Design, Synthesis, and Testing of Insulin Hexamer-Stabilizing Agents

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The addition of zinc to insulin solution leads to a long-acting insulin preparation because the zinc stabilizes the less soluble hexameric form of the hormone. It is clear from the crystal structure of dizinc insulin that there is a space at the center of the hexamer, between the two zinc atoms, that could accommodate a small organic molecule. It should thus be possible to design a structure that could further stabilize the insulin hexamer by binding at this site. Computer graphic techniques have been used to design several molecules capable of forming multiple bonds to the six histidine residues surrounding the site. Synthesis and testing of one of these compounds, benzene-1,4-disulfonic acid, show a significant increase in weight-average molecular weight of insulin in solution, and control experiments with related structures suggest that this effect is due to the proposed binding mechanism.

Since the first long-acting insulin was developed in 1936,[1] the use of long-acting insulins in therapy has become well established,[2] and many attempts have been made to further extend its duration of action. Noteworthy among these is the use of insulin–albumin microbeads to release biologically active insulin for periods of up to 3 weeks,[3] although it appears unlikely that this dosage form will be clinically useful. This paper describes an alternative approach to the design of longer acting insulins.

Insulin exists in solution as monomers, dimers, tetramers, hexamers, and polymers of hexamers in dynamic equilibrium.[4] If this equilibrium could be influenced toward the hexamer and higher aggregates, the time for dissociation to the active monomeric species would be increased. Insulin stabilized in this way could be developed into an injectable suspension form with time–action characteristics similar to ultralente insulins or even longer. With this in mind, we have used the three-dimensional crystal structure of porcine dizinc insulin[4] as the basis for the design of small molecules intended to bind selectively to the insulin hexamer. This general approach to drug design by the method of receptor fit has recently been reviewed by Goodford.[5]

The three-dimensional crystal structure of porcine insulin was first determined by Hodgkin and collaborators in 1969[6,7] to a resolution of 2.8 Å and subsequently refined to 1.9,[8] 1.5,[9] and 1.2 Å.[10] The final refinement allowed the positioning of many hydrogen atoms in the insulin molecule.

At the center of the insulin hexamer, two zinc atoms lie on the threefold axis about 16 Å apart, one 8 Å above and the other 8 Å below the local twofold axes. Each zinc atom coordinates to three equivalent N_3 nitrogens of the B10 histidines and to three water molecules. It has been postulated that the charge of the zinc ions is neutralized by spreading these charges over to the B13 glutamic acid carboxyl group via the imidazole ring and a water molecule connected by a hydrogen-bond network.[11]

Between the two zinc ions lies a hydrophilic cavity bounded by residues B9 serine, B10 histidine, and B13 glutamate, and their threefold related equivalents, with associated water structure. The basic shape of the space is ellipsoidal, with groups of three histidines at the top and bottom and an "equator" of glutamates at the center. Figure 1 shows stereoscopic views of the center of the hexamer viewed down and across the threefold axis.

Experimental Section

The Victorian College of Pharmacy Ltd. molecular modeling system MORPHEUS was used to examine the crystal structure and to perform superimpositions. MORPHEUS is based upon molecular modeling programs developed by P. Pauling, D. Richardson, and M. Lee at University College, London, with additional programs in the package written by C. Lowther, G. Quint, D. Richardson, and D. Winkler at the Victorian College of Pharmacy Ltd. Hard-copy plots of molecular structure were generated by PLUTO, written by S. Motherwell of Oxford University and modified by G. Jones and D. Winkler.

The influence of hexamer stabilizing agents on the weight-average molecular weight of insulin was determined by the method of Jeffrey,[12] who showed that glucose significantly reduced the apparent weight-average molecular weight distribution for insulin at both pH 2 and pH 7.

Bovine crystalline zinc insulin was supplied by the Australian Commonwealth Serum Laboratories. All reagents used were analytical grade except the insulin hexamer-stabilizing compounds, which were laboratory grade or were prepared synthetically. Glass-distilled water was used in the preparation of all buffer solutions, which were millipored through 0.45-μm filters. The pH 8 buffer, ionic strength 0.2, was composed of 0.1 M NaCl, 0.1 M Tris–adjusted to pH 8 with the HCl. Sodium azide (0.01%) was added as a preservative. The pH 2 buffer, ionic strength 0.15, was composed of 0.05 M HCl, 0.05 M glycine, and 0.1 M NaCl. Buffers containing test compounds and those containing glucose were prepared by weighing the appropriate amount of material and making the volume up to a predetermined value with either pH 2 or pH 8 buffer. Adjustment of pH was unnecessary. These solutions were millipored through 0.45-μm filters. Problems were encountered with insulin precipitating from buffers at pH 7.0, so it was decided to perform experiments at pH 8, where precipitation did not pose any problems. Zinc insulin solutions were prepared by dissolving about 24 mg of protein in 10 mL of buffer adjusted to pH 9.6. After the insulin dissolved, the pH was readjusted to 8 by dialyzing against 200 mL of buffer. The stock solution was then spun at 50 K for 30 min to remove any un-

