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Opioids Bind to the Amino Acids 84 to 118 of UDP-Glucuronosyltransferase UGT2B7

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ABSTRACT

The UDP-glucuronosyltransferase UGT2B7 is an important human UGT isoform that catalyzes the conjugation of many endogenous and exogenous compounds, among them opioids, resulting in the formation of D-glucuronides. The binding site of the aglycone is located in the N-terminal half of the protein. Using NMR analysis, we demonstrate that the opioid binding site in UGT2B7 is within the 84 to 118 N-terminal amino acids. Three maltose binding protein-UGT2B7 fusion proteins, 2B7F3 and 2B7F4 incorporating the amino acids 24 to 118 and 24 to 96 of UGT2B7, respectively, and 2B7F5 incorporating amino acids 84 to 118 of UGT2B7 were expressed in *Escherichia coli* and purified by affinity chromatography. NMR analysis showed that morphine was bound to the fusion protein 2B7F3 with a K_D value similar to the K_D values obtained for the previously produced fusion proteins, which included amino acids 24 to 180. Morphine did not bind to 2B7F4, but it did bind to 2B7F5. Both NMR 1-D spectra and NOESY experiments indicated that the 2B7F5 protein was mediating magnetization transfer within the morphine. These results allowed us to predict and model a binding site within the amino acids 96 to 101 of UGT2B7. A mutant fusion protein 2B7F3 with the substitution D99A was produced, and the NMR spectroscopy analysis of the protein supported the model. A marked reduction of morphine binding was observed when the charged aspartate was substituted with alanine.

Of the more than 30 mammalian isoforms of UDP-glucuronosyltransferase that have been identified, the human UGT2B7 isoenzyme is one of the most important. It is expressed in liver, kidney, intestine, colon, testis, and brain (King et al., 2000), and it catalyzes the glucuronidation of opioids, androsterone, catechol estrogens, hyodeoxycholic acid, nonsteroidal anti-inflammatory drugs, 3′-azido-3′-dideoxythymidine, and retinoic acid with high efficiency (Ritter et al., 1990; Jin et al., 1993; Coffman et al., 1998; Barbier et al., 2000; Samokyszyn et al., 2000). This enzyme and its simian homolog UGT2B9 (Green et al., 1997) are the only enzymes that catalyze the glucuronidation of both the 3-OH and 6-OH positions of opioids when present and thus provide for the formation of the pharmacologically active morphine-6-glucuronide and codeine-6-glucuronide (Coffman et al., 1997). The presence of UGT2B7 in the human central nervous system where the μ-opioid receptors are located is important because morphine 6-*O*-glucuronide is more active than morphine (Osborne et al., 1990).

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Activity studies of expressed chimeric UGT cDNAs have shown that the aglycone binding domain is likely to be seated within the first 298 amino acids of the N terminus of the protein, presumably in the region of amino acids 55 to 180, a region with the least homology of primary sequence between the UGT isoforms (Mackenzie, 1990). We have demonstrated previously using maltose binding protein-UGT2B7 fusion proteins that the opioids bind to the amino acids 24 to 142 of UGT2B7 (Coffman et al., 2001), and only one binding site was predicted from the data obtained. This was demonstrated by NMR spectroscopy and equilibrium dialysis. The dissociation constants (K_D), as determined experimentally, were found to be similar to the K_m values reported for the full-length and functional activity of full-length UGT2B7.

