Differential Protein Mobility of the γ-Aminobutyric Acid, Type A, Receptor α and β Subunit Channel-lining Segments*

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The γ-aminobutyric acid, type A (GABA_A) receptor ion channel is lined by the second membrane-spanning (M2) segments from each of five homologous subunits that assemble to form the receptor. Gating presumably involves movement of the M2 segments. We assayed protein mobility near the M2 segment extracellular ends by measuring the ability of engineered cysteines to form disulfide bonds and high affinity Zn^{2+}-binding sites. Disulfide bonds formed in α/β_E270 γ/254 but not in α_N275 β/γ/254 or α/β_γ/255. Diazepam potentiation and Zn^{2+} inhibition demonstrated that expressed receptors contained a γ subunit. Therefore, the disulfide bond in α/β_E270 γ/254 formed between non-adjacent subunits. In the homologous acetylcholine receptor 4-Å resolution structure, the distance between α carbon atoms of 20′ aligned positions in non-adjacent subunits is ~19 Å. Because disulfide trapping involves covalent bond formation, it indicates the extent of movement but does not provide an indication of the energetics of protein deformation. Pairs of cysteines can form high affinity Zn^{2+}-binding sites whose affinity depends on the energetics of forming a bidentate-binding site. The Zn^{2+} inhibition IC_{50} for α/β_E270 γ/254 was 34 μM. In contrast, it was greater than 100 μM in α_N275 β/γ/254 and α/β_γ/255 receptors. The high Zn^{2+} affinity in α/β_E270 γ/254 implies that this region in the β subunit has a high protein mobility with a low energy barrier to translational motions that bring the positions into close proximity. The differential mobility of the extracellular ends of the β and α M2 segments may have important implications for GABA-induced conformational changes during channel gating.

GABA_A receptors are allosteric proteins that mediate fast inhibitory neurotransmission in the central nervous system (1–3). They are members of the Cys-loop receptor ion channel gene superfamily that includes glycine, serotonin type 3 (5-HT_3), and nicotinic acetylcholine (ACh) receptors (4–6). GABA_A receptors are formed by five homologous subunits assembled around a central channel. Most endogenous receptors contain two α, two β, and one γ subunit arranged in a clockwise orientation αβγβγ when observed from the extracellular end of the channel (7, 8). However, expression of just α and β subunits also results in functional receptors with the favored stoichiometry being two α and three β in the order αβαββ (9–11). Each subunit has an ~200-amino acid, extracellular, N-terminal, ligand-binding domain and a C-terminal, channel-forming domain with four membrane-spanning segments (M1, M2, M3, and M4).

The channel is principally lined by the five α-helical M2 segments (12, 13). An index numbering system facilitates comparisons between M2 segments of superfamily members (14). The 0′ position is defined as the positively charged residue located near the cytoplasmic end of the channel, GABA_A β_250. The 20′ position, GABA_A β_270, is aligned with the acetylcholine receptor extracellular ring of charge (15) and is predicted by amino acid sequence analysis to be the extracellular end of M2 (16). Experimental evidence indicates that M2 extends two helical turns beyond the 20′ position (17). In the 4-Å resolution cryo-EM structure of the homologous Torpedo ACh receptor confirms this and demonstrates that the 20′ position lies at the level of the extracellular membrane surface (13). In the 4-Å resolution cryo-EM structure the narrowest region of the closed channel, inferred to be the gate, is near the midpoint, between the 9′ and 14′ positions (13). Cysteine accessibility studies in the 5-HT_3 receptor are consistent with this, although similar studies in the ACh receptor concluded that the gate was at the channel’s cytoplasmic end (18, 19). Evidence from ACh, GABA_A, and 5-HT_3 receptors indicates that the structure of the cytoplasmic end of the channel is relatively fixed and rigid (20–22). This would be consistent with evidence that the size and charge selectivity filters, and the major determinants of single channel conductance are located at the cytoplasmic end of the channel (12, 15, 23–25). In contrast, the extracellular end of the channel undergoes conformational motion due to both thermal protein motion and agonist-induced gating (17, 20, 21, 26). In the cryo-EM-derived structure, the extracellular ends of the M2 domains are loosely packed, suggesting that these domains might possess a high degree of flexibility/mobility (13). Consistent with this, substituted cysteine accessibility method studies of the GABA_A receptor β_1 subunit M2 domain concluded that the M2 segment extracellular halves were loosely packed and/or highly mobile (21).

We previously used disulfide trapping experiments in αβ receptors to probe thermal protein motion and proximity relationships between M2 segment, channel-lining residues in different subunits (26). The ability for a pair of cysteines to form a disulfide bond depends on the presence of an oxidizing environment and on the collision frequency. The collision frequency depends on the average separation distance of the sulphydryls, their relative orientation in the protein, and the flexibility/
mobility of the protein in the region of the Cys residues. We used copper phenanthroline (Cu:phen) to create an oxidizing environment. Cu:phen catalyzes the formation of reactive oxygen species, such as superoxide and hydroxyl radicals, from molecular oxygen (27). We showed that at the 20° level disulfide bonds formed between Cys substituted for the β20 but not between Cys substituted for the α20. In order for a disulfide bond to form, the Cys α carbons must come to within 5.6 Å of one another (28). Assuming that the 4Å resolution ACh receptor structure is a good model for the GABAA receptor structure, the average distance between the 20° α carbons of residues in adjacent and non-adjacent subunits is 12 and 19 Å, respectively (13). Because there are three β subunits in the αβ receptors used in our previous work, we could not distinguish whether the disulfide bond was forming between Cys substituted in adjacent or in non-adjacent positions. Thus, the extent of the thermal motion could not be determined (26). To resolve this issue the current experiments have been performed in αβγ receptors where the two β subunits are not adjacent.