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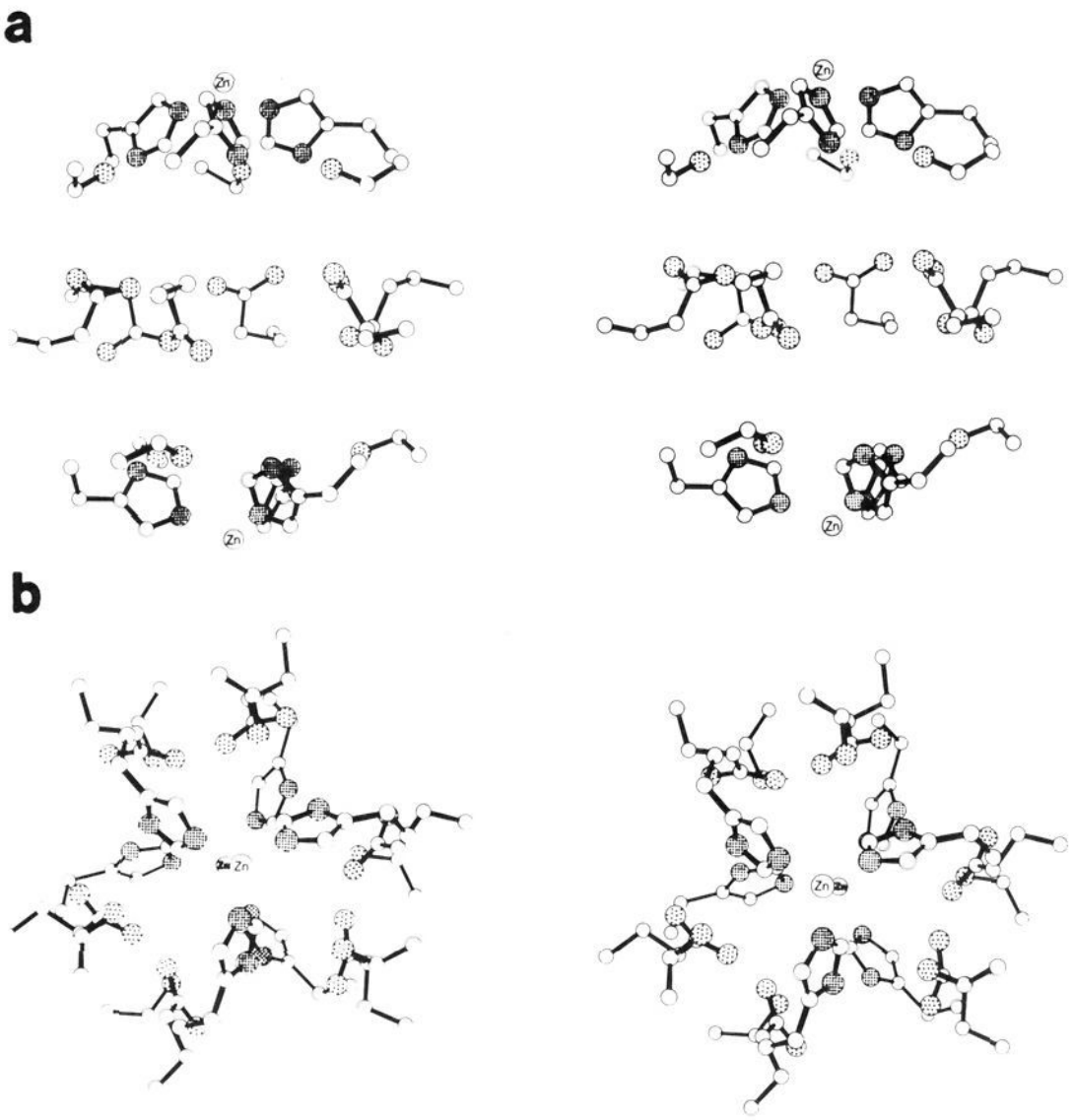


Figure 1. Center of the insulin hexamer viewed across the threefold axis (a) and down the threefold axis (b). Light and dark shadings represent oxygen and nitrogen atoms, respectively.