In this study, we designed four soluble fusion proteins. Three soluble fusion proteins were generated, containing the maltose binding protein (MBP) and the N-terminal amino acids 24 to 118, 24 to 96, and 84 to 118, respectively, of the UGT2B7 isoenzyme. Specific binding of morphine to the UGT2B7 domain of these fusion proteins was demonstrated by NMR spectroscopy analysis. Modeling techniques were then used to propose a plausible binding site focusing pri-

ABBREVIATIONS: UGT, UDP-glucuronosyltransferase; MBP, maltose binding protein; NOESY, nuclear Overhauser effect spectroscopy; 2B7F1, fusion protein of amino acids 24 to 180 from UGT2B7 and MBP; 2B7F2, fusion protein of amino acids 24 to 142 from UGT2B7 and MBP; 2B7F3, fusion protein of amino acids 24 to 118 from UGT2B7 and MBP; 2B7F4, fusion protein of amino acids 24 to 96 from UGT2B7 and MBP; 2B7F5, fusion protein of amino acids 84 to 118 from UGT2B7 and MBP.

marily on amino acids 96 to 101. To test the model, a mutation was produced in which aspartate 99 was changed to alanine. Binding studies using NMR spectroscopy were then carried out, and morphine binding to the wild-type protein was compared with the protein containing the mutant.

Materials and Methods

Materials. The pMalc2x vector, maltose binding protein, and amylose resin were obtained from New England Biolabs (Beverly, MA). The QuickChange Site-Directed Mutagenesis Kit was purchased from Stratagene (La Jolla, CA). Morphine sulfate was obtained from Merck (Whitehouse Station, NJ). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Construction of Expression Plasmids and Purification of the UGT2B7 Fusion Proteins. The MBP-tagged fusion proteins 2B7F1 and 2B7F2 containing the amino acids 24 to 180 and 24 to 142 of UGT2B7, respectively, were produced and characterized as described previously (Coffman et al., 2001). The cDNA encoding for the MBP-tagged fusion proteins 2B7F3 [UGT2B7(24–118)] and 2B7F4 [UGT2B7(24–96)] were generated by inserting stop codon mutations in the plasmid encoding for MBP-tagged UGT2B7(24–180). The cDNA encoding for the fragment of UGT2B7 that codes for amino acids 84 to 118 was subcloned by polymerase chain reaction from the cDNA encoding for 2B7F3. The primers included the restriction enzyme sites *Eco*R1 and *Pst*I on the 5′ and 3′ ends, respectively. The polymerase chain reaction product was ligated into the *Eco*R1/*Pst*I site of the bacterial expression vector pMalc2x, resulting in a plasmid that coded for the MBP-tagged fusion protein 2B7F5 [MBP UGT2B7(84–118)]. The cDNA of the mutation 2B7F3 D99A was generated from the cDNA of 2B7F3 using the primer CAGATTAAGAGATGGTCAGCCCTTCCA.

All mutation and ligation products were verified by DNA sequencing. The recombinant proteins were expressed and purified as described previously (Coffman et al., 2001). The purified proteins were analyzed on SDS gels and by circular dichroism spectroscopy as described previously (Coffman et al., 2001).

NMR Spectroscopy. The binding properties of morphine to the MBP-tagged fusion proteins were analyzed by examining the effect of the proteins on the NMR spectra and relaxation rates of the morphine. All spectra were collected on the INOVA-500 500 MHz spectrometer (Varian Inc., Palo Alto, CA) in the College of Medicine NMR Facility at the University of Iowa. Spectra were processed using VNMR 6.1C software (Varian).

The sample temperature was held at 25°C for all work reported here. A 6000-Hz spectral width and 90° pulse width of 7 μs were used in all spectra. All samples were prepared with 50 mM phosphate buffer at pH 8 in D_2O. Water signals were suppressed by low power saturation during all delays except for the acquisition time. Longitudinal relaxation rates were measured, and the K_D values were calculated, when possible, using methods described previously (Coffman et al., 2001). NOESY spectra were collected with mixing times of 100, 200, 400, and 600 ms on solutions containing morphine alone and for solutions containing morphine and the UGT2B7 fusion protein.

