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Research Article

Testing of Insulin Hexamer-Stabilizing Ligands Using Theoretical Binding, Microcalorimetry, and Nuclear Magnetic Resonance (NMR) Line Broadening Techniques

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In a study aimed at the development of a long-acting insulin preparation, Manallack and co-workers (5) reported on the design of small organic molecules which have the potential to bind to insulin and stabilize its hexameric aggregate. Two of the molecules that were designed with their computer graphics program were thought to be particularly promising as ligands: benzene-*p*-disulfonate and benzene-*p*-diphosphonate. In the present work, the insulin binding abilities of these molecules have been thoroughly tested. A theoretical binding program, GRID, was used to calculate the binding energetics of the molecules and to predict the most probable site of their binding. Microcalorimetry and NMR line broadening techniques were used to measure the actual binding reactions of the ligands. For both compounds, no evidence of binding to insulin was ever observed in either the microcalorimetry or the NMR studies. In contrast, a series of phenolic ligands commonly used as preservatives for insulin showed evidence of substantial binding using either method. An explanation has been proposed for this apparent discrepancy between computer predictions and actual experimental data: The theoretical programs do not take solvation effects of the aqueous medium into account. Solvation effects would tend to inhibit binding of the ionized ligand molecules due to charge delocalization and steric crowding.

KEY WORDS: calorimetry; benzene-*p*-disulfonate; benzene-*p*-diphosphonate; phenol.

INTRODUCTION

In the 1950s X-ray crystallographic methods were first used to determine the atom-by-atom structure of biological macromolecules (1). During the next 20 years, the structures of several enzymes and other proteins were elucidated. Attempts were made to design novel compounds which could fit into sites on these macromolecules (2), thus introducing the method of drug design by receptor fit (3).

Initial attempts to use this method relied extensively on modeling the shape of the protein at the ligand binding site, using physical models with metal or plastic parts. However, the fitting process is not just a matter of simple geometry because the chemical properties of the individual atoms are critically important, and the goodness of fit should be determined from both the geometry and the energetics of the protein–ligand interaction. It is not easy to use physical models to predict chemical interactions, especially when considering long-range electrostatic forces (4).

Early computer modeling attempts at drug design also relied extensively on binding geometry, with little regard to energetics. In a study aimed at the development of a long-acting insulin preparation, Manallack and co-workers (5) reported on the design of small organic molecules which have the potential to bind to insulin and stabilize its hexameric aggregate.[5] Using a computer graphics program, MORPHEUS, they docked potential ligand molecules into the central cavity of the hexamer and measured the distances between atoms of the ligand and atoms of insulin.

Results indicated that deprotonated acidic moieties were suitable for binding at either end of the cavity, where their negative charges could interact with the positively-charged zinc ions and B10 histidine side chains. The workers postulated that acidic groups bearing three oxygen atoms, such as sulfonate or phosphonate groups, would be better suited for binding than two-oxygen groups such as carboxylate, because each oxygen of a three-oxygen group (with its

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[5] The insulin monomer consists of two chains, A (21 amino acids) and B (30 amino acids), which are linked by two disulfide bonds. Insulin is stored in the pancreas as a hexamer which incorporates two zinc ions, each coordinated to three B10 histidine residues. As the monomer is the bioactive form, it was proposed (5) that stabilization of the hexamer via the binding of ligands at a natural cavity in the center of the hexamer may result in a longer-acting insulin preparation.

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partial negative charge) could interact with one of the three-fold-related histidine rings. Thus it was determined that the conjugate bases of benzene-*p*-disulfonic acid (disulf) and benzene-*p*-diphosphonic acid (diphos) were most promising as potential ligands for insulin. The structures of disulf and diphos are shown in Fig. 1.

The computer program GRID (6) has been developed to calculate the energetics of drug-protein interactions over an entire macromolecule, given a ligand probe at a particular location within the macromolecule structure. Both the energy and the shape of protein-ligand interactions can be considered simultaneously when designing drugs (6). In the present study, the GRID program was used to test the designed ligand molecules by locating energetically favorable binding sites for various ligand probe groups in the central cavity of the insulin hexamer. The program provided an indication of the site and strength of binding for several functional groups that may be incorporated into molecules designed to bind to the protein.

