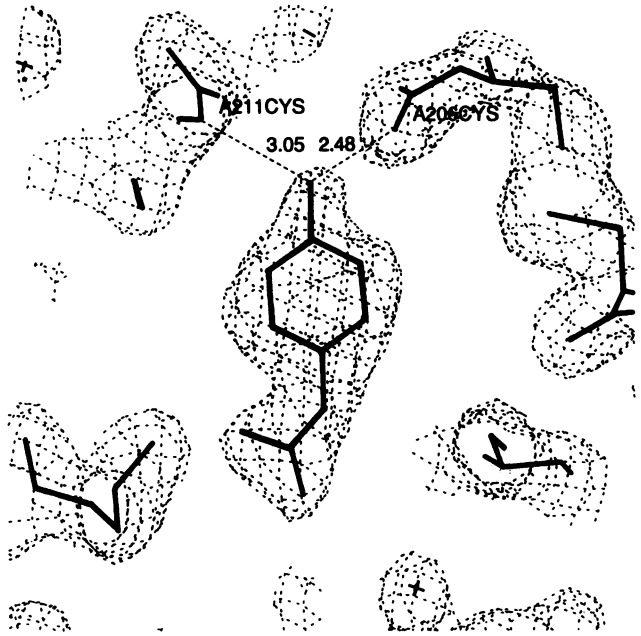

FIG. 2. $2|F_o - F_c|$ electron density (1.0σ) in the vicinity of the Tylenol molecule. Hydrogen bonds to the oxygen and nitrogen atoms of A6 and A11 Cys, respectively, are illustrated as dashed lines. The crystallographic R-factor is 16.8%.

fully refined coordinates from the first set of data excluding Tylenol, water molecules, and disordered side chains were then substituted and an omit map was generated to verify or reposition these suspect groups. The refinement converged at a residual of 0.173 for 6551 data between 8.0 and 1.9 Å resolution. Details of both refinements are given in Table 2.

At the end of the refinement, a single $1\sigma 2|F_o - F_c|$ peak and several $2.5\sigma F_o - F_c$ peaks were found in the phenol binding site in both structures. The existence of these peaks, which are too close to the Tylenol molecule to be interpreted as water molecules, strongly suggests that Tylenol is not present with an occupancy of unity. Rather, these peaks represent a cluster of water molecules that are present in those dimers lacking the guest molecule, similar to that observed in the T_3R_3 structure for which there is no guest molecule in the binding site (7). The thermal parameters of the Tylenol molecule are approximately twice that observed for phenol or *p*-hydroxybenzamide (unpublished results), adding further support to the contention that the site is not fully occupied.

RESULTS

The structure of the TR insulin dimer complexed with Tylenol is shown in Fig. 3, and three identical dimers related by the crystallographic threefold axis comprise the symmetric T_3R_3 insulin hexamer. With the exception of those residues that undergo the T → R transformation, the two monomers of the dimer are related by a pseudo-twofold axis, which is nearly perpendicular to the crystallographic threefold axis. A comparison of the two structures obtained from the refinement of the data from the two crystals, minimizing the displacements of backbone atoms in residues A1–A21 of both monomers and B1.1–B28.1 and B3.2–B28.2, resulted in an average displacement of 0.175 Å and a rms displacement of 0.193 Å; the inclusion of side chains increased the average and rms displacements to 0.344 and 0.649 Å, respectively. A comparison of the backbone atoms of the two insulin dimers complexed to Tylenol with that of the T_3R_3 dimer, which contains no guest molecule in the binding site (7), resulted in average and rms displacements of 0.199 and 0.248 Å for the first crystal and 0.161 and 0.197 Å for the second crystal. The R-state monomer is also very similar to that observed in the R_6 hexamer, with average and rms displacements of 0.393 and 0.462 Å, respectively, and while the largest displacements are observed at the N and C termini of the A- and B-chains, there is also a small but significant rotation of the A-chain, N-terminal α-helix.