The morphine-binding behavior of 2B7F3 was studied by adding 3.0-μl aliquots of 100 mM morphine to 650 μl of a solution containing 12 μM 2B7F3 and 7 μl of 100 mM morphine. For the 2B7F3 D99A mutant, 3.8-μl aliquots of 100 mM morphine were added to 650 μl of a solution containing 12 μM of the protein. In the experiment determining the 2B7F5 binding of morphine, 0.8-μl aliquots of 100 mM morphine were added to 650 μl of a solution containing 8 μM 2B7F5. For the naloxone displacement experiments, 0.8-μl aliquots of 10 mM naloxone were added to 650 μl of a solution containing 12 μM 2B7F5 and a 3.2-μl aliquot of 10 mM morphine. All aliquots were calibrated by weight.

Molecular Modeling. The modeling program used was SYBYL software version 6.4 (Tripos, St. Louis, MO) on an O2 workstation (SGI, Mountain View, CA). The methods for building the tertiary structure of the N-terminal domain of UGT2B7, amino acids 24 to 180, were as follows: the conformation for each amino acid was assigned manually using the secondary structure prediction from the PHD program (Rost and Sander, 1993). From this initial geometry, the model was minimized, and simulated annealing was performed to explore the conformational space of the molecule. The Biopolymer tool in SYBYL was used for building the peptide. Energy minimization and annealing were performed using the Kollman all-atoms force field and Kollman charges. Ten rounds of annealing were performed by heating to 2000 K. Using the motifs for the opioid-binding sites in CYP2D6 and the μ receptor (Modi et al., 1996: Sagara et al., 1996; Mansour et al., 1997), the motif (Asp, Glu), (Arg, Lys), (Ser, Thr, or Cys), and (Phe, Trp, Ala, Val, Leu, Ile) was identified in UGT2B7 from amino acids 93 to 105. The SYBYL docking tools were then used to explore this site.

Results

Expression and Characterization of Soluble MBP-Tagged Fusion Proteins. The three fusion proteins 2B7F3, 2B7F4, and 2B7F5 were expressed in the *Escherichia coli* system in the cytosol and were purified as described under *Materials and Methods*. The molecular masses of the fusion proteins were 54 kDa, 51 kDa, and 47 kDa, as verified on SDS-polyacrylamide gel electrophoresis. The purity was estimated to be approximately 95 to 98%. The circular dichroism spectra of the expressed proteins exhibited α-helix structure in 50 mM phosphate at pH 8 and room temperature, demonstrating that the conditions for NMR spectroscopy were nondenaturing.

NMR Spectroscopy of Morphine Binding to Expressed MBP-Tagged Fusion Proteins. The morphine peak with a chemical shift of 5.68 ppm (H-7) was used for the study of the longitudinal relaxation rate ($R_1 = 1/K_2$) and the calculation of the K_D in the presence of the fusion proteins 2B7F3 and 2B7F4. The results are summarized in Fig. 1 together with data published previously (Coffman et al., 2001). The results demonstrate clearly that the binding site is within amino acids 24 to 118 of UGT2B7. The fusion protein 2B7F4 does not bind morphine. Previously we showed that the MBP itself does not bind morphine (Coffman et al., 2001).

The fusion protein 2B7F5 was constructed with use of the peptide UGT2B7 (amino acids 84–118) attached to the carboxylic acid end of MBP. The amino acid sequence and the predicted secondary structure of the peptide are shown in Fig. 2. The longitudinal relaxation rate of the morphine peak with a chemical shift of 6.69 ppm (H-2) was used to study the dynamic behavior of morphine in the presence of 2B7F5. The rate was increased over the relaxation rates for the free morphine or morphine in the presence of bovine albumin serum. The increase was concentration-dependent (Fig. 3).

Fusion Protein						Morphine Binding	K_D μM
2B7F1	MBP	24	UGT2B7		180	+	90
2B7F2	MBP	24	UGT2B7	142		+	~100
2B7F3	MBP	24	UGT2B7	118		+	~100
2B7F4	MBP	24	UGT2B7	96		-	
MBP	MBP					-	

Fig. 1. Morphine binding to MBP-UGT2B7 fusion protein and to MBP.

