Residues in the Fifth Membrane-Spanning Segment of the Dopamine D2 Receptor Exposed in the Binding-Site Crevice†

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ABSTRACT: The binding site of the dopamine D2 receptor, like that of other homologous G-protein-coupled receptors, is contained within a water-accessible crevice formed among its seven membrane-spanning segments. Using the substituted-cysteine accessibility method, we previously mapped the residues in the third membrane-spanning segment (M3) that are exposed in the binding-site crevice [Javitch et al. (1995) *Neuron 14*, 825]. We have now mutated, one at a time, 24 consecutive residues in and flanking the fifth membrane-spanning segment (M5) to cysteine and expressed the mutant receptors in HEK 293 cells. Thirteen of these mutants reacted with charged, hydrophilic, lipophobic, sulfhydryl-specific reagents, added extracellularly, and were protected from reaction by another reversible dopamine antagonist, sulpiride. Thus, the side chains of these residues are exposed in the binding-site crevice. Of the 13 exposed residues, 10 are consecutive, from Phe189 to Phe198. This pattern of exposure is inconsistent with the expectation that M5, like M3, forms a fixed α-helix, one side of which is exposed in the binding-site crevice. The exposed region of M5, which contains the serines likely to bind agonist [Strader et al. (1989) *J. Biol. Chem. 264*, 13752], might loop out into the lumen of the binding-site crevice and be completely accessible to water and thus to MTSEA. Alternatively, the exposed region of M5 might be embedded in the membrane and also in contact with other membrane-spanning segments. At any instant, only a limited set of residues might be exposed in the binding-site crevice; however, M5 might move rapidly to expose different sets of residues.

The dopamine receptors, like the homologous receptors for the biogenic amines and for acetylcholine, bind neurotransmitters present in the extracellular medium and couple this binding to the activation of intracellular G-proteins (Civelli et al., 1991; Strader et al., 1994). The binding sites of these receptors are formed among their seven, mostly hydrophobic, membrane-spanning segments (Oprian, 1992; Strader et al., 1994) and are accessible to charged, water-soluble agonists, like dopamine. Thus, for each of these receptors, the binding site is contained within a water-accessible crevice, the binding-site crevice, extending from the extracellular surface of the receptor into the plane of the membrane. The surface of this crevice is formed by residues that contact specific agonists and/or antagonists and by other residues that may play a structural role and affect binding indirectly.

To identify the residues that form the surface of the binding-site crevice in the human D2 receptor, we have adapted a new approach, the substituted-cysteine accessibility method (SCAM)[1] (Akabas et al., 1992, 1994; Javitch et al., 1995). Consecutive residues in the membrane-spanning segments are mutated to cysteine, one at a time, and the mutant receptors are expressed in heterologous cells. If ligand binding to a cysteine substitution mutant is near-normal, we assume that the structure of the mutant receptor, especially around the binding site, is similar to that of wild type and that the substituted cysteine lies in an orientation similar to that of the wild-type residue. In the membrane-spanning segments, the sulfhydryl of a cysteine can face either into the binding-site crevice, into the interior of the protein, or into the lipid bilayer; only sulfhydryls facing into the binding-site crevice should be accessible to hydrophilic, lipophobic, sulfhydryl-specific reagents. For such reagents, we use derivatives of methanethiosulfonate (MTS): positively charged MTS ethylammonium (MTSEA) and MTS ethyltrimethylammonium (MTSET) and negatively charged MTS ethylsulfonate (MTSES) (Stauffer & Karlin, 1994). These reagents are about the same size as dopamine, with maximum dimensions of approximately 10 Å by 6 Å. They form mixed disulfides with the cysteine sulfhydryl, covalently linking $-SCH_2CH_2X$, where X is NH_3^+, $N(CH_3)_3^+$, or SO_3^-. We use two criteria for identifying an engineered cysteine as being exposed in the binding-site crevice: (i) the reaction with an MTS reagent alters binding irreversibly; (ii) this reaction is retarded by the presence of agonists or antagonists.

We previously found that antagonist binding to wild-type D2 receptor was irreversibly inhibited by MTSEA and MTSET and that Cys118, in the M3 membrane-spanning segment, was responsible for this sensitivity (Javitch et al., 1994). Therefore, we used the mutant C118S, which is insensitive to MTS reagents, as the starting point for further

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[1] Abbreviations: SCAM, substituted-cysteine accessibility method; M3, the third membrane-spanning segment; M5, the fifth membrane-spanning segment; MTS, methanethiosulfonate; MTSEA, MTS ethylammonium; MTSET, MTS ethyltrimethylammonium; MTSES, MTS ethylsulfonate.

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mutation. In our initial application of SCAM to the D2 receptor (Javitch et al., 1995), we found that 10 of 23 residues tested in the M3 segment were exposed in the binding-site crevice. From the pattern of exposure, we inferred that M3 forms an α-helix, one side of which faces the binding-site crevice.

Previous studies, first in the homologous β-adrenergic receptor and later in D2 receptor, have suggested that serine residues in M5 directly interact with the catechol hydroxyls of catecholamine agonists (Strader et al., 1989; Cox et al., 1992; Mansour et al., 1992). To identify all the residues in M5 that contribute to the binding-site crevice, we applied SCAM.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. Cysteine mutations were generated as described previously (Javitch et al., 1995). Mutations were confirmed by DNA sequencing. Mutants are named as (wild-type residue)(residue number)(mutant residue), where the residues are given in the single-letter code.

