Crystallographic Evidence for Dual Coordination around Zinc in the T_3R_3 Human Insulin Hexamer[†,‡]

Ewa Ciszak[§] and G. David Smith[*]

Medical Foundation of Buffalo, Inc., 73 High Street, Buffalo, New York 14203

Received September 22, 1993; Revised Manuscript Received November 22, 1993[⊗]

ABSTRACT: The T_3R_3 human insulin hexamer (T and R referring to extended and α-helical conformations, respectively, of the first eight residues of the B-chain), complexed to two zinc ions, crystallizes in space group $R3$ with hexagonal cell constants a = 80.64 and c = 37.78 Å. The structure has been refined to a residual of 0.172 using 9225 independent data to 1.6-Å resolution. The asymmetric unit consists of a TR dimer, and the insulin hexamer is generated by the action of the crystallographic 3-fold axis. The conformation of one insulin trimer is nearly identical to that of the T_6 hexamer, while the other trimer approximates that of the R_6 hexamer, except for the three N-terminal B-chain residues that adopt an extended rather than an α-helical conformation. Each of the two zinc ions, which lie on the crystallographic 3-fold axis and exhibit two different, disordered coordination geometries, is coordinated by the imidazole groups of three symmetry-related B10 histidine residues. The coordination sphere of the zinc in the T_3 trimer is either tetrahedral, with the fourth site filled by a chloride ion, or octahedral, completed by three water molecules. The coordination of the zinc in the 12-Å narrow channel in the R_3 trimer is tetrahedral, with either a second chloride ion or a water molecule completing the coordination sphere. The putative off-axial zinc binding sites that result from the T→R transition of monomer II do not contain zinc ion, but instead are filled with clusters of ordered water molecules. The observation that the T-state trimer contains zinc in both tetrahedral and octahedral geometries has important implications for the interpretation of spectroscopic results.

Insulin circulates in the bloodstream and interacts with the insulin receptor as a monomer (Berson & Yellow, 1966), but is synthesized and stored in the pancreas as a zinc-containing hexamer. The insulin molecule consists of a 21-residue A-chain and a 30-residue B-chain linked by two disulfide bonds; an additional intrachain disulfide bond exists in each A-chain. In the presence of zinc ion, insulin forms hexamers. These aggregated forms of insulin are used in therapeutic preparations for the control of diabetes and have also been the subject of numerous crystallographic investigations (Bentley et al., 1976, 1992; Smith et al., 1984; Baker et al., 1988; Derewenda et al., 1989; Smith & Dodson, 1992).

Insulin's crystal structure was first reported as the rhombohedral "2-Zn form" (Adams et al., 1969), named according to its zinc content (Schlichtkrull, 1958). The results of this work showed that a crystallographic 3-fold axis generated the hexamer from an insulin dimer. The two molecules that make up the dimer have nearly identical conformations and are related to each other by a local 2-fold axis, perpendicular to the crystallographic 3-fold axis. The conformation of each A-chain consists of two antiparallel α-helices connected by a short extended strand, A9Ser–A12Ser, while each B-chain consists of two extended strands connected by a central α-helical segment, B9Ser–B19Cys. Each of the two zinc ions lies on a crystallographic 3-fold axis and is coordinated by the side chains of three symmetry-related B10 histidine residues from each of the two insulin trimers.

The addition of chloride ion to the crystallizing medium produces a second rhombohedral crystal form, which also possesses a 3-fold-symmetric hexamer. In contrast to the 2-Zn form, in which the two independent monomers have nearly identical conformations, the first eight residues of one of the B-chains have undergone a change in conformation from extended to α-helical (Bentley et al., 1976; Smith et al., 1984). This change in conformation displaces the N-terminus of the B-chain by more than 30 Å, producing a continuous α-helix from B1Phe to B19Cys. As a result of the displacement of the N-terminus of the B-chain, three symmetry-equivalent elliptical cavities are produced in the vicinity of the B5His residues. Earlier crystallographic studies had suggested that these cavities were either fully or partially occupied by zinc ions coordinated by the side chain of B5His, a second orientation of the B10His side chain, and two water molecules (Bentley et al., 1976, 1992; Smith et al., 1984). This crystal form was called "4-zinc" insulin to reflect the fact that the hexamer could bind up to a maximum of four zinc ions.