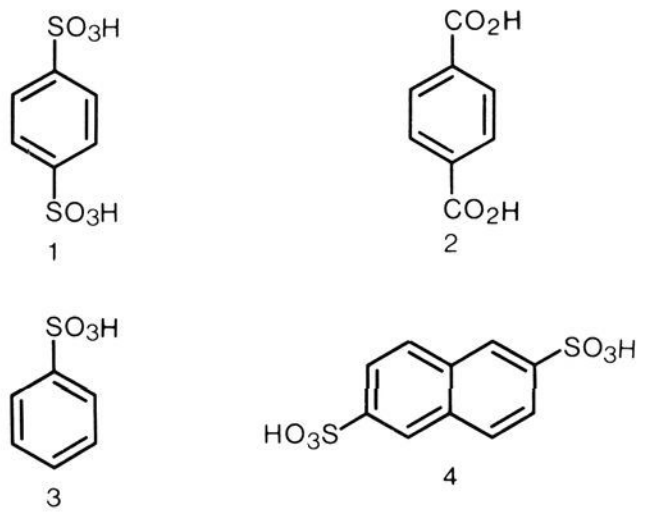
dissolved material. The concentration was checked at 276 nm on a spectrophotometer, employing an extinction coefficient of 1.05 ODU/cm (mg/mL).[13] Final concentrations of stock solutions were between 2.1 and 2.2 mg/mL.

The densities of buffer solutions were determined at 20 °C on a precision density meter (DMA-02, Anton Paar, Graz). The values used for the partial specific volumes of the protein were at pH 2, 0.72 mL/g,[13] and at pH 8, 0.73 mL/g.[14] Sedimentation equilibrium experiments were carried out at 20 °C by the meniscus depletion method of Yphantis using a Beckman Model E ultracentrifuge equipped with Rayleigh interference optics.[15] In all sedimentation equilibrium experiments, double sector cells (aluminum-filled epoxy resin) were employed containing in one sector equilibrium dialysate, and in the other dialyzed protein solution. A column height of 3 mm was used, and it should be noted that no fluorocarbon oil was used as a base fluid as a precaution against the possibility of any protein interaction with the oil. Sedimentation equilibrium was generally reached within 16 h, and fringe patterns were recorded photographically on Kodak spectroscopic IIG plates. Photographs were taken at the beginning of the experiment and at equilibrium, so that any leakage from cells could be checked. Fringe displacement measurements were taken at regular distances along the cell with a Nikon microcomparator. Apparent weight-average molecular weights were calculated for points throughout the cell by the expression

$$M_r(\text{app})_x = [\text{d ln } c/\text{d}(x^2)_x][2RT/(1 - \nu\rho)\omega^2]$$

where c is the protein concentration at a point in the cell located x centimeters from the axis of rotation, R is the gas constant, T is the absolute temperature, ν is the partial specific volume of the protein, ρ is the density of the solution, and ω is the angular velocity in radians/second. The computer program of Roark and Yphantis was used to calculate number, weight, and z and z + 1 average molecular weights throughout the cell.[16]

Benzene-1,4-disulfonic acid (**1**) was prepared by the method of Meerwein et al.,[17] terephthalic acid (**2**) and benzenesulfonic acid (**3**) were obtained from BDH Chemicals Ltd, Poole, UK, and naphthalene-2,6-disulfonic acid (**4**) was purchased from the Aldrich Chemical Co.



Results and Discussion

In designing molecules to fit the center of the hexamer, we worked backwards from the protein residues, building dummy atoms onto the B10 histidine ring nitrogens to represent optimum positions for the interacting atoms of the designed molecules. In order to keep the perturbation of the observed crystal structure to a minimum, we initially chose to map out a series of possible dummy atom sites by varying only the second torsion angle in the histidine sidechain (ABCD in Figure 2). Fortunately, this variation

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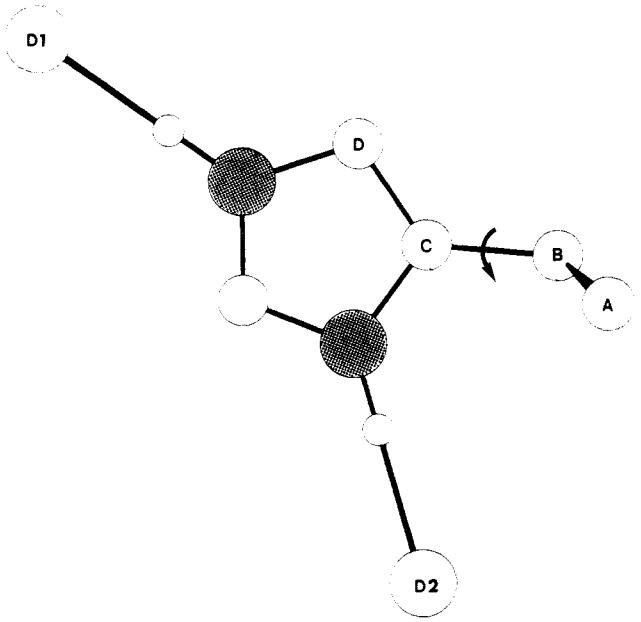