Transient Transfection. HEK 293 cells were grown in DMEM/F12 (1:1) containing 3.15 g/L glucose in 10% bovine calf serum at 37 °C and 5% CO_2. Thirty-five mm dishes of 293 cells at 60−80% confluence were cotransfected with 1 μg of pcD_2 or mutant pcD_2 and 0.2 μg of pRSVTag using 9 μL of lipofectamine (Gibco) and 1 mL of OPTIMEM (Gibco). Five hours after transfection, the plates were diluted with 1 mL of media containing 20% bovine calf serum. Twenty-four hours after transfection the media were changed. Forty-eight hours after transfection, cells were washed with phosphate-buffered saline (PBS; 8.1 mM NaH_2PO_4, 1.5 mM KH_2PO_4, 138 mM NaCl, 2.7 mM KCl, pH 7.2), briefly treated with PBS containing 5 mM EDTA, and then dissociated in PBS. Cells were pelleted at 1000*g* for 5 min at 4 °C, and resuspended for binding or treatment with MTS reagents.

[^{3}H]-N-Methylspiperone Binding. Whole cells were resuspended in 450 μL of buffer A (25 mM HEPES, 140 mM NaCl, 5.4 mM KCl, 1 mM EDTA, and 0.006% BSA, pH 7.4). Cells were then diluted 20-fold with buffer A. [^{3}H]-*N*-Methylspiperone (Dupont/NEN) binding was determined by a modification of reported procedures (Javitch et al., 1995). For saturation binding, duplicate borosilicate tubes contained six different concentrations of [^{3}H]-*N*-methylspiperone between 5 and 400 pM in buffer A with 300 μL of cell suspension in a final volume of 0.5 mL. The mixture was incubated at room temperature for 60 min and then filtered using a Brandel cell harvester through Whatman 934AH glass fiber filters (Brandel). The filter was washed twice with 5 mL of 10 mM Tris-HCl and 120 mM NaCl, pH 7.4 at room temperature. Specific [^{3}H]-*N*-methylspiperone binding was defined as total binding less nonspecific binding in the presence of 1 μM (+)-butaclamol (Research Biochemicals).

Reactions with MTS Reagents. For treatment with MTS reagents, whole cells were resuspended in 450 μL of buffer A. Aliquots (50 μL) of cell suspension were incubated with MTS reagents at the stated concentrations at room temperature for 2 min. Cell suspensions were then diluted 20-fold, and 300 μL aliquots were used to assay for [^{3}H]-*N*-methylspiperone (300 pM) binding as described above. The fractional inhibition was calculated as 1 − [(specific binding after MTS reagent)/(specific binding without reagent)]. We used the SPSS for Windows (SPSS, Inc.) statistical software to analyze the effects of the MTS reagents by one-way ANOVA according to Student−Newman−Keuls criteria ($p < 0.05$).

The second-order rate constant (k) for the reaction of MTSEA with each susceptible mutant was estimated by determining the extent of reaction after a fixed time, 2 min, with four concentrations of MTSEA, between 0.01 and 2.5 mM (all in excess over the quantity of reactive sulfhydryls). The fraction of initial binding, Y, was fit to e^{-kct}, where k is the second-order rate constant, c is the concentration of MTSEA, and t is the time (120 s).

Table 1: Characteristics of [^{3}H]-*N*-Methylspiperone Binding to the Cysteine-Substituted Dopamine D2 Receptor[a]

mutant	K_D (pM)	K_{MUT}/K_{C118S}	B_{max} (fmol/cm^2)	n
C118S	88 ± 9	1.0	180 ± 18	3
P187C	140 ± 24	1.6	78 ± 4	2
A188C	no binding detected			
F189C	67 ± 1	0.8	51 ± 9	2
V190C	110 ± 17	1.2	49 ± 18	2
V191C	77 ± 16	0.9	29 ± 1	2
Y192C	76 ± 8	0.9	44 ± 2	2
S193C	33 ± 6	0.4	80 ± 19	2
S194C	85 ± 10	1.0	100 ± 18	2
I195C	75 ± 7	0.9	77 ± 10	3
V196C	65 ± 16	0.7	81 ± 33	2
S197C	35 ± 8	0.4	20 ± 2	2
F198C	560 ± 55	6.4	53 ± 10	2
Y199C	110 ± 12	1.2	180 ± 26	2
V200C	160 ± 42	1.8	220 ± 49	2
P201C	690 ± 370	7.8	69 ± 17	2
F202C	80 ± 37	0.9	280 ± 63	3
I203C	57 ± 15	0.6	170 ± 17	3
V204C	92 ± 8	1.0	270 ± 33	3
T205C	92 ± 2	1.0	330 ± 3	2
L206C	83 ± 15	0.9	210 ± 10	3
L207C	74 ± 9	0.8	210 ± 10	3
V208C	120 ± 38	1.4	110 ± 55	2
Y209C	140 ± 36	1.6	170 ± 22	2
I210C	120 ± 40	1.4	200 ± 72	2

[a] Cells transiently transfected with the appropriate receptor were assayed as described in Experimental Procedures. Data were fit to the binding isotherm by nonlinear regression. The means and SEM are shown for n independent experiments, each with duplicate determinations. B_{max} values are presented as femtomoles per square centimeter of plate area.

