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Localization of Tolbutamide Binding Sites on Human Serum Albumin Using Titration Calorimetry and Heteronuclear 2-D NMR[†]

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ABSTRACT: The sulfonylureas are a class of oral hypoglycemic agents used to treat type II diabetes mellitus, and tolbutamide is a "first generation" member of this family. It is a nonpolar, weakly acidic drug that binds to serum albumin in the circulation. In the present study, we have examined the interactions of tolbutamide with human serum albumin by isothermal titration calorimetry and heteronuclear multiple-quantum coherence NMR spectroscopy. Calorimetric titrations revealed that tolbutamide binds to albumin at three independent sites with the same or comparable affinity. This result was independently confirmed by NMR experiments which resolved three resonances at ^{1}H chemical shifts of 2.07, 2.11, and 2.14 ppm, corresponding to [*methyl*-^{13}C]tolbutamide bound to three discrete binding sites. The binding affinity quantitated by calorimetry ($K_d = 21 \pm 9\ \mu$M at pH 7.4, 37 °C) was approximately 5 times lower than the most frequently reported value. Tolbutamide titrations of albumin complexed with three other drugs whose binding sites have been localized by X-ray crystallography (salicylate, clofibric acid, and triiodobenzoic acid) demonstrated direct competition for common binding sites. NMR experiments with samples containing [*methyl*-^{13}C]tolbutamide and these competing drugs permitted assignment of the resonances at 2.07 and 2.14 ppm to tolbutamide bound to the aspirin sites in albumin subdomains IIIA and IIA, respectively. These findings permit the first assignment of tolbutamide binding sites to specific locations on the albumin molecule within the context of the recently published crystal structure of human serum albumin. In addition, this information provides a molecular basis for predicting unfavorable drug interactions involving tolbutamide in patients with type II diabetes.

Human serum albumin accounts for approximately 60% of the protein in extracellular fluids and serves as the primary serum transport protein for a number of lipophilic metabolites and pharmaceutical agents (Spector, 1986; Carter & Ho, 1994). The affinity of albumin for a particular drug determines the rate at which unbound or free drug becomes available to diffuse from the circulation to sites of action and metabolism (Hervé *et al.*, 1994). It is the unbound form of the drug that interacts with active sites and exerts the agent's pharmacologic effect (Sellers & Koch-Weser, 1977).

Most drugs bind to albumin at a small number of discrete binding sites. Studies with acidic drugs such as warfarin (Sellers & Koch-Weser, 1971), phenytoin (Wesseling & Mols-Thurkow, 1975; Monks *et al.*, 1978), and sulfonamides (Anton, 1973) have demonstrated that acidic drugs may competitively displace one another from binding sites on albumin, increasing the unbound and pharmacologically active fraction of the displaced drug. Two distinct binding sites, denoted sites I and II, were initially characterized on the basis of competitive displacement of dansylamino acid probes (Sudlow *et al.*, 1975, 1976). More recently, X-ray crystallographic studies of albumin have revealed that some drugs bind in the hydrophobic pockets of subdomains IIA and IIIA (He & Carter, 1992).

Sulfonylureas are a class of oral hypoglycemic agents that are used to treat type II diabetes mellitus refractory to control by diet modification and exercise alone. Approximately 35% of diabetics in the United States are treated with one of these agents (Kennedy *et al.*, 1988). The sulfonylureas are weak acids that are extensively bound to serum albumin in the circulation (Marchetti *et al.*, 1991). The first generation sulfonylureas—tolbutamide, chlorpropamide, acetohexamide, and tolazamide—are thought to be more susceptible to displacement from albumin than newer, second generation sulfonylureas such as glyburide and glipizide (Brown & Crooks, 1976; Lebovitz, 1990). However, the binding sites for these agents have not been localized on the albumin molecule, and their interactions with albumin are poorly understood.

In the present investigation, we employed isothermal titration calorimetry and heteronuclear 2-D NMR to characterize the interactions between the first-generation agent tolbutamide and human serum albumin. Tolbutamide (Figure 1) was chosen for initial study for two reasons. First, a number of pharmaceutical agents have been demonstrated to displace tolbutamide from albumin (Christensen *et al.*, 1963; Judis, 1972; Brown & Crooks, 1976; Zini *et al.*, 1976; Fujii *et al.*, 1983). Such displacement has been observed to

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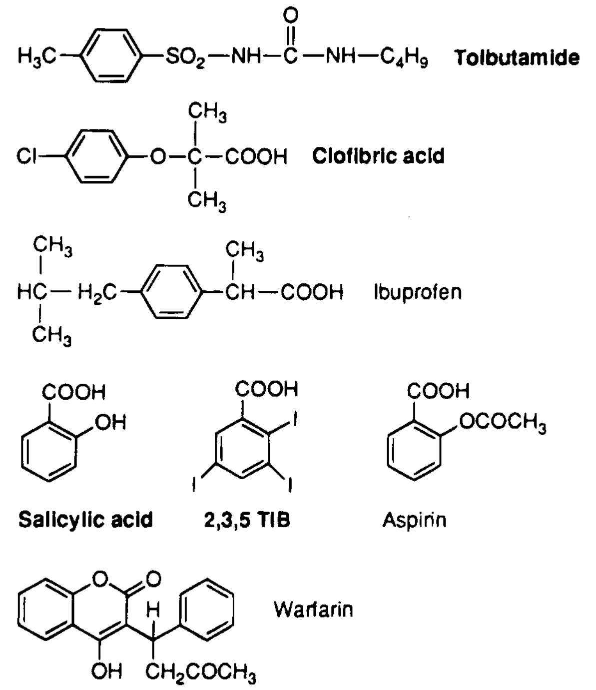