As additional crystallographic and spectroscopic studies were undertaken, it was found that insulin hexamers existed with the N-termini of all six B-chains in α-helical conformations and in the presence of only two zinc ions (Derewenda et al., 1989; Roy et al, 1989; Smith & Dodson, 1992; Wollmer et al., 1987). In order to avoid confusion in the naming of the hexameric forms according to zinc ion content, a nomenclature has been adopted that reflects the conformation of the first eight residues of the B-chains in the hexamer: T and R, referring to an extended and an α-helical conformation of B1Phe–B8Gly, respectively (Kaarsholm et al., 1989). Thus, the 2-zinc insulin hexamer is called T_6, while T_3R_3 refers to the 4-zinc insulin structure. The hexameric forms that are produced in the presence of phenolic derivatives are called R_6 (Derewenda et al., 1989; Smith & Dodson, 1992). We report here the crystal structure of a T_3R_3 human insulin hexamer that contains only two zinc ions. Both of the zincs exhibit a dual coordination pattern, there is no evidence for a second orientation of the B10 histidine side chain, and all three off-axial elliptical cavities contain only water molecules.

[†] Supported by NIH Grant DK-41387.

[‡] Atomic coordinates have been deposited with the Brookhaven Protein Data Bank under filename 1TRZ.

[*] Author to whom correspondence should be addressed.

[§] On leave from the Institute of Chemistry, Pedagogical University, ul. Oleska 48, 45-052 Opole, Poland.

[⊗] Abstract published in *Advance ACS Abstracts,* January 15, 1994.

0006-2960/94/0433-1512$04.50/0

Table 1: Summary of Measured Intensities

resolution range, d(Å)	no. of possible reflections	no. of measured reflections	% reflections with $F_o \geq 2\sigma(F_o)$
∞–2.5	3659	3165	98
2.5–2.0	3487	2984	93
2.0–1.8	2657	2191	78
1.8–1.7	1834	1463	60
1.7–1.6	2321	1798	47

EXPERIMENTAL PROCEDURES

Crystallization. Human insulin was provided by Lilly Research Laboratories as crystalline biosynthetic human insulin complexed with zinc. Buffer, salts, and other reagents were purchased and used without further purification. Crystals were grown from 0.05 M sodium citrate and 0.007 M zinc acetate in the presence of 0.75 M sodium chloride at pH 6.4 (Schlichtkrull, 1958). Colorless, sharp-edged rhombohedrons up to 1 mm in length grew in approximately 1 week. The crystals belonged to space group $R3$ and were indexed in a hexagonal cell with dimensions $a = 80.64$ and $c = 37.78$ Å; a volume of 2.128×10^5 Å^3 is consistent with the presence of a dimer in the asymmetric unit.

Data Collection. Intensity data were measured from a single insulin crystal of dimensions $0.7 \times 0.6 \times 0.5$ mm using a Rigaku R-AXIS II image plate system and an RU-200 rotating anode generator with graphite-monochromated Cu Kα radiation ($\lambda = 1.541\,78$ Å) at 290 K to a resolution limit of 1.60 Å. A total of 34 613 data was recorded on 35 frames, each frame corresponding to an oscillation angle of 2.25°. Integration, merging, and scaling of intensities yielded 11 601 independent data with an overall R_{merge} of 0.054 ($R_{merge} = \sum|\langle I\rangle - I_i|/\sum I_i$). The completeness of the data is given in Table 1. A total of 9225 reflections with $|F_o| > 2\sigma(F_o)$ between 10.0- and 1.6-Å resolution was used in the initial stages of the refinement and between 8.0 and 1.6 Å in the final cycles.

X-ray Analysis and Refinement. The crystals are isomorphous with the previously reported insulin crystals ($a = 80.95$ and $c = 37.64$ Å) (Bentley et al., 1976; Smith et al., 1984). In order to avoid biasing the initial maps and subsequent refinement by the inclusion of suspect atoms or residues, a minimal TR dimer was constructed from the refined TR structure (Smith et al., 1984) by excluding all water molecules, zinc and chloride ions, B13Glu side chains, and disordered side chains, including both orientations of the disordered B10His side chain. In addition, B1 and B2 of the R-state monomer and B29 and B30 of both monomers were excluded, resulting in a total of 767 protein atoms. The largest peaks from the initial $F_o - F_c$ map corresponded to a pair of zinc ions located on the 3-fold axis. The next largest peak was located approximately 2.0 Å from one zinc ion and was initially modeled as a chloride ion. The refinement was continued with the restrained least-squares program PROFFT (Hendrickson & Konnert, 1980; Finzel, 1987). Throughout the refinement, $2F_o - F_c$ and $F_o - F_c$ maps were calculated and examined with FRODO (Bush & Jones, 1988). Appropriate adjustments were made to main and side chains, and water molecules were added in accord with the criteria of good electron density and acceptable hydrogen bonds to other atoms. No interpretable electron density was observed for the terminal atoms of side chains of B21Glu of monomer II and B29Lys and B30Thr of both monomers. The side chains of A5Gln and B9Ser of monomer I were refined in two alternative conformations with occupancies of 50%. The refinement converged at a residual of $R = 0.172$ for 9225 reflections, with $|F_o| \geq 2\sigma(|F_o|)$ between 8.0- and 1.6-Å resolution ($R = 0.181$ for all data between 10.0- and 1.6-Å resolution). The final model consists of 791 protein atoms, 123 water molecules, and zinc, chloride, and sodium ions. Summary of the refinement is listed in Table 2. Atomic coordinates have been deposited with the Brookhaven Protein Data Bank.