RESULTS

Effects of Cysteine Substitution on Antagonist Binding. In a background of the mutant C118S, we mutated to cysteine, one at a time, 24 consecutive residues, Pro187 to Ile210, in and flanking M5. Each mutant receptor was transiently expressed in HEK 293 cells, and the K_D and B_{max} characterizing the equilibrium binding of the radiolabeled antagonist, [^{3}H]-*N*-methylspiperone, were determined. At 21 positions, the K_D of the cysteine substitution mutant was between 0.4 and 2 times the K_D of C118S (Table 1). For the mutants F198C and P201C, the K_D was 6−8-fold greater than that of C118S. For these 23 mutants, B_{max} ranged from 10% to 180% of that obtained with C118S (Table 1). Only the mutant A188C failed to bind [^{3}H]-*N*-methylspiperone.

The K_I of the antagonist sulpiride was determined in the 23 mutants which bound [^{3}H]-*N*-methylspiperone (Table 2). At 18 positions, the K_I was between 0.6 and 3 times the K_I

Table 2: Inhibitory Potency of (−)-Sulpiride on [^{3}H]-*N*-Methylspiperone Binding to the Cysteine-Substituted Dopamine D2 Receptor[a]

mutant	apparent K_I (nM)	n	$K_{I(MUT)}/K_{I(C118S)}$
C118S	8 ± 1	5	1.0
P187C	26 ± 7	2	3.1
F189C	180 ± 16	3	21.8
V190C	21 ± 5	2	2.5
V191C	16 ± 4	3	1.9
Y192C	17 ± 12	2	2.1
S193C	0.7 ± 0.1	3	0.1
S194C	17 ± 5	2	2.0
I195C	16 ± 4	2	1.9
V196C	7 ± 2	2	0.8
S197C	110 ± 38	3	13.7
F198C	39 ± 16	3	4.7
Y199C	9 ± 3	2	1.1
V200C	12 ± 2	2	1.5
P201C	2100 ± 980	3	257.6
F202C	7 ± 0	2	0.8
I203C	6 ± 1	2	0.8
V204C	9 ± 2	2	1.1
T205C	5 ± 0	2	0.6
L205C	8 ± 1	2	0.9
L207C	5 ± 1	2	0.6
V208C	16	1	2.0
Y209C	20 ± 4	2	2.5
I210C	8 ± 1	2	0.9

[a] Cells transiently transfected with the appropriate receptor were assayed with [^{3}H]-*N*-methylspiperone (80 pM) as described in Experimental Procedures in the presence of nine concentrations of (−)-sulpiride. The apparent K_I was determined by the method of Goldstein and Barrett (1987) using the IC_{50} value obtained by fitting the data to a one site competition model by nonlinear regression. The means and SEM are shown for n independent experiments, each with duplicate determinations.

of C118S. For mutants F189C, S197C, F198C, and P201C, the K_I was 5−260 times the K_I of C118S. The K_I of S193C was decreased 10-fold; i.e., the affinity was increased.

Reactions of the Mutants with MTSEA. We tested the effects of 2.5 and 0.25 mM MTSEA on the mutants. The higher concentration significantly blocked [^{3}H]-*N*-methylspiperone binding at 13 of 23 positions (Figure 1A). The lower concentration significantly blocked binding to 10 of these mutants (Figure 1B). To quantitate the susceptibility to MTSEA, we determined the second-order rate constants for the reaction with MTSEA (Table 3). The most reactive cysteines were those substituted for Tyr192, Ser193, Ser194, Ile195, and Phe198. Cysteines substituted for Phe189, Val190, Val191, Val196, and Ser197 were of intermediate reactivity with MTSEA. Cysteines substituted for Val200, Pro201, and Tyr209 were the least reactive. While the reversible antagonist sulpiride significantly retarded the reaction of MTSEA with each of the reactive mutants, the degree of protection varied from 10% to 65% (Figure 2).

Reactions with MTSET and MTSES. Nine of the 10 mutants most sensitive to MTSEA (Figure 1B) were susceptible to reaction with MTSET (Figure 3A); one of the sensitive mutants, S197C, did not react with MTSET. Also, the mutants reacted more rapidly with MTSEA than with MTSET, since 2 min of 0.25 mM MTSEA gave about the same inhibition as 2 min of 1 mM MTSET. MTSES, the negatively charged derivative, reacted with seven of the nine mutants that were susceptible to MTSET (Figure 3B); V191C and S193C did not react with MTSES.

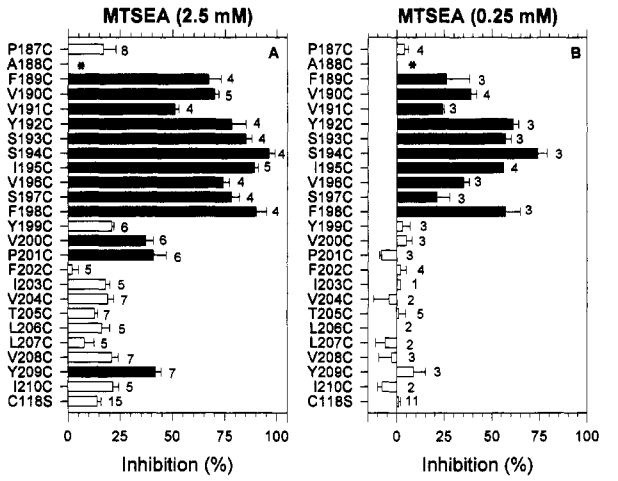

FIGURE 1: Inhibition of specific [^{3}H]-*N*-methylspiperone (300 pM) binding to intact cells transiently transfected with wild-type or mutant D2 receptors resulting from a 2-min application of (A) 2.5 mM MTSEA or (B) 0.25 mM MTSEA. The means and SEM are shown. The number of independent experiments for each mutant is shown next to the bars. Solid bars indicate mutants for which inhibition was significantly different ($p < 0.05$) than C118S by one-way ANOVA. An asterisk indicates no detectable binding.