FIGURE 1: Chemical structures of pharmaceutical agents employed in this study (boldface type) and related compounds. TIB, triiodobenzoic acid.

cause clinically significant and life-threatening hypoglycemia in diabetics (Christensen et al., 1963; Seltzer, 1989). A better knowledge of the molecular interactions and binding site locations for tolbutamide might help to predict and circumvent such adverse drug reactions. Second, tolbutamide contains an aromatic methyl group that, when selectively enriched with ^{13}C, could serve as a useful probe for isotope-directed 2-D NMR studies of the drug−protein complex. Because of the difficulty in obtaining 2-D NMR spectra for such a high molecular mass complex (67 kDa), the use of a NMR probe that contains significant internal mobility, favorable relaxation properties and a high degree of sensitivity is a prerequisite for such studies. For this purpose, therefore, we synthesized [99%, *methyl*-^{13}C]tolbutamide.

The calorimetric and NMR results were used in a concerted fashion to characterize the binding properties of tolbutamide and to localize two of its binding sites on human serum albumin.

MATERIALS AND METHODS

Materials. Essentially fatty acid-free and globulin-free human serum albumin, tolbutamide, 2,3,5-triiodobenzoic acid (TIB),[1] sodium salicylate, and clofibric acid were all purchased from Sigma Chemical Co. Prior to use, the albumin was dissolved in phosphate-buffered saline (PBS; 135 mM sodium chloride, 20 mM potassium phosphate, 0.1 mM ascorbic acid, 0.1 mM NaN_3) or TES buffer (Cistola & Small, 1991) and then repeatedly dialyzed in an Amicon-50 ultrafiltration cell with a YM-5 membrane to remove citrate salts present in the crystallized protein (final citrate $\leq$0.13% w/w total buffer salts). A portion of the albumin was delipidated a second time according to the defatting protocol of Chen (1967). The [99%, *methyl*-^{13}C]toluene used in the synthesis of [*methyl*-^{13}C]tolbutamide was purchased from Cambridge Isotope Laboratories.

Synthesis of [methyl-^{13}C]tolbutamide. Intially, [*methyl*-^{13}C]*p*-toluenesulfonate was prepared by reacting 1 g of [99%, *methyl*-^{13}C]toluene with concentrated sulfuric acid as described by Fieser (1957). [^{13}C-*methyl*]*p*-toluenesulfonate (2 g) was next converted to a sulfonyl chloride by the procedure of Ray and Sofer (1950). The sulfonyl chloride (0.715 g) was then reacted with concentrated NH_4OH, creating [*methyl*-^{13}C]*p*-toluenesulfonamide (Furniss *et al.*, 1978). Finally, [99%, *methyl*-^{13}C]tolbutamide was synthesized by reacting 0.244 g of [*methyl*-^{13}C]*p*-toluenesulfonamide and 0.16 g of *n*-butylisocyanate according to example five in the patent of Ruschig *et al.* (1961). The purity and carbon-13 content of the intermediate products and [*methyl*-^{13}C]tolbutamide was confirmed to be $\geq$95% by proton and carbon-13 NMR spectroscopy. The final yield of [*methyl*-^{13}C]tolbutamide was 0.235 g.

Preparation of NMR Samples. Albumin solutions of 1.2−1.5 mM (80−100 mg/mL) were prepared in PBS. The concentration of albumin was determined by measuring the absorbance at 280 nm and using an extinction coefficient of 5.5 for a 1% albumin solution (Shaklai & Bunn, 1984). A molecular mass of 66 250 Da (Spector, 1986) was used in all calculations.

A solution of [*methyl*-^{13}C]tolbutamide dissolved in chloroform was prepared and its concentration determined by measuring dry weights using a Perkin-Elmer AD-4 microbalance. An appropriate volume of this solution, necessary to achieve a final desired mole ratio of drug to albumin, was transferred to a glass vial, the organic solvent evaporated under dry nitrogen, and the [*methyl*-^{13}C]tolbutamide dissolved in 100 μL of H_2O and 1.2 equiv of 0.1 N KOH. To this vial, a 2 mL aliquot of the concentrated albumin solution was then transferred to form the drug−protein complex. After a 30 min incubation, the pH of the solution was measured at ambient temperature (22−23 °C) and adjusted to pH 7.4 (at 37 °C) using the relationship $\Delta pH/\Delta T = -0.0146$ (Lentner, 1984). The sample was then frozen in an ethanol/dry ice bath and lyophilized overnight. The lyophilized powder was redissolved in 2 mL of 99.996 atom % D_2O (Cambridge Isotope Labs), and a 0.5 mL aliquot was transferred to a 5 mm Wilmad 540-PPT NMR tube.

NMR samples containing the competing drugs TIB and clofibric acid were prepared as described above, with appropriate quantities of competing agent added to the albumin solutions before addition of [*methyl*-^{13}C]tolbutamide, to give a desired mole ratio of competing drug to albumin.

Titration Calorimetry. Titration calorimetry was performed using a Microcal OMEGA differential titration calorimeter (Wiseman *et al.*, 1989). Aqueous solutions of human albumin were prepared using TES or PBS buffer. Separately, solutions of tolbutamide, TIB, and clofibric acid were prepared at 3.5 mM concentrations in TES or PBS buffer. Thirty-five to forty-five 6 mL aliquots of ligand were injected into a 1.4 mL albumin sample (0.10−0.13 mM) at 3 min intervals until final ligand/albumin mole ratios of 5:1, 4:1, and 3:1 were achieved for tolbutamide, TIB, and clofibric acid, respectively. All titrations were performed at 37 ± 0.1 °C and pH 7.4. Competition studies were performed by first incubating albumin solutions with known

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[1] Abbreviations: HMQC, heteronuclear multiple-quantum correlation NMR spectroscopy; NOESY, nuclear Overhauser and exchange spectroscopy; PBS, phosphate-buffered saline; TES, *n*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; TIB, triiodobenzoic acid (an aspirin analogue).