Table 2: Refinement Statistics[a]

resolution (Å)	8.0–1.6
no. of reflections ($F_o \geq 2\sigma(F_o)$)	9225
R value	0.172
distances (Å)[a]	
bond (1–2)	0.018 (0.020)
angle (1–3)	0.049 (0.040)
planar (1–4)	0.057 (0.050)
chiral volume (Å^3)	0.159 (0.150)
planar groups (Å)	0.014 (0.020)
nonbonded (Å)	
single torsion	0.211 (0.300)
multiple torsion	0.201 (0.300)
possible H-bonds	0.239 (0.300)
torsion angles (deg)	
planar	2.3 (3.0)
staggered	15.8 (20.0)
orthonormal	17.1 (20.0)

[a] Target σ in parentheses.

RESULTS

TR Insulin Dimer. The asymmetric unit consists of an insulin TR dimer constructed from monomers I and II, shown in Figure 1. Three dimers assemble around two zinc ions and form an insulin T_3R_3 hexamer that possesses a crystallographic 3-fold axis. Unlike the 3-fold-symmetric T_6 or R_6 hexamers, in which the conformations of the two independent monomers are nearly identical, significant differences in conformation exist between the two independent monomers of the T_3R_3 hexamer, the result of a change in conformation of the first eight residues of one of the B-chains. In monomer I, this segment of the B-chain adopts an extended or T conformation, while in monomer II it has an α-helical or R conformation (Figure 2). However, this α-helix is disrupted at B3 Asn, a consequence of a ψ torsion angle of 94°; this places $O^{\delta 1}$ of the B3Asn side chain in a position where it can accept hydrogen bonds from the main-chain nitrogen atoms of both B6Leu and B5His. Thus, the B3Asn side chain acts as a substitute for the carbonyl oxygen atoms of B2Val and B1Phe, replacing their hydrogen-bonding interactions in the α-helix. The φ,ψ torsion angles of B1Phe and B2Val are similar to those of B3Asn and are located in the extended region of the Ramachandran plot. The C-terminus of the B-chain is also shifted away from the A-chain residues; contacts between the nitrogen of A19Tyr and the carbonyl oxygen of B25Phe are 3.09 and 4.59 Å for the T- and R-state monomers, respectively, as compared to 3.36 and 3.16 Å in the T_6 hexamer and 3.77 and 4.16 Å in the R_6 hexamer (Baker et al., 1988; Smith & Dodson, 1992). Another consequences of the T→R transition is a displacement of the C^α of B7Cys, resulting in a rearrangement of the A7Cys–B7Cys disulfide bond and a small rotation of the α-helical segment at the N-terminus.

The formation of the dimer is stabilized by multiple hydrogen-bonding interactions, as listed in Table 3. The hydrogen-bonding pattern in a short, antiparallel pleated sheet consisting of residues B24Phe–B26Tyr is similar to that observed in all known T_6 and R_6 hexameric forms. The change in conformation of the N-terminal B-chain residues displaces several residues to positions in which they can now make hydrogen bonds to the residues of an adjacent monomer and

