

- Ueda, T., Kikuchi, A., Ohga, N., Yamamoto, J., & Takai, Y. (1990) *J. Biol. Chem.* 265, 9373-9380.
- Ui, M. (1984) *Trends Pharmacol. Sci.* 5, 277-279.
- Vogel, U. S., Dixon, R. A. F., Schaber, M. D., Diehl, R. E., Marshall, M. S., Scolnick, E. M., Sigal, I. S., & Gibbs, J. B. (1988) *Nature* 335, 90-93.
- West, M., Kung, H., & Kamata, T. (1990) *FEBS Lett.* 259, 245-248.
- Wolfman, A., & Macara, L. G. (1990) *Science* 248, 67-69.
- Yamamoto, J., Kikuchi, A., Ueda, T., Ohga, N., & Takai, Y. (1990) *Mol. Brain Res.* 8, 105-111.
- Yamamoto, K., Kondo, J., Hishida, T., Teranishi, Y., & Takai, Y. (1988) *J. Biol. Chem.* 263, 9926-9932.
- Yamamoto, T., Kaibuchi, K., Mizuno, T., Hiroyoshi, M., Shirataki, H., & Takai, Y. (1990) *J. Biol. Chem.* 265, 16626-16634.
- Zahraoui, A., Touchot, N., Chardin, P., & Tavitian, A. (1988) *Nucleic Acids Res.* 16, 1204.

X-ray Structure of an Unusual Ca^{2+} Site and the Roles of Zn^{2+} and Ca^{2+} in the Assembly, Stability, and Storage of the Insulin Hexamer[†]

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ABSTRACT: Metal ion binding to the insulin hexamer has been investigated by crystallographic analysis. Cadmium, lead, and metal-free hexamers have been refined to *R* values of 0.181, 0.172, and 0.172, against data of 1.9-, 2.5-, and 2.5-Å resolution, respectively. These structures have been compared with each other and with the isomorphous two-zinc insulin. The structure of the metal-free hexamer shows that the His(B10) imidazole rings are arranged in a preformed site that binds a water molecule and is poised for Zn^{2+} coordination. The structure of the cadmium derivative shows that the binding of Cd^{2+} at the center of the hexamer is unusual. There are three symmetry-related sites located within 2.7 Å of each other, and this position is evidently one-third occupied. It is also shown that the coordinating B13 glutamate side chains of this derivative have two partially occupied conformations. One of these conformations is two-thirds occupied and is very similar to that seen in two-zinc insulin. The other, one-third-occupied conformation, is seen to coordinate the one-third-occupied metal ion. The binding of Ca^{2+} to insulin is assumed to be essentially identical with that of Cd^{2+} . Thus, we conclude that the Ca^{2+} binding site in the insulin hexamer is unlike that of any other known calcium binding protein. The crystal structures reported herein explain how binding of metal ions stabilizes the insulin hexamer. The role of metal ions in hexamer assembly and dissociation is discussed.

When free of divalent metal ions, the protein hormone insulin exists in solution as an equilibrating mixture of monomer, dimer, tetramer, hexamer, and higher aggregates. The hexamer has been shown to contain two high-affinity binding sites for Zn^{2+} , and in the presence of this ion, the distribution of insulin species in solution is driven toward the hexamer state. A wide variety of divalent metal ions will substitute for Zn^{2+} at these sites (Blundell et al., 1972).

Insulin storage vesicles are known to contain high concentrations of Ca^{2+} and Zn^{2+} ions (Howell et al., 1975), and there is evidence that Ca^{2+} is taken up by rhombohedral zinc insulin crystals in vivo (Howell et al., 1978). From the available in vivo evidence, it appears likely that Ca^{2+} interactions with the zinc proinsulin and zinc insulin hexamers are important and that the form of insulin within mature storage vesicles is crystalline (Greider et al., 1969). The three-dimensional structure of the two-zinc insulin hexamer, $(\text{In})_6(\text{Zn}^{2+})_2$,¹ has

been solved to a resolution of 1.5 Å (Baker et al., 1988). The hexamer is a torus-shaped molecule with dimensions 35 Å by 50 Å (Figure 1). The six insulin subunits are arranged as a trimer of asymmetric dimers about a 3-fold symmetry axis. The two high-affinity zinc sites are located on the 3-fold axis separated by a distance of 15.9 Å. Each site is formed by three His(B10) imidazole ligands (one from each dimer) as shown in Figure 2; octahedral coordination at each site is completed by water molecules.

The work of Schlichtkrull (1956) on the stoichiometries of divalent metal ion binding in crystalline rhombohedral insulin hexamers gave the first indication of the presence of a third, high-affinity metal ion binding site within the insulin hexamer. During the determination of the structure of $(\text{In})_6(\text{Zn}^{2+})_2$, isomorphous replacement studies with Cd^{2+} , Pb^{2+} , and UO_2^{2+} ions indicated the presence of a metal binding site in the vicinity of the center of the hexamer (Adams et al., 1967; Blundell et al., 1972). The X-ray diffraction study of Emdin et al. (1980) showed that when crystals of $(\text{In})_6(\text{Zn}^{2+})_2$ were soaked in solutions containing high concentrations of Zn^{2+} , the crystals bind Zn^{2+} at a variety of sites, including the

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¹ Abbreviations: NMR, nuclear magnetic resonance; FT, Fourier transform; $(\text{In})_6(\text{M}^{2+})_m(\text{M}^{2+})_n$, metal-substituted insulin hexamers where *m* and *n* designate the stoichiometries of metal ions bound to the His(B10) and Glu(B13) sites, respectively; $(\text{In})_6$, metal-free insulin hexamer.

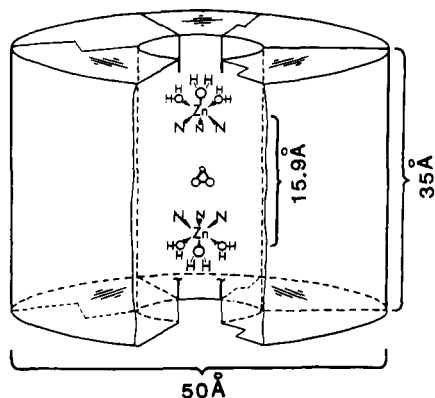


FIGURE 1: Organization of the insulin hexamer. Jagged lines between monomers on the top and bottom of the hexamer indicate a dimer-dimer interface. The straight lines between monomers on the top and bottom of the hexamer indicate a monomer-monomer interface within a dimer. The "hash marks" indicate the top of monomers. Zn^{2+} ions are indicated on the 3-fold axis in the positions they occupy in two-zinc insulin. Each of the zinc ions is coordinated by three His(B10) side chains and by three water molecules. Six Glu(B13) side chains cluster together around the Ca^{2+}/Cd^{2+} sites at the center of the hexamer.