Table 1: Titration Calorimetry Results for Binding of Tolbutamide, TIB, and Clofibric Acid to Human Serum Albumin at 37 °C[a]

parameter	tolbutamide	TIB	clofibric acid
n	3.0 ± 0.1	2.13 ± 0.03	0.98 ± 0.03
K_d (μM)	21 ± 9	21 ± 3	17 ± 1
$\Delta G°$ (kJ/mol)	−27 ± 1	−26.7 ± 0.3	−27.2 ± 0.2
$\Delta H°$ (kJ/mol)	−6.0 ± 0.6	−35.3 ± 0.7	−27.8 ± 0.5
$T\Delta S°$ (kJ/mol)	21 ± 2	−8.5 ± 0.8	−0.6 ± 0.6

[a] Results for HSA and ligands in TES buffer. Each result represents the mean ± standard deviation of three experiments except for tolbutamide, which represents four.

mole ratios of TIB, salicylate, or clofibric acid before titration with tolbutamide.

Raw calorimetric data were processed using the software package ORIGIN (Wiseman *et al.*, 1989). After manually setting the peak-by-peak baselines of the raw data, the signal in calories/s for each injection was integrated and plotted as heat evolved per mole of ligand injected (ΔQ/ΔX) versus the mole ratio of total ligand to protein in the sample cell (see Figure 1). The heat of dilution for the titration of ligand into buffer without protein was then subtracted from the y-axis value of each point of the curve. This corrected plot was fit by an iterative Marquardt algorithm to a binding model employing a single set of independent sites. The association (K_a) and dissociation (K_d) constants, molar binding stoichiometry (n), and molar binding enthalpy ($\Delta H°$) were determined directly from the fitted curve. The Gibbs free energy and molar entropy of binding were calculated using the equations $\Delta G° = -RT \ln K_a$ and $\Delta S° = (\Delta H° - \Delta G°)/T$, respectively, where R is the gas constant and T is absolute temperature in Kelvin.

NMR Spectroscopy. Two-dimensional $^1H-^{13}C$ HMQC (Müller, 1979; Bax *et al.*, 1990) and NOESY-HMQC [Figure 2B of Fesik et al. (1987)] experiments were recorded at 37 °C on Varian Unity 500 and 600 spectrometers interfaced to Sun SPARC workstations and equipped with Nalorac and Varian 5 mm indirect detection probes, respectively. On the Unity 500, the 90° pulse widths were 9.1 μs for ^1H (at 30 W) and 11.5 μs for ^{13}C (at 200 W); on the Unity 600, these widths were 8.8 and 15.0 μs, respectively. The fixed delays during the pulse sequence were set to 3.3 ms, a compromise value ~20% less than $1/2J_{CH}$, in order to minimize transverse relaxation during the pulse sequence. Broad-band carbon-13 decoupling during aquisition was accomplished using WALTZ-16 (Shaka *et al.*, 1983) centered at 26 ppm for experiments performed on the Varian 500 and 40.06 ppm for experiments on the Varian 600. Proton and carbon chemical shifts were referenced to external sodium 3-(trimethylsilyl)propionate-*2,2,3,3*-d_4 in D_2O at 37 °C (0.0 ppm).

RESULTS

Calorimetric Studies To Quantitate Drug–Albumin Binding. The interactions of tolbutamide, TIB, and clofibric acid with albumin were analyzed by isothermal titration calorimetry, and the binding parameters are summarized in Table 1. Tolbutamide was found to bind to three sites with equal or comparable affinity. An example of a calorimetric binding isotherm for tolbutamide and albumin is shown in Figure 2. The molar binding enthalpy contributed 22% of the total molar Gibbs free energy of binding. The binding of tolbutamide to albumin was also examined in the buffer used for NMR experiments, PBS. The parameters in PBS (n = 2.9 ± 0.2, K_d = 18 ± 2, $\Delta H°/\Delta G°$ = 0.20) were essentially identical to those obtained in TES buffer (Table 1). Also, no differences were noted in drug binding to singly- vs doubly-defatted albumin.