Table 3: Rates of Reaction of MTSEA with the Cysteine-Substituted Dopamine D2 Receptor[a]

mutant	k_{MTSEA} (M^{-1} s^{-1})	k_{MUT}/k_{WT}	n
F189C	7.4 ± 2.7	0.2	3
V190C	8.8 ± 2.7	0.2	4
V191C	3.1 ± 0.4	0.1	3
Y192C	32 ± 2.4	0.8	3
S193C	25 ± 3.7	0.7	3
S194C	47 ± 14	1.2	3
I195C	27 ± 5.9	0.7	4
V195C	6.1 ± 1.0	0.2	3
S197C	6.1 ± 0.9	0.2	3
F198C	32 ± 9.5	0.8	3
V200C	1.8 ± 0.1	0.05	3
P201C	1.5 ± 0.3	0.04	3
Y209C	2.0 ± 0.3	0.05	3

[a] The second-order rate constant (k) was determined as described in Experimental Procedures. The means and SEM of n independent experiments, each performed with triplicate determinations, are shown. k_{MUT}/k_{WT} was obtained by dividing each k value by the k determined for the wild-type receptor in which Cys118 reacts.

DISCUSSION

Residues Exposed in the Binding-Site Crevice. In using SCAM to identify the residues exposed in the binding-site crevice, we make the following assumptions: (1) The highly polar MTS reagents react only at the water-accessible surface of the protein. (2) In the membrane-spanning segments, the lining of the binding-site crevice is the only water-accessible surface. (3) The addition of $-SCH_2CH_2X$ to a cysteine at the surface of the binding-site crevice should alter binding irreversibly, and competitive antagonists and agonists should retard the reaction with MTSEX. On the basis of these assumptions, we infer that 13 of the 23 residues tested in M5 are exposed in the binding-site crevice (Figure 1).

The extent of protection by sulpiride varied significantly among the mutants (Figure 2). Protection of a substituted cysteine is most simply explained by its proximity to the sulpiride and [^{3}H]-*N*-methylspiperone binding site. Nevertheless, not every one of these residues need contact sulpiride; sulpiride could protect residues deeper in the crevice by

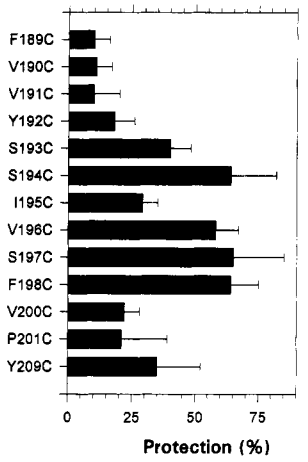

FIGURE 2: Sulpiride protection of cysteine substitution mutants. Dissociated cells were incubated in buffer A for 20 min at room temperature in the presence or absence of (±)-sulpiride, and then MTSEA was added, in the continued presence or absence of sulpiride, for 2 min at a concentration chosen to inhibit 50−75% of specific [^{3}H]-*N*-methylspiperone binding in the absence of sulpiride. Concentrations of MTSEA were as follows: 2.5 mM, V191C, V200C, P201C, Y209C; 1 mM, F189C, V190C, V196C; 0.25 mM, Y192C, S193C, S197C; 0.1 mM, S194C, I195C, F198C. For most mutants, sulpiride was used at a concentration of 10 μM. To compensate for changes in the K_I, sulpiride concentrations were adjusted for several mutants as follows: 1 μM, S193C; 100 μM, F189C, S197C, and P201C. Cells were washed by filtration though 96-well multiscreen plates containing GFB filters (Millipore). In the wash buffer, sodium was replaced by choline in order to facilitate removal of residual sulpiride. [^{3}H]-*N*-Methylspiperone binding to the washed cells was performed in buffer A in the multiscreen plates in a final volume of 0.25 mL. The means and SEM of three to five independent experiments, each performed with triplicate determinations, are shown. Protection was calculated as 1 − [(inhibition in the presence of sulpiride)/(inhibition in the absence of sulpiride)]. In each mutant, protection by sulpiride was significant ($p < 0.05$) by paired t-test.

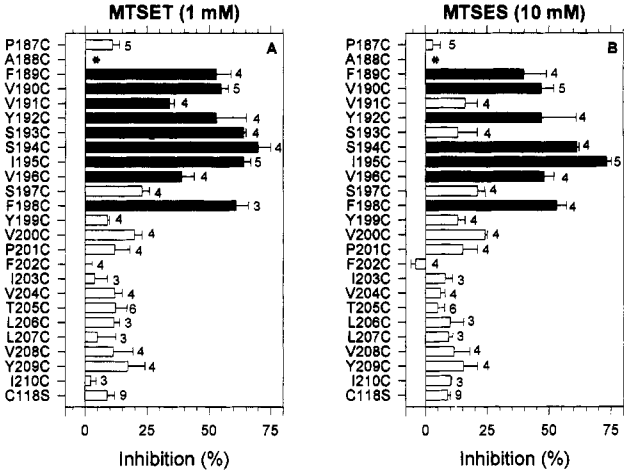

FIGURE 3: Inhibition of specific [^{3}H]-*N*-methylspiperone (300 pM) binding to intact cells transiently transfected with wild-type or mutant D2 receptors resulting from a 2-min application of (A) 1 mM MTSET or (B) 10 mM MTSES. On the basis of the relative rate constants for reaction with simple thiols in solution, namely, 10:4:1 for MTSET, MTSEA, and MTSES, respectively (Stauffer & Karlin, 1994), we used equireactive concentrations of 1 mM MTSET (A), 2.5 mM MTSEA (Figure 1A), and 10 mM MTSES (B). The means and SEM are shown. The number of independent experiments for each mutant is shown next to the bars. Solid bars indicate mutants for which inhibition was significantly different ($p < 0.05$) than C118S by one-way ANOVA. An asterisk indicates no detectable binding.

