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Analysis of Opioid Binding to UDP-Glucuronosyltransferase 2B7 Fusion Proteins Using Nuclear Magnetic Resonance Spectroscopy

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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. In this study, we demonstrate that the opioid binding site in UGT2B7 is within the first 119 amino-terminal amino acids. Two maltose binding protein fusion proteins, 2B7F1 and 2B7F2, incorporating the first 157 or 119 amino acids, respectively, of UGT2B7 were expressed in *Escherichia coli* and purified by affinity chromatography. NMR spectroscopy using one-dimensional spectra, the inversion recovery method, and the transferred nuclear Overhauser effect spectroscopy was used to study the binding properties of opioids to the fusion proteins. Morphine was found to bind at a single site within the first 119 amino acids and to undergo a conformational change upon binding, as demonstrated by transferred nuclear Overhauser effect spectroscopy. Dissociation constants were obtained for morphine, naloxone, buprenorphine, and zidovudine, and the results were confirmed by equilibrium dialysis determinations. Two possible opioid binding sites, based on the nearest neighbors from opioid binding to the μ-receptor and to cytochrome 2D6, are proposed.

The UDP-glucuronosyltransferases (UGTs) (EC 2.4.1.17) are protein products of a multigene family that catalyze the transfer of the glucuronic acid moiety of UDP-glucuronic acid (UDPGlcUA) to numerous endo- and xenobiotics of many chemical classes to form β-D-glucuronides with enhanced hydrophilicity (Mackenzie et al., 1997). In mammals, the UGTs are membrane-bound proteins of approximately 530 amino acid residues residing in the endoplasmic reticulum. The putative transmembrane domain is located near the carboxyl terminus of the protein such that only a small length of the protein resides in the cytosol (Meech and Mackenzie, 1997). The major part of the protein is located in the lumen of the endoplasmic reticulum, including the substrate binding domains and the catalytic site. Furthermore, the residues residing in the lumen may be associated with the inner membrane without traversing the membrane (Meech and Mackenzie, 1998). This has presented a challenge for the purification of UGTs both from animal tissue and from cell culture preparations, especially in the large amounts necessary for structure determination by X-ray crystallography or NMR spectroscopy. To date, no tertiary structure, specific binding constants, or catalytic sites have been established for any UGT isoform.

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 amino acids 55 to 180, a region with least homology of primary sequence between the UGT isoforms (Mackenzie, 1990). It has been demonstrated by photoaffinity labeling of UGT fusion proteins with azido-UDPGlcUA that a UDP binding site is present in the 299-to-466 amino acid region, and that a glucuronic acid binding site is present in the N-terminal region (amino acids 14–150) (Pillot et al., 1993). The results of mutation studies have suggested that the region Arg-Gly-His-Glu conserved in all UGTs is important for catalytic activity (Senay et al., 1997).

Today, more than 30 mammalian isoforms of UDP-glucuronosyltransferase have been identified. The human UGT2B7 isoenzyme is one of the most important. It is expressed in liver, kidney, intestine, colon, testis, prostate, and brain (King et al., 2000). UGT2B7 is an important human isoform that catalyzes with high efficiency the glucuronida-

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ABBREVIATIONS: UGT, UDP-glucuronosyltransferase; UDPGlcUA, UDP-glucuronic acid; AZT, zidovudine; MBP, maltose binding protein; CD, circular dichroism; NOESY, nuclear Overhauser effect spectroscopy; TRNOESY, transferred nuclear Overhauser effect spectroscopy.

tion of opioids, androsterone, catechol estrogens, hyodeoxycholic acid, nonsteroidal anti-inflammatory drugs, AZT, and retinoic acid (Coffman et al., 1998; Ritter et al., 1990; Jin et al., 1993; Barbier et al., 2000; Samokyszyn et al., 2000). This enzyme is, so far, the only enzyme, besides the simian homolog UGT2B9 (Green et al., 1997), that is capable of catalyzing the glucuronidation of both the 3-OH and 6-OH positions of opioids and forming the pharmacologically active morphine-6-glucuronide and codeine-6-glucuronide (Coffman et al., 1997).