The Tylenol molecule is bound by the R-state monomer through a pair of hydrogen bonds from the phenolic hydroxy group to the carbonyl oxygen of A6.2 Cys (2.48 Å) and the nitrogen of A11.2 Cys (3.05 Å), similar to that observed for other guest molecule phenolic derivatives. Since the electron density maps are not capable of distinguishing between the *cis* and *trans* orientations of the methyl group of the acetamide moiety, the Tylenol molecule was initially modeled in

Table 2. Refinement statistics

		Crystal 1	Crystal 2
Resolution, Å		8.0–1.9	8.0–1.9
No. of reflections ($F_o \geq 2\sigma(F_o)$)		6316	6551
R value		0.168	0.173
No. of reflections (all data)		6687	6947
R value		0.174	0.180
	Target σ	Distances, Å	
Bond distances, 1–2	0.020	0.019	0.019
Angle distances, 1–3	0.040	0.047	0.046
Planar distances, 1–4	0.050	0.049	0.047
Chiral volume, Å^3	0.150	0.174	0.165
Planar groups, Å	0.020	0.013	0.013
Nonbonded distances, Å			
Single torsion	0.50	0.199	0.187
Multiple torsion	0.50	0.222	0.226
Possible H bonds	0.50	0.261	0.326
Torsion angles, °			
Planar	3.0	2.2	2.3
Staggered	20.0	18.5	18.8
Orthonormal	25.0	19.5	18.2

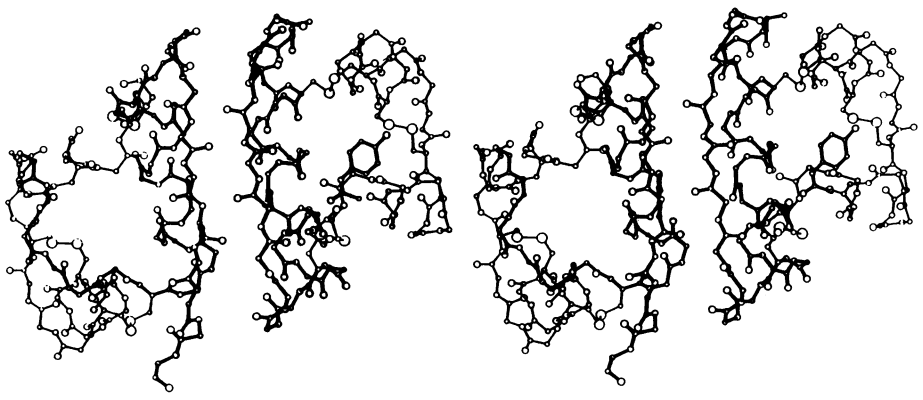

FIG. 3. Stereo drawing of the backbone of the TR insulin dimer complexed to Tylenol. B-chains are drawn with thick bonds, while the A-chains and the disulfide bridges are drawn with thin bonds. The R-state monomer is on the right.

the *trans* conformation observed in the small molecule crystal structure determination of Tylenol (18). After several cycles of refinement, it became obvious that this conformation not only placed the carbonyl oxygen in a hydrophobic region of an adjacent insulin dimer but also resulted in a very close contact between the methyl group and the side chain of a symmetry-related B13.1 Glu. As a result, the conformation was changed from *trans* to *cis*, which results in three contacts between the acetamide moiety of Tylenol and the insulin hexamer, shown in Fig. 4. The carbonyl oxygen is 2.54 Å from the carboxyl group of B13.1 Glu of an adjacent dimer, while the methyl group is 3.08 and 3.18 Å from the side chains of B17.1 Leu and B5.2 His of an adjacent dimer, respectively. The closest contact of the Tylenol amido nitrogen is to the carbonyl oxygen of B10.2 His at a distance of 3.55 Å. The absence of strong hydrogen-bonded interactions to this portion of the guest molecule may explain in part the high thermal motion of Tylenol as compared to that observed for other phenolic derivatives.

In the small molecule crystal structure of Tylenol (18) the acetamide group is rotated ≈18° out of the plane of the phenol group and the methyl group is *trans*. Tylenol, bound to insulin, not only has the methyl group in a *cis* conformation but also has the acetamide group rotated ≈50° out of the plane of the phenol group. This conformation was clearly indicated by the electron density maps and since there are no strong hydrogen-bonded interactions between the acetamide moiety and the insulin hexamer, the conformation is evidently induced by the spatial constraints of the phenolic binding site. Attempts to use the conformation observed in the crystal structure of Tylenol produce unacceptably short contacts between the acetamide moiety and the insulin hexamer.