The extent of the movements that would be required to explain the disulfide bond formation in our original studies highlights a potential limitation of disulfide trapping experiments. Because disulfide bonds are covalent they may trap relatively rare conformational states of the protein. In the aspartate chemotaxis receptor, a protein of known crystal structure, thermal protein movement allowed disulfide bond formation between pairs of engineered Cys whose α carbons were separated in the crystal structure by 15 Å (28). Thus, disulfide trapping may provide insight into the extent of thermal motion, but it does not necessarily measure the average separation distances. To address this issue, in the present work we have measured the Zn2⁺ binding affinity of receptors containing pairs of engineered Cys. Pairs of Cys can form bidentate, high affinity Zn2⁺-binding sites if they are positioned appropriately. The Zn2⁺ affinity of these sites will depend on the orientation of the Cys sulfur atoms, their average separation distance, and the energy needed to distort the average protein structure to bring the Cys into position to bind the Zn2⁺ ion. The Zn2⁺ affinity will lie between the picomolar range, the affinity of Zn2⁺ for peptides containing four Cys Zn2⁺ finger-binding protein sequences (29), and the 10–1000 μM range, the Zn2⁺ affinity of single Cys (10, 30, 31). In crystal structures of high affinity Zn2⁺-binding sites the Cys α carbons are separated by about 5–7 Å. The non-covalent nature of this interaction provides a better estimate of the average separation and/or the energy required to distort the average conformation to the structure necessary for high affinity binding.

Disulfide trapping has been used to study protein mobility and proximity relationships between residues in both water-soluble and integral-membrane proteins (28, 32–34). Engineer-heavy metal-binding sites has been used to study proximity relationships in ion channels and transporters (10, 35–37).

Here we report that in αβγ receptors disulfide bonds form when all five subunits contain 20' engineered Cys residues, but for the single Cys mutants they only form when the engineered Cys is in the β subunit, not when it is in α or γ. Consistent with this, a high affinity Zn2⁺-binding site is only formed when the engineered Cys is in the β subunit. These results provide insights into the extent and asymmetric nature of the thermal motion near the extracellular ends of the GABAA receptor channel-lining M2 segments and have implications for the channel gating process.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis and Oocyte Expression—**All cysteine substitution mutants were made using PCR as described previously (21). mRNA was synthesized in vitro using the Amplicap T7 High Yield Message Maker kit (Epigenetics Technologies, Madison, WI). mRNA was dissolved in diethylpyrocarbomate-treated water and stored at −80 °C. *Xenopus laevis* were purchased from Nasco Science (Fort Atkinson, WI). Stage V–VI oocytes were defolliculated by incubation in 2 mg/ml Type 1A collagenase (Sigma) for 75 min. Oocytes were washed in OR2 (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES; pH adjusted to 7.5 with NaOH) and kept in OR3 (70% Leibovitz L-15 medium (Invitrogen) supplemented with 10 mM HEPES, 50 μg/ml tetracycline, and 50 μg/ml gentamicin). Oocytes were injected 24 h after isolation with 50 nl of a 1:1 mixture of rat α1, β1, γ2s subunit mRNA (200 pg/ml) and were kept in the same medium for 2–5 days at 17 °C.

**Reagents—**A 100 mM stock solution of GABA (Sigma) in water was aliquoted and stored at −20 °C. 1 mM stock solutions of diithiothreitol (DTT; Sigma) and o-phenanthroline (Sigma) were made in nominally calcium-free frog Ringer’s solution (CFFR: 115 mM NaCl, 2.5 mM KCl, 1.8 mM MgCl₂, and 10 mM HEPES, pH 7.5) and Me2SO, respectively, and stored for no more than 1 month at −20 °C. A stock solution of 100 mM CuSO₄ was made in water. CuSO₄ and o-phenanthroline were mixed in CFFR directly before use to a final concentration of 100 μM CuSO₄ and 400 μM o-phenanthroline, expressed as 100:400 μM Cu:phen. A 100 mM stock solution of N-ethylmaleimide (NEM, Sigma) was made in CFFR directly before use. A 10 mM diazepam stock solution was made in Me2SO and stored at −20 °C. A 100 mM ZnCl₂ stock solution was made in water with 1 mM EDTA to prevent the precipitation of Zn2⁺ (OH₂). Tricine (Sigma) was diluted directly into 1X buffer at a concentration on 10 mM. Stock solutions of 500 mM N-2-(acetamido)iminodiacetic acid (Sigma), pH 7.3, and 100 mM diethylthiuram monosulfide (Sigma), pH 7.3, were made in water.

**Electrophysiology—**Two-electrode voltage clamp recordings were conducted at room temperature in a 250-μl chamber continuously perfused at 1 ml/min with CFFR solution. For the GABA dose-response curves, CFFR was replaced by a buffer containing 100 mM NaCl, 2.8 mM KCl, 0.3 mM BaCl₂, and 5 mM HEPES, pH 7.3. Currents were recorded from oocytes using two-electrode voltage clamp recording at a holding potential of −60 mV. The ground electrode was connected to the bath via a 3 kΩ KCl/agar bridge. Glass microelectrodes filled with 3 M KCl had a resistance of <2 MΩ. Data were acquired and analyzed using a Tektronix TDS 301 two-channel digitizer (Dagan Instruments, Minneapolis, MN) and PClamp 8 software (Axon Instruments, Union City, CA). The amount of potentiation by diazepam was calculated by the equation, % potentiation = (Iznm + DI) × 100, where Izm and DI are the zinc-induced currents with and without diazepam, respectively. The zinc potentiation is presented as the mean ± S.E.