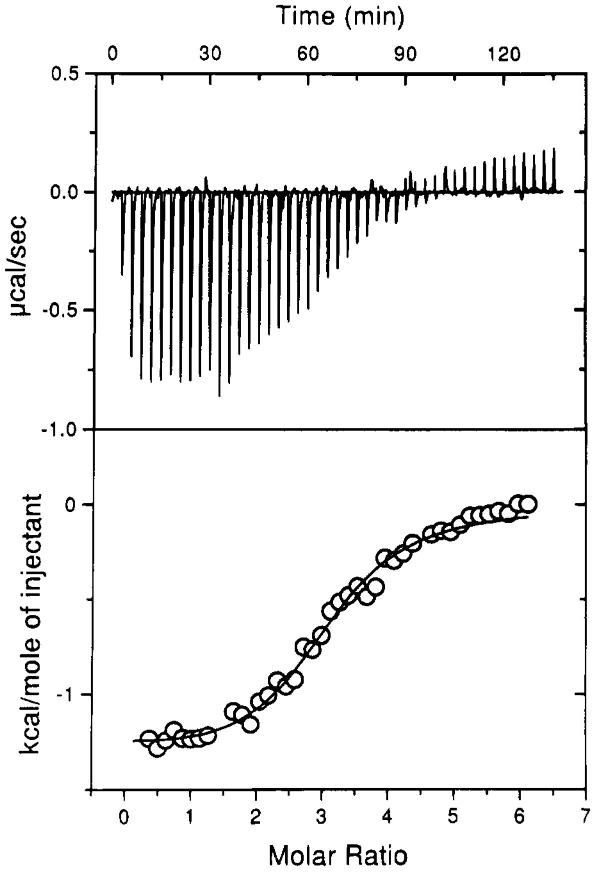

FIGURE 2: Calorimetric titration of human serum albumin with tolbutamide at 37 °C. The top panel shows the raw heat signal for 45 injections of 6 μL aliquots of 3.5 mM tolbutamide into a reaction cell containing 1.4 mL of 0.13 mM HSA, all at 37 °C. The bottom panel shows the integrated heat of each injection after correction for heat of dilution of ligand and normalized to the amount of tolbutamide injected (open circles). The curve through the points represents the best fit to a single set of independent binding sites model. The parameters defining this fitted curve are n = 3.0, K_d = 20 μM, and $\Delta H°$ = −5.5 kJ/mol.

The aspirin analogue, TIB, and the lipid-lowering agent, clofibric acid, were observed to bind to albumin with molar binding stoichiometries of 2 and 1, respectively (Table 1). These agents were chosen for study because, unlike tolbutamide, their binding site locations are known from X-ray crystallographic studies (He & Carter, 1992). As shown in Table 1, the molar binding enthalpies accounted for most or all of the Gibbs free energies of binding for both drugs, and binding was associated with a net unfavorable binding entropy at 37 °C.

Two-Dimensional NMR Experiments To Monitor Tolbutamide Binding. Samples containing varying mole ratios of [*methyl*-^{13}C]tolbutamide and albumin were prepared, and the binding interactions were monitored by two-dimensional HMQC NMR spectroscopy. In this isotope-directed experiment, the ^1H resonances for the ^{13}C-enriched ring-methyl group of tolbutamide were selectively enhanced, and the resonances for protons attached to ^{12}C in the ligand and protein were suppressed. Because the TES buffer has several methylene protons that gave rise to sizeable natural abun-

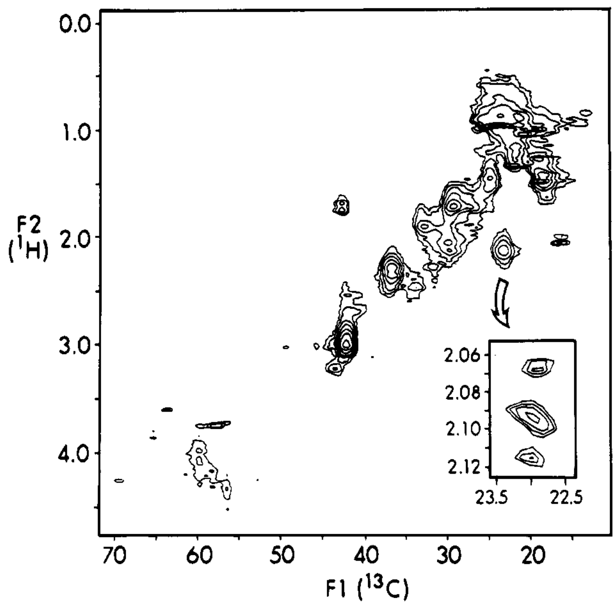

FIGURE 3: Aliphatic region of a two-dimensional HMQC spectrum of [*methyl*-^{13}C]tolbutamide bound to human serum albumin at 37 °C. The sample used to collect this spectrum contained 3 mol of [*methyl*-^{13}C]tolbutamide/mole of albumin at a protein concentration of 1.2 mM. This spectrum was collected on a Varian Unity 600 spectrometer (599.945 MHz for ^{1}H). Both the entire spectrum and the inset were processed with pseudo-echo apodization, but a higher degree of resolution enhancement was used for the inset. Line-broadening factors of −4 and −9 Hz were applied to F_2 for the parent spectrum and inset, respectively. For the parent spectrum, Gaussian time constants of 0.04 and 0.003 s were used for F_2 and F_1, respectively. The inset was processed with Gaussian time constants of 0.08 s for F_2 and 0.003 s for F_1. This spectrum was aquired with the following parameters: 256 transients, 1024 time-domain points, and 6400 Hz spectral width. In the carbon dimension (F_1), 64 increments were collected using a 12 070 Hz spectral width.

dance peaks in HMQC spectra, all samples for NMR studies were prepared in PBS. As indicated above, the energetics of tolbutamide binding to albumin in PBS were essentially identical to those in TES buffer.

Figure 3 shows the aliphatic region of a HMQC spectrum of a 3:1 tolbutamide/albumin complex. It demonstrates that the resonances corresponding to the [*methyl*-^{13}C] moiety of tolbutamide bound to albumin are well resolved from those corresponding to natural abundance carbon-13 in the protein. The Figure 3 inset illustrates the spectrum processed with resolution enhancement and shows that three peaks of comparable intensity were resolved. The best resolution was achieved along the ^{1}H axis.[2] The methyl resonances had ^{1}H chemical shifts of 2.07 ± 0.02, 2.11 ± 0.01, and 2.14 ± 0.02 ppm.[3] None of these peaks represented unbound tolbutamide, since the NMR spectrum of [*methyl*-^{13}C]-tolbutamide in PBS in the absence of protein exhibited a proton chemical shift of 1.83 ppm. The possibility that these resonances represented the three individual methyl protons in tolbutamide bound to a single site was also ruled out on the basis of the competition studies described below. Therefore, the three ^{1}H resonances at 2.07, 2.11, and 2.14 ppm represented tolbutamide bound to three distinct binding environments or sites on albumin, providing independent confirmation of the binding stoichiometry of 3 determined by calorimetry.