Each of two zinc ions lies on the threefold axis where they are coordinated by three symmetry-related B10 His side chains. Because of the T→R transition, the two surfaces that bind zinc are not equivalent; one surface is constructed from three T-state monomers while R-state monomers make up the other trimer. The presence of three α-helical segments of the N termini of the B-chains in the R-state trimer produces a narrow channel coincident with the crystallographic threefold axis and provides the only access to the coordinated metal. Because the width of the channel is relatively narrow, the metal ion is required to adopt tetrahedral rather than the octahedral coordination observed in the T_6 hexamer (6). Bond distances between zinc and the three symmetry-related $N^{\epsilon 2}$ of B10.2 His in the R-state trimer are 2.06 and 2.12 Å, while distances of 2.39 and 2.33 Å are observed to the fourth ligand, a chloride ion, for crystals one and two, respectively.

In the T-state trimer, the metal ion resides in a shallow dimple and is in contact with the surrounding solvent. There is sufficient space to accommodate an octahedral coordination sphere, as was observed in the T_6 insulin hexamer (6) where three water molecules complete the coordination sphere of the zinc ion. In the T_3R_3 insulin hexamer (7), the T-state zinc ion exhibits a "dual-coordination" scheme whereby the metal ion is octahedrally coordinated in 50% of the hexamers but is tetrahedral in the other 50%. In the present studies, the electron density maps clearly show that the coordination sphere of the T-state zinc ion is completed by water and is octahedral in crystal 1 but tetrahedral in crystal 2. Distances from zinc to B10 His $N^{\epsilon 2}$ are 2.23 and 2.11 Å, while contacts to the water molecules are 2.45 and 2.05 Å, respectively, for crystals 1 (octahedral) and 2 (tetrahedral).

Hydrogen bonds that stabilize the formation of the dimer in the T_3R_3 hexamer have been described in detail (7). The conformation of the B25.1 side chain (χ^1, χ^2 = 66.1°, 84.5°) places the phenyl ring over the A-chain of the monomer and a hydrogen bond exists between the nitrogen of B25.1 and the carbonyl oxygen of A19.1 (3.10 Å). In contrast, the side chain of B25.2 (χ^1, χ^2 = −83.7°, 61.3°) lies over the dimer-forming interface but the distance between the corresponding nitrogen and carbonyl oxygen atoms has increased to 4.80 Å. In the T_6 hexamer (6), both B22 Arg side chains are disordered in two well-defined conformations where they form salt bridges to the carboxyl groups of A21 Asn or A17 Glu. In the T_3R_3 hexamer complexed with Tylenol, there is no evidence for disorder of the B22 Arg side chains. Salt bridges exist between B22.1 and A17.1 Glu and between B22.2 and A21.2 Asn.

Several side chains adopt orientations that normally would be considered to be unlikely. The C^{γ} side chains of both A4 Glu residues nearly eclipse the nitrogen atom of that residue. Although this is a high-energy conformation, the energy loss is compensated by the folding of the negatively charged carboxyl group back toward the positively charged N terminus of the A-chain, A1 Gly, with distances of 3.18 and 3.00 Å for monomers 1 and 2, respectively. The A10 Ile side chains provide another interesting set of conformations. $C^{\gamma 1}$ of A10.1 Ile (T-state) is nearly *trans* to the carbonyl group of that residue, a frequently observed conformation with χ^1, χ^2 = −61°, 166°. In that conformation, the closest contact of this side chain is to a water molecule at 3.39 Å. In contrast, H^{α} and H^{β} of A10.2 Ile (R-state) are eclipsed, which also eclipses both C^{γ}s with the nitrogen and the carbonyl group ($\chi^{1,1}$ = 133°, $\chi^{1,2}$ = 5°). This high-energy conformational state results in a single contact of 3.02 Å between the side chain and a water molecule. The reason for this unlikely conformation is the proximity of the A10.2 Ile side chain to a B5.2 His side chain in an adjacent dimer of the hexamer. Rotation of the χ^1 torsion angle to any of the preferred, staggered conforma-