In the current study, we have designed two soluble fusion proteins containing the maltose binding protein (MBP) and either the N-terminal 157 amino acids or 119 amino acids of the UGT2B7 isoenzyme. Specific binding of opioids to the UGT2B7 domain of these fusion proteins was demonstrated by NMR spectroscopy and equilibrium dialysis. Binding constants were calculated from the parameters obtained experimentally.

Experimental Procedures

Materials

The pMalc2x vector, maltose binding protein, and amylose resin were obtained from New England Biolabs (Beverly, MA). [^{3}H]Diprenorphine (57 Ci/mmol) and [^{3}H]naloxone (56 Ci/mmol) were from Amersham Pharmacia Biotech (Piscataway, NJ). All other chemicals were from Sigma Chemical Co. (St. Louis, MO). Dialysis membranes were purchased from Amika Corp. (Columbia, MD). Protein assay reagents were provided by Bio-Rad (Hercules, CA).

Construction of Expression Plasmids and Production of Recombinant Protein

The cDNA encoding a fragment of UGT2B7 that codes for amino acids (24–180) was subcloned by polymerase chain reaction from the cDNA encoding the full-length UGT2B7 (Coffman et al., 1998). The primers included the restriction enzyme sites *Eco*RI and *Sal*I on the 5′ and 3′ ends, respectively. The polymerase chain products were ligated into the *Eco*RI/*Sal*I site of the bacterial expression vector pMalc2x and the sequence was verified by DNA sequencing. The recombinant protein was expressed in Topp3 *Escherichia coli* cells obtained from Stratagene (La Jolla, CA). The carboxyl end of the MBP was joined to the N terminus of the UGT2B7 fragment, creating a fusion protein, 2B7F1. The cDNA encoding for 2B7F2, a fusion protein truncated at amino acid 142, was generated by insertion of a stop codon mutation in the plasmid encoding for 2B7F1.

The expression strain was grown at 25°C in LB media containing 100 μg/ml ampicillin to an A_{600} of 0.6 to 1.0. The protein expression was then induced with 1 mM isopropyl-1-thio-β-galactoside and the cells were harvested after 4 to 7 h. The cells were lysed and the supernatant was collected following the procedure described in the manufacturer's manual. Affinity purification of the MBP-tagged fusion proteins 2B7F1 and 2B7F2 was performed under nondenaturing conditions on amylose resin, as described in the manufacturer's manual. The fusion proteins were concentrated and necessary buffer exchanges were performed using Amicon stirred cell concentrators with YM 30 filters. All protein concentrations were measured by the Bio-Rad method or by measuring the absorption at 280 nm.

Circular Dichroism (CD) Spectroscopy

The CD spectra of 2B7F1, 2B7F2, and MBP were acquired on an AVIV 62DS spectrapolarimeter (Lakewood, NJ) at 25°C. The far-ultraviolet spectra (200–260 nm) were measured using a 1-cm path length quartz cell and averaged over three accumulations. The mean molar residue ellipticity was calculated after subtraction of the buffer contribution (50 mM phosphate buffer, pH 8). Preliminary experiments showed that the spectra were pH-dependent, showing less helical content at acid pH (pH 4.3) than at a pH above 7.4 (pH 7.4–9.3). Therefore, all subsequent experiments were performed at pH 8.

NMR Spectroscopy

All spectra were collected on the Varian INOVA-500 500-MHz spectrometer (Palo Alto, CA) in the College of Medicine NMR Facility at the University of Iowa (Iowa City, IA.) Spectra were processed using Varian's VNMR 6.1B software. 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. One-dimensional spectra were baseline corrected using the spline-function algorithm in VNMR 6.1B. 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 but the acquisition time.

Proton NMR spectra for lineshape experiments were obtained using a 3-s relaxation delay and a 3-s acquisition time. For the solution of morphine in buffer, 256 transients were averaged. Transients (4096) were averaged in the solutions containing morphine with 2B7F1, 2B7F2, or MBP. Spectra were zero filled at 131,072 points and apodized by a 3-Hz exponential line-broadening function before measurements.