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84 TELENFIMQQIKRW S D L P K D TFWLYFSQVQEIMSI
   HHHHHHHHHHHHHH . . . L . . HHHHHHHHHHHHHHH
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Fig. 2. The amino acid sequence of UGT2B7(84–118) and the predicted secondary structure. The secondary structure predicted by the PHD program from CUBIC (Columbia University) is also shown (Rost and Sander, 1993). Residues in which the predicted accuracy was <82% were left unassigned. H = α-helix, L = loop.

Concentration-dependent changes in the chemical shifts were observed for the H-1, H-2, H-7, and H-8 peaks. The data for H-2 are presented in Fig. 4. The results shown in Figs. 3 and 4 show that morphine binds to fusion protein 2B7F5 within amino acids 84 to 118 of UGT2B7. It was, however, not possible to calculate a dissociation constant for morphine because a phase change occurred in the solution at higher morphine concentrations. The relaxation rate of morphine, at a fixed morphine concentration and in the presence of 2B7F5, was decreased by the addition of naloxone, indicating that morphine was displaced by naloxone at a specific binding site (Fig. 5). The diagonal and cross-diagonal peaks in the NOESY spectrum of free morphine were of opposite signs, whereas in the presence of 2B7F5, the peaks were all of the same sign, demonstrating the binding of morphine to the protein. In the NOESY spectrum of morphine in the presence of 2B7F5 there were many new off-diagonal peaks, compared with the spectrum of the free morphine. This indicates that the protein is mediating magnetization transfer within the morphine (Fig. 6). A simple analysis of the NOESY spectra (Gronenborn and Clore, 1982) yielded conflicting distance estimates. This implies that a more detailed dynamical model must be applied (Curto et al., 1996).

Molecular Modeling of a Possible Opioid-Binding Site in UGT2B7(84–118). The data from the analysis by NMR spectroscopy demonstrated that morphine binds to the peptide 2B7F5. The predicted secondary structure of this peptide (Fig. 2) contains two α-helices connected by a string of amino acids with undefined structure. Using the methods described under *Materials and Methods*, we were able to define this area as a loop with a pocket formed by amino acids 96 to 101 and to dock morphine and buprenorphine in the pocket.

The model shows that hydrogen bonding was possible between Arg 96 and the hydroxy groups in the 3 or 6 position of the opioids. The distance from the opioid nitrogen in morphine and buprenorphine to the COO– Asp 99 was close enough (~ 4 Å) to form a weak ionic bond. We were able to dock morphine with either the 3-OH or the 6-OH group exposed. The docking of buprenorphine showed a possible additional hydrogen bonding between the Ser 98 and the hydroxy group on the buprenorphine C19.

Figure 7 shows the morphine and buprenorphine docked to the pocket. The peptide is shown with its Connolly surface, and both the Connolly surfaces and the stick representations are shown for the ligands. Morphine is shown docked with its 6-OH group exposed in Fig. 7A, showing that it is possible for morphine to bind with its 6-OH group exposed. Docking with

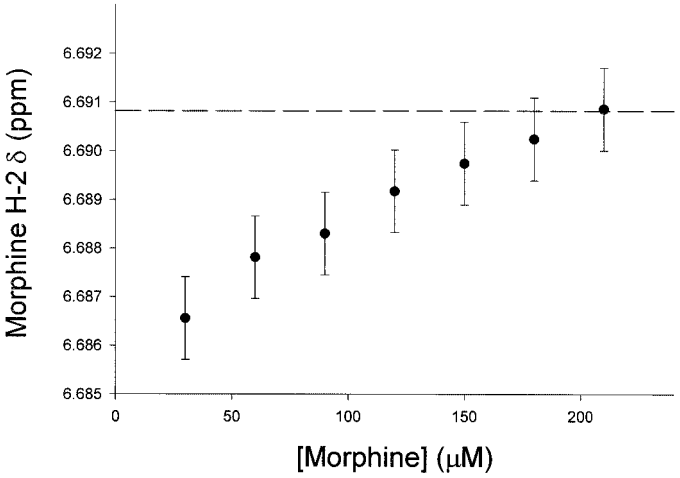

Fig. 4. The chemical shift of morphine H-2 increases as aliquots of morphine are added to a solution containing 8 μM 2B7F5. This is further evidence of morphine binding. The dashed line indicates the chemical shift of H-2 in D_2O.