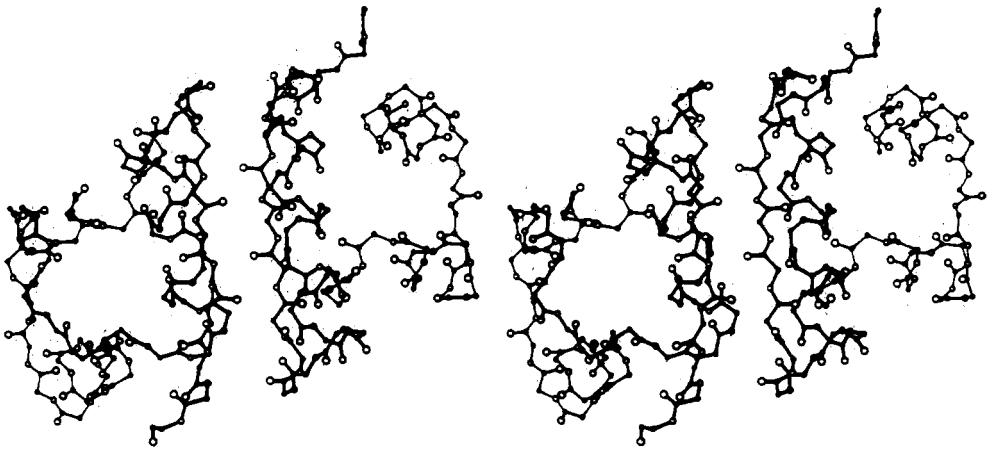

FIGURE 1: Stereo drawing of the main-chain atoms of the TR insulin dimer viewed along the local 2-fold axis. A- and B-chains are drawn with thin and thick bonds, respectively, and monomer I (T conformation) is on the left side of the dimer.

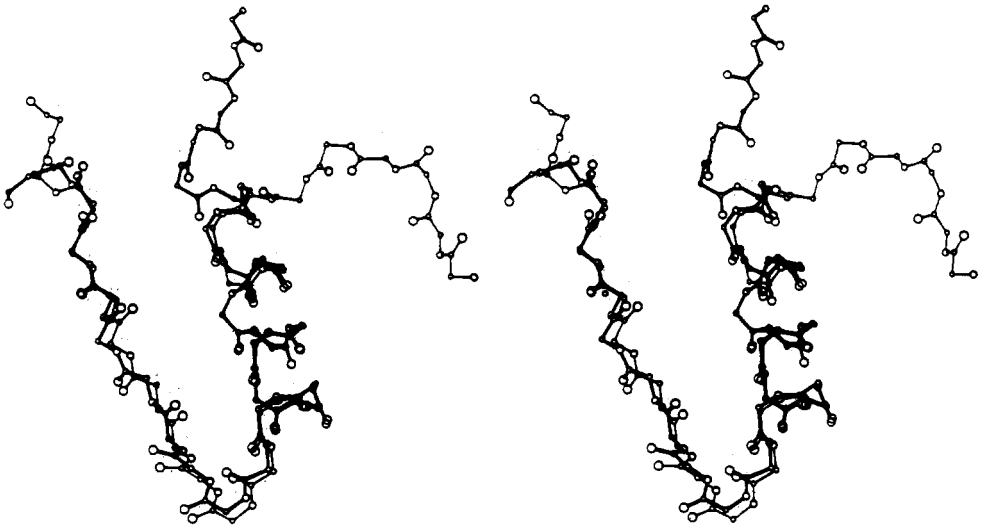

FIGURE 2: Superposition of the B-chain of monomer I (thin bonds) onto that of monomer II (thick bonds). Backbone atoms of B11–B19 were used in the least-squares optimization of the two chains; the average displacement between pairs of atoms used in the optimization procedure was 0.177 Å.

Table 3: Hydrogen-Bonding Interactions between Monomers I and II

monomer I		monomer II		
residue	atom	residue	atom	distance (Å)
B16Tyr	O^{η}	B5His	O	2.82
B13Glu	$O^{\epsilon 1}$	B9Ser	$O^{\gamma a}$	2.90
B13Glu	$O^{\epsilon 2}$	B9Ser	$O^{\gamma a}$	2.85
B26Tyr	O	B24Phe	N	2.92
B26Tyr	N	B24Phe	O	2.93
B24Phe	O	B26Tyr	N	2.80
B24Phe	N	B26Tyr	O	2.89

[a] The hydroxy group of B9Ser is disordered with 50% occupancy.

therefore add stability to the structure. These additional hydrogen-bonding interactions include B13Glu and B16Tyr of monomer I to B9Ser and B5His of monomer II, respectively. The change in conformation of the B-chain also results in the formation of three elliptical cavities at the dimer–dimer interface in regions that were occupied by the side chains of B6Leu of the T_6 hexamer. In R_6 hexameric forms of insulin, crystallized in the presence of phenolic derivatives, these sites are known as the phenol binding sites.