Longitudinal relaxation rates were measured using the inversion recovery method (Vold et al., 1968). The number of transients averaged per trace ranged from 2048 at low morphine concentrations to 128 at the highest morphine concentrations. Recovery delays ranging from 0.01 to 10 s were used. The relaxation time (T_1) was extracted for the hydrogens on the carbons in positions C7 and C8 (H7 and H8) of morphine by the standard functions in the VNMR software.

K_D values for species other than morphine were measured by monitoring their effect on the binding of morphine to the fusion protein. The relaxation times (T_1) of the hydrogen atom on carbons 7 and 8 in the morphine molecule (H7 and H8) were first measured in a solution containing 12 μM protein and 60 μM morphine. An aliquot of a second aglycone was then added and the T_1 values were measured again. Concentrations of the aglycones were estimated to give a clear change in the value of morphine T_1 if binding occurred.

Transferred nuclear Overhauser enhancements were measured using spectral parameters of 1024 complex points in the f2 dimension and 512 complex points in the f1 dimension. Gaussian functions, shifted by 30° for f2, were used for apodization in both dimensions. Interproton distances were calculated by relaxation matrix analysis (Borgias and James, 1989; Campbell and Sykes, 1991).

Analysis of Relaxation Data. In the fast exchange limit for a single-site binding model, the measured relaxation rate, ($R_1 = 1/T_1$) is calculated as follows:

$$R_{1,\mathrm{obs}} = \chi_{\mathrm{bound}} R_{1,\mathrm{bound}} + \chi_{\mathrm{free}} R_{1,\mathrm{free}} = (1 - \chi_{\mathrm{free}}) R_{1,\mathrm{bound}} + \chi_{\mathrm{free}} R_{1,\mathrm{free}}. \quad (1)$$

The R_1 values are the appropriate relaxation rates and the χ values are the mole fractions of bound (ap) and free aglycone (a) in the presence of protein (p). This equation can be solved for

$$\chi_{\mathrm{free}} = (R_{1,\mathrm{obs}} - R_{1,\mathrm{bound}})/(R_{1,\mathrm{free}} - R_{1,\mathrm{bound}}), \quad (2)$$

using the equilibrium constant expression

K_D = [a] [p]/[ap] for the binding reaction and [ap]

= [a] + [p] for the conservation relations

It is then found that

$$\chi_{\mathrm{free}} = \{C_a - C_p - K_D + [(C_p - C_a + K_D)^2 + 4 K_D C_a]^{1/2}\}/2C_a. \quad (3)$$

Once a set of $R_{1,\mathrm{obs}}$ has been determined as a function of C_a a value for χ_{free} is determined for each concentration of a using eq. 2. The K_D

value is then determined by a nonlinear least-squares fit of eq. 3 to the experimental determined χ_{free}. $R_{1,bound}$ of 56 s^{-1} was used for 2B7F1, and 52 s^{-1} for 2B7F2.

Analysis of Competitive Binding Data. Binding constants for aglycones in the competitive binding experiments were calculated indirectly from the mole fraction of displaced morphine. Changes in the morphine R_1 were used to monitor competitive binding of a second ligand as described above. $R_{1,m}$ is the relaxation rate of the morphine in the presence of protein only, and $R_{1,ma}$ is the relaxation rate of morphine in the presence of the protein and another aglycone. From these values and knowing the analytic concentrations of the three species and the K_D value for morphine, the K_D values for binding other aglycones can be estimated. The mole fraction χ_{free} of free morphine in a solution containing only morphine and protein is calculated using eq. 3. After addition of the second aglycone (a), the observed morphine R_1 is converted to a mole fraction of free morphine $\chi_{f,\ ma}$ using eq. 2. The concentration of the morphine-protein complex in solutions without the second aglycone, $[mp]_{ref} = C_m(1 - \chi_{f,\ ref})$. On adding the second aglycone, the concentration of bound morphine, $[mp]_{ma} = C_m(1 - \chi_{f,\ ma})$.

Assuming that all the aglycones bind to the same site, and that only one molecule binds to each site, the concentration of the protein complex with the second aglycone is equal to the difference between the bound concentrations measured for morphine in the presence and absence of the second aglycone $[ap] = [mp]_{ref} - [mp]_{ma}$.