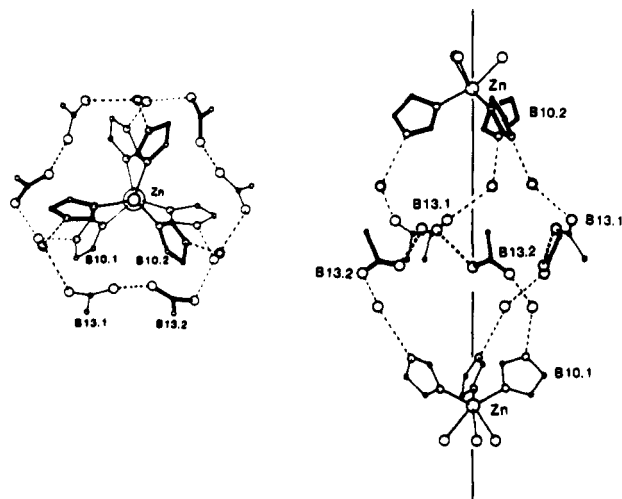


FIGURE 2: At the core of the insulin hexamer, a network of hydrogen bonds links the His(B10) and Glu(B13) side chains via ordered water molecules. The only protein groups shown in this figure are the His(B10) imidazoles and Glu(B13) carboxylates with molecule 1 protein groups shown in thin lines and molecule 2 in bold lines. Zinc ions and water molecules are shown as spheres. Carboxylate oxygens of Glu(B13) side chains from the same dimer are only 2.6 Å from each other. It is assumed that these functions hydrogen bond each other and that one of the side chains is protonated, i.e., in the acid form. (Left) View down the 3-fold axis. (Right) View perpendicular to the 3-fold axis.

Glu(B13) carboxylates at the center of the hexamer.

The ^{113}Cd FT NMR studies of Sudmeier et al. (1981) established that the two-zinc insulin hexamer is a calcium binding protein. A variety of physical-biochemical studies have since been undertaken to characterize in more detail the calcium binding site of $(In)_6(Cd^{2+})_2$. It has been shown that Ca^{2+} binds at the center of the hexamer in a cage formed by the six Glu(B13) carboxylates. Calcium ion binds to this site with a stoichiometry of one Ca^{2+} ion per hexamer, and with a dissociation constant of $80 \mu M$ (Sudmeier et al., 1981; Storm & Dunn, 1985; Alameda et al., 1985; Kaarsholm & Dunn, 1987; Dunn et al., 1987; Palmieri et al., 1988; Coffman & Dunn, 1988). Cd^{2+} and Ca^{2+} ions bind competitively and with the same stoichiometry to the B13 carboxylates, indicating that the coordination geometries are likely to be the same for these two ions. This proposal is consistent with their very similar ionic radii of 0.97 and 0.99 Å (Weast, 1968), also with the

generally similar coordination found for Cd^{2+} and Ca^{2+} exhibited both by small-molecule chelators and by other calcium binding proteins (Cotton & Wilkinson, 1972; Norman et al., 1987).

The hexamer 3-fold symmetry axis passes through the center of the 10-Å diameter cavity formed by the Glu(B13) carboxylates (Blundell et al., 1972; Baker et al., 1988). This cavity is located midway between the two His(B10) zinc sites (which reside on the 3-fold axis). In the hexamer crystal structure, at pH 6.2, the six Glu(B13) carboxylates are arranged in a hydrogen-bonded pairing, in which it is presumed that half of the Glu(B13) side chains are protonated to the carboxylic acid form (Baker et al., 1988) (Figure 2). A carboxylic acid residue (Glu or Asp) at residue B13 is conserved in all but one of the zinc binding insulins that have been sequenced (Halldén et al., 1986). The exception is hagfish insulin, in which the B13 residue is Asn; this insulin is unusual in that it probably does not form hexamers (Peterson et al., 1975; Cutfield et al., 1979).

Although the stoichiometry of one Ca^{2+} ion per hexamer suggested a single Ca^{2+} locus within the cavity (Storm & Dunn, 1985), inspection of the three-dimensional structure of the hexamer indicates considerable reorganization of the hexamer would be required to form a single site involving all six B13 carboxylates. Furthermore, the preliminary isomorphous replacement data for the Cd^{2+} - and Pb^{2+} -substituted hexamers each indicated the presence of three, closely spaced metal ion loci in the Glu(B13) cavity (Dodson et al., unpublished results). Therefore, we have undertaken detailed X-ray structure studies of three hexameric insulin derivatives to investigate further the binding of metal ions at the His(B10) and Glu(B13) sites. These crystalline derivatives are the metal-free hexamer, $(In)_6$ at 2.5-Å spacing, and the Cd^{2+} - and Pb^{2+} -substituted hexamers, $(In)_6(Cd^{2+})_2Cd^{2+}$ and $(In)_6(Pb^{2+})_2(Pb^{2+})_3$ at 1.9- and 2.5-Å spacing, respectively.

As will be shown herein, the structural changes in the His(B10) site of the hexamer resulting either from removal of Zn^{2+} or by substitution of Cd^{2+} or Pb^{2+} for Zn^{2+} are minor. However, substitution of Cd^{2+} or Pb^{2+} into the (metal-free) Glu(B13) cavity induces a conformational change which disrupts the intradimer Glu-Glu hydrogen-bonding interaction (Figure 2) and forms metal binding sites which span the dimer-dimer interface via coordination of carboxylate pairs from adjacent dimers. Given that the binding of Ca^{2+} to B13 carboxylates is the same as Cd^{2+} , we speculate on the structural role of Ca^{2+} in hexamer assembly and stability. We also note that the structure of the Ca^{2+} binding site of the insulin hexamer is unlike Ca^{2+} sites reported for other Ca^{2+} binding proteins.

MATERIALS AND METHODS

Materials. The pig insulin was a gift from the NOVO Research Laboratories, Copenhagen. Metal ion salts, buffer salts, and other reagents for preparing metal-substituted insulin hexamers were purchased as the highest purity grades available, and used without further purification.

Crystallization. Rhombohedral two-zinc insulin crystals were grown according to the recipe of Schlichtkrull (1956), slightly modified to yield the larger dimensions needed for X-ray analysis. The cadmium insulin crystals were grown from zinc-free insulin and cadmium salts using conditions analogous to those appropriate for two-zinc insulin crystals.

It is possible to remove zinc ion from the two-zinc insulin crystals without seriously impairing their X-ray diffraction properties. This is done by diffusing EDTA through the crystals [at concentrations ranging from 0.1% to 1% (by weight)] and exchanging the solution every 2–4 h until the

Table I: Crystallographic Statistics

	two-zinc ^a	cadmium	lead	zinc-free
no. of crystals	6	2	1	1
resolution (d_{\min}) (Å)	1.5	1.9	2.5	2.5
no. of reflections	13524	6490	3084	2984
reflections $>2\sigma$	94.6	92.4	73.2	91.2
R_{merge}^b	0.040	0.065	0.075	0.072
R_{factor}^c	0.153 ^d	0.181 ^e	0.172 ^e	0.172 ^e
(1-2) bond distances (Å)	0.024	0.014	0.012	0.016
(1-2) angle distances (Å)	0.054	0.035	0.045	0.040
(1-4) distances (Å)	0.064	0.038	0.043	0.047
planarity (Å)	0.019	0.011	0.009	0.013
chiral volumes (Å)	0.155	0.142	0.128	0.155
nonbonded contacts (Å)				
single torsion	0.180	0.189	0.196	0.202
multiple torsion	0.491	0.356	0.347	0.349
possible hydrogen bonds	0.303	0.354	0.416	0.289
conformation of torsion angles (deg)				
planar (0°, 180°)	3.3	1.8	1.5	2.2
staggered ($\pm 60^\circ, 180^\circ$)	17.0	17.4	19.1	17.5
orthonormal ($\pm 90^\circ$)	22.9	24.8	21.9	24.1
isotropic temp factors				
main-chain bond (Å ²)	5.560	0.956	0.666	0.770
main-chain angle (Å ²)	6.327	1.717	1.223	1.339
side-chain bond (Å ²)	11.605	1.229	0.688	0.988
side-chain angle (Å ²)	13.104	2.010	1.165	1.612