To supplement their theoretical modeling work, Manallack and co-workers (5) performed a sedimentation equilibrium study to test the binding ability of the ligand molecules. The results appeared promising, although some "poorer" ligands showed unexpectedly strong binding, and there were considerable problems with irreproducibility of the data (7).

In the present work, microcalorimetry and NMR line broadening were used to test the binding abilities of the two most promising ligands, disulf and diphos. Each of these techniques can be used to determine if a binding interaction is indeed occurring, and if so, thermodynamic and kinetic parameters of the interaction can be derived from the microcalorimetry and NMR data, respectively.

MATERIALS AND METHODS

The GRID Program

Program GRID is a computational procedure for determining energetically favorable binding sites on macromolecules of known structure. When the program is run, a probe functional group is moved around a specified grid within the target molecule. For each point of the grid, energy calculations are made for interactions between the probe, located at that point, and each heavy atom of the target molecule. The total energy for each point is calculated as the sum of three interaction energies:

$$E_{TOTAL} = E_{LJ} + E_{EL} + E_{HB} \quad (1)$$

where E_{LJ}, E_{EL}, and E_{HB} are the Lennard-Jones, electrostatic, and hydrogen bonding energies, respectively. Coordinates of those points on each z plane having the most favorable total energies are output by GRID in tabular form (6).

In the present study, x,y,z coordinate positions of the heavy atoms in the insulin hexamer were input in the format specified for the Protein Data Bank (PDB) at Brookhaven (8). A preliminary program, GRIN, first checked for possible errors in the PDB data, then appended a set of energy parameters to each heavy atom in the protein structure. The protein atoms, with their attached energy parameters, were used as one of the inputs to GRID. Other inputs were the energy parameters of the probe group, and coordinates of the specified grid over which calculations would be performed.

GRID energy calculations were performed on various parts of the insulin hexamer, including the entire protein structure. However, almost all of the most favorable interactions for a variety of probe groups occurred in the central cavity of the hexamer. This region of the protein has been previously described in detail by X-ray crystallographic studies (9). Interactions of a variety of probe nuclei in the central cavity were studied on the Victorian College of Pharmacy and the University of Kansas Department of Medicinal Chemistry VAX 11/750 computers.

Benzene-*p*-disulfonic acid (Disulf)

Benzene-*p*-diphosphonic acid (Diphos)

Fig. 1. Potential ligand molecules designed by Manallack (7).

Microcalorimetry Studies

Crystalline porcine zinc insulin was kindly supplied by Eli Lilly and Company and was used without further purification. Disulf was prepared according to the method of Meerwein and co-workers (10), and diphos was prepared by the method of Chantrell *et al.* (11). The two compounds were verified by proton and carbon-13 NMR. All solutions were prepared using double-distilled water.

Separate aqueous solutions of insulin and ligand were prepared for this work. The insulin solutions were prepared so that after mixing with ligand the final insulin concentration would be held constant at approximately 3.6 mg/ml (0.1 m*M* hexamer). The concentrations of the ligand solutions were varied. All solutions were adjusted to pH 7.4. Since no buffer was used in the solutions, the final pH after mixing was checked periodicially; it was not found to deviate beyond ±0.1 pH unit.

The calorimetry studies were performed on an LKB Flow Microcalorimeter (LKB Produkter AB, Bromma, Sweden), connected to a Keithley 150B Microvolt Ammeter (Keithley Instruments Inc., Cleveland, Ohio) and a chart recorder. In the following paper (12), we have used flow microcalorimetry to study binding of phenolic compounds to insulin. The general calorimeter setup and calorimetry data workup described in that paper were the same as those employed in the disulf and diphos studies.

NMR Line Broadening Studies

Crystalline bovine zinc insulin was purchased from Sigma and used without further purification. All ligand molecules used were reagent grade or prepared synthetically. Phenol and resorcinol were purchased from Fisher. Metacresol was purchased from Sigma.

All solutions were prepared with 99% D_2O, purchased from Sigma. pD was adjusted using very small amounts of

concentrated NaOD and DCl solutions, also purchased from Sigma. pD values were obtained directly from a pH electrode. It was assumed that the difference between a measured value and the true pD was negligible.

Insulin solutions at a concentration of 0.5 m*M* hexamer were prepared by placing 36 mg of insulin into 2 ml of D_2O and adjusting the pD to 10.5. Once a clear solution had been obtained, the pD was reset to the desired value. Aliquots (0.2 ml) of these solutions (0.1 μmol each) were successively added to samples of the ligand solutions.