Figure 2. Histidine with dummy atoms built onto the nitrogen atoms at a distance of 2.8 Å.

proved sufficient to locate a range of suitable positions for histidine binding functions, and further conformational variations of the histidine side chain or the protein backbone were not undertaken.

The dummy atoms were built onto the nitrogen atoms of the ring at a distance of 2.8 Å (i.e., approximately H-bonding distance), and the torsion angle ABCD was altered in small increments to yield two series of possible oxygen positions for dummy atoms D1 and D2. This was completed for all six histidine residues, resulting in two major sets of oxygen positions. The first set of positions, for atom D2, consisted of two roughly circular regions of about 4.0-Å radius around the threefold axis, the two circles being approximately 5.2 Å apart. The second set, for atom D1, yielded two circles of about 1.3-Å radius around the threefold axis and approximately 9.2 Å apart (Figure 3). However, if allowance is made for the considerable flexibility of N–H···O hydrogen bonds, each of these sets of positions can be regarded as the midpoint of a range of possible locations, and we therefore decided to model an intermediate set of positions between the two. These intermediate positions, together with the trigonal symmetry of the histidine residues, suggest that a pair of either sulfonic or phosphonic acid groups, separated by a distance corresponding approximately to the length of a benzene ring, could provide optimal binding to all six histidine residues. Benzene-1,4-disulfonic acid (**1**) was therefore proposed as a potential histidine binding agent. Modeling of this molecule into the hexamer cavity gives a moderately good match, even without alteration to the histidine torsion angles (Figure 4). The distance between the histidyl nitrogens and sulfonic acid oxygens is optimal at 2.79 Å, although other atoms make slightly closer contacts (e.g., atom C1, Figure 5), which may reduce the strength of this interaction. In addition, the H-bond angle for the N–H–O bond is 82° compared to the normal range of 180–110°.[18] One must keep in mind, however, that this is the nonperturbed site and that only small changes in the torsion angles of the histidine side chain, or elsewhere in the insulin structure, are likely to be needed to improve the fit. The H-bond angle of 82° may also be less of a problem if

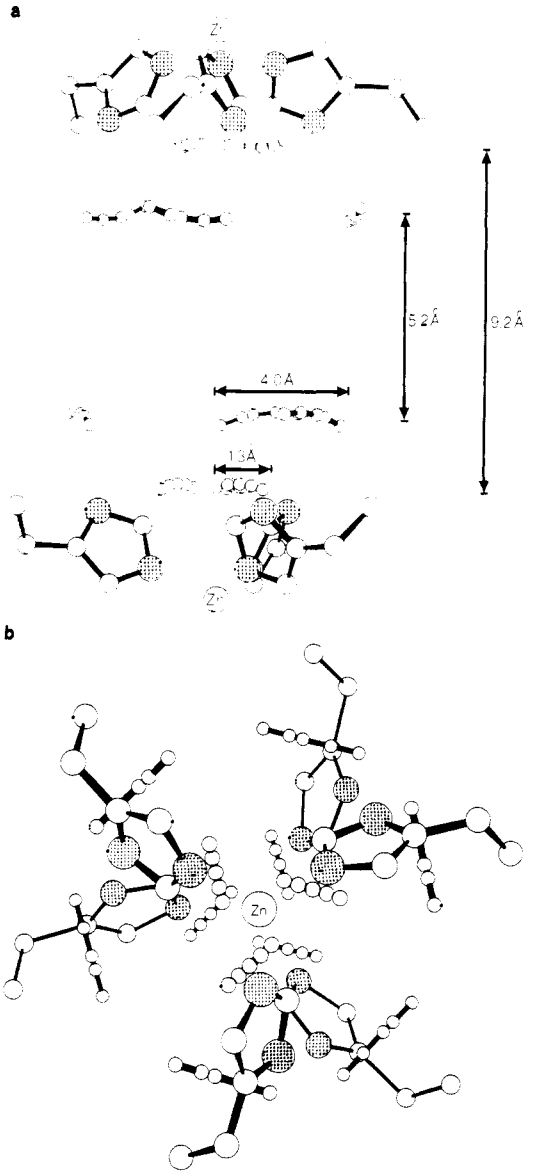

Figure 3. Dummy atom positions from the two nitrogen atoms of the B10 histidine residues viewed across (a) and down (b) the threefold axis.

the nature of the interaction is partly ionic, thus not requiring an optimum H bond.