Using the conservation relations for the second aglycone and the protein,

$$[a] = C_a - [ap] = C_a - [mp]_{ref} + [mp]_{ma} \quad (4)$$

$$[p] = C_p - [ap] - [mp]_{ma} = C_p - [mp]_{ref}.$$

and

(5) $K_{D,a} = [a]\,[p]$

$$/[ap] = (C_a - [mp]_{ref} + [mp]_{ma})(C_p - [mp]_{ref})/([mp]_{ref} - [mp]_{ma}).$$

This leads to an experimentally accessible expression for the K_D of the second aglycone ($K_{D,a}$) seen below in eq. 5.

Determination of Opioid Binding to MBP-2B7 Fusion Proteins Using Equilibrium Dialysis

Equilibrium dialysis for [^{3}H]diprenorphine binding to 2B7F1 and 2B7F2 fusion proteins were conducted in 50 mM sodium phosphate buffer pH 6.5, with 0.2 μM protein and varying concentrations of diprenorphine (1–100 μM). The samples were dialyzed against an equal volume of 50 mM sodium phosphate buffer, pH 6.5, for six h at room temperature. Dissociation constants were determined using nonlinear regression analysis. The dissociation constant for buprenorphine binding was determined by its ability to displace diprenorphine binding. Equilibrium dialysis for [^{3}H]naloxone binding to the fusion proteins were conducted similarly, except sodium phosphate buffer pH 8.0 was used and the concentrations of naloxone were varied from 5 to 500 μM.

Results

Expression and Characterization of Soluble Fusion Proteins with N-Terminal Fragments of UGT2B7. The two fusion proteins 2B7F1 and 2B7F2 were designed without the signal peptide (amino acids 1–23) to prevent membrane association in the *E. coli* system. The proteins were expressed in the cytosol and purified as described under *Experimental Procedures* at a yield of 50 to 75 mg/l. The molecular masses of the fusion proteins, 61 and 55 kDa, respectively, were verified on SDS-polyacrylamide gel electrophoresis. The purity was estimated to about 95 to 98%. Both fusion proteins reacted with sheep anti-rabbit PNP-UGT antibody, which reacts with the UGT2B7 holoprotein (Coffman et al., 1998) (data not shown). The amino acid sequence and the predicted secondary structure of the UGT2B7 fragment of the fusion proteins are shown in Fig. 1.

The maltose binding protein and the two fusion proteins were analyzed by CD spectroscopy (Fig. 2). All three proteins exhibited α-helix structures. These data were used for verification that the conditions used for performing NMR-spectroscopy and equilibrium dialysis were nondenaturing.

NMR Spectroscopy of Opioid Binding to the Expressed Fusion Proteins. An NMR experiment was performed to establish whether NMR spectroscopy would give the proper information necessary to establish binding and to calculate binding constants. The result of this experiment is shown in Fig. 3. The proton spectra of 2B7F1, morphine alone and morphine in the presence of 2B7F1 are shown. The peaks, which represent hydrogen 7 and 8, correspond to chemical shifts of 5.7 and 5.4 ppm, respectively. These peaks are well outside the range where the observed protein spectra could obscure data. These peaks are broadened in the presence of the protein, showing that morphine binds to the protein in fast exchange. The peak with chemical shift of 5.68 ppm (H7) was used for the study of the relaxation rates of morphine in the presence of the fusion proteins. H7 was chosen over H8 for analysis because the baseline was less variable in this region.