Ligand solutions were prepared at a concentration of 15 m*M* in D_2O. The pD was adjusted to the same value as the insulin solution with which the ligand would be mixed. A 0.4-ml sample (6 μmol) of each ligand solution was mixed with aliquots of insulin for the NMR determinations.

^1H NMR spectra were obtained at 300 MHz on a Varian VXR-300 spectrometer (Varian NMR Instrument Division, Palo Alto, California). All measurements were made using a 5-mm NMR sample tube. ^{31}P NMR spectra were obtained at 121.4 MHz on the Varian VXR-300 spectrometer and at 161.9 MHz on a Varian VXR-400 spectrometer. A 5-mm sample tube was used for experiments on the VXR-300, and a 10-mm tube was used for the VXR-400. Three milliliters of D_2O was added to the latter, along with the insulin and ligand solutions, to generate an adequate sample volume for measurement. In all of the experiments, D_2O was used as the NMR lock solvent.

RESULTS

Theoretical Binding Studies

GRID binding energies for several nonmetal probe groups at various z-planes on the two-zinc insulin hexamer are shown in Table 1. The last line in the table shows results for zinc ions binding in the central cavity of the zinc-free insulin hexamer.

Each binding energy in the table represents the most favorable interaction noted for a given probe group on a particular z-plane. The results indicate that probe groups possessing a full or partial negative charge (carboxyl oxygen, carbonyl oxygen, etc.) show favorable interaction energies when located near the B10 histidine residues and positively charged zinc ions ($z = \pm 8$). On the other hand, probe groups possessing positive charge (protonated amine, carbonyl carbon, etc.) show the most favorable interactions near the negatively charged B13 glutamate residues at the center of the cavity ($z = 0$, $z = \pm 3$). Probe groups without formal charge (methyl, water) have relatively constant binding energies throughout the central cavity.

For the zinc cation probe, the most favorable binding energies were found in the center of the cavity, near the B13 glutamate residues, from $z = -3$ to $z = +3$. In their description of the five-zinc insulin hexamer, Emdin and associates (13) noted that three zinc binding sites occurred in the glutamate region of the central cavity, with each zinc ion coordinated to two of the glutamate side chains. For the B10 histidine region of the cavity ($z = \pm 8$), the GRID results indicated strong zinc binding, although not as strong as the glutamate sites. The program accurately predicted that the best location for zinc binding on these planes was on the threefold axis itself, i.e., at points (0,0,8) and (0,0,−8), which is consistent with the observed zinc locations in the two-zinc hexamer (9).

Table I. GRID Binding Energies for Various Probe Groups Within the Central Cavity of the Insulin Hexamer

Probe group	Highest total binding energy (kcal)				
	$z = 0$	$z = \pm 3$	$z = \pm 6$	$z = \pm 8$	$z = \pm 10$
Carboxyl oxygen	1.2	5.7	2.8	16.6	7.3
Carbonyl oxygen	0.8	5.1	1.6	9.6	4.6
Hydroxyl	4.5	7.3	3.6	8.1	6.6
Water	6.6	8.3	4.6	9.4	7.6
Methyl	3.3	4.1	3.7	3.5	3.1
Carbonyl carbon	6.5	6.8	4.2	3.8	3.4
Amino	14.1	17.0	8.1	7.8	7.9
Zinc (II)	76.1	62.1	36.2	33.0	27.3

Microcalorimetry Studies

Measured heats of binding disulf and diphos onto two-zinc insulin are shown in the last two lines in Table II. Also included in the table are heats of binding of several phenolic ligand molecules (12), for comparison with the disulf and diphos results.

As Table II indicates, much smaller heats of reaction were measured for disulf and diphos than for the phenolic ligands, at comparable ligand concentrations. There are two possible explanations for this behavior. First, the enthalpy of binding may be approximately zero (within experimental error) for disulf and diphos. Second, the disulf and diphos molecules may not bind to insulin to any appreciable extent, in spite of the predictions of the computer graphics and GRID studies. To distinguish between these possibilities, NMR line broadening studies were performed.