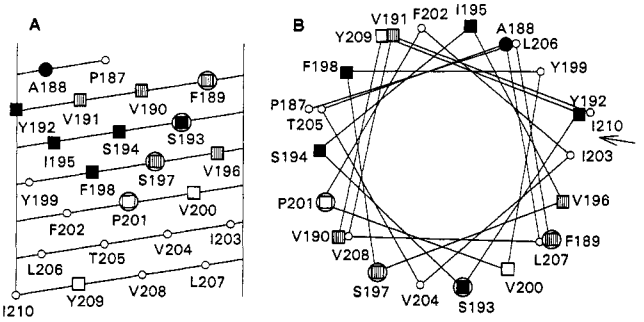

FIGURE 4: Helical net (A, left) and helical wheel (B, right) representations of the residues in and flanking the M5 segment of the dopamine D2 receptor, summarizing the effects of MTSEA on [^{3}H]-*N*-methylspiperone binding. Reactive residues are represented by squares, where the fill indicates the range of the second-order rate constants in M^{-1} s^{-1} for reaction with MTSEA (see Table 3): solid square = $k > 20$; striped square = $10 > k > 3$; open square = $2 > k > 1$. The symbol is enclosed in a large circle for those mutants in which the K_I for sulpiride is changed 10-fold or more by cysteine substitution itself. Small open circles indicate that MTSEA had no effect on binding. The filled circle indicates no binding after cysteine substitution. The arrow indicates where the helix is split to create the helical net.

binding above them and blocking the passage of MTSEA from the extracellular medium to the cytoplasmic end of the crevice. The residues that are more poorly protected may be located further from the actual binding site, toward the margins of the binding-site crevice. However, we cannot rule out indirect effects through propagated structural changes for either the inhibition by the MTS reagents or the protection by sulpiride. Whatever the mechanism of irreversible inhibition by the MTS reagents, the effect itself is proof that the reaction had occurred.

Secondary Structure of M5. Our observation that 10 sequential cysteine substitution mutants, from F189C to F198C, are accessible to MTSEA is surprising. This is inconsistent with the expectation that M5, like M3 (Javitch et al., 1995), forms an α-helix with one side facing the binding-site crevice and the rest of the surface facing the lipid bilayer or other residues (Figure 4). It is unlikely that the pattern of exposure results from the reaction of MTSEA with non-water-accessible sulfhydryls for two reasons: (i) MTSEA is 3-orders of magnitude more soluble in water than in 1-octanol (Akabas et al., 1992). (ii) The reaction of the MTS reagents, as exemplified by the reaction of methylmethanethiosulfonate with 2-mercaptoethanol, is 9- to 10 orders of magnitude faster with the ionized thiolate than with the un-ionized thiol (Roberts et al., 1986), and only water-exposed cysteine thiols are likely to ionize. Therefore, even if MTSEA did react to some extent with sulfhydryls exposed to lipid or to the interior of the protein, its reactivity would be much less than with water-accessible sulfhydryls. We observed, however, that MTSEA reacts with cysteines substituted for four consecutive residues, Tyr192 to Ile195, with rates comparable to its rate of reaction with the highly reactive Cys118 and not orders of magnitude more slowly.[2]

One possible explanation for the continuous stretch of accessible residues is that the structure of some of these cysteine substitution mutants is sufficiently perturbed to expose an endogenous cysteine that was previously inaccessible. C118S, the mutant used as background for all successive mutations, is 100-fold less reactive toward MTSEA than is wild-type D2 receptor. C118S is not, however, completely insensitive to the reagent; additional

cysteine(s) react(s) at very high concentrations of MTSEA (Javitch et al., 1994). To test whether the structure of some of the cysteine substitution mutants might be sufficiently perturbed to increase the exposure of the endogenous cysteines to MTSEA, we substituted all five endogenous membrane-spanning cysteines (C56V + C118S + C126S + C168S + C385S). We expressed four sequential mutants, V191C to S194C, one at a time, in this background devoid of any other membrane-spanning cysteines. Although the level of expression was significantly reduced in the "Cys-less" receptor background, the results of reaction with MTSEA were identical to those observed in the background of C118S (data not shown). Thus, the reactions of these four mutants, and likely of the others as well, are with the substituted cysteine and not an endogenous cysteine.

There are two additional explanations for the observed wide pattern of exposure. First, the exposed region of M5, which contains the serines likely to bind agonist (Strader et al., 1989; Cox et al., 1992; Mansour et al., 1992), might loop out into the lumen of the binding-site crevice and be completely accessible to water and thus to MTSEA (Figure 5A). Such a loop is found in porin where it extends into the central pore and determines the functional properties of the channel (Cowan et al., 1992). Second, M5 might be completely embedded in the membrane and also in contact with other membrane-spanning segments. At any instant, only a limited set of residues might be exposed in the binding-site crevice; however, M5 might move rapidly, either by rotation, translocation, or a conformational change, to expose different sets of residues (Figure 5B).

The most reactive mutants are located sequentially from Tyr192 to Ile195 plus Phe198. While these residues surround the serines thought to interact with agonists (Ser193, Ser194, and Ser197), the increased reactivity of MTSEA is seen at consecutive positions and not just on one side of a putative α-helix. Furthermore, it is these most reactive, consecutive residues which are best protected by sulpiride (Figure 2). This is consistent with their exposure within a loop near the dopamine binding site.