^aThe two-zinc insulin structure has been described by Baker et al. (1988). ^b $R_{\text{merge}} = (\sum |I_i| - |I_{\text{av}}|) / \sum |I_{\text{av}}|$. ^c $R_{\text{factor}} = (\sum |F_{\text{obs}}| - |F_{\text{calc}}|) / \sum |F_{\text{obs}}|$. ^dCalculated on all data. ^eCalculated on all data of higher than 10.0-Å resolution.

zinc loss is complete, as judged by precession photography. The extent of removal of the zinc ions is variable; lower EDTA concentrations generally cause slower removal but often with less damage to the crystal. Extended soaking times result in seriously impaired diffraction properties.

The X-ray diffraction patterns of cadmium and two-zinc insulin crystals were found to be closely isomorphous, with small but distinct intensity changes associated with the increased atomic number and the increased number of Cd²⁺ binding sites. Small differences between Friedel pairs could be seen caused by the anomalous scattering of the cadmium ion. The Cd²⁺ crystals also could be obtained by addition of cadmium acetate to insulin crystals from which zinc ion had been removed by treatment with EDTA (G. G. Dodson, unpublished results).

The crystalline lead insulin hexamer, (In)₆(Pb²⁺)₂(Pb²⁺)₃, was prepared by the addition of lead acetate to zinc-free insulin crystals. At 0.01 M Pb²⁺, the His(B10) site is fully substituted, and the Glu(B13) site essentially so (Adams et al., 1969). There are other, weaker, lead binding sites on the hexamer; the occupancy of these sites increases with lead concentration (Adams et al., 1969).

Data Collection. Diffraction data were collected on a PDP 11 controlled Hilger and Watts four-circle diffractometer using nickel-filtered Cu K_α radiation from a sealed-tube source, running at 40 kV and 20 mA. X-ray reflections were recorded by using a 30-step Ω scan, with a step size of 0.03°; the first and last three steps were used to estimate the background intensity. The lead and zinc-free insulin crystals typically gave the largest mosaic spread. Data were collected in overlapping shells with at least 2 equiv measured for each reflection. The crystals were monitored for decay and slippage by measuring three standard reflections after every 50th intensity. Lorentz, polarization, and absorption corrections were applied (North et al., 1968). The data were subsequently sorted, scaled together, and merged (see Table I).

X-ray Analysis and Refinement. The detailed atomic structure of two-zinc insulin at 1.5-Å spacing was already

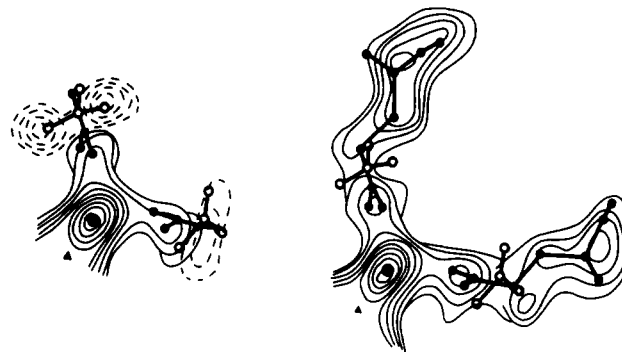


FIGURE 3: B13 side chains have two conformations in cadmium insulin. (Left) $|F_{\text{Cd,obs}}| - |F_{\text{Zn,obs}}|, \alpha_{\text{Zn,calc}}$; (Right) $|F_{\text{Cd,obs}}| - |F_{\text{Zn-2/3B13,calc}}|, \alpha_{\text{Zn-2/3B13,calc}}$ where $F_{\text{Cd,obs}} = \text{observed } (\text{In})_6(\text{Cd}^{2+})_2(\text{Cd}^{2+})$ structure factor amplitudes, $F_{\text{Zn,obs}} = \text{observed } (\text{In})_6(\text{Zn}^{2+})_2$ structure factor amplitudes, $\alpha_{\text{Zn,calc}} = \text{phases calculated from the refined two-zinc insulin coordinates}$, and $F_{\text{Zn-2/3B13,calc}}$ and $\alpha_{\text{Zn-2/3B13,calc}} = \text{structure factor amplitudes and phases calculated from the refined two-zinc insulin coordinates with Glu(B13) residues set to two-thirds occupancy}$. Molecule 1 and molecule 2 Glu(B13) residues shown here coordinating the same Cd²⁺ ion are from neighboring dimers. The two-thirds-occupied Glu(B13) conformation that is almost identical with that in two-zinc insulin is indicated with open circles; the new one-third-occupied conformation that coordinates the Cd²⁺ is shown with filled circles. The one-third-occupied Cd²⁺ is shown as a large filled circle at the point of highest electron density. The crystallographic 3-fold axis is indicated by a triangle. Contouring is in intervals of 0.2 eÅ⁻³. First positive contour, (solid lines) is at 0.4 eÅ⁻³. First negative contour (broken lines) is at -0.6 eÅ⁻³.

known (Baker et al., 1988). The rhombohedral crystal structures of zinc-free, cadmium, and lead insulins were all essentially isomorphous to two-zinc insulin. The structure analyses of these species all began from the two-zinc insulin coordinates.

The starting model for refinement against the zinc-free insulin data was the two-zinc insulin coordinates tailored by removal of protein groups with multiple conformations, partially occupied solvent molecules, zinc ions, His(B10) side chains, and solvent molecules near the zinc site.

For cadmium and lead insulins, the metal ion occupancies were estimated both from peak heights in difference maps phased on the refined two-zinc insulin structure and from conventional difference maps computed after several cycles of refinement with the metal ions set to zero occupancy.

A Cd²⁺ ion is located near the B13 glutamate residues at the center of the hexamer. This site is close to the crystallographic 3-fold axis, and symmetry-related ions are only 2.7 Å apart. This and peak heights in Fourier maps indicate that the B13 Cd²⁺ site is only one-third occupied.

Various constructed Fourier maps show that there are two conformations for the B13 side chains in cadmium insulin (see Figures 3 and 4A). One of the conformations, very similar to that seen in two-zinc insulin, is two-thirds occupied. The other, one-third-occupied conformation, is seen to bind the one-third-occupied cadmium ion. This modeling of two B13 side-chain conformations is supported by the refined *B* factors (see Table II).