NMR Line Broadening Studies

NMR line broadening will occur when the molecule of interest undergoes chemical exchange with another molecule in the solution. If the exchange is moderately slow relative to the chemical shift difference between peaks corresponding to free and bound ligand, the following equation for linewidth (LW) applies (14,15):

$$\mathrm{LW} = \frac{1}{\pi T_{2,\mathrm{obs}}} = \frac{f_F}{\pi T_{2,F}} + \frac{f_B}{\pi \tau_B} \qquad (2)$$

Table II. Heats of Reaction with Zinc Insulin (0.1 m*M*)

Ligand	Concentration (mmol/liter)	Heat (kcal/mol)
m-Cresol	5	−0.87
Methylparaben	3	−1.42
Phenol	5	−1.21
Resorcinol	5	−5.14
Diphos	2	−0.17
Disulf	5	0.12

where $T_{2,obs}$ is the observed spin–spin relaxation time; $T_{2,F}$ is the spin–spin relaxation time of the free nucleus; f_F and f_B are the mole fractions of free and bound ligand, respectively; and τ_B is the lifetime of a bound ligand nucleus. Under the slow exchange condition, separate peaks are observed for bound and free ligands (16,17).

If exchange is fast relative to the chemical shift difference, a different equation applies for linewidth (14,15):

$$LW = \frac{1}{\pi T_{2,obs}} = \frac{f_F}{\pi T_{2,F}} + \frac{f_B}{\pi T_{2,B}} \quad (3)$$

where $T_{2,B}$ is the spin–spin relaxation time of the bound peak. Under these conditions, only one peak is observed for both the free and bound nuclei. Its chemical shift ($\Delta\omega$) is a population-weighted average of the chemical shifts of the free ($\Delta\omega_F$) and bound ($\Delta\omega_B$) species (14,15):

$$\Delta\omega = f_F\Delta\omega_F + f_B\Delta\omega_B \quad (4)$$

For both Eq. (2) and Eq. (3), linewidth is a linear function of the fraction of ligand bound if the ligand:protein ratio is kept high. Related equations apply in intermediate exchange conditions and these also show that linewidth should vary with the fraction of ligand bound, regardless of the rate of exchange.

*Benzene-*p*-disulfonate and Benzene-*p*-diphosphonate.* Due to the planes of symmetry present in these molecules, the NMR spectra of disulf and diphos are quite simple. The ^1H spectrum of disulf is a singlet in the aromatic region. Likewise, the ^{31}P spectrum of diphos (with broadband proton decoupling) is also a singlet. The four-ring protons of diphos are all chemically equivalent, as they are in disulf, but in diphos the ring protons can couple with the two phosphorus nuclei. Consequently, the ^1H spectrum of diphos is a complex multiplet in the aromatic region.

The linewidths of peaks in the disulf and diphos spectra were measured as the insulin concentration in the solutions was raised. Results for diphos and disulf are shown in Figs. 2 and 3, respectively (pD = 10.2). In all cases no measurable line broadening was observed over the entire insulin concentration range studied. Hence, the data showed that no significant interactions were occurring between these ligands and insulin.

Insulin–diphos mixtures were also studied over a pD range from 7.5 to 13.0 (Fig. 4). No significant difference was observed in the ligand linewidth for the presence versus the absence of insulin over the entire pD range. Thus, the data again indicated that no reaction was occurring between the ligands and insulin, in spite of the computer predictions. An increase in the linewidth did occur in both the presence and the absence of insulin as pD was lowered to around neutrality. This broadening will be discussed further in a later publication.

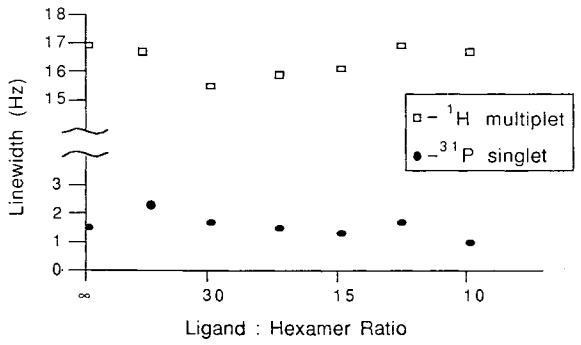


Fig. 2. Peaks widths of diphos as a function of insulin concentration.

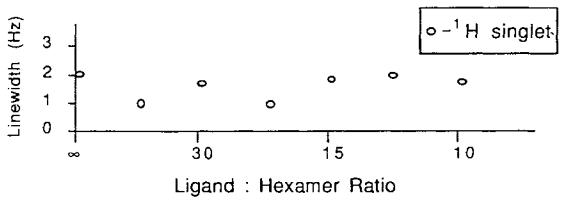


Fig. 3. Peak width of disulf as a function of insulin concentration.