Figure 4 shows the dependence of the relaxation rate on morphine concentration in the presence of a fixed 2B7F1 concentration. The relaxation rate decreases monotonically with increasing morphine concentration, asymptotically approaching the value for free morphine. This relationship was calculated using varying K_D values for morphine binding to the protein using eqs. 2 and 3 under *Experimental Procedures*. In addition to the one-site model of eqs. 2 and 3, a two-site model was investigated. This model did not give convergent fits, indicating that morphine is most probably bound to a single site. The resulting curves were fitted to the experimental data. The best fit obtained from the single-site model allowed for a calculation of a K_D value of 91 μM. A K_D value of 100 μM for morphine binding to 2B7F2 was also

```
                                        Motif A
    24 GKVLVWAAEY SHWMNIKTIL DELIQRGHEV TVLASSASIL
PHD    LLEEEE.LL. ..HHHHHHHH HHHHHHLL.E EEEE......

               Motif B
    64 FDPNNSSALK IEIYPTSLTK TELENFIMQQ IKRWSDLPKD
PHD    ..LLLLLL.. .......LL. HHHHHHHHHH HH.H...L..

                                                Motif C
   104 TFWLYFSQVQ EIMSIFGDIT RKFCKDVVSN KKFMKKVQES
PHD    ..HHHHHHHH HHHHHHHHHH HHHHHHHH.. HHHHHHHH..


   144 RFDVIFADAI FPCSELLAEL FNIPFVYSLS FSPGYTF
PHD    LL.EEE.LLL L.HHHHHHHH ..LL.EEEEE .LLL...
```

Fig. 1. Amino acid sequence of the fusion protein 2B7F1. The following motifs are underlined: motif A, the possible binding site for the glucuronic acid moiety of UDPGlcUA; motif B, the proposed retinoic acid binding site; motif C, the proposed steroid binding site. The arrow indicates the carboxyl end of 2B7F2. The secondary structure predicted by the PHDsec program from CUBIC (Columbia University, New York, NY) is also shown (Rost and Sander, 1993). Residues, where the predicted accuracy was <82%, were left unassigned. H, α-helix; E, β-sheet; L, loop.

obtained experimentally. The relaxation rate observed for morphine in the presence of MBP was the same as for free morphine, indicating that no binding occurs to MBP.

Specific binding constants for other aglycones were calculated using the morphine displacement method described under *Experimental Procedures*. Figure 5 shows how the relaxation rate of morphine bound to 2B7F1 decreases with the addition of increasing amounts of naloxone. Similar curves were obtained for other aglycones. The dissociation constants determined for buprenorphine, AZT, and naloxone based on displacement studies are shown in Table 1. In addition, androsterone glucuronide or glucose were added to the morphine-2B7F1 solution, but these compounds had no effect on the morphine relaxation rate, indicating that the K_D values for these compounds are considerably larger than 1 mM at the morphine binding site or that no competitive binding resulted (Table 1).

Binding Constants of Opioids Measured by Equilibrium Dialysis. Equilibrium dialysis studies allowed for the determination of a K_D value of 7.5 μM with diprenorphine and 2B7F1. The K_D value for naloxone was calculated to be 50 μM for binding to either fusion proteins. Using the displacement method, a K_D value of 10 μM was found for buprenorphine. These values are in very good agreement with those established by NMR spectroscopy.

Transferred Nuclear Overhauser Effects. The NOESY spectrum of 1 mM morphine (Fig. 6A) showed positive diagonal peaks, and negative off-diagonal peaks as expected for a

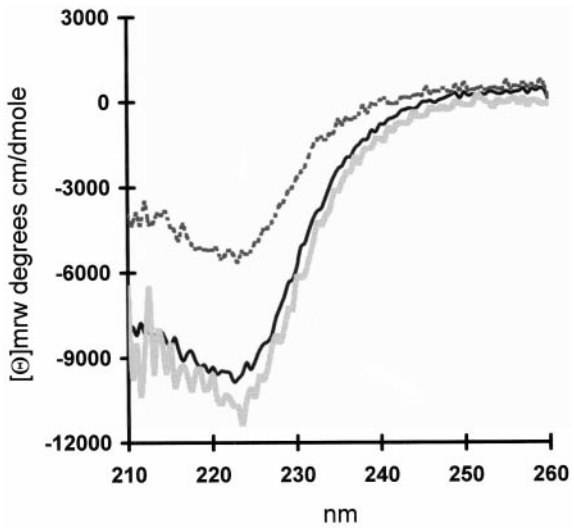

Fig. 2. CD spectra of maltose binding protein (dashed line), 2B7F1 (solid black line), and 2B7F2 (solid gray line). The conditions were 1 nM protein in 50 mM phosphate buffer at pH 8 for all three spectra.