For comparison with **1**, terephthalic acid (**2**), benzenesulfonic acid (**3**), and naphthalene-2,6-disulfonic acid (**4**) were modeled into the site. These experiments showed, as expected, that terephthalic acid and benzenesulfonic acid bind to a maximum of four and three histidine residues, respectively, while the naphthalene derivative **4** brings the sulfonic acid groups into too close a contact with the histidine residues to allow binding without a significant conformational change in the hexamer. Thus, if the postulated binding model for **1** is correct, each of these three molecules would be expected to produce significantly less effect on the weight-average molecular weight of insulin than that due to compound **1**.

Sedimentation equilibrium experiments were performed on insulin at pH 2 or pH 8 in the presence or absence of 1–4 or glucose. The experiments on glucose were included as a control to reproduce Jeffrey's work[12] on the dissociation of insulin by glucose under similar conditions. Each experiment was compared to a sedimentation equilibrium control run performed on insulin and conducted within a period of 3 days.

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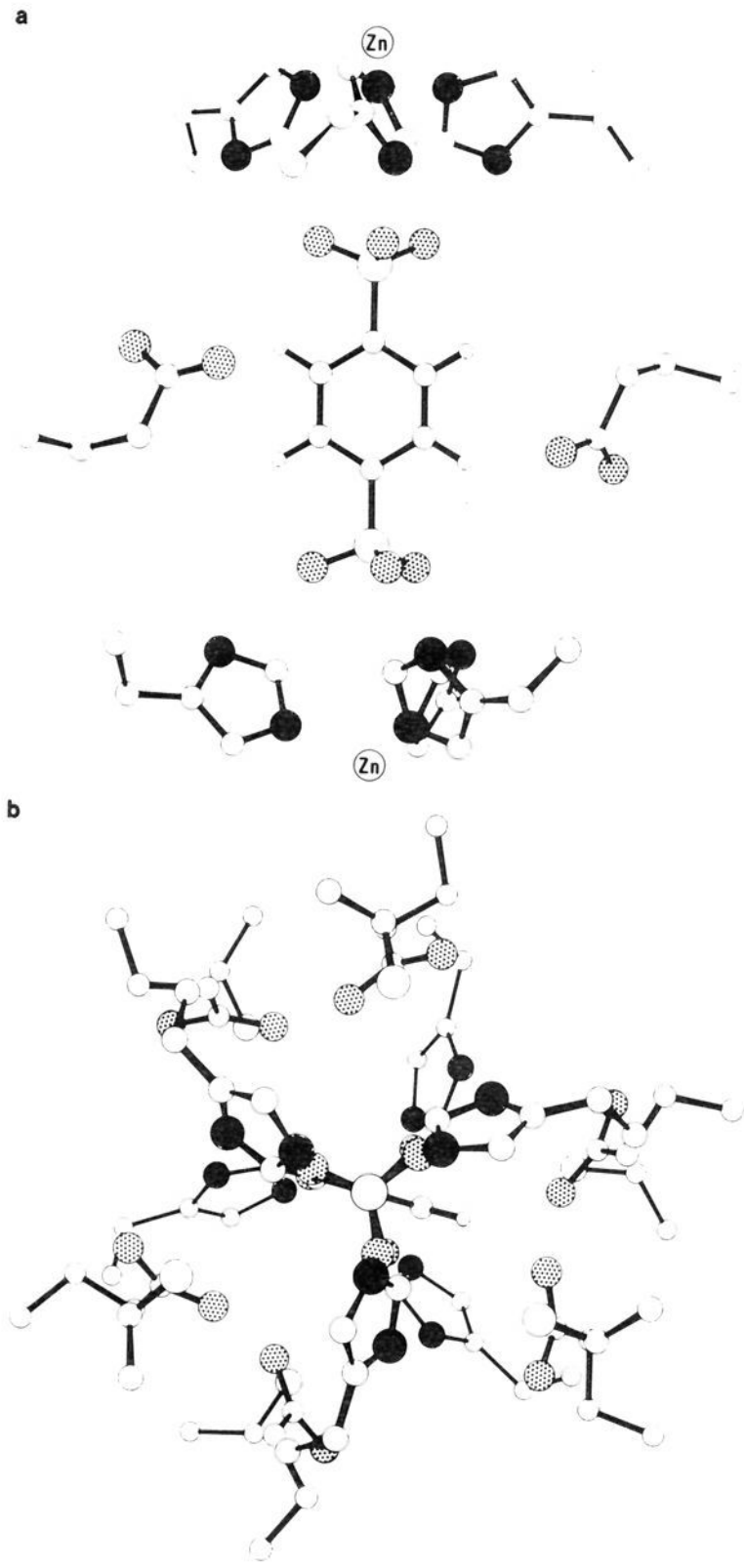