Phenolic Ligands. For comparison with the disulf and diphos results, line broadening studies were conducted on phenol, meta-cresol, and resorcinol, which have been shown to bind to insulin (12,18). ^1H NMR assignments for these molecules are summarized in Table III.

In line broadening studies (at pD 7.5), phenolic molecules showed strong evidence of binding to insulin. Results for selected peaks of meta-cresol are shown in Fig. 5. In this graph, the ligand concentration was always well in excess of the insulin; it was therefore assumed that the fraction of ligand bound was equal to the hexamer:ligand ratio. Insulin was added to the solution until a ligand-to-hexamer ratio of 15:1 was achieved; beyond that, interference from the growing insulin peaks obscured the broadened ligand peaks. In addition to broadening, changes in chemical shift occurred for almost all of the ligand peaks, further evidence that binding was occurring. Some of the peaks migrated into adjacent peaks and could not be followed over the entire insulin concentration range.

The binding of phenolic molecules to insulin is relatively weak in nature (12); hence, it can be assumed that the fast-exchange situation applies (15). Using Eq. (3), an approximate value of the bound spin–spin relaxation time for each nucleus ($T_{2,B}$) can be calculated from the slope of a straight line drawn through the data points of a graph such as Fig. 5. Values of $T_{2,B}$ obtained in this manner are shown in Table IV. The nuclei all have very short bound spin–spin relax-

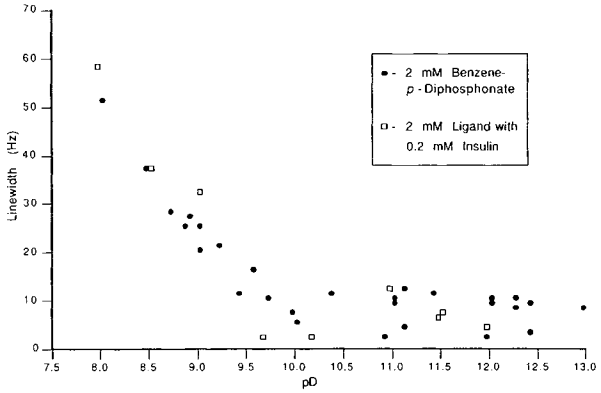
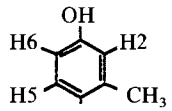
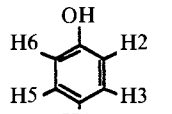
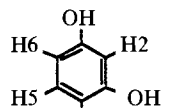


Fig. 4. Linewidth in the presence and absence of insulin as a function of pD.

Table III. Proton NMR Assignments for Phenolic Ligands

Ligand	Proton	δ (ppm)	Multiplicity[a]
 Meta-Cresol	H2	6.58	Singlet
	H4	6.52	Doublet
	H5	7.01	Triplet
	H6	6.63	Doublet
	CH_3	2.10	Singlet
 Phenol	H2, H6	6.78	Doublet
	H3, H5	7.19	Triplet
	H4	6.84	Triplet
 Resorcinol	H2	6.32	Singlet
	H4, H6	6.39	Doublet
	H5	7.07	Triplet

[a] The observed multiplicity arises from ortho coupling; meta couplings were unresolved.

ation times, which is not surprising given the long correlation time of such a large ligand–macromolecule complex. The values are similar for all three molecules, indicating that the ligands all utilize the same mechanism of binding to insulin. Since the molecules do not possess formal charge, except for a slight charge on the phenolic moiety, there would be little driving force for binding in the central cavity of the hexamer (see GRID results, earlier). Instead, it is likely that these molecules bind to some site on the exterior surface of the hexamer.