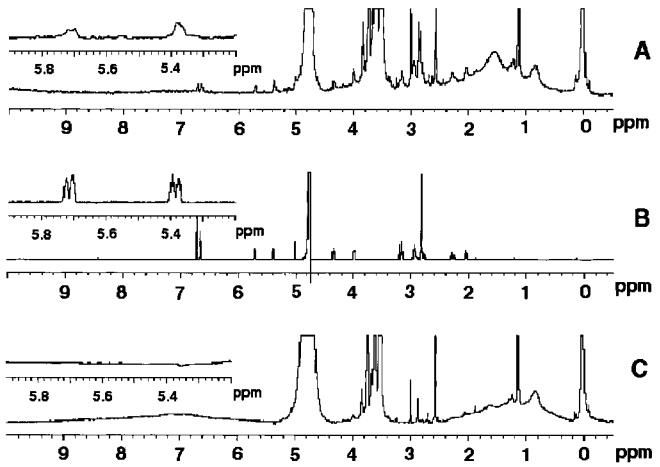

Fig. 3. Proton NMR spectra of 80 μM morphine in the presence of 60 μM 2B7F1 (A), 1 mM morphine (B), and 60 μM 2B7F1 (C). The samples were in phosphate buffer at pH 8.

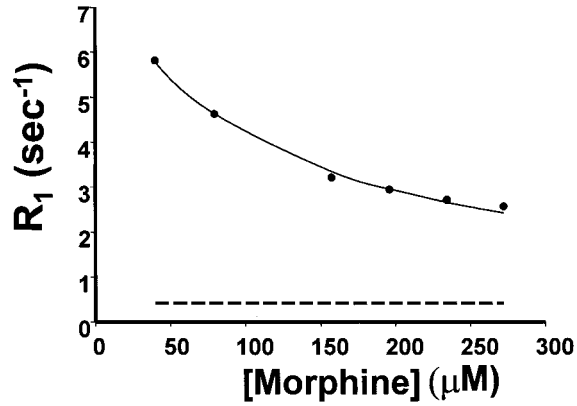

Fig. 4. Relationship between the relaxation rate and concentration of morphine in the presence of 60 μM 2B7F1. The solid dots represent experimentally determined relaxation rates. The S.E. values are smaller than the radius of the dots. The dashed line is the relaxation rate of free morphine. The samples were in 50 mM phosphate buffer at pH 8.

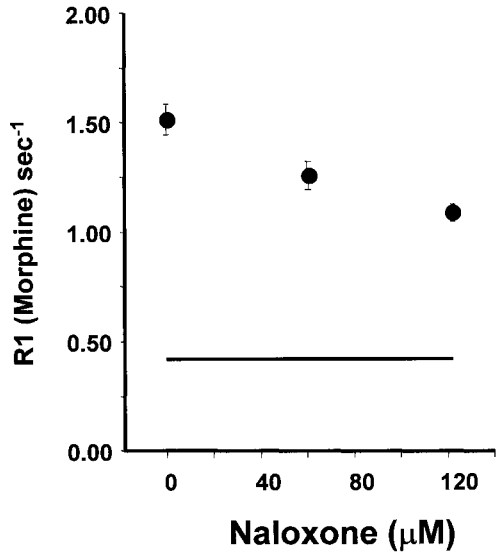

Fig. 5. Relationship between the relaxation rate of morphine in the presence of 2B7F1 and increasing concentration of naloxone. The concentrations of morphine and 2B7F1 were constant at 61 and 12 μM, respectively. The samples were in 50 mM phosphate buffer at pH 8.

TABLE 1

Dissociation constants of opioid binding to fusion proteins 2B7F1 and 2B7F2 as measured by NMR spectroscopy

	2B7F1	2B7F2
	μM	
Morphine	91	100
Naloxone	50	N.D.
Buprenorphine	10	N.D.
AZT	100	N.D.
Glucose	>1000	N.D.
Androsterone glucuronide	>1000	N.D.