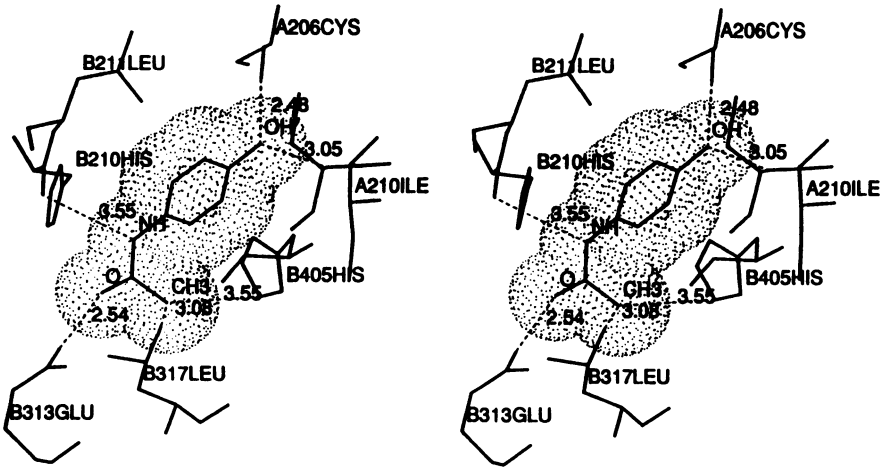

FIG. 4. Stereo drawing of the immediate environment of the Tylenol molecule within the phenolic binding site. Hydrogen bonds and close contacts are illustrated as dashed lines, and a Connolly surface (12) is drawn around the Tylenol molecule. Labels on the amino acids refer to the A- or B-chain, residue number, and amino acid name—e.g., B317Leu refers to the B-chain, monomer 3, residue 17, leucine; monomer 3 is related by symmetry to the T-state monomer 1, while monomer 4 is related to the R-state monomer 2. Heteroatoms of the Tylenol molecule are also labeled.

tions results in contacts between the Ile and His side chains, which range from 2.1 to 3.1 Å, while either of the two alternate eclipsed conformations produces contacts between the C^{γ} carbon atoms and the His side chain of 2.5–2.6 Å. At the same time, the side chain of B5.2 His is restricted to its observed conformation (χ^1, χ^2 = −165°, 129°) by the proximity of side chains in adjacent residues in the α-helical segment of the B-chain and by the A10.2 Ile side chain in a neighboring dimer.

The A-chain residues A8 Thr, A9 Ser, and A10 Ile reside between the intrachain disulfide link (A6–A11) and are preceded by the interchain disulfide link (A7–B7). Because of the T → R transition, C^{α} of B7.2 Cys is displaced by 1.45 Å, which produces a 180° change in the torsion angle of the A7–B7 disulfide bond. The net effect is a 20° rotation of the N-terminal α-helical segment of the A-chain, which terminates at A8 Thr, producing displacements of the C^{α}s of ≈1 Å. Because of the presence of the two disulfide bonds and the unusual conformation of A10.2 Ile, which is necessary to relieve potential interdimer contacts, any conformational changes to accommodate the rotation of the N-terminal α-helical segment must be centered in this A8–A10 tripeptide segment. The increase in these residues' thermal parameters by ≈20% and the decrease in the quality of their electron densities suggest that there may be a small degree of disorder in the backbone atoms of this tripeptide segment and that the observed conformation represents an average of a family of very closely related conformations.

DISCUSSION

While the A-chain amino acid sequences of porcine and human insulin are identical, two amino acid substitutions exist in bovine insulin at A8 (Thr to Ala) and A10 (Ile to Val). Slow-acting forms of bovine insulin have been found to have superior therapeutic properties as compared to human insulin (19). The high-energy conformation of the A10.2 Ile side chain, which is imposed by the presence of the B5.2 His side chain in an adjacent dimer, must reduce the stability of the human insulin hexamer somewhat. However, in beef insulin this residue is Val, which lacks the $C^{\delta 1}$ carbon atom. A visual inspection following the replacement of the A10.2 Ile residue by Val in the present study shows that a Val side chain can adopt a lower energy conformation ($\chi^1 \cong 180°$) while maintaining a distance of 3.3 Å from the imidazole ring of B5.2 His. The resulting increase in hexamer stability may explain in part the more nearly constant plasma levels of slow-acting beef insulin preparations as compared to human insulin.

It was noted earlier that because of high thermal motion and the presence of ΔF peaks, the Tylenol molecule does not appear to occupy the binding site at 100% occupancy. There are several factors that would be expected to reduce the binding of Tylenol to the insulin hexamer as compared to that of phenol. First, the solubility of Tylenol is considerably less than that of other phenolic derivatives such as phenol or resorcinol. Second, the shape of the phenolic binding site within the hexamer requires the acetamide group to be rotated out of the plane of the phenyl group, bringing about a loss of delocalization in the guest molecule and hence an increase in energy. Third, the lack of strong interactions between the acetamide moiety and the two dimer surfaces adds little to stabilize the hexamer. Finally, the results of this work do not suggest that there may be a pharmacological interaction of insulin and Tylenol in diabetics who have taken the latter. Since complex formation does not appear to be complete at the concentrations used in the crystallization experiments, which is many orders of magnitude larger than the plasma levels in individuals who have taken these drugs through their normal routes, it seems unlikely that any appreciable complexation of Tylenol by insulin will occur in the bloodstream.