**Diazepam-induced Current Enhancement—**The GABA dose-response relationship was determined on each cell. A GABAA EC50 concentration was applied in the absence and presence of 1 μM diazepam. To prevent disulfide bond formation receptors were kept reduced by the application of 10 mM DTT at the beginning of the experiment and between pulses of reagents. The amount of potentiation by diazepam was calculated by the equation, % potentiation = (Iznm + DI) × 100, where Izm and DI are the zinc-induced currents with and without diazepam, respectively. The GABA EC50 concentration was applied in the absence and presence of 1 μM diazepam. To prevent disulfide bond formation receptors were kept reduced by the application of 10 mM DTT at the beginning of the experiment and between pulses of reagents. The amount of potentiation by diazepam was calculated by the equation, % potentiation = (Iznm + DI) × 100, where Izm and DI are the zinc-induced currents with and without diazepam, respectively.

**Diazepam-induced Current Enhancement—**Once GABA EC50 concentration was applied in the absence and presence of 1 μM diazepam. To prevent disulfide bond formation receptors were kept reduced by the application of 10 mM DTT at the beginning of the experiment and between pulses of reagents. The amount of potentiation by diazepam was calculated by the equation, % potentiation = (Iznm + DI) × 100, where Izm and DI are the zinc-induced currents with and without diazepam, respectively. The % inhibition is given as mean ± S.E.

**NEM Inhibition of Zn2⁺ Binding—**After a control pulse of 30 μM GABA plus Zn2⁺, receptors were treated with 100 μM NEM for 5 min. We then reapplied GABA, first alone and then in the presence of Zn2⁺. (In some cases the effect of NEM on Zn2⁺-induced inhibition was tested on separate cells. Because this did not alter the outcome, the results of all experiments were combined.) Receptors were kept reduced by the application of 10 mM DTT at the beginning of the experiment and between pulses of reagents. Prior to every pulse of GABA or GABA plus Zn2⁺, receptors were reduced with DTT (10 mM, 5–10 min) and either washed (GABA pulses) or treated with Zn2⁺ (GABA plus Zn2⁺ pulses) for 1 min. The degree of inhibition by Zn2⁺ both before and after NEM treatment was determined by the equation, % inhibition = (Izm/IA) × 100, where IA and Izm are the zinc-induced currents with and without Zn2⁺, respectively. The % inhibition is given as mean ± S.E.

**Disulfide Bond-induced Inhibition—**Once GABA EC50 concentration was applied in the absence and presence of 1 μM diazepam. To prevent disulfide bond formation receptors were kept reduced by the application of 10 mM DTT at the beginning of the experiment and between pulses of reagents. Prior to every pulse of GABA or GABA plus Zn2⁺, receptors were reduced with DTT (10 mM, 5–10 min) and either washed (GABA pulses) or treated with Zn2⁺ (GABA plus Zn2⁺ pulses) for 1 min. The degree of inhibition by Zn2⁺ both before and after NEM treatment was determined by the equation, % inhibition = (Izm/IA) × 100, where IA and Izm are the zinc-induced currents with and without Zn2⁺, respectively. The % inhibition is given as mean ± S.E.
rate of disulfide bond formation. Therefore, the extent of inhibition attributed to the presence of disulfide bonds was calculated by the equation, \% inhibition = \{1 - (I_{\text{max}}/I_{\text{max}}^0)\} \times 100, where \(I_{\text{max}}^0\) is the GABA-induced current following application of DTT, and \(I\) is the current prior to DTT treatment. A saturating concentration of GABA was used for all experiments, except where specified. The range of GABA concentrations was between 0.3 and 10 mM. The maximal currents and % effect for each reagent are presented as the mean ± S.E.

Reoxidation Rates—A control pulse of a low concentration of GABA (below the GABA EC50) was followed by a 10-min application of 10 mM DTT. This led to an increase in the size of the currents. Pulses of GABA were then applied at 5- to 10-min intervals to monitor the return of the currents to their unreduced levels. The peak currents were fit with the equation, \(I = I_0 \exp(-t/r) + I_n\), where \(I_0\) is the current at time \(t\), \(I_n\) is the initial current, \(I\) is the final current, and \(r\) is the time at which 63% of the total current decay occurred.

\[\text{Zn}^{2+}: \text{Dose-response Curves—}\] A control pulse of GABA was followed by coapplications of GABA with increasing concentrations of \(\text{Zn}^{2+}\). Prior to every pulse of GABA or GABA plus \(\text{Zn}^{2+}\), receptors were reduced with DTT (10 mM, 5–10 min) and either washed or treated with \(\text{Zn}^{2+}\) for 1 min. Currents were normalized to the initial, control current, and fit with the Hill equation, \(I/I_{\text{max}} = I/I_0 (1 + (IC_{50}/\text{Zn}))^{n}\), where \(I\) is the current, \(I_{\text{max}}\) is the control current, \(IC_{50}\) is the \(\text{Zn}^{2+}\) concentration that produces half-maximal inhibition, \(Zn\) is the \(\text{Zn}^{2+}\) concentration, and \(n\) is the Hill coefficient. Fits were performed in Prism 3.02 (GraphPad Software, San Diego, CA).

To avoid artifacts caused by potential submicromolar levels of heavy metal contamination, \(\text{Zn}^{2+}\) dose-response curves were performed in the presence of heavy metal chelators as described (38, 39). 10 mM Tricine was present in all solutions in which the final \(\text{Zn}^{2+}\) concentrations were in the range of 1 nM to 1 mM. Under these conditions, [Free \(\text{Zn}^{2+}\)] was equal to [Added \(\text{Zn}^{2+}\)/2000. 1 mM N-2-acetamidoiminoacetic acid was added to all \(\text{Zn}^{2+}\)-containing solutions in which the final \(\text{Zn}^{2+}\) concentrations were <1 nM. Under these conditions, [Free \(\text{Zn}^{2+}\)] was equal to [Added \(\text{Zn}^{2+}\)/17,000. Solutions requiring a final \(\text{Zn}^{2+}\) concentration of greater than 1 \(\mu\)M had no chelator added. To solutions requiring no free \(\text{Zn}^{2+}\), the high affinity chelator diethylthiatricarbocyanine was added to a concentration of 10 \(\mu\)M. A concentration of 20–30 \(\mu\)M GABA was used in all experiments. Tricine, N-2-acetamidoiminoacetic acid and diethylthiatricarbocyanine had no significant effects on control GABA-induced currents (data not shown). The relationship between free \(\text{Zn}^{2+}\) and added \(\text{Zn}^{2+}\) was determined by the chemical speciation program, Geochem.