N.D., not determined.

small molecule. In the TRNOESY spectrum (Fig. 6B) of a solution containing 1 mM morphine and 10 μM 2B7F1 both the diagonal and off-diagonal peaks are negative. These data provide further evidence of specific binding of morphine to the fusion protein, because the change of sign of the diagonal peaks relative to the off-diagonal peaks is due to a large increase in the average rotational correlation time for morphine (Balaram et al., 1972; Anderson et al., 1987). As shown in Fig. 6, A and B, the patterns of cross peaks are quite different in the two spectra. This indicates that a conformational change occurs in morphine upon binding to the protein. Furthermore, it can be seen, when the two spectra are compared, that several new peaks are present in the TRNOESY spectrum (Fig. 6B) that were not observable in the NOESY spectrum (Fig. 6A). Thus, distances between the morphine hydrogens have changed upon binding to 2B7F1 due to a conformational change in the morphine molecule (Balaram et al., 1972; Anderson et al., 1987).

Discussion

This study was designed to explore the aglycone binding region of UGT2B7. For this purpose the generation of a fusion protein containing the first 157 amino acids from the amino terminus of UGT2B7 and maltose binding protein were produced. In the current study the fusion protein was isolated from the cytosol of the *E. coli*. This fusion protein was used to explore the interaction of opioid compounds, which have been shown to be excellent substrates for UGT2B7, and presumably, good ligands with a UGT2B7 aglycone binding domain or domains. Among the opioids, morphine is a very suitable compound to use in NMR spectroscopic binding studies, because the chemical shifts for hydrogen H7 and H8 of morphine lie in a region free from interference of other substances, binding of morphine to 2B7F1 occurs in fast exchange and the morphine binding constant is in a range where displacement of the ligand can be readily observed.

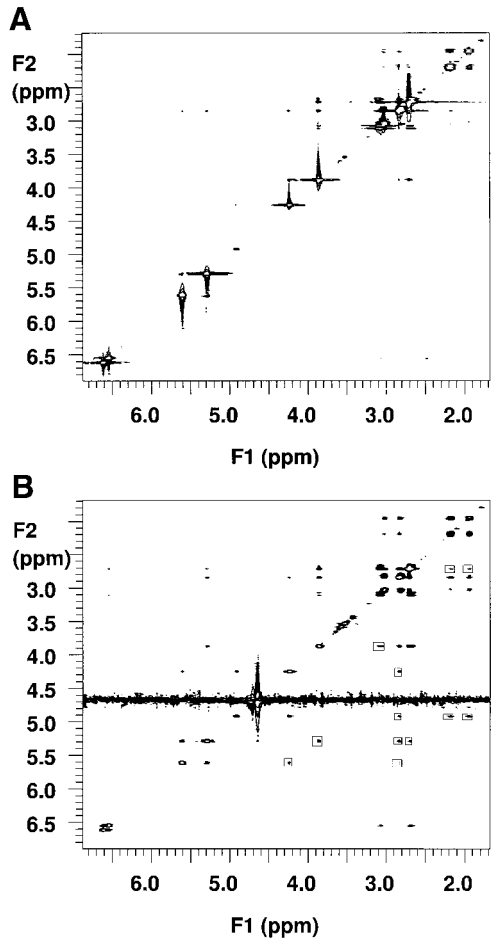

Fig. 6. A, NOESY spectrum of 1 mM morphine. B, TRNOESY spectrum of 1 mM morphine in the presence of 12 μM 2B7F1. The mixing time was 300 ms for each spectrum. Occupied rectangular boxes in B indicate transferred nuclear Overhauser effect peaks that are absent in A.

Binding of morphine to the fusion proteins was confirmed in four different ways using NMR spectroscopy techniques: broadening of the chemical shifts peaks in the one-dimensional spectrum, increase in relaxation rate compared with free morphine, displacement of morphine by other ligands, and the presence of transferred nuclear Overhauser enhancement. There was no observed interaction of morphine with MBP alone, and compounds such as androsterone glucuronide and glucose gave no measurable binding parameters with 2B7F1.