Zinc Coordination. Each of the 3-fold-symmetric trimers binds a zinc ion at full occupancy on the 3-fold axis through $N^{\epsilon 2}$ of the three symmetry-related B10His side chains; Zn–$N^{\epsilon 2}$ bond distances are 2.02 and 2.05 Å in the T and R trimers, respectively, and are in good agreement with those observed in previous insulin structures or in complexes of zinc with organic compounds (Orpen et al., 1989; Balschmidt et al., 1991). Because of the conformational differences between monomers I and II, the two zinc binding sites are no longer equivalent. Zn1 lies at the bottom of a shallow depression on the surface of the T-state trimer (Figure 3a). In the T_6 hexameric insulin structure, this shallow depression is large enough to easily accommodate an octahedrally coordinated zinc ion, with the three remaining sites occupied by water molecules. In spite of the fact that the T-state trimer surface of the T_3R_3 hexamer is not significantly different from that observed for the T_6 hexamer, the electron density maps revealed one large axial peak and three smaller symmetry-related peaks adjacent to the zinc ion. Since the density of the three smaller peaks was approximately one-half that of a well-defined water molecule, they were interpreted as water molecules with occupancies of 0.5; the density of the larger axial peak was larger than that of a water molecule and was therefore interpreted as a chloride ion, also with an occupancy of 0.5. The presence of these peaks suggests that statistical disorder of the coordination of the zinc ion is present, so that in 50% of the hexamers, the coordination sphere is filled by three water molecules with Zn1–OW1 bond distances of 2.54 Å; in the other 50% of the hexamers, a chloride ion is the fourth

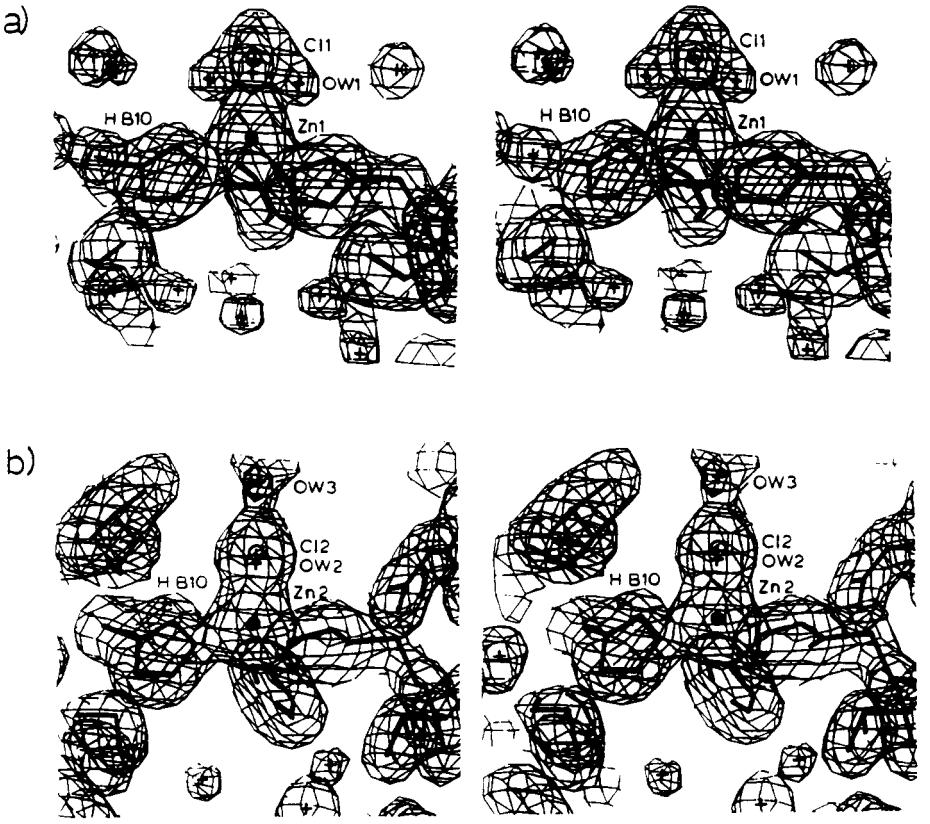

FIGURE 3: Stereoviews of the $2F_o - F_c$ electron density map superimposed on the atoms in the vicinity of the zinc ion binding suites on (a) the T_3 and (b) the R_3 side of the T_3R_3 insulin hexamer. Contours are drawn at one standard deviation. The zinc ions are represented as filled circles, chloride ions as crossed circles, and water molecules as crosses. For clarity, only one of the 3-fold-related ion/molecules are labeled.

ligand in a tetrahedral coordination sphere, with a Zn1–Cl1 bond distance of 2.31 Å.