Statistics—All statistical analyses were performed in Prism 3.02 using a one-way analysis of variance followed by the Newman-Keuls multiple comparison test.

RESULTS

A \(\gamma\) Subunit Is Present in Functional Receptors—In \(\alpha\beta\gamma\) receptors \(\beta\) subunits are found only in non-adjacent positions, whereas in \(\alpha\beta\) receptors there is a pair of \(\beta\) subunits in adjacent positions (see the introduction). Therefore, if a disulfide bond formed between \(\beta\) subunits, we could only know that this bond occurred between non-adjacent subunits if we also knew that a \(\gamma\) subunit was present in the receptor. We tested for the presence of a \(\gamma\) subunit using two approaches, diazepam potentiation and \(\text{Zn}^{2+}\) inhibition. In \(\alpha\beta\gamma\) receptors, 1 \(\mu\)M diazepam is reported to potentiate currents induced by an EC50 concentration of GABA by more than 100%, whereas \(\alpha\beta\) receptors are unaffected by diazepam (40, 41). \(\text{Zn}^{2+}\) also enables us to distinguish between \(\alpha\beta\) and \(\alpha\beta\gamma\) receptors, because \(\alpha\beta\) receptors have a \(\text{Zn}^{2+}\) IC50 of ~0.5 \(\mu\)M, whereas \(\alpha\beta\gamma\) receptors are insensitive to \(\text{Zn}^{2+}\) (42, 10). The high affinity \(\text{Zn}^{2+}\)-binding site is formed in \(\alpha\beta\) receptors by the \(\beta\)His-287 (M2 17') in the adjacent \(\beta\) subunits. In \(\alpha\beta\gamma\) receptors there are no adjacent \(\beta\) subunits and therefore no high affinity \(\text{Zn}^{2+}\)-binding site.

We tested the effects of diazepam on two populations of the mutant \(\alpha\beta\gamma\) receptors. Half of the cells were injected with a 1:1:1 and half with a 1:1:10 molar ratio of \(\alpha\), \(\beta\), and \(\gamma\) mRNA. If the cells injected with a 1:1:1 ratio of mRNA expressed the \(\gamma\) subunit in all cell surface receptors, then there should be no increase in the amount of diazepam potentiation in the cells injected with 1:1:10 compared with 1:1:1. To assure that the presence of spontaneously formed disulfide bonds did not interfere with the effects of diazepam and \(\text{Zn}^{2+}\), the reducing
agent DTT was applied for several minutes before the application of all reagents. As seen in Fig. 1 (A and B), the degree of diazepam-induced potentiation in the two populations of receptors was not significantly different (1:1:1, 179 ± 70% (n = 3); 1:1:10, 108 ± 18% (n = 3)). Therefore, in our hands, the majority of the GABA-induced current from oocytes injected with an equimolar ratio of the three subunits arises from receptors containing a γ subunit. Similar results were obtained with other Cys mutant receptors used in this study.

To further support the conclusion that the functional cell surface receptors used in this study contained a γ subunit, we examined the extent of inhibition by Zn²⁺. Two different mutants were used for the experiments with Zn²⁺: the double Cys mutant, α²₀°Cβ₂₀°C, and the triple Cys mutant, α₂₀°Cβ₂₀°Cγ₂₀°C. In both cases we injected equimolar amounts of mRNA for each subunit. 5 μM Zn²⁺ should inhibit more than 50% of the current in the α₂₀°Cβ₂₀°C mutant while having no effect on the current of the α₂₀°Cβ₂₀°Cγ₂₀°C mutant. Surprisingly, the two mutants showed similar amounts of Zn²⁺-induced inhibition: 85 ± 4% (n = 2) and 69 ± 8% (n = 5) for α₂₀°Cβ₂₀°C and α₂₀°Cβ₂₀°Cγ₂₀°C, respectively (Fig. 1, C and D). Because the engineered cysteines in these mutants could potentially bind Zn²⁺, we restated the effects of Zn²⁺ after exposing the reduced receptors to the alkylating agent NEM (100 μM, 5 min). Alkylation should abolish the ability of cysteine to bind heavy metals. NEM diminished currents in both mutants. However, following alkylation with NEM, 5 μM Zn²⁺ inhibited the remaining currents in α₂₀°Cβ₂₀°C and α₂₀°Cβ₂₀°Cγ₂₀°C by 60 ± 2% (n = 3) and 6 ± 2% (n = 4), respectively. Therefore, we infer that double α₂₀°Cβ₂₀°C cells retained a high affinity for Zn²⁺ following alkylation, because the native high affinity, Zn²⁺-binding site composed of β₂₀C-His-267 (17') in adjacent β subunits (42, 10) are insensitive to NEM. In contrast, in the triple mutant, α₂₀°Cβ₂₀°Cγ₂₀°C, after NEM alkylation Zn²⁺ no longer inhibited significantly, because in the presence of the γ subunit there are not adjacent β subunits to form a high affinity Zn²⁺-binding site. The overall conclusion from the experiments with Zn²⁺ and diazepam is that, when injected with a 1:1:1 ratio of α, β, and γ mRNA, the large majority of cell surface receptors contain a γ subunit. The important implication of this finding for the experiments described below is that two α subunits are not in adjacent positions nor are the two β subunits.