Table 2: Titration Calorimetry Results for Tolbutamide Binding Competition Studies with Selected Ligands[a]

competing agents	mol/mol albumin[b]	tolbutamide		
		n	K_d (μM)	ΔH° (kJ/mol)
none[c]		3.0 ± 0.1	21 ± 9	−6.0 ± 0.6
TIB	0.5:1	2.31 ± 0.02	23 ± 3	−6.5 ± 0.1
	1:1	1.77 ± 0.03	33 ± 3	−7.7 ± 0.1
	2:1	1.3 ± 0.2	28 ± 11	−9 ± 2
salicylate	0.5:1	2.5 ± 0.2	20 ± 3	−5.2 ± 0.4
	1:1	2.1 ± 0.1	21 ± 4	−5.2 ± 0.1
	2:1	1.36 ± 0.03	22 ± 10	−4.8 ± 0.8
clofibric acid	0.5:1	2.49 ± 0.08	18 ± 4	−6.0 ± 0.3
	1:1	2.21 ± 0.03	16 ± 2	−6.1 ± 0.3

[a] Each result represents the mean ± standard deviation of three experiments except 2:1 TIB/albumin and 0.5:1 salicylate/albumin, which represent four experiments. [b] Moles of competing agent per mole of albumin. [c] Titration of apo-albumin with tolbutamide.

NMR spectra were also used to qualitatively assess the relative affinities of these three sites for tolbutamide. If the affinities were approximately equal, then these sites should be equally occupied at tolbutamide/albumin mole ratios that are below saturation. NMR spectra of complexes with sample mole ratios of 1:1 and 2:1 exhibited spectra very similar to that shown in the Figure 3 inset, with three discrete methyl resonances at the same chemical shifts. These results confirmed the calorimetry result that all three sites have comparable affinity for tolbutamide.

Competition Studies To Localize Tolbutamide Binding Sites. To determine if drugs that bind to albumin in the subdomain IIA and IIIA binding cavities block the binding of tolbutamide to albumin, samples containing known mole ratios of TIB, salicylate, and clofibric acid to albumin were prepared and calorimetrically titrated with tolbutamide. The effects of increasing mole ratios of competitor/albumin on tolbutamide binding are summarized in Table 2. Salicylate, TIB, and clofibric acid each caused stoichiometric reductions in the number of tolbutamide binding sites with incremental increases in mole ratio of competing agent to albumin. The decrease in tolbutamide molar binding stoichiometry occurred with little or no change in the tolbutamide dissociation constant or molar binding enthalpy, suggesting that TIB, salicylate, and clofibric acid decreased tolbutamide binding by occupying sites on albumin to which tolbutamide binds.

Two-dimensional HMQC experiments were performed with samples of albumin containing known amounts of TIB and clofibric acid as competing agents in an attempt to localize tolbutamide binding sites on albumin. X-ray crystallography studies of drug−albumin complexes have demonstrated that aspirin and analogues, such as salicylate and iodinated salicylate (TIB), equally partition between the binding cavities of subdomains IIA and IIIA, while clofibric acid binds only in the subdomain IIIA binding cavity (He & Carter, 1992). Figure 4 shows HMQC spectra of samples containing 3 mol of [*methyl*-^{13}C]tolbutamide/mol of albumin (panel A), 2:1:1 TIB/[*methyl*-^{13}C]tolbutamide/albumin (panel

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[2] The digital resolution along the F_1 (^{13}C) axis was coarse, limiting the degree to which these peaks could be resolved along this axis. Attempts to increase the digital resolution in F_1 resulted in an unacceptable loss of signal intensity, presumably because of the high molecular weight of the complex and rapid relaxation during the t_1 period of the pulse sequence.

[3] To assess reproducibility, four independent spectra of the 3:1 [*methyl*-^{13}C]tolbutamide/albumin complex were collected at both 500 and 600 MHz. The reported chemical shifts represent the mean ± standard deviation for these four spectra. Two of the four are shown in Figures 3 and 4A.