Figure 4. Compound 1 binding to the center of the hexamer in the nonperturbed state viewed across (a) and down (b) the threefold axis. Several residues have been omitted for clarity.

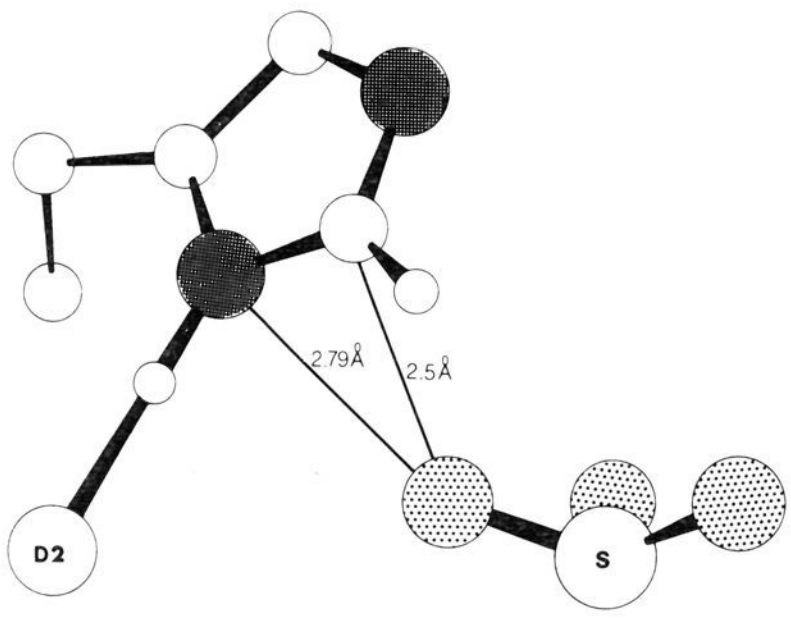

Figure 5. Contacts between the sulfonic acid group of compound 1 and the B10 histidine ring.

Sedimentation Equilibrium Results at pH 2. Three sedimentation equilibrium experiments were performed on bovine insulin at pH 2. They were carried out in the presence and absence of glucose and in the presence of **1**. A 7.5-g sample of glucose per 100 mL was dialyzed against insulin overnight, and the result of a sedimentation equilibrium experiment on this solution displayed the same dissociative property as reported in the previous study[12] (Figure 6). Compound **1** at a concentration of 50 mg/100 mL was also dialyzed against insulin. The apparent weight-average molecular weight distribution for this experiment increased for points throughout the cell (Figure 6). This suggests that the aggregation of insulin is being shifted toward higher polymeric species. In view of the relatively low concentration used, it is unlikely that the result is due to solvent structure changes but rather to direct interaction of **1** with the protein. It is not possible to distinguish which equilibria are being influenced nor if the interaction is at the proposed site.