**Inter-subunit Disulfide Bond Formation in the 20’ Cysteine Mutants**—In response to the initial GABA applications, cells expressing α₂₀°Cβ₂₀°Cγ₂₀°C receptors, which contain engineered Cys in all five subunits, had maximal GABA-induced currents (İmax) of 457 ± 81 nA (n = 11) that were much smaller than those of wild-type, 3058 ± 405 nA (n = 5) (Fig. 2A, 2B, and 3A). Following application of DTT (10 mM, 10 min) the İmax for the wild-type receptors was unchanged (3221 ± 386 nA (n = 5)), whereas that of the mutant receptor increased nearly 10-fold (α₂₀°Cβ₂₀°Cγ₂₀°C: 4297 ± 320 nA (n = 11)). Therefore, we infer that DTT reduced intersubunit disulfide bonds that formed spontaneously in α₂₀°Cβ₂₀°Cγ₂₀°C.

We next determined whether the oxidizing agent Cu:phen, which catalyzes disulfide bond formation, could reverse the effects of DTT in α₂₀°Cβ₂₀°Cγ₂₀°C. A 3-min application of 100-400 μM Cu:phen decreased the α₂₀°Cβ₂₀°Cγ₂₀°C currents by 89 ± 12% (n = 3). This was not significantly different than their initial levels. In contrast, a similar Cu:phen application had no effect on wild-type currents (n = 3) (Fig. 2, A and B). A subsequent DTT application restored the mutant receptor currents to within 5 ± 2% (n = 3) of the levels produced by the first DTT application.

To ensure that the effect of DTT was not due to chelation of contaminating heavy metals in the buffer, we tested whether the metal chelator EGTA could also potentiate currents in α₂₀°Cβ₂₀°Cγ₂₀°C. DTT potentiated currents by 981 ± 162%, whereas, when applied for several minutes, 1 mM EGTA only potentiated currents by 35 ± 16% (n = 3). Therefore, we conclude that α₂₀°Cβ₂₀°Cγ₂₀°C formed one or two spontaneous disulfide bonds, which could be reduced by DTT and reformed by Cu:phen.

**Disulfide Bond Formation in Receptors with Single 20’ Cys Mutant Subunits**—Further experiments were aimed at gaining insight into the subunits involved in disulfide bond formation in α₂₀°Cβ₂₀°Cγ₂₀°C. We first examined mutant receptors containing a Cys in only one subunit: α₂₀°Cβ₂₀°Cγ₂₀°C, and α₂₀°Cβ₂₀°Cγ₂₀°C. Disulfide bonds formed spontaneously in α₂₀°Cβ₂₀°C. The initial İmax before DTT application, 1078 ± 214 nA (n = 6), was smaller than in the wild-type receptors, and after reduction with DTT İmax increased to 3251 ± 395 nA (n = 6) similar.
to wild-type currents (Figs. 2C and 3A). Furthermore, application of 100-400 μM Cu:phen returned currents to within 3 ± 23% of the initial untreated levels (n = 3), and a second DTT application increased currents to within 33 ± 5% (n = 2) of those after the initial DTT application.

In contrast to aβ2/CYγ, in aβ2/Cβγ the initial I_{max} was 3422 ± 664 nA (n = 4) comparable to that of wild-type receptors. Application of DTT (3784 ± 813 nA, n = 4) or Cu:phen (2655 ± 761 nA, n = 4) did not significantly alter GABA currents (Fig. 3A). As expected, given that there is only one γ subunit per receptor, there was no evidence for disulfide bond formation in aβ2/Cγ receptors. The initial I_{max} was 2779 ± 572 nA (n = 4). Currents were unaltered by application of either DTT (2317 ± 617 nA, n = 4) or Cu:phen (2317 ± 640 nA, n = 3) (Fig. 3A). From the experiments on receptors containing a single mutant subunit we conclude that in the 20°C position intrasubunit disulfide bonds formed between β subunits, but not between α subunits.

**Disulfide Bond Formation in Receptors with Two Subunits Containing 20°C Cys Mutants**—We tested mutants containing Cys in two different subunits for their ability to form disulfide bonds. The initial I_{max} of α20°Cβγ20°C, α20°Cβ20°Cγ, and αβ20°Cγ20°C were 1781 ± 209 nA (n = 10), 1545 ± 215 nA (n = 11), and 567 ± 106 (n = 11) respectively. All were significantly less than the wild-type receptor I_{max}. Reduction with DTT increased the currents to levels similar to those of wild type bringing the currents to 3414 ± 265 nA, 3907 ± 195 nA, and 2846 ± 245 nA for α20°Cβγ20°C, α20°Cβ20°Cγ, and αβ20°Cγ20°C, respectively (Fig. 3A). Cu:phen reversed the effects of DTT, and a second DTT application duplicated the effects of the first DTT application (data not shown). We conclude that disulfide bonds formed in all three double mutants. The extent to which disulfide bond formation at the 20°C level inhibits GABA-induced currents can be quantified for each mutant using the equation % inhibition = [(I_{DTT} – I_{DTT})/I_{DTT}] × 100, where I and I_{DTT} represent the GABA currents before and after DTT, respectively. Because the currents of untreated receptors (spontaneously oxidized) were of the same magnitude as those of receptors treated with Cu:phen, we used the initial currents in our calculation. The mutants containing a disulfide bond fall into three significantly different groups based on the extent of inhibition (Fig. 3B). Group 1 contains the mutant α20°Cβγ20°C with 49 ± 4% inhibition. Group 2 contains αβ20°Cγ and α20°Cβ20°Cγ with 68 ± 6% and 61 ± 5% inhibition, respectively. Group 3 contains αβ20°Cγ20°C and α20°Cβ20°Cγ20°C with 81 ± 3% and 89 ± 2% inhibition, respectively. From the results above we infer that 1) the two β subunits in a receptor can form a disulfide bond with one another (Fig. 3B, bar #3); 2) the disulfide bond in α20°Cβγ20°C must be between an α and a γ subunit (Fig. 3B, bar #6), because a disulfide bond does not form in either of the single mutants α20°Cβγ or αβ20°Cγ (Fig. 3B, bars #2 and #4); and 3) some portion of the disulfide bonds found in αβ20°Cγ20°C must be between a β and a γ subunit, because there is more inhibition in the double mutant than the single β mutant (Fig. 3B, compare bars #3 and #7).