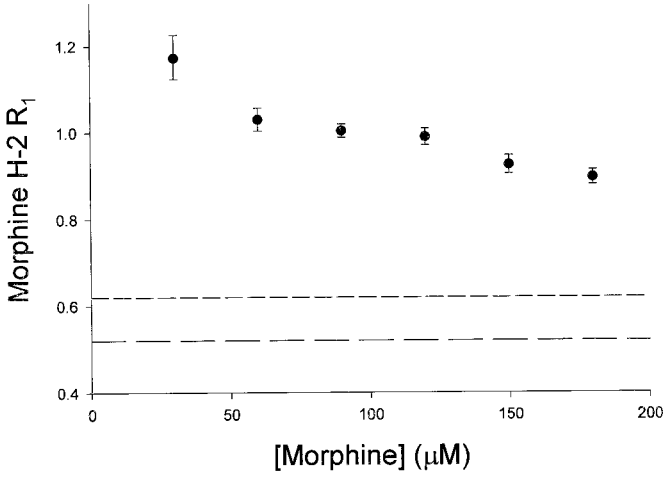

Fig. 3. The longitudinal relaxation rate of H-2 in morphine decreases when aliquots of morphine are added to a solution containing 8 μM 2B7F5. This indicates that morphine is binding to the fusion protein. The line with long dashes indicates the relaxation rate of H2 in a 1 mM solution of morphine, and the short dashes indicate the relaxation rate for 90 μM morphine in the presence of bovine serum albumin (same mass as used for 2B7F5). All three solutions are in the same buffer system.

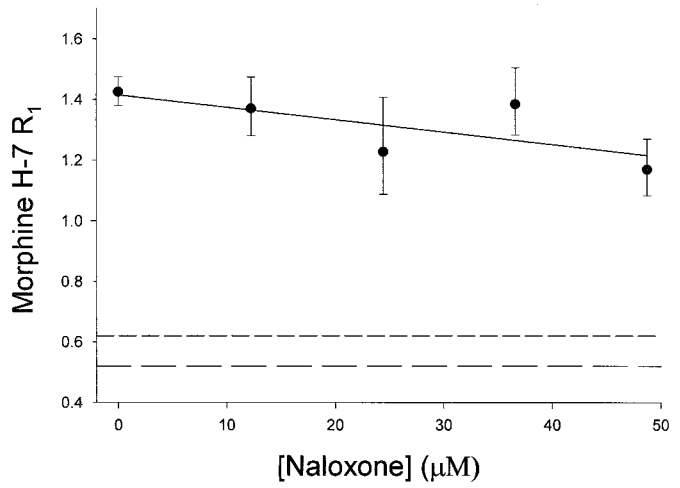

Fig. 5. The longitudinal relaxation rate of H-7 on morphine decreases when aliquots of naloxone are added to a solution containing 12 μM 2B7(84–118) and 49 μM morphine. This indicates that naloxone displaces morphine from a specific binding site on the protein. The line is a result of a linear regression with the R1 intercept equal to 1.42 ± 0.070 and the slope equal to −0.0041 ± 0.0023. (R^2 = 0.51).

the 3-OH group exposed was also feasible (data not shown). Buprenorphine is docked with its 3-OH group exposed (Fig. 7B). The phenolic ring of buprenorphine is in the same position as the phenolic ring of morphine when morphine is docked with its 3-OH group exposed. It is shown that the buprenorphine molecule nearly fills the space in the pocket, making many surface-to-surface interactions possible.