Based largely on the structure of bacteriorhodopsin (Henderson et al., 1990), which is not homologous to the D2 receptor, but also on biophysical data from rhodopsin (Findlay & Pappin, 1986; Schertler et al., 1993), which is homologous to the D2 receptor, the membrane-spanning segments of G-protein-coupled receptors are widely believed to be α-helical (Baldwin, 1993). However, others have inferred deviations from regular α-helical structure in the membrane-spanning segments of rhodopsin (Findlay & Pappin, 1986). A recent refinement of the low-resolution structure of rhodopsin, determined by cryoelectron microscopy, shows four clearly resolved tracks of density likely to

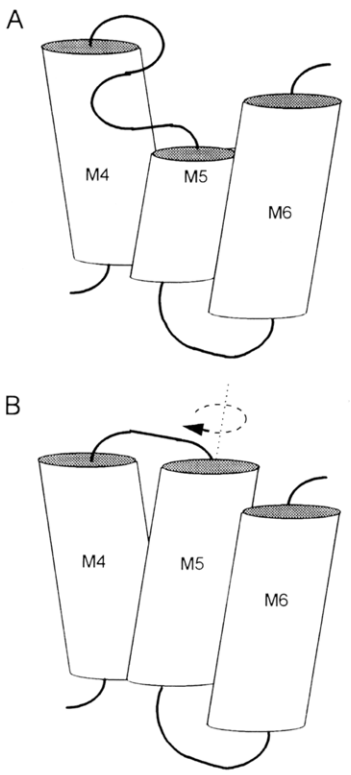

FIGURE 5: Schematic representation of alternative models to explain the exposure of 10 consecutive residues to MTSEA. In (A), the N-terminal portion or extracellular end of M5 loops into the binding-site crevice where it is completely exposed to water and to MTSEA. In (B), M5 moves, either by rotation, translocation, or a conformational change, to expose consecutive residues to the binding-site crevice. The cylinders represent membrane-spanning segments.

be α-helices and a less resolved continuous arc-shaped feature, which the authors interpreted to be highly tilted α-helices (Unger & Schertler, 1995). The sharpened 3D map shows a protuberance at one end, which the authors believe to be too big to be a resolved feature and rather to represent overlapping signals of tilted helices. Nonetheless, this protuberance might possibly represent the exposed portion of M5 looping into the retinal binding-site crevice.

The alternative explanation is that M5 is completely embedded in the membrane but can move sufficiently, either by rotation, translocation, or a conformational change, to expose consecutive residues to the binding-site crevice (Figure 5B). Although the findings of Careaga and Falke (1992) support the notion that α-helices can move in the membrane to a significant extent, they studied the rotation and translocation of α-helices exposed to lipid and protein, not α-helices exposed to lipid, protein, and a water-filled space.

Pro201 is conserved in the putative fifth membrane-spanning segment of nearly all G-protein-coupled receptors. A structural consequence of membrane-spanning prolines can be the creation of kinks in helices (von Hejne, 1991), and proline *cis*−*trans* isomerization has been suggested to contribute to the function of transport proteins (Brandl & Deber, 1986). Such a conformational change in M5 might be involved in the function of G-protein-coupled receptors and might alternately expose different sets of residues to the binding-site crevice.

Whether M5 loops into the lumen or is embedded in the membrane, its orientation and/or secondary structure may fluctuate. Agonist binding to part of M5 may induce a

[2] The second-order rate constants for the reaction of MTSEA with these substituted cysteines are 3-5 orders of magnitude less than the rate constants for the reaction of MTSEA with free sulfhydryls in solution or with the binding-site cysteines of the nicotinic acetylcholine receptor (Stauffer & Karlin, 1994). However, the rates are comparable with those observed with substituted cysteines exposed in the channel of the cystic fibrosis transmembrane conductance regulator (Akabas, personal communication) and are about one-tenth of those observed with substituted cysteines exposed in the closed state in the channel of the nicotinic acetylcholine receptor (Pascual and Karlin, personal communication). Steric hindrance and suppression of the ionization of the cysteine sulfhydryl are two factors that could lower the rate constants for the reaction of MTSEA in the binding-site crevice.

conformational change or stabilize one of multiple conformations, and this may be a critical step in the transduction of binding into receptor activation. Such a conformational change in M5 could be communicated to the intracellular loop at the C-terminal end of the segment, the major site of G-protein interaction (Strader et al., 1987).

In related G-protein-coupled receptors, residues which align with the exposed Val190, Ser193, Ser194, Ser197, and Pro201 have been implicated in the binding of agonists and/or antagonists (Strader et al., 1989; Wess et al., 1991; Ho et al., 1992; Link et al., 1992; Pollock et al., 1992; Weitz et al., 1992; Fathi et al., 1993; Fong et al., 1993; Tomic et al., 1993; Yamano et al., 1993). We found that sulpiride's affinity is affected by mutation of Phe189 as well as Ser193, Ser197, and Pro201 (Table 2). An effect of mutation on binding does not prove that a residue contacts ligand; the effect may be indirect (Schirmer et al., 1995). However, cysteines substituted for each of these four residues react with MTSEA and are exposed in the binding-site crevice. It is curious that these four exposed residues which most affect sulpiride's affinity lie along an arc on the helical net representation (Figure 4). One interpretation of these results is that the secondary structure of M5 is α-helical in the conformation which binds sulpiride.