**The Rate of Disulfide Bond Formation Is Fastest in αβ20°Cγ20°C**—To learn more about the relative proximity and mobility of the different subunits around the channel, we measured the rates of spontaneous disulfide bond formation in α20°Cβ20°Cγ, α20°Cβγ20°C, αβ20°Cγ20°C, and αβ20°Cγ. As shown for αβ20°Cγ20°C in Fig. 4A, a test pulse of GABA was

**FIG. 3.** The effects of disulfide bonds on the GABA-induced currents of 20°C cysteine-substitution mutants. A, the initial GABA current for all 20°C mutants except for α20°Cβγ and αβ20°C were significantly lower than the wild-type GABA current (*, p < 0.05; n ≥ 4; black bars). Upon reduction with DTT (10 mM, 10 min; gray bars) the GABA current of wild-type and mutant receptors were similar. B, the % inhibition produced by disulfide bonds was calculated for all mutants (see text; n ≥ 4). There were three significantly different groups among those mutants that showed inhibition (p < 0.05): α20°Cβγ20°C (●), αβ20°Cγ and α20°Cβ20°Cγ (●), and αβ20°Cγ20°C and α20°Cβ20°C20°C (●). For each mutant, only subunits containing engineered cysteines are shown. The numbers at the bottom of panel B are the bar numbers referred to under “Results.”
followed by a 10-min application of 10 mM DTT. Pulses of GABA were then applied every 5–10 min, depending on the mutant. Over the course of several minutes the currents returned to their initial values, indicating the spontaneous reformation of the disulfide bonds. The peak currents of all the pulses were fit with the single exponential equation (Fig. 4B). The $\tau$ values for the mutants were as follows ($n \geq 4$): $a20^C C20^C Cy$, $3 \pm 1$ min; $a20^C C20^C Cb$, $23 \pm 7$ min; $a20^C C20^C Cb$, $15 \pm 2$ min; and $a20^C Cb$, $17 \pm 4$ min. $a20^C C20^C Cy$ is the only mutant to have a $\tau$ significantly different from the other mutants. The difference in disulfide bond formation rates between $a20^C C20^C Cy$ and $a20^C Cb$ implies that at least some of the disulfide bonds in $a20^C C20^C Cy$ are between Cys in $\alpha$ and $\beta$ subunits. This implies a higher collision rate between the $\alpha$ and $\beta$ Cys than between the non-adjacent $\beta$ Cys in $a20^C Cb$. This faster rate may be due to disulfide bond formation between adjacent $\alpha$ and $\beta$ subunits.

$a20^C Cb$ Forms a High Affinity, Zn$^{2+}$-binding Site—The intersubunit disulfide bond that forms between $\beta$20$^C$ Cys in $a20^C Cb$ receptors indicates that the combined movement of the two non-adjacent $\beta$ M2 segments was sufficient to traverse the channel diameter. The frequency of this event is unknown, because disulfide bonds can trap the receptor in a rare conformation. To address this issue we sought to determine whether these Cys could form a high affinity Zn$^{2+}$-binding site. Due to the significantly lower energy involved in the Zn$^{2+}$Cys interaction compared with a covalent disulfide bond, Zn$^{2+}$ would be unable to trap the rare conformations that could be trapped with a disulfide bond. These experiments were carried out with reduced receptors to ensure that the Cys were fully available to bind Zn$^{2+}$. In addition, the buffer contained heavy metal chelators to remove any trace metals that might compete with Zn$^{2+}$ for binding to the Cys.

100 $\mu$M Zn$^{2+}$ inhibited wild-type $\alpha 20^C \beta$ receptors by 7 ± 6% ($n = 3$). In contrast, in $a20^C Cb$ receptors, 10 $\mu$M Zn$^{2+}$ inhibited 96 ± 1% of the current. The Zn$^{2+}$ IC$_{50}$ for $a20^C Cb$ was 34 ± 5 nM ($n = 3$; Fig. 5, A and B), several orders of magnitude smaller than the wild-type $\alpha 20^C \beta$ IC$_{50}$. In wild-type $\alpha 20^C \beta$ receptors, the $\beta 17^C$ histidine, $\beta 326$, from adjacent subunits forms a high affinity bidentate Zn$^{2+}$-binding site (10). To determine whether $\beta 17^C$ participated in the formation of an intrasubunit Zn$^{2+}$-binding site with $\beta 20^C$, we constructed the double mutant, $\beta 20^C \beta 20^C / H17^C / H17^S$ and expressed it with wild-type $\alpha$ and $\gamma$ subunits. The Zn$^{2+}$ IC$_{50}$ values of $\alpha \beta E20^C / H17^C / H17^S$ and $\alpha \beta 20^C C20^C / H17^C / H17^S$ were comparable (data not shown). We conclude that Zn$^{2+}$-inhibition of $a20^C Cb$ was due to Zn$^{2+}$ binding to an intersubunit, bidentate, Zn$^{2+}$-binding site formed between the engineered Cys in the two non-adjacent $\beta$ subunits.