There are two sets of symmetry-related Pb²⁺ sites at the hexamer center (see Figure 4B). The symmetry-related ions are separated by 4.5 Å, and the shortest distance between ions from the two sets is 2.7 Å. On the basis of both geometry and Fourier maps, it was assumed that the sum of the occupancies of these two Pb²⁺ sites is 1.0.

Atomic coordinates of the zinc-free, cadmium, and lead insulins were refined with the restrained least-squares procedure of Hendrickson (1985), modified to incorporate fast Fourier transform algorithms (Agarwal, 1978). Positional

Table II: Glu(B13) Temperature Factors (\AA^2)

	C^α	C^β	C^γ	C^δ	$O^{\epsilon 1}$	$O^{\epsilon 2}$
$(\text{In})_6(\text{Zn}^{2+})_2$						
molecule 1	7.2	11.8	27.7	62.1	28.1	33.0
molecule 2	8.9	12.7	15.6	53.8	47.2	33.7
$(\text{In})_6(\text{Cd}^{2+})_2(\text{Cd}^{2+})$ occupancy one-third						
molecule 1	16.1	16.2	16.3	16.3	16.4	16.4
molecule 2	13.9	13.7	13.4	13.3	13.0	13.3
$(\text{In})_6(\text{Cd}^{2+})_2(\text{Cd}^{2+})$ occupancy two-thirds						
molecule 1	12.5	13.7	14.9	15.7	16.4	16.0
molecule 2	9.7	11.3	13.6	15.3	16.3	16.5

Table III: Metal Ion Occupancies and Temperature Factors^a

	His(B10)		Glu(B13) ^b		Glu(A17)	
	Mol 1	Mol 2			Mol 1	Mol 2
two-zinc	1.0 (11)	1.0 (10)				
cadmium	1.0 (34)	1.0 (22)	0.33 (31)			
lead	1.0 (19)	1.0 (16)	0.70 (18)	0.30 (22)	0.50 (19)	0.20 (20)
zinc-free	0.33 (12)	0.27 (11)				

^aTemperature factors, in units of \AA^2 , are given in parentheses. ^bThe Cd^{2+} and Pb^{2+} sites at Glu(B13) are not associated with any one molecule but are bound equally to molecules (Mol) 1 and 2.

parameters and isotropic B factors were refined for each atom; occupancies were not refined. The major difference between the refinement of zinc-free, cadmium, and lead insulins described here, and that of two-zinc insulin (Baker et al., 1988), is that we have applied restraints to the B factors, which was not done in the earlier work. Map fitting was performed using FRODO (Jones, 1985) on an Evans and Sutherland PS330. Convergence in each of the three related series (zinc-free, cadmium, and lead insulins) was rapid. Water molecules were deleted if their B values became very large or if they were seen not to lie in electron density during map fitting. Otherwise no special attention was given to the solvent structure away from the metal sites. Refinement statistics are given in Table I.

RESULTS

Zinc-Free Insulin. During refinement against the zinc-free insulin data, it became clear that not all the metal ions had been removed by EDTA treatment. Residual zinc occupancy was estimated by refining several starting models that were identical with the two-zinc insulin coordinates but had different partial occupancies for the zinc ions. The zinc occupancy was taken to be correct when the difference map was flat at the zinc site and if the refined B value was close to the starting value. It is clear from the difference maps that the metal ion is largely replaced by a water molecule. Like the zinc ion, this water molecule is located on the crystallographic 3-fold axis but is shifted further from the hexamer center so that the His(B10) $\text{N}^{\epsilon 2}$ to water oxygen distance is ca. 2.8 \AA .

The B factors for zinc ions with occupancy 0.33 (ca. nine electrons) and 0.27 (ca. eight electrons) are 12 and 11 \AA^2 , respectively (Table III); these values are similar to those obtained in two-zinc insulin. The B factors for the water molecules that largely replace the zinc ions in zinc-free insulin are 28 and 15 \AA^2 , respectively for molecules 1 and 2; these are physically sensible values.

Although the zinc ions are mostly removed, there is no sign of disorder in the His(B10) side chains. The ring conformation is essentially the same as that found in the metal-bound hexamers, a finding consistent with the view that these groups are held in an ideal and well-defined coordinating arrangement by the framework of the hexamer.

Glu(B13) Binding Site. For both the zinc-free insulin hexamer and the two-zinc insulin hexamer, where there is no

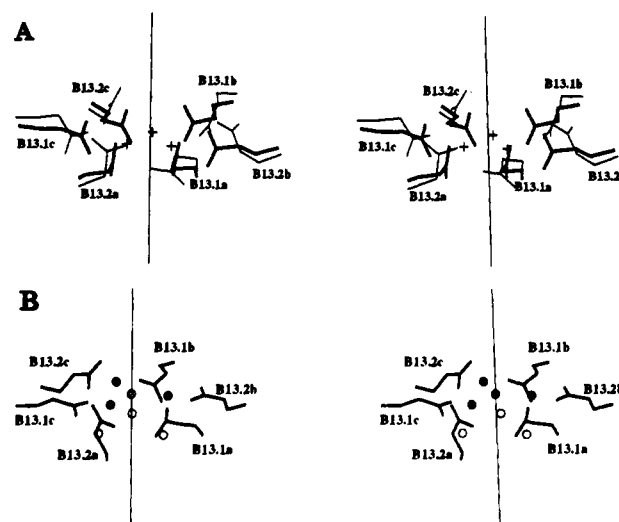


FIGURE 4: Metal ion binding to Glu(B13) viewed perpendicular to the 3-fold axis. (A) Cadmium insulin. The three, one-third-occupied, symmetry-related Cd^{2+} sites are marked with crosses. The one-third-occupied Glu(B13) conformations that ligand the metal ions are drawn in bold lines. The two-thirds-occupied side chains, essentially identical with those in two-zinc insulin, are shown in thin lines. Nomenclature of the Glu(B13) residues is, for example, B13.1a = residue from molecule 1 of dimer A, etc. The crystallographic 3-fold axis is indicated by the long line. (B) Lead insulin. Pb^{2+} ions are indicated by filled circles (0.7 occupancy) and by open circles (0.3 occupancy). The putative Cl^- ion, located on the crystallographic 3-fold axis, is shown as a gray circle. The top three, symmetry-related, 0.7 occupied Pb^{2+} sites are 4.5 \AA distant from each other. The 0.3 occupied Pb^{2+} sites are also ca. 4.5 \AA from their symmetry equivalents, and are 2.7 \AA from the nearest 0.7 occupied Pb^{2+} ion.

metal binding at the Glu(B13) carboxylates, the glutamyl side chains have the same conformation and make the same interactions. Thus, coordination of Zn^{2+} at the His(B10) sites does not alter the glutamic acid interactions located some 8 \AA away. The conformations of the B13 carboxylate oxygens are fixed by H-bonding contacts to protein atoms and water molecules (see Figure 2).