Mutagenesis of Aspartate 99 in the Proposed Opioid-Binding Site. The results of the NMR spectroscopy analysis of morphine binding to 2B7F3D99A is shown in Fig. 8. The relaxation rates for 2B7F3D99A are much lower than those found for the wild-type protein. This indicates that substitution of the charged aspartate 99 with an uncharged alanine results in a marked reduction in morphine binding. This observation lends further credence to the model proposed.

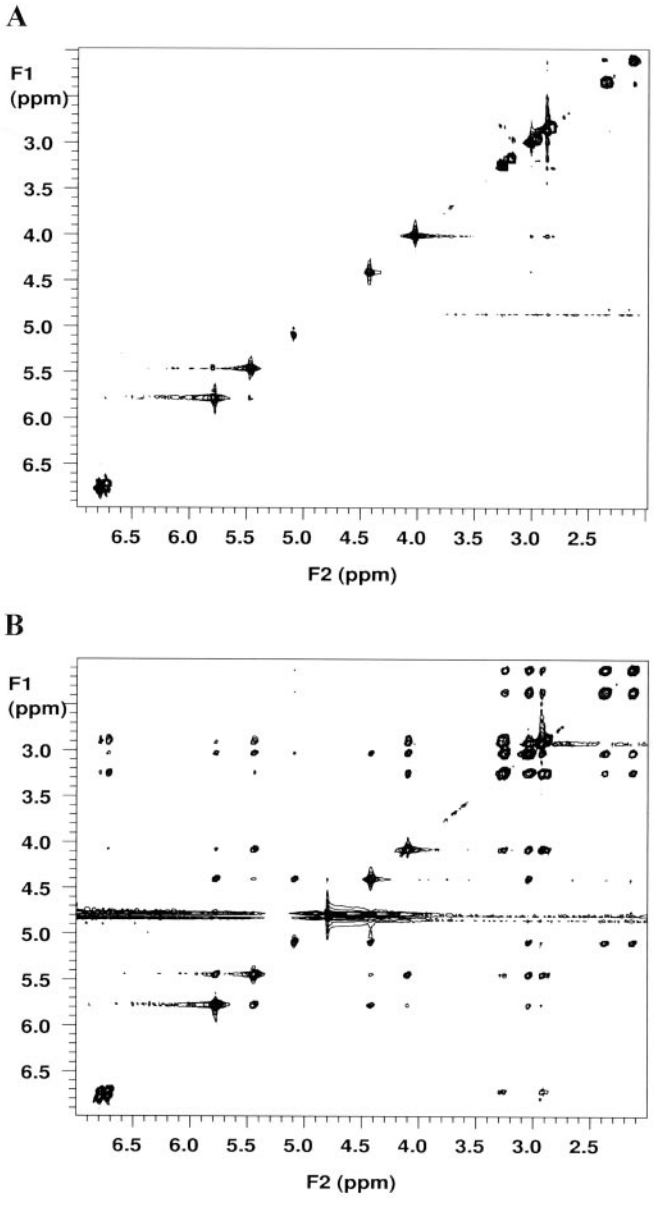

Fig. 6. NOESY spectra (300 ms mixing time) of 1 mM morphine in buffer (A) and 300 μM morphine in buffer plus 12 μM 2B7F5 (B). The cross peaks and diagonal peaks have opposite signs in the spectrum of morphine alone and the same sign when the protein is present. This indicates that the morphine is binding to 2B7F5, causing a large effective rotational correlation time for morphine. The large number of morphine-morphine cross peaks indicates that the protein is mediating magnetization transfer within morphine.

Discussion

This study was designed to further explore the aglycone binding region of UGT2B7. It has previously been shown that opioid compounds bind to a single site on the first 119 amino acids of the N-terminal end of UGT2B7. Two possible binding sites were suggested for this region, one in the domain from amino acids 93 to 105, and one in the domain from amino acids 116 to 132 (Coffman et al., 2001).