$\alpha 20^C Cb$ receptors also showed some increased sensitivity to Zn$^{2+}$ compared with wild-type receptors ($n = 4$; Fig. 5B). However, because the predicted IC$_{50}$ would be greater than 100 $\mu$M a complete Zn$^{2+}$ dose-response relationship was not determined. The increase over wild-type sensitivity was abolished if, in addition to adding a Cys at $a20^C$, the glutamate at the $\beta 20^C$ position was replaced with an asparagine: 100 $\mu$M Zn$^{2+}$ inhibited GABA-induced currents in $\alpha 20^C Cb$ by 32 ± 4% ($n = 4$), but only altered the $a20^C Cb / N20^C$ currents by 1 ± 2% ($n = 3$). Therefore, in $\alpha 20^C Cb$, a low affinity, bidentate, Zn$^{2+}$-binding site formed at the $20^C$ position between the engineered cysteine in the $\alpha$ subunit and the native glutamate in the $\beta$ subunit, but not solely between $a20^C$ Cys.

An Intersubunit Disulfide Bond Does Not Form in $\alpha 17^C Cb$—The 17$^C$ position is one $\alpha$ helical turn down from the 20$^C$ position. Because the distance across the channel between 17$^C$ residues should be shorter than it is between 20$^C$ residues (13), we tested the ability of $\alpha 17^C Cb$ to form a disulfide bond.

![Image](image.png)

**Fig. 5.** The engineered cysteines in $a20^C Cb$ form a high affinity, bidentate Zn$^{2+}$-binding site. A, receptors were exposed to increasing Zn$^{2+}$ concentrations. Prior to every pulse of GABA or GABA plus Zn$^{2+}$, receptors were reduced with DTT (10 mM, 5 min) and either washed or treated with Zn$^{2+}$ for 1 min. A pulse of 30 $\mu$M was then applied in the presence of Zn$^{2+}$. All experiments were done in the presence of metal chelators (see “Experimental Procedures”). Bars above traces indicate application of reagent. Leak currents have been subtracted. Current is not shown during application of Zn$^{2+}$ alone or DTT. Holding potential: -60 mV. B, Zn$^{2+}$ dose-response curves for $a20^C Cb$ (triangles) and $\alpha 20^C Cb$ (squares). Data were normalized to the current in the absence of Zn$^{2+}$ and plotted against the Zn$^{2+}$ concentration. The IC$_{50}$ for $a20^C Cb$ was determined by fitting the dose-response curve with the Hill equation (line). A fit for $a20^C Cb$ was performed to obtain a partial curve.

DTT and Cuphen had no effects on maximal GABA-induced currents in $\alpha 17^C Cb$ (DTT: +6 ± 2%, $n = 3$; Cuphen: -6 ± 3%, $n = 3$). Receptors were also unaffected by DTT using an EC$_{10}$ concentration of GABA, which is more sensitive to modifications that affect gating (wild-type: +46 ± 1% ($n = 2$); mutant: +19 ± 6% ($n = 3$)). Thus, it appears that $\beta-\beta$ disulfide bonds do not form between non-adjacent $\beta$ subunits at the 17$^C$ position in $\alpha 20^C$ receptors.

**DISCUSSION**

We used disulfide trapping and Zn$^{2+}$ binding to study the mobility of the GABAA receptor M2 segments in $\alpha 20^C$ receptors. In these receptors there are two $\alpha$, two $\beta$, and one $\gamma$ subunits. The two $\alpha$ subunits are in non-adjacent positions around the channel axis as are the $\beta$ subunits (7–11). Our experiments showed that disulfide bonds formed between Cys residues substituted for $\beta$-Glu-270 (20') but not between Cys substituted for the aligned $\alpha_1$ subunit residue $\alpha_1$-Asn-275 (20'). Disulfide bond formation between the engineered $\beta$ Cys implies that the collision frequency between the engineered $20'$ Cys is significantly higher than between the $\alpha$ engineered 20' Cys. In the
ACh receptor 4-Å structure the 20' residues have a similar orientation to and distance from the channel axis (13), suggesting that these are not the bases for the disparity between α and β. Thus, at this level in the channel the β subunit M2 segments must be more mobile and/or more flexible than the α subunit M2 segments in αβγ receptors. This implies that the β M2 segments are less tightly packed with the rest of the protein than the α M2 segments (43, 21).

In the ACh receptor 4-Å structure the α carbons of non-adjacent 20' residues are ~19 Å apart (Fig. 6) (13). Because disulfide trapping involves formation of a covalent bond, it does not provide information on the energetics of bringing the 20' residues to within ~5 Å necessary to form the disulfide bond. High affinity Zn<sup>2+</sup> binding involves a non-covalent interaction with pairs of engineered Cys residues. The α carbon separation of the two Cys residues is comparable in a bidentate Zn<sup>2+</sup>-binding site and in a disulfide bond (29, 44–47). However, unlike disulfide bonds, the energetics of apposing two Cys residues can be measured through the affinity of the resultant binding site for Zn<sup>2+</sup>. The higher the Zn<sup>2+</sup> affinity the lower the energy barrier to bringing the two Cys residues close enough to form a bidentate-binding site. Bound Zn<sup>2+</sup> ions usually display tetrahedral coordination (44, 47, 48). The affinity of a site for Zn<sup>2+</sup> depends on the number of chelating Cys residues. The Zn<sup>2+</sup> affinity of sites containing a single Cys residue is generally in the tens of micromolar to millimolar concentration range (29, 31, 49), whereas the Zn<sup>2+</sup> affinity of proteins containing four Cys residues chelating a Zn<sup>2+</sup> ion range from 10<sup>−18</sup> to 10<sup>−12</sup> M (38, 50–52). The Zn<sup>2+</sup> affinity for β, E20'C containing receptors was 34 nM. To achieve this affinity the Zn<sup>2+</sup> must be bound by both Cys.