In cadmium insulin hexamers, the B13 carboxylate oxygens bind directly to Cd^{2+} ; as illustrated in Figure 4A, this interaction gives a new conformation to the B13 side chains. Two pairs of carboxylate oxygens, one from each dimer, present a quartet of coordinating atoms to the metal ion. Each site has only one-third occupancy, and each of these three sym-

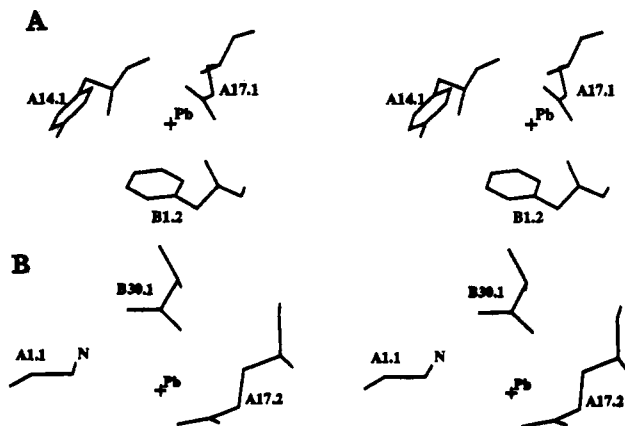


FIGURE 5: Pb²⁺ binding to Glu(A17). (A) Molecule 1: This Pb²⁺ site is 0.5 occupied. In addition to coordinating the Glu(A17) carboxylate, the metal ion is in close contact with Phe(B1) across the dimer interface. The side chain of Tyr(A14) is 4.3 Å from the Pb²⁺ ion. The three residues shown here are all from the same hexamer. (B) Molecule 2: Here the Pb²⁺ ion has an occupancy of only 0.2. Unlike molecule 1, this site is located between hexamers, rather than between dimers from the same hexamer. The metal ion is now liganded by two carboxylate functions: Glu(A17) and the B-chain C-terminus from a neighboring hexamer. The A-chain N-terminus (from the same hexamer as the B-chain C-terminus) is 3.6 Å from the metal ion.

metry-related sites is located almost exactly on one of three pseudo-2-fold symmetry axes which pass through the hexamer center in a plane perpendicular to the 3-fold symmetry axis.

Possibly owing to disorder, the Glu(B13) side chains could not be located with precision in the lead insulin crystal, especially for molecule 1. The limited resolution, the presence of two partially occupied Pb²⁺ sites, and the series termination effects from the large lead ions all mitigate against resolving the Glu(B13) side chains.

Difference density indicates that a solvent molecule, possibly chloride, is located on the 3-fold axis near the center of the hexamer. This peak is approximately 2.5 Å from each of the three symmetry-related Pb²⁺ ions in the major B13 site (see Figure 4B). When a water molecule, i.e., oxygen atom, is refined at this position, its *B* value becomes very low, less than 3.0 Å². A chloride ion, however, refined to a more reasonable *B* value of 12 Å².

No water molecules coordinated to Cd²⁺ or Pb²⁺ have been identified at the Glu(B13) site. In the cadmium insulin structure, this is a consequence of the one-third occupancy which reduces the electron density of the associated water structure to the noise level. It may also be that the water molecules interacting with the Cd²⁺ ions are poorly ordered. In lead insulin, there is a similar problem of partial occupancy, compounded by series termination effects and limited resolution of the data.

Glu(A17) Binding Sites. Two other, partially occupied, Pb²⁺ sites appear in the crystal; both involve Glu(A17) and are approximately related to each other by the local 2-fold symmetry axis. In one case, the Pb²⁺ ion is bound to the carboxylate of Glu(A17) of molecule 1 and is packed against the Phe(B1) ring of molecule 2 (Figure 5A). The occupancy of this site is about half (ca. 40 electrons). The second Glu(A17) site (Figure 5B) also involves the carboxylate function of the B-chain C-terminus from the adjacent, 3-fold screw-related, hexamer in the crystal. Although there are two carboxylate groups at this site, the Pb²⁺ occupancy is actually somewhat lower, about 0.2 (ca. 16 electrons). In both cases, the Pb²⁺ ions replace well-defined water molecules (seen in the two-zinc insulin crystals) at positions about 0.5 Å nearer

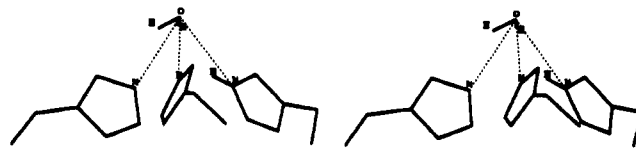


FIGURE 6: Model of how, in the absence of metal ion, a water molecule can form hydrogen bonds with three His(B10) side chains, one of which is likely to be protonated. The 3-fold axis is vertical.

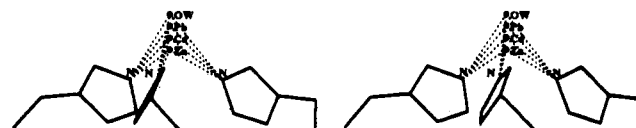


FIGURE 7: His(B10) side chains have the same conformation in all the structures described here. Different His(B10) ligands shift along the 3-fold axis in accord with their different ionic radii (see Table IV).

to the Glu(A17) carboxylate. Comparison with the two-zinc insulin structure suggests that the carboxylate function is appropriately placed to coordinate Pb²⁺ ion without much movement.

DISCUSSION

The His(B10) sites of the insulin hexamer bind Zn²⁺ very much more strongly than Ca²⁺, but the ease with which other divalent metal ions (e.g., Co²⁺, Ni²⁺, Cu²⁺, Fe²⁺, Cd²⁺, and Pb²⁺) are substituted for Zn²⁺ is more characteristic of a small-molecule chelator than of most other zinc metalloproteins (Schlichtkrull, 1956; Dunn, 1975; Storm & Dunn, 1985; Bertini et al., 1986). The Glu(B13) sites exhibit a different specificity; while Zn²⁺ binds very weakly to this site, the only metal ions which have been shown to bind tightly are Cd²⁺, Ca²⁺, and Pb²⁺ (Blundell et al., 1972; Emdin et al., 1980; Storm & Dunn, 1985; Dunn et al., 1987). This specificity arises in part from the chemical nature of the ligands (carboxylate oxygens) and the site constraints which limit the number of ligands from the protein to a pair of carboxylates.

Because the divalent metal ions under consideration, Zn²⁺, Ca²⁺, Cd²⁺, and Pb²⁺, are all spherical, there is no crystal field interaction to favor a particular ligand geometry. Consequently, the coordination geometries at the His(B10) and Glu(B13) sites are dictated by ligand-ligand interactions (both steric and electronic) and the strictures on ligand placement imposed by the three-dimensional structure of the protein.

His(B10) Sites of (In)₆. The His(B10) site of metal-free hexamer is a preformed site which binds a water molecule and is poised for Zn²⁺ coordination. At the pH of the metal-free hexamer crystal (pH 6.2), it is most likely that at least one out of every three His(B10) imidazolyl groups is protonated. Placement of a water molecule at approximately the Zn²⁺ position provides a means for dissipating this charge (Figure 6).

Binding of Cd²⁺ and Pb²⁺ to the His(B10) Sites. When His(B10)-coordinated Zn²⁺ is replaced by Cd²⁺ or Pb²⁺, the increased ionic radii (0.97 and 1.20 Å, respectively, compared to a value of 0.74 Å for Zn²⁺) is accommodated by increased N-Cd²⁺ and N-Pb²⁺ bond lengths (see Figure 7 and Table IV). Bond angles are only slightly affected, and the surrounding protein environment radiating out from the site is essentially unchanged by these substitutions. As a consequence of the increased radii of these metal ions, the distance between metal ions increases because each substituting metal ion moves out from the center of the hexamer along the 3-fold symmetry axis.