Statistical disorder of the coordination of the second zinc ion, Zn2, bound to the R-state trimer is also observed (Figure 3b). Zn2 lies at the bottom of a narrow 12-Å channel composed of three symmetry-related segments of the B-chains, B1Phe–B10His, which restricts the coordination to tetrahedral. The observed electron density at the fourth coordination site along the 3-fold axis is elongated. The peak located adjacent to the zinc ion is the third largest peak in the entire $2F_o - F_c$ electron density map, is located 2.32 Å from Zn2, and corresponds to a chloride ion, Cl2. A third peak on the 3-fold axis (OW3) is 4.8 Å from Zn2 and 2.5 Å from Cl2. The presence of this third peak suggests that the "chloride ion" peak may represent the positions of two different ligands with occupancies of 0.5, a chloride ion and a water molecule. Thus, in one-half of the hexamers, the tetrahedral coordination sphere of the zinc ion is filled by a chloride ion (Zn2–Cl2, 2.32 Å); in the other 50%, a water molecule occupies this site (Zn2–OW2, 2.04 Å), where it forms a hydrogen bond (2.78 Å) to OW3.

Previous studies of the T_3R_3 hexamer found two orientations for the B10His side chains of the R-state trimer (Bentley et al., 1975, 1992; Smith et al., 1984). The first orientation placed the imidazole ring within bonding distance of the axial zinc ion, while the second placed the ring in the vicinity of a symmetry-related B5 histidine side chain of monomer II. Three symmetry-related large peaks in the electron density map were interpreted as additional off-axial zinc ions, each with tetrahedral coordination to B5His, the second orientation of B10His, and two water molecules. However, the results of the present study provide no evidence for the presence of a zinc ion in the off-axial binding site or for the existence of a second orientation of the B10His side chain. Instead, the cavities are filled with a cluster of water molecules that interact with protein atoms, similar to that observed in the structure of the zinc-free B13Gln (Bentley et al., 1992). Contacts between protein atoms and water molecules include the side chain of B5His of monomer II ($N^{\delta 2}$–OW10, 3.20 Å), the *N*-amino and carbonyl groups of a symmetry-related A11Cys of monomer I (O–OW10, 2.60 Å; N–OW55, 2.74 Å), and each other (OW10–OW65, 2.47 Å; OW55–OW65, 2.71 Å).

The Hexamer Center. The B9Ser and B13Glu residues, as well as several peaks that correspond to either water molecules or sodium ions, are located in the center of the hexamer between the two zinc ions and are shown schematically in Figure 4. The side chains of B13Glu of monomer I ($\chi^1, \chi^2, \chi^3 = -151°$, 50°, 46°) do not project toward the hexamer center, but rather accept hydrogen bonds from the vicinal hydroxy group of B9Ser of monomer II, contributing to dimer stabilization. The side chain of B9Ser is observed in two distinct orientations, which permits it to donate its proton to either $O^{\epsilon 1}$ (2.90 Å) or $O^{\epsilon 2}$ (2.85 Å). In monomer II, the side chains of B13Glu are directed toward the center of the hexamer ($\chi^1, \chi^2, \chi^3 = -61°, -149°, 24°$), and the separation between symmetry-related carboxyl groups is 4 Å. At a pH of 6.4 of the crystallizing medium, it is most likely that these carboxyl groups are fully ionized and represent a high-affinity binding site for a positively charged ion or water molecules. Although the potential of the B13Glu residues to act as additional metal ion binding sites has been studied (Hill et al., 1991), the electron density in this region in the present study provides no evidence for a large peak that could be interpreted as a zinc ion. However, three symmetry-related peaks are nearly equidistant from the B13Glu $O^{\epsilon 2}$ of monomer II (2.63 Å) and a symmetry-related $O^{\epsilon 1}$ (2.93 Å), but due to the action of the 3-fold axis, each peak is separated from its symmetry-related mates by