The results of this investigation clearly show that the T_3R_3 insulin hexamer is capable of binding guest molecules larger than simple *p*- or *m*-substituted phenols such as resorcinol or *m*-cresol.** Although Tylenol is bound at <100% occupancy, these results clearly demonstrate that it is possible to induce the T → R transition by means of a nontoxic phenolic derivative. The identification of other guest molecules that are also nontoxic and have a higher binding affinity to the insulin hexamer may ultimately result in a new generation of longer-acting therapeutic insulin preparations.

**Ciszak, E. & Smith, G. D., American Crystallographic Association Meeting, August, 1992, Pittsburgh, abstr. PC46.

The authors wish to thank Walter A. Pangborn for assistance in the data collection, Robert H. Blessing for stimulating discussions, Amy Taylor for technical assistance, and Lilly Research Laboratories for a generous gift of recombinant human insulin. This work has been supported by National Institutes of Health Grant DK-41387.

1. Schlichtkrull, J. (1958) *Insulin Crystals* (Munksgaard, Copenhagen).
2. Smith, G. D., Swenson, D. C., Dodson, E. J., Dodson, G. G. & Reynolds, C. D. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 7093–7097.
3. Bentley, G., Dodson, E. J., Dodson, G. G., Hodgkin, D. & Mercola, D. (1976) *Nature (London)* **261**, 166–168.
4. Kaarsholm, N. C., Ko, H.-C. & Dunn, M. F. (1989) *Biochemistry* **28**, 4427–4435.
5. de Graff, R. A. G., Lewit-Bentley, A. & Tolley, S. P. (1981) in *Structural Studies on Molecules of Biological Interest*, eds. Dodson, G. G., Glusker, J. & Sayre, D. (Oxford Univ. Press, Oxford, U.K.), pp. 547–556.
6. Baker, E. N., Blundell, T. L., Cutfield, J. F., Cutfield, S. M., Dodson, E. J., Dodson, G. G., Hodgkin, D. C., Hubbard, R. E., Isaacs, N. W., Reynolds, C. D., Sakabe, K., Sakabe, N. & Vijayan, N. M. (1988) *Philos. Trans. R. Soc. London B* **319**, 369–456.
7. Ciszak, E. & Smith, G. D. (1994) *Biochemistry* **33**, 1512–1517.
8. Derewenda, U., Derewenda, Z., Dodson, E. J., Dodson, G. G., Reynolds, C., Smith, G. D., Sparks, C. & Swenson, D. (1989) *Nature (London)* **338**, 589–596.
9. Smith, G. D. & Dodson, G. G. (1992) *Proteins* **14**, 401–408.
10. Shoichet, B. K., Bodian, D. L. & Kuntz, I. D. (1992) *J. Comp. Chem.* **13**, 380–397.
11. Allen, F. H., Kennard, O. & Taylor, R. (1983) *Acc. Chem. Res.* **16**, 146–153.
12. Connolly, M. L. (1983) *J. Appl. Crystallogr.* **16**, 548–558.
13. Hendrickson, W. A. & Konnert, J. H. (1980) in *Computing in Crystallography* eds. Diamond, R., Ramaseshan, S. & Venkatesan, K. (Indian Acad. Sci., Bangalore, India), pp. 13.01–13.23.
14. Finzel, B. C. (1987) *J. Appl. Crystallogr.* **20**, 53–55.
15. Bush, B. L. & Jones, T. A. (1988) PS300 FRODO-Molecular Graphics Program for the PS300; modified by Sack, J. (Rice University, Houston, TX).
16. Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thornton, J. M. (1993) *J. Appl. Crystallogr.* **26**, 283–291.
17. Howell, P. L. & Smith, G. D. (1992) *J. Appl. Crystallogr.* **25**, 81–86.
18. Haisa, M., Kashino, S., Kawai, R. & Maeda, H. (1976) *Acta Crystallogr.* **B32**, 1283–1285.
19. Seigler, D. E., Olsson, G. M., Agramonte, R. F., Lohman, V. L., Ashby, M. H., Reeves, M. L. & Skyler, J. S. (1991) *Diabetes Nutr. Metab.* **4**, 267–273.