In addition to the three His(B10) imidazole ring nitrogen atoms, each Cd²⁺ ion is also coordinated to three water

Table IV: His(B10) N^{ε2} to Ligand Distances (Å)

ligand	molecule 1	molecule 2	av	expected ^a
Zn ²⁺	2.05	2.05	2.05	2.05
Cd ²⁺	2.20	2.30	2.25	2.28
Pb ²⁺	2.70	2.30	2.50	2.51
Zn ²⁺ (Zn-free)	2.00	2.00	2.00	2.05
H ₂ O (Zn-free)	2.70	3.00	2.85	2.82

^aExpected metal ion–N^{ε2} distances were calculated by taking the (In)₆(Zn)₂ distance (2.05 Å) as the standard and adding the difference between Zn²⁺ and Cd²⁺ or Pb²⁺ ionic radii; radii used are Zn²⁺, 0.74 Å; Cd²⁺, 0.97 Å; and Pb²⁺, 1.20 Å (Weast, 1968). Expected His N^{ε2} to H₂O distance taken from Gorbitz (1989).

molecules, producing a distorted octahedral ligand field. This arrangement is analogous to ligation of Zn²⁺. The octahedral arrangement is unusual for Cd²⁺; owing to the larger ionic radius of this ion, Cd²⁺ complexes typically contain seven or eight ligands (Cotton & Wilkinson, 1972). In the case of lead insulin, no solvent can be seen coordinating the metal ions. This may be a result of the lower resolution of the (In)₆-(Pb²⁺)₂(Pb²⁺)₃ data.

Binding of Cd²⁺ and Pb²⁺ to the Glu(B13) Sites. Perhaps the most unusual aspect of the Glu(B13) site coordination chemistry is that one Cd²⁺ ion is bound at the hexamer center (Figure 4A) while three Pb²⁺ ions are located in this region (Figure 4B). The three Pb²⁺ ions are distributed over six sites organized as pairs too close for simultaneous occupancy; one set of three Pb²⁺ ions has occupancies of about 0.7, and the other set of three Pb²⁺ ions has occupancies of about 0.3. By contrast, the one Cd²⁺ ion populates three symmetry-related positions, each with one-third occupancy. This one-third occupancy of the three Cd²⁺ sites is considered to arise from the random distribution of one Cd²⁺ ion over the three equivalent positions within one hexamer. The Cd–Cd distance in adjacent sites, at 2.7 Å, is almost certainly too short to accommodate the Coulombic repulsions which would arise from the simultaneous placement of a Cd²⁺ ion in each position. It seems likely that with only a single Cd²⁺ ion per Glu(B13) cavity, migration of Cd²⁺ from one site to the next is a rapid process.

In the Pb²⁺ derivative, the symmetry-related Glu(B13) metal ion positions are located further apart, ca. 4.5 Å, and it is evident that this separation is sufficient to allow simultaneous occupation by three Pb²⁺ ions over six sites. Although the limited resolution, disorder, and series termination effect in the Pb²⁺ derivative preclude any definitive conclusions about the nature and number of solvent molecules coordinated to Pb²⁺, the density map suggests there may be a chloride ion located on the 3-fold axis midway between the three major Pb²⁺ sites.

According to the structural pictures provided by these X-ray diffraction studies, when either Cd²⁺ or Ca²⁺ binds within the Glu(B13) cavity, coordination involves disordering of the B13 side chains between two conformations. The intradimer hydrogen bonds between two sets of carboxylate–carboxylic acid pairs are ruptured, one of the side chains in the carboxylic acid form ionizes, and two of the side chains reorganize to form an interdimer coordination site for the metal ion. Since this new site spans the dimer–dimer interface, metal ion coordination must provide a new interaction which should facilitate assembly and help stabilize the insulin hexamer.

We report here the structure of (In)₆(Cd²⁺)₂Cd²⁺, an analogue of the species of physiological interest, (In)₆-(Zn²⁺)₂Ca²⁺. The reason why the Ca²⁺ structure has not been determined is as follows; our initial attempts to prepare crystals of (In)₆(Zn²⁺)₂Ca²⁺ by soaking (In)₆(Zn²⁺)₂ in Ca²⁺ solutions appeared to be unsuccessful. No significant changes were

observed in the structure factor amplitudes unless the Ca²⁺ concentration was so high that the diffraction properties were seriously impaired. We now realize, from the structure of (In)₆(Cd²⁺)₂Cd²⁺, that the (In)₆(Zn²⁺)₂Ca²⁺ diffraction pattern should be extremely similar to that of (In)₆(Zn²⁺)₂. The difference in structure would be the substitution of one-third of a Ca²⁺ ion for one-third of a water molecule, a resulting change of less than three electrons. Thus, it is not surprising that there were no detectable X-ray intensity changes from the Ca²⁺-soaked crystals.

¹H FT NMR studies of Cd²⁺ and Ca²⁺ binding show that when these metal ions are introduced to the Glu(B13) site, the ¹H NMR signals from distant side-chain residues are perturbed (Palmieri et al., 1988). These effects are particularly obvious for those aromatic side chains (Phe and Tyr) which form hydrophobic clusters along the monomer–monomer and dimer–dimer interfaces. One possible explanation for some of the perturbed chemical shifts in the NMR spectrum comes from the Pb²⁺ site located by the carboxylate of Glu(A17) of molecule 1. At this site, the metal ion is positioned against the aromatic ring of Phe(B1) and is quite close to the ring of Tyr(A14). Even low partial occupancy by Cd²⁺ or Ca²⁺ at this site in solution would cause significant perturbation of the NMR spectrum. Palmieri et al. (1988) conclude that most of these spectral perturbations arise from motions which radiate out from the Glu(B13) site along the subunit interfaces, causing small shifts in the relative positions of the aromatic rings. If this interpretation is correct, then these conformational changes are too small to be detected at the resolution of the structures reported herein.

Comparison with Other Ca²⁺ Binding Proteins. The binding sites of Ca²⁺ binding proteins for which three-dimensional structures have been determined mostly fall into two or three general classes: there are two closely related types of the helix–loop–helix “EF-hand” motifs (Kretsinger, 1972) that are widespread among proteins of the calmodulin family (Babu et al., 1987; Herzburg & James, 1985; Sundaralingam et al., 1985; Szebenyi & Moffat, 1986, 1987), and there are loop motifs represented by such Ca²⁺ binding proteins as trypsin, concanavalin A, phospholipase A₂, and site 3 of thermolysin (Szebenyi & Moffat, 1987). There is no clear sequence similarity for this latter group. In addition to the above groups, there is a Ca²⁺ site in α-amylase which is located between two domains (Buisson et al., 1987; Boel et al., 1990), and three Ca²⁺ ions have been located in the satellite tobacco necrosis virus (STNV) capsid (Jones & Liljas, 1984). The most common Ca²⁺ ligands are the side chains of Glu and Asp residues, main-chain carbonyl oxygen atoms, and water molecules. Sometimes the carboxylates donate both oxygens as ligands, other times only one. Occasionally, these sites include Ser or Thr hydroxyls. The total number of ligands is usually seven or eight. In the EF-hand motif, the ligands usually are closely spaced, creating a tight loop which entwines the metal ion.