The binding constants obtained for morphine using NMR spectroscopy are very similar for both fusion proteins, indicating that the opioid binding site is within the first 24 to 142 amino acids of UGT2B7. The dissociation constants for morphine, naloxone, and buprenorphine binding to 2B7F1 are similar to the K_m values obtained from kinetic studies with the full-length UGT2B7 (Coffman et al., 1998). Also there is very good agreement between results using the two different methods, NMR spectroscopy and equilibrium dialysis. Using NOESY-TRNOESY NMR spectroscopy it was possible to show that the morphine molecule binds specifically to the fusion protein and this opioid seems to undergo a conformational change upon binding. Due to the weakness of some of the signals it was not possible to determine all the coordinates of the new conformation state; however, it has been observed for nalorphine bound to anti-opioid monoclonal antibodies that the *N*-allyl group is forced into an axial isomeric configuration, whereas the equatorial confirmation is predominant in solution (Glasel and Borer, 1986).

Previous structure-function analyses (Pillot et al., 1993; Meech and Mackenzie, 1997; Senay et al., 1997) have established that the binding sites for the UDP moiety of UDPGlcUA are probably in a region conserved among all UGTs in the carboxyl-terminal end. However, a study by Pillot et al. (1993) suggested that the glucuronic acid moiety of UDPGlcUA is bound to a conserved region of the N terminus of UGTs. Most UGTs have the conserved motif GHEV/T (Fig. 1, motif A) in the N-terminal region, and Senay et al. (1997) have suggested that this motif is important for structural or catalytic function. A similar motif, GVKAT, is present in the solanidine UDP-glucose glucosyltransferase (Moehs et al., 1997). This suggests that the UGT protein is folded in such a manner that UDPGlcUA spans the carboxyl and N-terminal regions of the molecule, and thus brings the glucuronic acid moiety into proximity with the N-terminal aglycone binding site. This hypothesis is supported by other studies with UDP-galactose-4-epimerase (PDB 1KVQ), where the sugar is bound to lysine 84 and valine 86 in the motif GVKAV. In this protein, the uracil ring of UDP-glucose is bound to alanine

216 and phenylalanine 218 in the motif LAIF, which is present at the carboxyl end of nearly all UGTs.

Moehs et al. (1997) have suggested, using sequence homology, that the region amino acids 134 to 168 in UGT2B7 (Fig. 1, motif C) is a possible steroid-binding site. Our data show that this region is not necessary for opioid binding based on the observation of morphine binding to 2B7F2 with similar affinity compared with 2B7F1. Likewise, the region of amino acids 72 to 104 of UGT2B7 (Fig. 1, motif B) has been identified by Radominska-Pandya et al. (1999) as a possible binding site for retinoic acid. The results of our study are consistent with this hypothesis.

Opioids are especially unique as substrates for studying protein-ligand interactions. They are capable of binding to certain proteins by forming an ion pair between the basic nitrogen of the opioid and a negatively charged group, such as from aspartic acid or glutamic acid. The binding of codeine to CYP2D6 has been studied by NMR and a binding site has been proposed to have the opioid nitrogen, forming an ion pair with a charged carboxyl oxygen of an aspartic acid monomer (Modi et al., 1996). In addition, modeling and mutation studies have led to a proposed binding site of opioids to the μ-receptor in the brain, which also involves an aspartic acid monomer (Sagara et al., 1996; Mansour et al., 1997). The proposed binding sites of both CYP2D6 and the μ-receptor also contain a basic monomer, a hydroxyl- or sulfur-containing monomer and hydrophobic amino acids (Modi et al., 1996; Sagara et al., 1996; Mansour et al., 1997). Such a motif (D, E), (R, K), (S, T, or C), and (F, W, A, V, L, I) was identified on UGT2B7 in the nonconserved region of the N-terminal domain from amino acid 93 to 105. This motif is within the proposed retinoic acid binding site (Radominska-Pandya et al., 1999) and is located within the loop of a predicted short helix-loop-long helix secondary structure (Fig. 1). Another possible site for this motif is amino acids 116 to 132 located within the end of the long helix. Using the Helical Wheel (http://marqusee9.berkeley.edu/kael/helical.htm), it can be shown that in this region, D 129, S132, and K125 are in proximity. Our NMR studies are consistent with a one-opioid binding site model. Studies are in progress to distinguish between the two sites proposed here.

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