It is unclear why Y209C reacts with MTSEA, albeit very slowly, without the exposure of any intervening residues after Pro201. In M3, we found that residues are exposed to the putative cytoplasmic end of the membrane-spanning segment (Javitch et al., 1995). If the more cytoplasmic portion of M5 is α-helical, one might predict that an intervening residue would be exposed. While it is conceivable that modification takes place without an effect on binding, the absence of an effect may relate to local steric constraints which prevent access and orientation of the MTSEA for reaction with the sulfhydryl groups. Perhaps when the smaller cysteine side-chain replaces the side chain of Tyr209, a cavity is created (Ericksson et al., 1992), and this allows the access of MTSEA to the substituted cysteine. Alternatively, the structure may deviate from that of an α-helix in this region as well.

Except for Ala188, none of the specific side chains in M5 are indispensable for the binding of the antagonist [^{3}H]-*N*-methylspiperone. The mutant A188C either did not reach the surface of the cell or it did not bind [^{3}H]-*N*-methylspiperone. In other G-protein-coupled receptors, the residues that align with Ala188 are highly variable, but do not include cysteine (Probst et al., 1992). It is possible that in A188C an aberrant disulfide bond is formed between the substituted cysteine and Cys107 or Cys182, the endogenous cysteines which normally form a critical disulfide bond in related G-protein-coupled receptors (Karnik & Khorana, 1990). Such an aberrant bond would likely impair the structure of the receptor.

Comparison of Reactions with MTSEA and MTSET. When adjusted for the rate constants for their reactions with simple thiols in solution (Stauffer & Karlin, 1994), the reaction of MTSEA with cysteines in the binding-site crevice is accelerated approximately 10-fold relative to that of MTSET (Figures 1 and 3A). MTSEA, like dopamine, contains an ethylammonium group, and it could be the affinity of this group for the dopamine-binding site that accelerates the reaction of MTSEA, especially with the most reactive mutants.

Electrostatic Potential in the Binding-Site Crevice. In M3, we found that MTSES reacted only with cysteines substituted for exposed residues more extracellular than Asp114 (Javitch et al., 1995). In M5, MTSES reacted with the mutants F189C, V190C, Y192C, S194C, I195C, V196C, and F198C (Figure 3B). At these reactive residues, the negatively charged MTSES (10 mM) inhibited binding nearly as much as the positively charged MTSET (1 mM) (Figure 3). Because 1 mM MTSET and 10 mM MTSES are equireactive with simple thiols in solution (Stauffer & Karlin, 1994), we infer that the electrostatic potential near these residues is close to zero. The decreased reaction of V191C and S193C with MTSES may reflect the presence of a more negative electrostatic environment in their vicinity.

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REFERENCES

Akabas, M. H., Stauffer, D. A., Xu, M., & Karlin, A. (1992) Acetylcholine receptor channel structure probed in cysteine-substitution mutants, *Science 258*, 307−310.

Akabas, M. H., Kaufmann, C., Archdeacon, P., & Karlin A. (1994) Identification of acetylcholine receptor channel-lining residues in the entire M2 segment of the alpha subunit, *Neuron 13*, 919−927.

Baldwin, J. M. (1993) The probable arrangement of the helices in G protein-coupled receptors, *EMBO J. 12*, 1693−1703.

Careaga, C. L., & Falke, J. J. (1992) Structure and dynamics of *Escherichia coli* chemosensory receptors: engineered sulfhydryl studies, *Biophys. J. 62*, 209−219.

Civelli, O., Bunzow, J. R., Grandy, D. K., Zhou, Q.-Y. E., & Van Tol, H. H. M. (1991) Molecular biology of the dopamine receptors, *Eur. J. Pharmacol. 207*, 277−286.

Cowan, S. W., Schirmer, T., Rummel, G. Steiert, M., Ghosh, R., Pauptit, R. A., Jansonius, J. N., & Rosenbusch, J. P. (1992) Crystal structures explain functional properties of two *E. coli* porins, *Nature 358*, 727−733.

Cox, B. A., Henningsen, R. A., Spanoyannis, A., Neve, R. L., & Neve, K. A. (1992) Contributions of conserved serine residues to the interactions of ligands with dopamine D2 receptors, *J. Neurochem. 59*, 627−635.

Brandl, C. J., & Deber, C. M. (1986) Hypothesis about the function of membrane-buried proline residues in transport proteins, *Proc. Natl. Acad. Sci. U.S.A. 83*, 917−921.

Ericksson, A. E., Basse, W. A., Zhang, X. L., Heinz, D. W., Blaber, M., Baldwin, E. P., & Matthews, B. W. (1992) Response of a protein structure to cavity-creating mutations and its relation to the hydrophobic effect, *Science 255*, 178−183.

Fathi, Z., Benya, R. V., Shapira, H., Jensen, R. T., & Battey, J. F. (1993) The fifth transmembrane segment of the Neuromedin B receptor is critical for high affinity Neuromedin B binding, *J. Biol. Chem. 268*, 14622−14626.

Findlay, J. B. C., & Pappin, D. J. C. (1986) The opsin family of proteins, *Biochem. J. 238*, 625−642.

Fong, T. M., Cascieri, M. A., Yu, H., Bansal, A., Swain, C., & Strader, C. D. (1993) Amino-aromatic interaction between histidine 197 of the neurokinin-1 receptor and CP 96345, *Nature 362*, 350−353.

Goldstein, A., & Barett, R. W. (1987) Ligand dissociation constants from competition binding assays: errors associated with ligand depletion, *Mol. Pharmacol. 31*, 603−609.