In the present study, three new fusion proteins containing fractions from the N-terminal end of UGT2B7 and maltose binding protein were produced and isolated. NMR experiments show that morphine binds to the fusion protein 2B7F3 with a K_D value similar to the K_D values obtained for the previously produced fusion proteins 2B7F1 and 2B7F2 (Fig. 1) (Coffman et al., 2001). This result indicates that the binding site for opioids is within amino acids 24 to 118 of the UGT2B7 protein. The second fusion protein, 2B7F4, in which the UGT2B7 peptide stops at amino acid 96, did not bind morphine.

The fusion protein 2B7F5, containing the maltose binding protein and amino acids 84 to 118 of UGT2B7, was then produced and isolated, and the binding of morphine to 2B7F5 was confirmed four different ways using NMR spectroscopy techniques. In the presence of the protein 2B7F5, there was (1) an increase in the morphine relaxation rate; (2) a change in the chemical shift in the peaks for morphine H-1, H-2, H-7, and H-8; (3) the negative sign on both diagonal and cross-diagonal peaks in the NOESY spectrum; and (4) displacement of morphine by naloxone.

The change in the chemical shifts (Fig. 4) and the appearance of new peaks in the NOESY spectrum (Fig. 6B) when both morphine and protein 2B7F5 are present indicates that the protein mediates the transfer of magnetization within the morphine. This could take place if either or both the protein and morphine changed conformation upon the binding of morphine to 2B7F5. Another possibility is a transfer between two morphine molecules bound in two different positions, for example the 3-OH or 6-OH group being exposed. Studies are in progress to distinguish between these possibilities.

The secondary structure of the UGT2B7 peptide is predicted to be a loop between two α-helices (Fig. 2). The two α-helices both are amphipathic [Helical Wheel tool (http://marqusee9.berkeley.edu/kael/helical.htm)], suggesting that a hydrophobic environment exists close to the loop. The loop (amino acids 93–105) between the helices contains the motif (Asp, Glu), (Arg, Lys), (Ser, Thr, or Cys), and (Phe, Trp, Ala, Val, Leu, Ile), which are the elements necessary for morphine binding as deduced from the opioid-binding sites of CYPD6 and the μ receptors (Modi et al., 1996; Sagara et al., 1996; Mansour et al., 1997). Molecular modeling techniques were then used to illustrate how morphine or buprenorphine were able to fit into a pocket in the loop formed by the amino acids R96-W97-S98-D99-L100-P101 (Fig. 7). There were few steric hindrances, and it was possible to bring the opioid molecules close enough to the peptide moiety for possible hydrogen bonding and ionic bonding to take place. It is important to note that it is possible to dock morphine with either its 3-OH