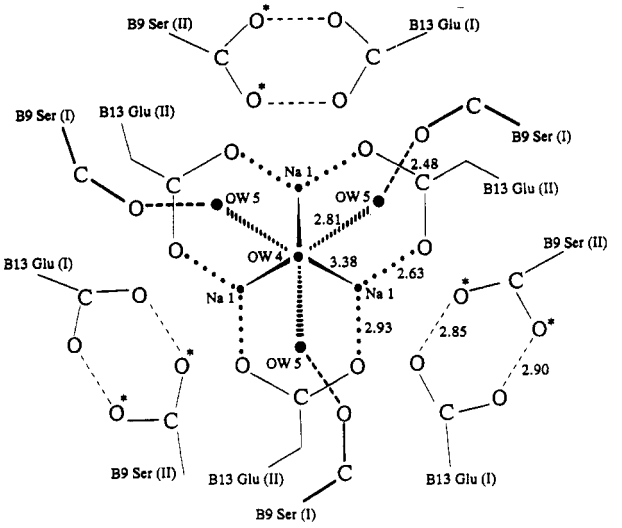

FIGURE 4: Schematic drawing of the center of the insulin hexamer viewed along the crystallographic 3-fold axis. Hydrogen bonds are illustrated as dashed lines, while dotted lines represent possible interactions to sodium ion. The asterisk is drawn next to the hydroxy group of the side chain of B9Ser of monomer II, which is observed in two discrete orientations.

2.6 Å. Since it is likely that a water molecule would donate each of its protons to carboxyl oxygen atoms, a 2.6-Å contact is unreasonably short in the absence of a hydrogen bond. Because the presence of a sodium ion would reduce the negative charge in this region and add stability to the hexamer, we have chosen to interpret this peak as a sodium ion (Na1) with 1/3 occupancy, which implies that each hexamer contains a single sodium ion in only one of the three possible sites. The absence of any additional electron density in the immediate vicinity of the sodium ion suggests that this region contains a disordered array of water molecules, some of which might act as ligands to the sodium ion. The higher thermal motion of the B13Glu carboxyl groups of monomer II as compared to monomer I is indicative of greater positional mobility and is consistent with a disordered array of water molecules. Another peak interpreted as a water molecule, OW4, is located at the center of the hexamer on the 3-fold axis and is 3.38 Å from Na1. Three other symmetry-related peaks that surround OW4 are located in the vicinity of the side chains of B9Ser of monomer I and have been interpreted as water molecules (OW5). This trio of water molecules forms hydrogen bonds to the O^{γ} of B9Ser of monomer I (2.48 Å) and to OW4 (2.81 Å) located on the 3-fold axis.

DISCUSSION

The T_3R_3 insulin hexamer, also known as 4-zinc insulin, is one of the preparations used in diabetes therapy since it is slow to dissociate to the biologically relevant monomer. Since the binding of zinc to each trimer is a major factor in stabilizing the hexamers, the molecular basis for the slow-acting aspect of this form of insulin can be explained by the T→R transition and the accompanying formation of a 12-Å-deep channel. One of the zinc ions lies at the bottom of this channel, where it is isolated from the enviroment. Prior to zinc's dissociation from the hexamer, the R-state trimer must undergo a change in conformation to that of T in order to make the zinc ion accessible to the surrounding solvent. In the absence of zinc, the hexamer can then begin to dissociate to dimers and monomers. While previous crystallographic results had suggested that the presence of the off-axial zinc ions added considerable stability to the T_3R_3 hexamer (Bentley et al., 1976; Smith et al., 1984), the results of the present study show that the T_3R_3 hexamer is stable in the absence of zinc in these sites, in agreement with the results obtained from spectroscopic studies of B5Ala insulin (Thomas & Wollmer, 1989), in which the absence of the B5His side chain precludes the formation of the off-axial zinc ion binding sites. This also confirms the results of spectroscopic studies, from which the critical stoichiometry for the high-affinity interaction between zinc and the hexamers was found to be 2 (Kaarsholm et al., 1989).

The dual coordination of zinc in tetrahedral and octahedral geometries in the T-state trimer has important implications for spectroscopic studies of the hexamer bound to various transition metals. Although the presence of the R-state α-helices that form the boundary of the metal ion channel restricts the coordination to tetrahedral, no spatial requirements are present at the metal ion binding site in the T trimer. The observation that the T-state trimer binds zinc in both tetrahedral and octahedral geometries strongly suggests that these species are also present in the solution from which crystallization occurred. The spectroscopic signature of tetrahedral cobalt(II) has been assumed to be indicative of an R-state trimer, while octahedral coordination represents a T state (Roy et al., 1989; Thomas et al., 1989). If the behavior of the cobalt(II)-substituted hexamers is similar to that of zinc-containing hexamers, the dual tetrahedral and octahedral coordination of the T-state-bound metal atom could be very misleading in estimating the extent of the T→R conformational transition.