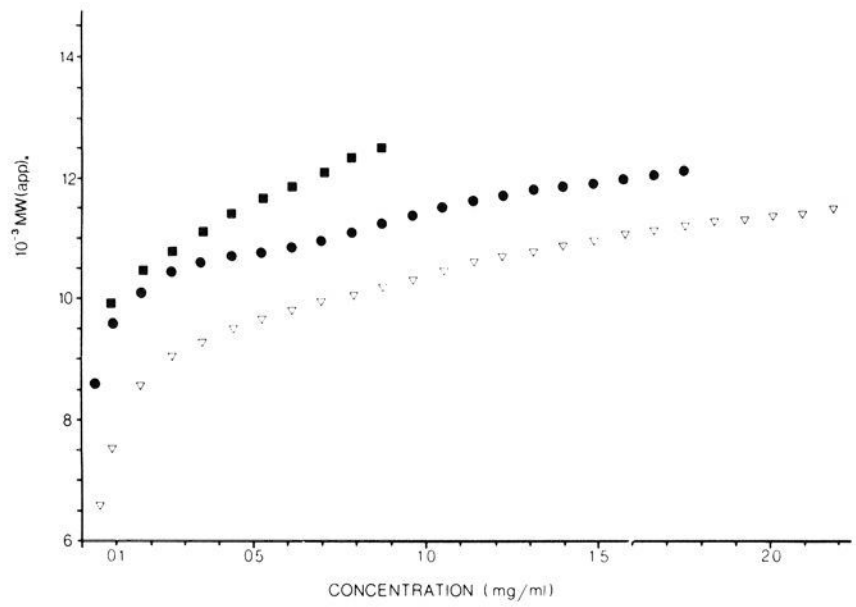

Figure 6. Apparent weight-average molecular weight vs. concentration for insulin at pH 2 showing the effect of glucose and compound **1**. Sedimentation equilibrium experiments were performed in glycine–NaCl buffer, ionic strength 0.15 at 20 °C: (●) 44 000 rpm; (▽) 44 000 rpm, 7.5 g glucose/100 mL; (■) 48 000 rpm, 50 mg of **1**/100 mL. Each curve represents the result of a single ultracentrifuge experiment.

Sedimentation Equilibrium Results at pH 8. Compound **1** was tested at two different concentrations against insulin. At the lowest concentration ratio of 3 mg/100 mL, there was no significant effect on insulin aggregation, but a 50 mg/100 mL aggregation of insulin was again observed (Figure 7). Compounds **2**–**4** were tested against insulin at the same molar concentration as that of **1** (i.e., **2**, 32 mg/100 mL; **3**, 30 mg/100 mL; **4**, 52 mg/100 mL). Both **2** (Figure 8) and **4** (Figure 9) increased the weight-average molecular weight of insulin, but to a lesser degree than **1**. The monosulfonic acid **3** had no effect. These results suggest that the binding of **1** to the insulin hexamer is as postulated in Figure 4, although the quantitative variation in the sedimentation equilibrium data from one experiment to another (see, for example, the control curves in Figures 7–9) precludes anything but a qualitative assessment of the binding of these ligands at this stage.

Conclusions

The present results illustrate the use of computer graphics for the rational design of small molecules intended to bind to the cavity at the center of the insulin hexamer. The use of computer graphics in this fashion is still a relatively novel technique in the field of drug design but will undoubtedly find increasing use by drug researchers as more protein structures become available.[5]

An obvious extension of these studies is to supplement the computer graphic analysis with energy calculations in order to identify more precisely the optimal analogues for

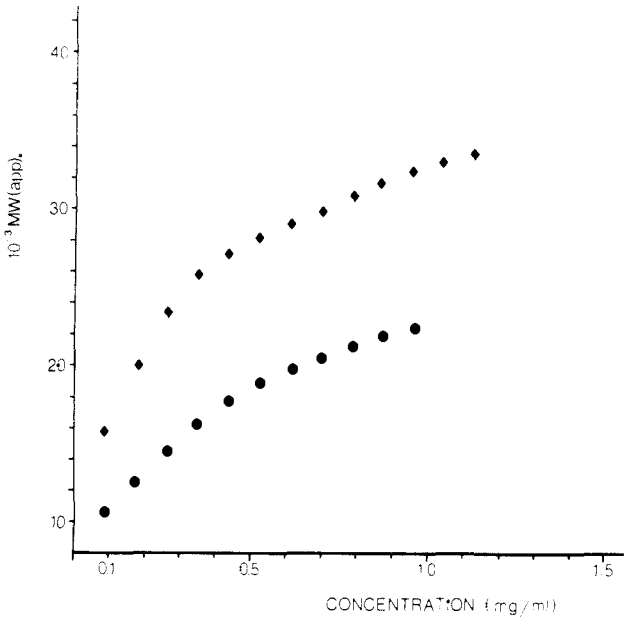

Figure 7. Apparent weight-average molecular weight vs. concentration for insulin at pH 8 showing the effect of compound **1**. Sedimentation equilibrium experiments were performed in Tris-NaCl buffer, ionic strength 0.2 at 20 °C: (●) 40 000 rpm; (♦) 34 000 rpm, 50 mg of 1/100 mL.