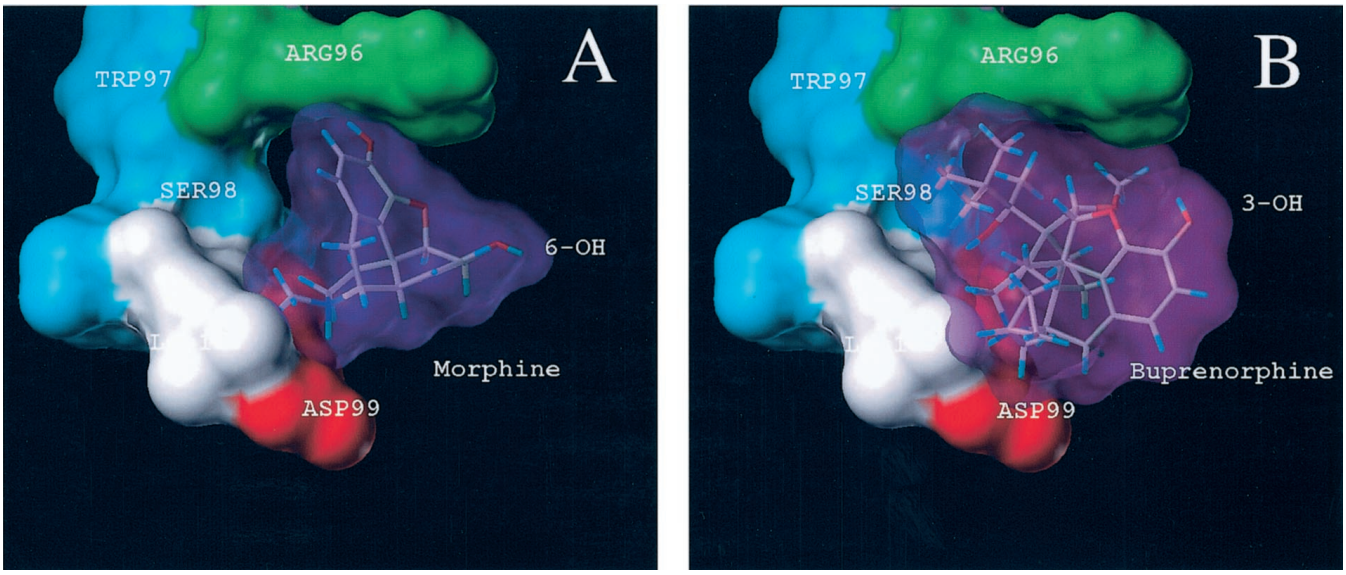

Fig. 7. Morphine and buprenorphine docked to UGT2B7 in the pocket formed by amino acids 96 to 101. Connolly surfaces are shown for the peptide. Both Connolly surfaces and a stick representation are shown for the opioids. A, morphine with its 6-OH group exposed. B, buprenorphine with its 3-OH group exposed.

or 6-OH positions exposed, depending on the position of the phenolic ring. UGT2B7 is the only UGT isoenzyme that is able to catalyze the glucuronidation of opioid 6-OH positions, as in morphine and codeine, the latter having a methyl group on the C3 position. Also, buprenorphine, having a greater affinity for UGT2B7, fits tightly in the pocket, which is consistent with a low K_D value observed previously (Coffman et al., 2001) and with the low K_m value for the enzymatic reaction using the holoprotein UGT2B7 (Coffman et al., 1998).

Further support for the proposed model was demonstrated by studies conducted with a fusion protein of amino acids 24 to 118 of UGT2B7 in which a mutation was produced at amino acid 99. In the model, amino acid 99, an aspartic acid, is proposed to be a factor in the binding of the morphine nitrogen. Insertion of an alanine, which has no charge, for an aspartate, which is negatively charged, led to a marked reduction in morphine binding. Further studies to test the validity of the model are currently underway.

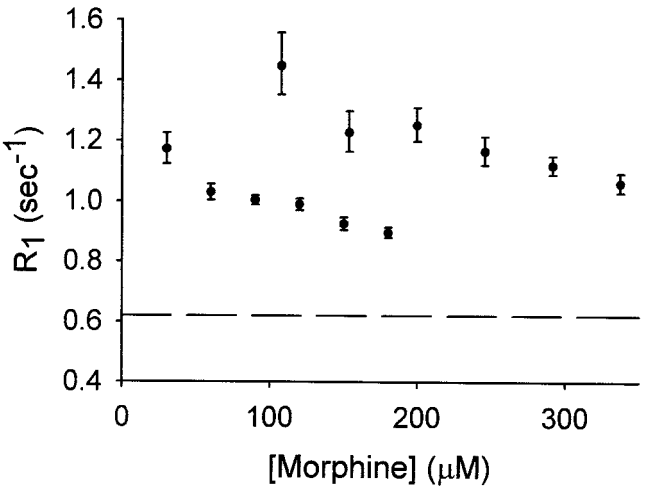

Fig. 8. The longitudinal relaxation rate of morphine H-2 is faster in 2B7F3 (top data set) than in the D99A mutant of 2B7F3 (bottom data set). The dashed line indicates the H-2 relaxation in a solution containing bovine serum albumin.

Acknowledgments

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