Henderson, R., Baldwin, J. M., Ceska, T. A., Zemlin, F., Beckmann, E., & Downing, K. H. (1990) Model for the structure of

bacteriorhodopsin based on high-resolution electron cryo-microscopy, *J. Mol. Biol. 213*, 899 −929.

Ho, B. F., Karschin, A., Branchek, T., Davidson, N., & Lester, H. A. (1992) The role of conserved aspartate and serine residues in ligand binding and in function of the $5HT_{1A}$ receptor: a site-directed mutation study, *FEBS Lett. 312*, 259−262.

Javitch, J. A., Li, X., Kaback, J., & Karlin, A. (1994) A cysteine residue in the third membrane-spanning segment of the human dopamine D2 receptor is exposed in the binding-site crevice, *Proc. Natl. Acad. Sci. U.S.A. 91*, 10355−10359.

Javitch, J. A., Fu, D., Chen, J., & Karlin, A. (1995) Mapping the binding-site crevice of the dopamine D2 receptor by the substituted-cysteine accessibility method, *Neuron 14*, 825−831.

Karnik, S. S., Sakmar, T. P., Chen, H.-B., & Khorana, H. G. (1988) Cysteine residues 110 and 187 are essential for the formation of correct structure in bovine rhodopsin, *Proc. Natl. Acad. Sci. USA. 85*, 8459−8463.

Link, R., Daunt, D., Barsh, G., Cruscinski, & Kobilka, B. (1992). Cloning of two mouse genes encoding α_2-adrenergic receptor subtypes and identification of a single amino acid in the mouse α_2-C10 homolog responsible for an interspecies variation in antagonist binding, *Mol. Pharmacol. 42*, 16−27.

Mansour, A., Meng, F., Meador-Woodruff, J. H., Taylor, L. P., Civelli, O., & Akil, H. (1992) Site-directed mutagenesis of the human dopamine D2 receptor, *Eur. J. Pharmacol. 227*, 205−214.

Oprian, D. D. (1992) The ligand-binding domain of rhodopsin and other G protein-linked receptors, *J. Bioenerg. Biomembr. 24*, 211−217.

Pollock, N. J., Manelli, A. M., Hutchins, C. W., Steffey, M. E., MacKenzie, R. G., Frail, D. E. (1992) Serine mutations in transmembrane V of the dopamine D1 receptor affect ligand interactions and receptor activation, *J. Biol. Chem. 267*, 17780−17786.

Probst, W. C., Snyder, L. A., Schuster, D. I., Brosius, J., & Sealfon, S. C. (1992) Sequence alignment of the G-protein coupled receptor superfamily, *DNA Cell Biol. 11*, 1−20.

Roberts, D. D., Lewis, S. D., Ballou, D. P., Olson, S. T., & Shafer, J. A. (1986) Reactivity of small thiolate anions and cysteine-25 in papain toward methyl methanethiosulfonate, *Biochemistry 25*, 5595−5601.

Schertler, G. F. X., Vila, C., & Henderson, R. (1993) Projection structure of rhodopsin, *Nature 362*, 770−772.

Schirmer, T., Keller, T. A., Wang, Y. F., & Rosenbusch, J. P. (1995) Structural basis for sugar translocation through maltoporin channels at 3.1 Å resolution, *Science 267*, 512−514.

Stauffer, D. A., & Karlin, A. (1994) The electrostatic potential of the acetylcholine binding sites in the nicotinic receptor probed by reactions of binding-site cysteines with charged methanethiosulfonates, *Biochemistry 33*, 6840−6849.

Strader, C. D., Dixon, R. A. F., Cheung, A. H., Candelore, M. R., Blake, A. D., & Sigal, I. S. (1987) Mutations that uncouple the β-adrenergic receptor from G_S and increase agonist affinity, *J. Biol. Chem. 262*, 16439−16443.

Strader, C. D., Candelore, M. R., Hill, W. S., Sigal, I. S., & Dixon, R. A. F. (1989) Identification of two serine residues involved in agonist activation of the β-adrenergic receptor, *J. Biol. Chem. 264*, 13572−13578.

Strader, C. D., Fong, T. M., Tota, M. R., Underwood, D., & Dixon, R. A. F. (1994) Structure and function of G protein-coupled receptors, *Annu. Rev. Biochem. 63*, 101−132.

Tomic, M., Seeman, P., George, S. R., & O'Dowd, B. F. (1993) Dopamine D1 receptor mutagenesis: role of amino acids in agonist and antagonist binding, *Biochem. Biophys. Res. Commun. 191*, 1020−1027.

Unger, V. M., & Schertler, G. F. X. (1995) Low resolution structure of bovine rhodopsin determined by electron cryo-microscopy, *Biophys. J. 68*, 1776−1786.

von Heijne, G. (1991) Proline kinks in transmembrane α-helices, *J. Mol. Biol. 218*, 499−503.

Weitz, C. J., & Nathans, J. (1992) Histidine residues regulate the transition of photoexcited rhodopsin to its active conformation, metarhodopsin II, *Neuron 8*, 465−472.

Wess, J., Gdula, D., & Brann, M. R. (1991) Site-directed mutagenesis of the m3 muscarinic receptor: identification of a series of threonine and tyrosine residues involved in agonist but not antagonist binding, *EMBO J. 10*, 3729−3734.

Yamano, Y., Ohyama, K., Chaki, S., Guo D.-F., & Inagami, T. (1992) Identification of amino acid residues of rat angiotensin II receptor for ligand binding by site directed mutagenesis, *Biochem. Biophys. Res. Commun. 187*, 1426−1431.

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