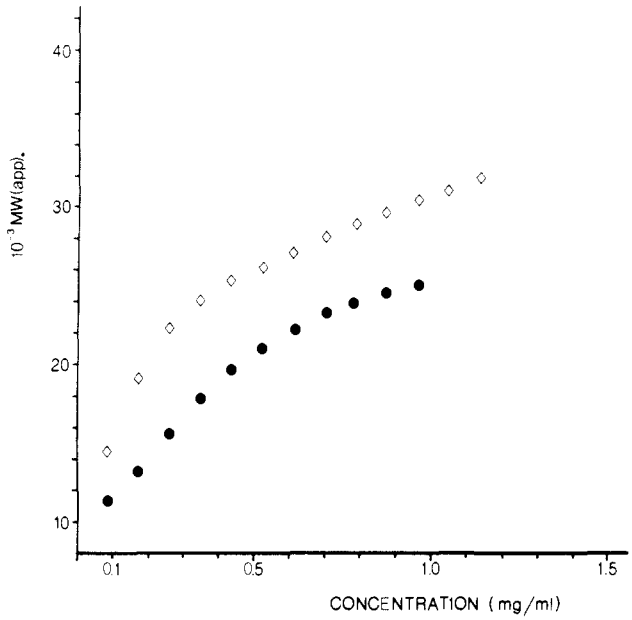

Figure 8. Apparent weight-average molecular weight vs. concentration for insulin at pH 8, showing the effect of compound **2**. Sedimentation equilibrium experiments were performed in Tris-NaCl buffer, ionic strength 0.2 at 20 °C: (●) 36 000 rpm; (◊) 36 000 rpm, 32 mg of 2/100 mL.

synthesis. We recognize, however, that even the most extensive theoretical studies of drug–receptor interactions yet available have not led to reliable predictions of the relative strengths of ligand–macromolecule interactions.[5] In the meantime, synthesis and testing of the proposed ligands remain the simplest method of evaluating the accuracy of our computer graphic-based predictions.

The results of the sedimentation equilibrium on insulin in the presence of **1**–**4** are encouraging but do not definitely establish the mode of interaction between the stabilizing molecules and the insulin protein: NMR studies or X-ray crystallography will be needed to solve this problem. Previous ^{1}H NMR studies performed on insulin have obtained resolvable signals from the histidine residues,[19] and monitoring this signal would provide a convenient probe for molecules binding to this site.

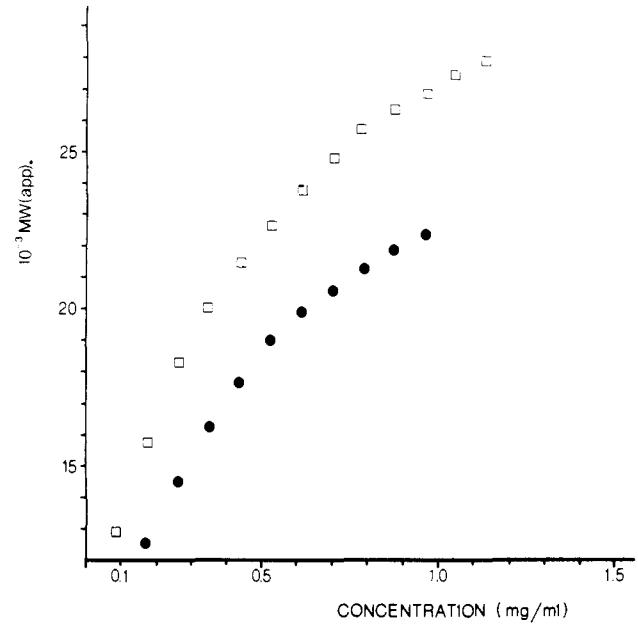

Figure 9. Apparent weight-average molecular weight vs. concentration for insulin at pH 8 showing the effect of compound **4**. Sedimentation equilibrium experiments were carried out in Tris-NaCl buffer, ionic strength 0.2 at 20 °C: (●) 34 000 rpm; (□) 34 000 rpm, 52 mg of 4/100 mL.

We are also carrying out additional design studies to investigate binding of ligands to the glutamate and serine residues and to explore possible binding sites in the interdimer clefts, which may be spacious enough to add supplemental groups onto existing or novel molecules to increase the stabilizing effect. Another avenue of investigation would be to examine the center of the tetrazinc insulin crystal structure.[20] This conformation may reveal additional residues available for binding. The possibility of stabilizing insulin in the tetrazinc conformation may also lead to a longer acting insulin.

The present experiments were performed on insulin at a concentration 40 000 times that in the bloodstream, in which the concentration of hexamer would be vanishingly small. It is therefore unlikely that compound **1** would have any effect at physiological levels, although it could probably be useful to stabilize insulin in its crystalline state. If future research on this project leads to molecules with a substantially higher binding affinity for the hexamer cavity, it may be possible to stabilize the hexameric form of the hormone in the bloodstream.

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Registry No. **1**, 31375-02-7; **2**, 100-21-0; **3**, 98-11-3; **4**, 581-75-9; Zn insulin, 8049-62-5.

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