DISCUSSION

Theoretical Binding Studies

Before attempting to interpret the results of the GRID program, it is important to consider the potential shortcomings of the method. Several of these, such as the neglect of

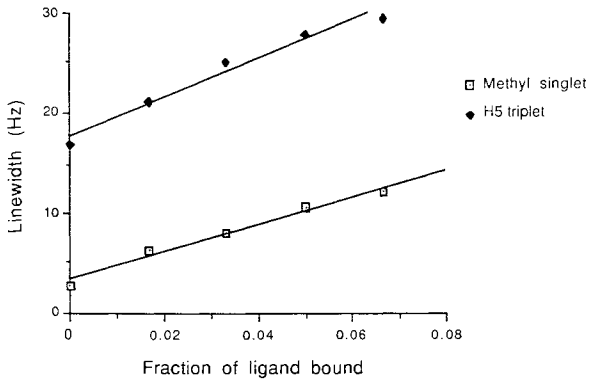


Fig. 5. Broadening of meta-cresol proton nuclei upon binding to insulin.

Table IV. Spin–Spin Relaxation Times for Various Proton Nuclei of Bound Phenolic Ligands

Compound	Nucleus	$T_{2,B}$ (msec)
Meta-Cresol	Methyl singlet	2.3
	H5 triplet	1.6
Phenol	H3, H5 triplet	1.9
	H2, H6 doublet	1.9
Resorcinol	H5 triplet	1.8
	H4, H6 doublet	2.6

entropy contributions, the assumption of pairwise additivity between atoms, the concept of "extended" atoms, and the assumed rigidity of the macromolecule structure, have been previously discussed by Goodford (6). One critical shortcoming in the present case of insulin–ligand interactions in an aqueous medium is that the GRID program does not adequately consider the effects of solvent molecules. Thus, the electrostatic and hydrogen-bonding energies calculated by GRID are probably overestimates of the actual values.

Ligand molecules designed in response to the GRID results would be expected to possess functional groups which take advantage of the "hot spots" shown in Table I. Manallack (7) studied molecules possessing negative charges at neutral pH (disulf, diphos, terephthalic acid, etc.) or positive charges [hexaminecobalt(III) chloride and tris(ethylenediamine)cobalt (III) chloride]. However, none of the compounds possessed both positive and negative charges. Further, most of the negatively charged compounds were charged at either end of the molecule, but the GRID results indicate that these negative charges were not separated sufficiently to allow maximum interaction with both zinc ions. These considerations should be borne in mind when future ligands are designed to bind to the central cavity.

For the zinc ion probes, considerably higher binding energies were observed for the glutamate sites ($z = \pm 3$) than the histidine sites ($z = \pm 8$). This result is opposite to what would be expected, since the histidines are known to be the strongest zinc-binding sites (19). As mentioned earlier, the presence of water molecules will probably reduce the actual affinities of the glutamate sites. A more important factor, however, is that when zinc ions actually bind to the histidines, the hexamer itself is also formed. The formation of the hexamer produces many dimer–dimer interactions, involving hydrophobic, hydrogen-bonding, and van der Waals forces. These interactions were not considered by the GRID program, since the insulin structure was input as the already-formed hexamer. Thus, binding of zinc to the histidine sites is probably much more energetically favorable than the GRID program predicted.

NMR Line Broadening Studies

The observed differences between the phenolic ligands and the designed ligands clearly indicate that the former are capable of binding to insulin, while the latter are not. Conditions which might have caused the phenolic peaks to broaden in the absence of binding include interference from the insulin peaks and changes in the viscosity of the solution. However, since the same insulin concentrations, pD, etc.,

were used for the disulf and diphos studies, and those peaks did not broaden, it can be concluded that the broadening of the phenolic molecules' peaks is definite evidence of their interactions with insulin.

The results of this and the previous studies done on the designed ligands appear to deliver somewhat contradictory assessments. The computer graphics and GRID modeling predicted that such molecules would be capable of binding in the central cavity of the hexamer. Sedimentation equilibria results, on the other hand, were inconclusive (7). The microcalorimetry and NMR line broadening studies employed in the present work failed to show any evidence of binding at all.

A possible explanation has been proposed for this discrepancy between computer predictions and actual experimental results. The acidic functional groups of these ligand molecules will be negatively charged at neutral or basic pH and, therefore, will possess a large solvation shell in the aqueous medium. Likewise, the zinc ions and glutamate residues in the central cavity will also be heavily solvated. Such solvation shells would both delocalize the charges in question and sterically hinder and ligand from entering the central cavity. The GRID and computer graphics programs do not account for solvation effects; they merely consider each functional group as the "naked" group, without regard to the solvent.

It seems likely, then, that disulf, diphos, and the other designed ligands show little binding to the insulin hexamer because of solvation effects. The phenolic molecules, which do not possess large solvation shells, are much more capable of binding.

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