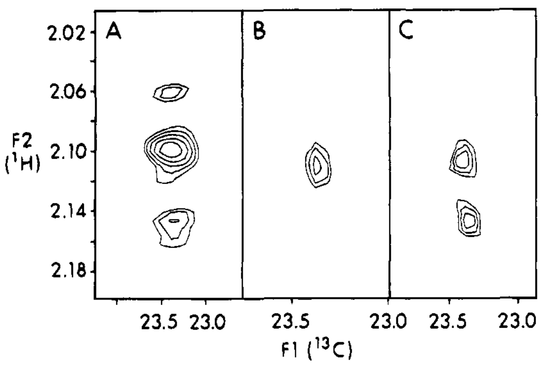

FIGURE 4: Two-dimensional HMQC spectra of [*methyl*-^{13}C]-tolbutamide complexed with human serum albumin in the absence and presence of competing drugs, all at 37 °C. Spectrum A represents a sample containing 3 mol of [*methyl*-^{13}C]tolbutamide/mole of HSA; it corresponds to the same sample, but different spectrum, from that shown in Figure 3. Spectrum B represents a sample containing a 2:1:1 mole ratio of TIB/[*methyl*-^{13}C]tolbutamide/HSA, and spectrum C, 1:2:1 clofibric acid/[*methyl*-^{13}C]-tolbutamide/HSA. The concentration of HSA was 1.2 mM for spectrum A and 1.5 mM for spectra B and C. Spectrum A was processed with a pseudo-echo weighting in F_2 (line-broadening factor, −8 Hz, and Gaussian time constant, 0.07 s). In F_1, Gaussian weighting (0.005 s) was employed. Spectra B and C were processed with pseudo-echo weighting in both dimensions (B: −5 Hz, 0.05 s; C: −6Hz, 0.05 s). Spectrum A was collected on a Varian Unity 600 spectrometer (599.945 MHz for ^{1}H), and spectra B and C were collected on a Varian Unity 500 spectrometer (499.843 MHz for ^{1}H). The spectral parameters were as follows: (A) 192 transients, 1024 time-domain points zero filled to 4096 (F_2), spectral width 6400 Hz (F_2), 72 increments zero-filled to 1024, spectral width, 6035 Hz (F_1). (B and C) 400 transients, 1024 time-domain points zero-filled to 4096 (F_2), spectral width, 5200 Hz (F_2), 100 increments zero-filled to 1024, spectral width, 4022 Hz (F_1).

B), and 1:2:1 clofibric acid/[*methyl*-^{13}C]tolbutamide/albumin (panel C). In the presence of saturating amounts of TIB (Figure 4B), two of the three tolbutamide resonances were selectively suppressed. In the presence of saturating amounts of clofibric acid (Figure 4C), one of the three tolbutamide resonances was selectively suppressed. In both cases, the intensities of the remaining peaks increased compared to those for a sample containing 1:1 [*methyl*-^{13}C]tolbutamide/albumin, indicating a redistribution of tolbutamide to the open binding sites. On the basis of the structural locations of the TIB and clofibrate binding sites as determined by X-ray crystallography, the resonances at 2.07 and 2.14 ppm were then assigned to tolbutamide bound at albumin subdomains IIIA and IIA, respectively (Figure 5).

The location of the third tolbutamide binding site could not be determined at this time. However, clues as to the identity of nearest neighbor residues could be obtained from the ^{13}C-edited NOESY spectrum shown in Figure 6. The sample used to collect this spectrum was identical to that shown in Figure 4B and contained 2:1:1 TIB/[^{13}C-*methyl*]-tolbutamide/albumin. Hence, two of the three tolbutamide binding sites were essentially occupied by TIB, and only the third unidentified site was occupied by tolbutamide. The cross-peaks shown along the horizontal axis designated by the arrow in Figure 6 represent through-space correlations (<5 Å) between the tolbutamide methyl protons and nearby protons on amino acid side chains in this binding site. The most intense cross-peaks, at x-axis values of 7.63 and 7.17 ppm, represent internal correlations between the methyl and ring protons on tolbutamide. However, the weaker correlations at 6.49, 6.93, and −0.08 ppm represent aromatic and aliphatic protons on adjacent protein side chains. Although the assignments for these cross-peaks remain to be established, the most likely amino acid types can be ascertained from the chemical shift values: 6.49 ppm (Tyr ring 3/5), 6.93 ppm (Tyr ring 2/6 or Phe ring), and −0.08 ppm (Leu methyl).

DISCUSSION

Isothermal titration calorimetry and heteronuclear 2-D NMR spectroscopy were used in a complementary fashion to characterize the molecular interactions between tolbutamide and human serum albumin. The calorimetry data were used to quantitate the energetics of tolbutamide binding, and the NMR spectra were used to monitor the occupancy of distinct tolbutamide binding sites. The results of these investigations make it possible to address questions about tolbutamide binding stoichiometries and affinities, binding site locations, and the potential clinical significance of these findings.

Binding Stoichiometry and Affinity. By calorimetry, tolbutamide was found to bind to three sites with equal or comparable affinity, and this stoichiometry was independently confirmed by the resolution of three discrete tolbutamide resonances by two-dimensional NMR experiments with carbon-13 enriched drug. Further, the detection of three resonances of approximately equal intensities at a 1:1 sample mole ratio of [*methyl*-^{13}C]tolbutamide/albumin is consistent with tolbutamide binding at three independent sites with approximately equal affinities for drug.

Earlier studies of tolbutamide binding to human serum albumin have reported binding at a single affinity class of sites with stoichiometries of 1.36 (Judis, 1972) and 2.46 (Zini *et al.*, 1976) and binding at two affinity classes of sites with stoichiometries of 2.27 and 8.22 for high and low affinity classes, respectively (Crooks & Brown, 1974). Crooks and Brown (1973) demonstrated that components of the tromethamine buffer used by Judis competed with tolbutamide for albumin binding sites and decreased its stoichiometry. It is also possible that the binding stoichiometry was underestimated in these equilibrium and dynamic dialysis studies because of artifactual binding of the drug to either dialysis cell components or membranes, with consequent reduction of tolbutamide concentration in the dialysis medium. Disparities in the number of affinity classes may be related to different models that have been employed to interpret the dialysis data. Although unlikely, it is possible that a second low affinity class of binding sites for tolbutamide exists that cannot be detected by calorimetry and NMR. However, the weak affinity reported (K_d = 1504 μM; Crooks & Brown, 1974) indicates that it is of little or no physiological significance.

The calorimetrically determined dissociation constant for tolbutamide (21 ± 9 μM) is in fairly good agreement with the dialysis results of Judis (1972) and Zini et al. (1976), who reported dissociation constants of 25 and 37 μM, respectively. However, Crooks and Brown (1974) observed a stronger binding interaction, and their measured binding affinity (K_d = 4.5 μM) is the most widely cited result [e.g., see Carter and Ho (1994)]. The tolbutamide binding affinity has been demonstrated to be sensitive to the concentration of sodium chloride in buffer, with affinity decreasing as the concentration of NaCl is increased (Brown & Crooks, 1974).