With one metal ion apparently moving rapidly between three symmetry-related sites and coordinating covalently separate protein molecules, the B13 Ca²⁺ binding site in insulin is quite different from Ca²⁺ binding sites in other known protein structures. This difference reflects a difference in biological function. The only protein ligands of Cd²⁺ at the center of the insulin hexamer are two B13 carboxylates, which are contributed from different dimers. Thus, the Ca²⁺ binding site is formed as a consequence of dimer aggregation to form tetramer and hexamer, its physiological role is probably to stabilize the hexamer during storage and to facilitate hexamer

formation from proinsulin. In contrast, metal coordinating groups in other Ca²⁺ binding proteins are from the same molecule and are usually close together in sequence. The exception to this is STNV in which, like insulin, the Ca²⁺ ions apparently stabilize an aggregate by coordinating groups from neighboring molecules.

Role of Metal Ions in Hexamer Assembly and Dissociation. Both the His(B10) sites and the Glu(B13) sites of the insulin hexamer span the dimer–dimer interfaces. Because the His(B10) site is constructed from three histidine residues, one from each dimer, this site is completely formed only in the assembled hexamer. Assuming a dimer to tetramer to hexamer assembly pathway, then at the tetramer stage of assembly, the two His(B10) sites are only partially formed, each consisting of just two imidazole ligands.

The situation is different for the Glu(B13) site. At the tetramer stage of assembly, one Glu(B13) site (across the dimer–dimer interface) is possible; the remaining two sites are formed when the hexamer is assembled. Hence, during assembly of the insulin hexamer, the first high-affinity metal binding site created is the Glu(B13) site of the tetramer.

The kinetic studies of Coffman and Dunn (1988) support an assembly mechanism in which the first metal ion binding step occurs concomitant with tetramer formation, and this metal ion interaction takes place at the Glu(B13) site. Such an interaction will provide a thermodynamic driving force which stabilizes the tetramer (relative to dimer). Conversion of tetramer to hexamer creates the Glu(B13) cavity, in which a total of six glutamate side chains are seen in close proximity in the crystal structure at pH 6.2. If a divalent metal ion is bound to the B13 site at the tetramer stage of assembly, then electrostatic repulsions among the Glu(B13) carboxylates will be attenuated by the presence of the metal ion, and hexamerization should be facilitated.

Preformed His(B10) sites of the hexamer rapidly bind Zn²⁺ at a rate that is limited by the rate of dissociation of inner-sphere-coordinated water (Coffman & Dunn, 1988; Wilkens & Eigen, 1964). Thus, uptake of Zn²⁺ by these sites undoubtedly is concomitant with (and limited by the rate of) hexamer formation. Since the His(B10) sites bridge the dimer–dimer interfaces of the hexamer, Zn²⁺ binding also stabilizes the hexamer with respect to dimer and tetramer.

It is likely that in the storage vesicles proinsulin hexamers, as (Proin)₆(Zn²⁺)₂Ca²⁺, are assembled in a sequence of steps analogous to that described above for insulin (Blundell et al., 1972; Steiner et al., 1981; Howell et al., 1975). The proteolytic processing of proinsulin hexamers to insulin hexamers occurs within the storage vesicle. This conversion greatly lowers the solubility of the hexamer, and as conversion takes place in vivo, crystallization of (In)₆(Zn²⁺)₂Ca²⁺ occurs (Blundell et al., 1972; Steiner et al., 1989). This crystallization is believed to serve two purposes: (1) the efficient storage of insulin; (2) the protection of insulin molecules from further proteolytic processing.

The His(B10) sites are located at the bottom of shallow depressions at opposite ends of the insulin hexamer. Three water molecules coordinated to each zinc ion extend out into these shallow depressions, while the imidazole ligands are arranged inside the central, water-filled channel running along the 3-fold axis (Figures 1 and 2). The His(B10) sites are fashioned for rapid release of Zn²⁺ via chelation. The studies of Dunn et al. (1980), Storm and Dunn (1985), and Kaarsholm and Dunn (1987) show that small-molecule tridentate chelators (Che) react with the His(B10) zinc ions by first rapidly replacing the coordinated water molecules to form a

(In)₆(Zn²⁺–Che)₂Ca²⁺ complex; then Zn²⁺ is removed from the protein in a slower, rate-limiting dissociation of chelator-coordinated Zn²⁺ from the protein.

When insulin is secreted from the pancreatic β-cells, the information presently available suggests that the crystals of (In)₆Zn²⁺)₂Ca²⁺ contained within the secretory vesicles are simply released from the cell via exocytosis (Formby et al., 1984). Since monomeric insulin is the biologically active form recognized by the insulin receptors, the rapid delivery of active hormone to the target cells requires that crystalline hexamers dissolve and dissociate rapidly. From the physical–chemical information available, it is not clear whether or not spontaneous crystal dissolution and hexamer dissociation is sufficiently rapid to account for the kinetics of the insulin response in vivo. Transit times for the delivery of active insulin to the liver from the pancreas are estimated to be on the order of a few seconds (Gold & Grodsky, 1984). In vitro kinetic studies of the sequestering and removal of Zn²⁺ from (In)₆(Zn²⁺)₂ or (In)₆(Zn²⁺)₂Ca²⁺ by tridentate chelators indicate Zn²⁺ can be removed within a few seconds (Dunn et al., 1980; Storm & Dunn, 1985; Kaarsholm & Dunn, 1987). Light-scattering studies (Coffman and Dunn, unpublished results) indicate that removal of Zn²⁺ is rate limiting for hexamer dissociation, and microspectrophotometry studies of insulin crystals indicate the action of zinc chelators greatly accelerates crystal dissolution (Ottonello and Dunn, unpublished results). For these reasons, we speculate that an endogenous chelator could play an active in vivo role in the conversion of crystalline insulin to the active hormone.

According to these arguments, the binding of Zn²⁺ or Ca²⁺ to the Glu(B13) site drives tetramer assembly, and this interaction, together with Zn²⁺ binding to hexameric His(B10) sites, drives the association of tetramer with dimer to form hexamer. During the release of crystalline insulin from the β-cells, the His(B10)-bound zinc ions must be removed in order for efficient dissolution of the crystals and rapid dissociation of hexamers. The physical arrangement of the ligand field at the B10 site seems designed to allow efficient sequestering and removal of Zn²⁺ via the intervention of endogenous chelator. These structural studies establish that the insulin hexamer is an intricate and reversible chelator with unusual Zn²⁺ and Ca²⁺ sites. These unusual sites play important in vivo functions in the assembly, storage, and release of insulin.

Registry No. L-His, 71-00-1; L-Glu, 56-86-0; Ca, 7440-70-2; Zn, 7440-66-6; Cd, 7440-43-9; Pb, 7439-92-1; insulin, 9004-10-8; insulin zinc, 8049-62-5.