The allosteric behavior of insulin has been studied extensively by spectroscopic and crystallographic techniques, which have shown that the presence of lyotropic anions such as chloride or thiocyanate can only induce a conformational transition from T_6 to T_3R_3. However, it is becoming clear that the T_3R_3 hexamer does not represent a single uniform moiety, since the results of crystallographic studies on the structure of this hexameric form have shown that it can exist with or without a metal ion in the off-axial binding site, that the R-state α-helix can include residues B1–B19 or B3–B19, and finally that the T-state axial metal ion can be coordinated either tetrahedrally or octahedrally. It appears that small differences in the pH or in the concentrations of the metal ion and lyotropic anion in the crystallizing medium may produce these differences in the T_3R_3 hexamer. The presence of phenolic derivatives can drive the T→R transition to completion, resulting in an R_6 hexamer complexed with the phenolic derivative. On the basis of the results obtained from the structure of the T_3R_3 hexamer, it seems likely that small changes in pH and in the concentrations of various reagents in the crystallizing medium may also produce subtle changes in the R_6 hexameric form.

ACKNOWLEDGMENT

The authors thank Walter A. Pangborn for assistance in the data collection, Robert H. Blessing and G. Guy Dodson for stimulating discussions, and Lilly Research Laboratories for the generous gift of recombinant human insulin.

REFERENCES

Adams, M. J., Blundell, T. L., Dodson, E. J., Dodson, G. G., Vijayan, M., Allen, F. H., Davies, J. E., Galloy, J. J., Johnson, O., Dennard, O., Baker, E. N., Harding, M. M., Hodgkin, D. C., Rimmer, R., & Sheet, S. (1969) *Nature (London) 224*, 491–496.

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.

Balschmidt, P., Hansen, F. B., Dodson, E. J., Dodson, G. G., & Korber, F. (1991) *Acta Crystallogr. B47*, 975–986.

Bentley, G. A., Dodson, E. J., Dodson, G. G., Hodgkin, D., & Mercola, D. (1976) *Nature 261*, 166–168.

Bentley, G. A., Brange, J., Derewenda, Z., Dodson, E. J., Dodson, G. G., Markussen, J., Wilkinson, A. J., Wollmer, A., & Xiao, B. (1992) *J. Mol. Biol. 228*, 1163–1176.

Berson, S. A., & Yalow, R. S. (1966) *Am. J. Med. 40*, 676.

Bush, B. L., & Jones, T. A. (1988) *PS300 FRODO-Molecular Graphics Program for the PS300*, modified by J. Sack, et al., Houston, TX.

Derewenda, U., Derewenda, Z., Dodson, E. J., Dodson, G. G., Reynolds, C., Smith, G. D., Sparks, C., & Swenson, D. (1989) *Nature 338*, 589–596.

Finzel, B. C. (1987) *J. Appl. Crystallogr. 20*, 53–55.

Hendrickson, W. A., & Konnert, J. H. (1980) in *Computing in Crystallography* (Diamond, R., Ramaseshan, S., & Venkatesan, K., Eds. pp 13.01–13.23, Indian Academy of Sciences, Bangalore, India.

Hill, C. P., Dauter, Z., Dodson, E. J., Dodson, G. G., & Dunn, M. F. (1991) *Biochemistry 30*, 917–924.

Kaarsholm, N. C., Ko, H.-C., & Dunn, M. F. (1989) *Biochemistry 28*, 4427–4435.

Orpen, G. A., Brammer, L., Allen, F. H., Kennard, O., Watson, D. G., & Taylor, R. (1989) *J. Chem. Soc., Dalton Trans.*, S1–S83.

Roy, M., Brader, M. L., Lee, R. W., Kaarsholm, N. C., Hansen, J. F., & Dunn, M. F. (1989) *J. Biol. Chem. 264*, 19081–19085.

Schlichtkrull, J. (1958) *Insulin Crystals*, Munksgaard, Copenhagen.

Smith, G. D., & Dodson, G. G. (1992) *Proteins 14*, 401–408.

Smith, G. D., Swenson, D. C., Dodson, E. J., Dodson, G. G., & Reynolds, C. D. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7093–7097.

Thomas, B., & Wollmer, A. (1989) *Biol. Chem. Hoppe*-Seyler *370*, 1235–1244.

Wollmer, A., Rannefeld, B., Johansen, B. R., Hejnaes, K. R., Balschmidt, P., & Hansen, F. B. (1987) *Biol. Chem. Hoppe*-Seyler *368*, 903–911.