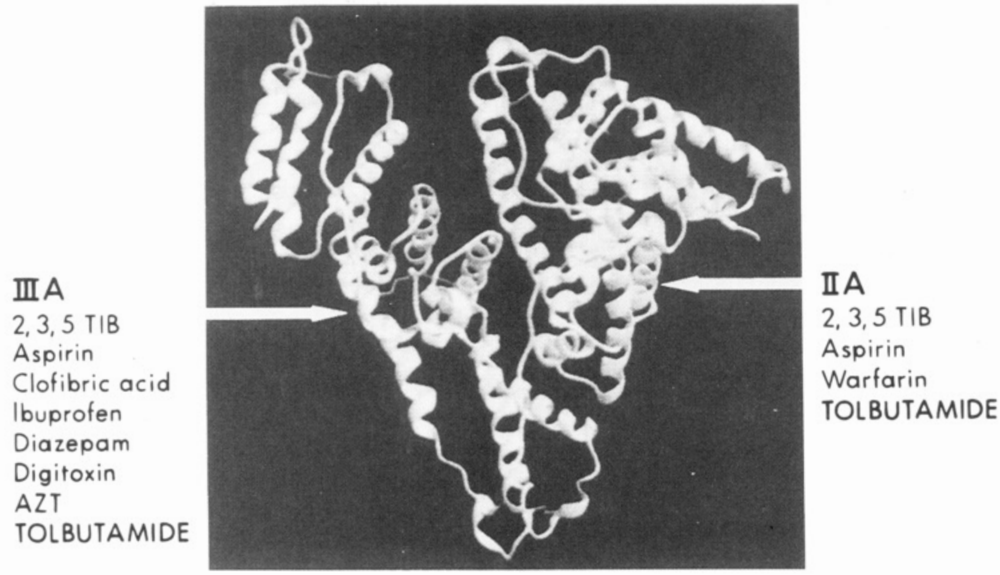

FIGURE 5: Ribbon diagram of human serum albumin illustrating overall topology and drugs known to bind at sites in subdomains IIA and IIIA. This diagram is derived from He and Carter's (1992) description of the 2.8 Å resolution crystal structure of albumin (used by permission from publisher). The binding site locations of TIB, aspirin, warfarin, clofibrate, ibuprofen, diazepam, digitoxin, and AZT were determined by X-ray crystallography. The binding site locations of tolbutamide were determined in the present study.

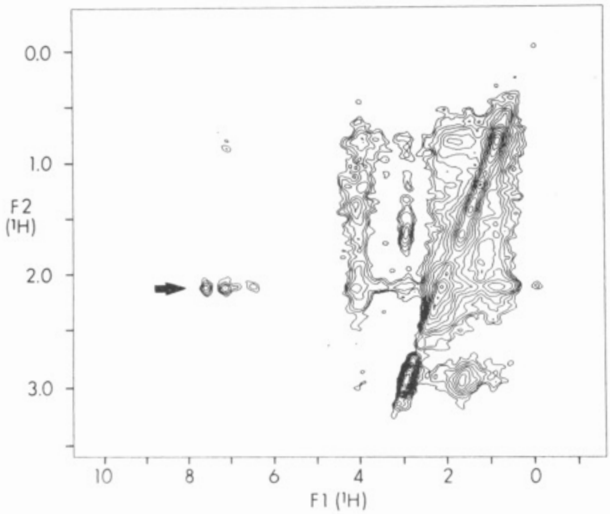

FIGURE 6: Two-dimensional NOESY-HMQC spectrum of a complex containing 2 mol of TIB and 1 mol of [*methyl*-^{13}C]-tolbutamide per mole albumin at 37 °C. The arrow at $F_2 = 2.11$ ppm indicates cross-peaks representing through-space correlations between the tolbutamide methyl protons and adjacent protons on the ligand and on protein side chains in the binding site. The sample used for this spectrum was identical to that used in Figure 4B. The spectral parameters were as follows: number of transients, 128; spectral width (F_2), 6200 Hz; time-domain points (t_2), 1024 zero-filled to 2048; number of increments (t_1), 140; spectral width (F_1), 6200 Hz.

Also, serum albumin undergoes a neutral-to-base (N−B) conformational change between pH 6 and 9, and the pK values of histidine residues involved in this transition are strongly dependent on the Ca^{2+} content of the solution (Labro & Janssen, 1986). The previously cited tolbutamide binding studies were performed in buffers without NaCl and Ca^{2+}, whereas the experiments in the present study were performed in buffer containing physiologic concentrations of these three key ions. Further, unlike partitioning techniques, titration calorimetry does not require separation of free ligand from bound in order to quantitate both species and avoids such perturbations of the ligand−protein equilibrium. Therefore, the dissociation constant measured in the present study is likely to provide the most accurate indication of albumin's affinity for tolbutamide under conditions relevant to human plasma.

Binding Site Locations. The drug competition results obtained by NMR and calorimetry, interpreted in the context of the crystal structure of human serum albumin complexed with other drugs (He & Carter, 1992), permit the first definitive assignment of tolbutamide binding sites to specific structural domains of the albumin molecule. Calorimetric competition experiments with TIB, salicylate, and clofibric acid revealed that these drugs cause a stoichiometric decrease in tolbutamide binding without affecting the binding affinity or enthalpy. These results suggested that the reduction in tolbutamide binding stoichiometry was due to direct competition for mutual binding sites. NMR competition studies with TIB and clofibric acid demonstrated the selective suppression of tolbutamide binding to two of its three sites, and that those two sites corresponded to the aspirin binding sites located in subdomains IIA and IIIA.