REFERENCES

- Adams, M. J., Dodson, G., Dodson, E., & Hodgkin, D. C. (1967) *Conform. Biopolym., Pap. Int. Symp. 1*, 9–16.
- Adams, M. J., Blundell, T. L., Dodson, E. J., Dodson, G. G., Vijayan, M., Baker, E. N., Harding, M. M., Hodgkin, D. C., Rimmer, R., & Sheet, S. (1969) *Nature* 224, 491–495.
- Agarwal, R. C. (1978) *Acta Crystallogr. A* 34, 791–809.
- Alameda, G. K., Evelhoch, J. L., Sudmeier, J. L., & Birge, R. R. (1985) *Biochemistry* 24, 1757–1762.
- Babu, Y. S., Bugg, C. E., & Cook, W. J. (1987) in *Proceedings of the Fifth International Symposium on Calcium Binding Proteins in Health and Disease* (Norman, A. W., Vanaman, T. C., & Means, T. A., Eds.) pp 305–311 Academic Press, New York.
- Baker, E. N., Blundell, T. L., Cutfield, J. F., Cutfield, S. M., Dodson, E. J., Dodson, G. G., Hodgkin, D. M. C., Hubbard, R. E., Isaacs, N. W., Reynolds, C. D., Sakabe, K., Sakabe, N., & Vijayan, N. M. (1988) *Philos. Trans. R. Soc. London, Ser. B* 319, 369–456.

- Bertini, I., Luchinat, C., Maret, W., & Zeppezauer, M., Eds. (1986) *Zinc Enzymes, Progress in Inorganic Biochemistry and Biophysics*, Vol. 1, pp 1-640, Birkhauser, Basel.
- Blundell, T., Dodson, G., Hodgkin, D., & Mercola, D. (1972) *Adv. Protein Chem.* 26, 279-402.
- Boel, E., Brady, L., Brzozowski, A. M., Derewenda, Z., Dodson, G. G., Jensen, V. J., Peterson, S. B., Swift, H., Thim, L., & Woldlike, H. F. (1990) *Biochemistry* 29, 6244-6249.
- Buisson, G., Duée, E., Haser, R., & Payan, F. (1987) *EMBO J.* 6, 3909-3916.
- Coffman, F. D., & Dunn, M. F. (1988) *Biochemistry* 27, 6179-6187.
- Cotton, F. A., & Wilkinson, G. (1972) in *Advanced Inorganic Chemistry*, 3rd ed., pp 309-332, 503-516, Wiley-Interscience, New York.
- Cutfield, J. F., Cutfield, S. M., Dodson, E. J., Dodson, G. G., Emdin, S. O., & Reynolds, C. D. (1979) *J. Mol. Biol.* 132, 85-100.
- Dunn, M. F. (1975) *Struct. Bonding* 23, 61-122.
- Dunn, M. F., Pattison, S. E., Storm, M. C., & Quiel, E. (1980) *Biochemistry* 19, 718-725.
- Dunn, M. F., Palmieri, R., Kaarsholm, N. C., Roy, M., Lee, R. W.-K., Dauter, Z., Hill, C., & Dodson, G. G. (1987) in *Proceedings of the Fifth International Symposium on Calcium Binding Proteins in Health and Disease* (Norman, A. W., Vanaman, T. C., & Means, A., Eds.) pp 372-383, Academic Press, New York.
- Emdin, S. O., Dodson, G. G., Cutfield, J. M., & Cutfield, S. M. (1980) *Diabetologia* 19, 174-182.
- Formby, B., Schmid-Formby, F., & Grodsky, G. M. (1984) *Diabetes* 33, 229-234.
- Gold, G., & Grodsky, G. M. (1984) *Experientia* 40, 1105-1114.
- Görbitz, C. H. (1989) *Acta Crystallogr.* B45, 390-395.
- Greider, M. H., Howell, S. L., & Lacy, P. E. (1969) *J. Cell Biol.* 41, 162-166.
- Halldén, G., Gafvelin, G., Mutt, V., & Jornvall, H. (1986) *Arch. Biochem. Biophys.* 247, 20-27.
- Hendrickson, W. A. (1985) *Methods Enzymol.* 115, 252-270.
- Herzberg, O., & James, M. N. G. (1985) *Nature* 313, 653-659.
- Howell, S. L., Montague, W., & Tyhurst, M. (1975) *J. Cell Sci.* 19, 395-409.
- Howell, S. L., Tyhurst, M., Duvefelt, H., Andersson, A., & Hellerström, C. (1978) *Cell Tissue Res.* 188, 107-118.
- Jones, T. A. (1985) *Methods Enzymol.* 115, 157-171.
- Jones, T. A., & Liljas, L. (1984) *J. Mol. Biol.* 177, 735-767.
- Kaarsholm, N. C., & Dunn, M. F. (1987) *Biochemistry* 26, 883-890.
- Kretsinger, R. H. (1972) *Nature (London), New Biol.* 240, 85-88.
- Norman, A. W., Vanaman, T. C., & Means, A. R., Eds. (1987) *Proceedings of the Fifth International Symposium on Calcium Binding Proteins in Health and Disease*, Academic Press, New York.
- North, A. C. T., Phillips, D. C., & Mathews, F. S. (1968) *Acta Crystallogr.* A24, 351-359.
- Palmieri, R., Lee, R. W.-K., & Dunn, M. F. (1988) *Biochemistry* 27, 3387-3397.
- Peterson, J. D., Steiner, D. F., Emdin, S. O., & Falkmer, S. (1975) *J. Biol. Chem.* 250, 5183-5191.
- Schlichtkrull, J. (1956) *Acta Chem. Scand.* 10, 1455-1458.
- Steiner, D. F., Bell, G. I., Hammer, R. E., Modson, O., Carroll, R. J., & Chan, S. J. (1989) in *Perspectives on the Molecular Biology and Immunology of the Pancreatic β -Cell: Current Communications in Molecular Biology* (Hanahan, D., McDevitt, H. O., & Cahill, S. G. E., Jr., Eds.) pp 19-36, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Storm, M. C., & Dunn, M. F. (1985) *Biochemistry* 24, 1749-1756.
- Sudmeier, J. L., Bell, S. J., Storm, M. C., & Dunn, M. F. (1981) *Science* 212, 560-562.
- Sundaralingam, M., Bergstrom, R., Strasburg, G., Rao, S. T., Roychowdhury, P., Greaser, M., & Wang, B. C. (1985) *Science* 227, 945-948.
- Szebenyi, D. M. E., & Moffat, K. (1986) *J. Biol. Chem.* 261, 8761-8777.
- Szebenyi, D. M. E., & Moffat, K. (1987) in *Proceedings of the Fifth International Symposium on Calcium Binding Proteins in Health and Disease* (Norman, A. W., Vanaman, T. C., & Means, T. A., Eds.) pp 323-332, Academic Press, New York.
- Weast, R. C., Ed. (1968) *CRC Handbook of Chemistry and Physics*, pp F152-F153, The Chemical Rubber Co., Cleveland, OH.
- Wilkens, R. G., & Eigen, M. (1964) *Adv. Chem. Ser.* 49, 55-80.