The observation of direct competition for binding sites between tolbutamide and the selected competing agents contrasts with the previous conclusion that displacement of first generation sulfonylureas by other anionic drugs is due to noncompetitive inhibition of binding (Brown & Crooks, 1976). However, the previous studies were performed using human and bovine albumins that were not defatted and in buffer without NaCl or Ca^{2+}, making it difficult to separate the effects of fatty acids and buffer composition from those of competing agents. The conclusion of a noncompetitive mechanism also assumes that the competing agent decreases albumin's affinity for displaced drug. The present calorimetry experiments clearly demonstrate that the acidic compounds studied have little or no effect on the binding affinity of tolbutamide. Moreover, the selective elimination of individual [*methyl*-^{13}C]tolbutamide resonances and the redistribution of tolbutamide to other binding sites observed by NMR is also consistent with direct competition for common binding sites. A number of other investigations of the interactions of acidic drugs have observed competitive displacement (Solomon *et al.*, 1968; Tillement *et al.*, 1973; Anton, 1973; Maruyama *et al.*, 1985), including studies which have investigated tolbutamide binding to albumin (Christensen et al., 1963; Sudlow *et al.*, 1975, 1976; Sjöholm *et al.*, 1979; Fujii *et al.*, 1983).

Assignment of tolbutamide binding sites to specific subdomains helps to clarify previous attempts to localize these sites. Sudlow *et al.* (1975) identified two distinct binding sites on albumin for acidic drugs based on their abilities to displace the fluorescent dansylamino acid probes 5-(dimethylamino)naphthalene-1-sulfonamide (DNSA, site I) and dansylsarcosine (site II). Tolbutamide was identified as a site I drug on the basis of competitive displacement of DNSA (Sudlow et al., 1975, 1976). Sudlow's sites I and II also came to be referred to as the warfarin site and diazepam site, respectively. Sjöholm *et al.* (1979) investigated the ability of unlabeled drugs to block binding of radiolabeled warfarin, diazepam, and digitoxin to albumin immobilized on polyacrylamide microparticles and found tolbutamide to displace both warfarin and diazepam. More recently, Kurono *et al.* (1987) classified tolbutamide as an "R-type drug" which binds at Sudlow's site II and has no effect on the reaction of 3,5-dinitroaspirin with Trp-214 at the "U-site" (Sudlow's site I). In addition to the contradictory nature of these findings, the binding site designations were made without knowledge of the three-dimensional structure of albumin. The current NMR studies clearly identify three independent tolbutamide binding sites and localize two of them to the aspirin sites in subdomains IIA and IIIA. It has been concluded that Sudlow's site I corresponds to the subdomain IIA binding cavity and site II to the subdomain IIIA binding cavity (Carter & Ho, 1994).

The observation that tolbutamide binds at multiple sites on albumin, and in particular subdomains IIA and IIIA, might be explained at least in part by consideration of its molecular structure (Figure 1). Drugs that bind only at subdomain IIA, such as the coumarin anticoagulant warfarin and the uricosuric agent sulfinpyrazone, tend to be heterocyclic molecules with ionizable groups near the center of the molecule. In contrast, agents that bind only to subdomain IIIA, such as ibuprofen and clofibric acid, have an extended configuration with ionizable groups located at one end of the molecule (Sudlow *et al.*, 1976; Carter & Ho, 1994). Tolbutamide has the extended configuration common to IIIA drugs, but its nonpolar moieties (toluene and *n*-butyl group) are located at the ends of the molecule and its ionizable groups at the center, like IIA drugs. Thus, tolbutamide appears to have structural features of both classes of drugs.

The location of the third tolbutamide binding site remains unknown. Clearly, the subdomain IIA/IIIA and Sudlow I/II paradigm for drug–albumin interactions is insufficient to account for this additional site, and further crystallographic and/or NMR studies will be necessary to localize it. To this end, the ^{13}C-edited NOESY spectrum in Figure 6 provides clues as to the residue types that are adjacent to the tolbutamide methyl group in this unknown site. If the general location of this binding site could be narrowed down to a certain domain or subdomain by inspection of the existing X-ray crystal structure (without tolbutamide), it may be possible to pinpoint the site using a combination of isotope-directed 2-D NMR and site-directed mutagenesis.

Clinical Significance. Accurate measurements of tolbutamide's albumin binding properties and knowledge of its binding site locations are important to prevent adverse drug reactions leading to life-threatening hypoglycemia. Tolbutamide and other first generation sulfonylureas are most frequently prescribed for older patients (Kennedy *et al.*, 1988), many of whom take several medications for multiple medical problems (Jackson & Bressler, 1988). Investigators at the Aberdeen Diabetic Clinic found that 51% of patients were taking one to five drugs with the potential to interact with their antidiabetic therapy (Logie *et al.*, 1976). A recent British study reported that 20% of patients taking a sulfonylurea experienced at least one episode of symptomatic hypoglycemia in the previous 6 months; 76% of patients reporting hypoglycemic episodes were taking other prescription medications compared to 51% of patients who did not report hypoglycemia (Jennings *et al.*, 1989).

Localization of tolbutamide binding sites provides a basis for systematically predicting which drugs have the potential to displace tolbutamide from albumin and potentiate its hypoglycemic effect. In addition to establishing the risk of displacement interactions with the drugs illustrated in Figure 5, the success of heteronuclear 2-D NMR in monitoring the distribution of [*methyl*-^{13}C]tolbutamide at its individual albumin binding sites suggests that this approach may be sensitive enough to extend to other drugs whose binding sites have not been identified but that are prescribed most frequently for diabetics (Rendell *et al.*, 1983). The synthesis of other isotope-enriched sulfonylureas should make it possible to utilize this combined calorimetric and NMR approach to study drug binding interactions of additional members of the sulfonylurea family, and other pharmaceutical agents in general.

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