It is difficult to know what the theoretical maximum affinity of two ideally positioned Cys residues is for Zn<sup>2+</sup> in part because there are no structural Zn<sup>2+</sup>-binding sites with just two Cys ligands. With this number we could calculate the amount of energy lost to protein distortion by Zn<sup>2+</sup> binding to the β20'Cys receptors to give the measured affinity of 34 nM. In proteins containing two Cys residues the Zn<sup>2+</sup> affinity ranges from nanomolar to micromolar (10, 36, 38). Thus, the 34 nM affinity that we have measured is toward the higher end of measured affinities for two Cys binding. This implies that there is a relatively small energy barrier to bringing the two Cys from their 19-Å separation distance in the ACh receptor structure to the optimal separation for Zn<sup>2+</sup>-binding.

Disulfide Bonds between α-β, α-γ, and β-γ 20' Cys Mutants—Although the α20'Cβγ mutant did not form an intersubunit disulfide bond, the α20'Cys was able to form disulfide bonds with the β20'Cys and with the γ20'Cys. We infer the formation of these α-β and α-γ disulfide bonds, because the extent of inhibition following oxidation was different in the double Cys mutant α20'Cβ20'Cγ than in the single Cys mutant α20'C20'Cγ and there was disulfide bond formation in α20'C20'C20'C (Fig. 3). In both cases the disulfide bonds could form either between adjacent or between non-adjacent subunits. At present we cannot distinguish between these possibilities. If the disulfide bonds form between α20'Cys and a Cys in an adjacent β or γ subunit the M2 segments to which the residues are attached would need to both rotate and move ~7 Å based on the ACh receptor structure (Fig. 6). Therefore, while the αM2 20' region, in conjunction with the βM2 or γM2 regions, is sufficiently flexible to move the 7 Å necessary to disulfide bond with a Cys on an adjacent subunit, the 14 Å required for disulfide bonding with a non-adjacent subunit, i.e., the other α subunit, appears to be too great a distance for the αM2 segments to overcome.

Gating and Spontaneous Protein Movement—The mobility of the extracellular ends of M2 that we have detected may be related to the channel gating process. In the 4-Å ACh receptor structure the channel gate is in the region between the 9' and 14' levels (13). Transduction of agonist binding in the extracellular domain to the gate may proceed through the extracellular end of M2. The strong inhibitory effect of a single disulfide bond at the 20' position ranging from 49% inhibition in α20'Cβ20'Cγ to 81% inhibition for αβ20'Cγ20'C demonstrates the importance of this region in channel gating. The motion that we have detected may represent the unsynchronized fluctuation of the extracellular ends of the M2 segments between their closed and open state conformations. Channel opening would require the concerted movement of all five M2 segments away from the channel axis into their open state conformation, an event that rarely occurs in the absence of agonist. The movement of the M2 segments may be similar to the spontaneous conformational fluctuations that the voltage sensing S4 segments undergoes in voltage-dependent K<sup>+</sup> channels as they sense the membrane potential on the two sides of the membrane (53–55).

Our observations of asymmetric motion in the β and α subunits raises the question of whether channel gating involves a larger movement of the β subunit M2 segments than of the α M2 segments. The GABA<sub>A</sub> β subunits form the principle portion of the agonist-binding sites, analogous to the ACh receptor α subunits (7). A greater fraction of the agonist surface area interacts with the principle subunit-binding site (7, 56). Whether this causes a greater movement in the β M2 segment is unknown. Differences in the potential coupling of the β and α extracellular domains to the membrane-spanning domains have been observed (57, 58). Whether these differences relate to the extent of M2 segment movement during gating is unknown at present. Perhaps consistent with our finding of greater conformational change in the principle subunit, Unwin and colleagues (59), based on differences between the extracellular domain structure of the Torpedo ACh receptor, which is
probably in the closed state, and acetylcholine binding protein (AChBP), which is probably in the activated state, have suggested that agonist binding causes a larger shift in the ACh subunit structure.

In the region of the gate in the 4 Å structure, the M2 domains from the different subunits appear to make close contact with one another. Specifically, hydrophobic side chains from the 9' to the 14' positions interact to form “a tight hydrophobic girdle around the pore” (13). This girdle, which should impede the mobility of the individual M2 domains, may explain why, at the more proximal 17' position, the βM2 α-helix is less mobile than at the more distal 20' position. The constriction at the central portion of the channel may also help to explain why, in a previous study, no disulfide bonds formed between aligned residues from the 17' to the 6' positions.

The mobility that we have demonstrated in the 20' region with disulfide linkage is also consistent with the results of studies with unnatural amino acids. Using α-hydroxy acids in place of amino acids they converted the backbone peptide amide to an ester linkage. They inferred that there was more backbone conformational changes in the extracellular half of the ACh receptor M2 segment (20). Using linear free energy relationship analysis, it has also been shown that the extracellular half of M2 appears to move as a unit in the ACh-induced gating process (2, 60).

The range of motion that we have inferred for the β subunit M2 α-helices is not without precedent. In a study of protein backbone flexibility in the Escherichia coli tε-galactose chemosensory receptor, a protein of known crystal structure, Falke and colleagues found two cysteines that could traverse 15 Å to form a disulfide bond (28, 61, 62). The time constant for the formation of this disulfide bond in the chemosensory receptor mutant was 3630 s in the presence of 1.5:4.5 mM Cu:phen, more than ten times slower that the formation time constant in αβ20'Cγ, where disulfide formation went to completion within 360 s in the presence of 100:400 mM Cu:phen (data not shown). The difference in the formation rates are probably even greater because in the chemosensory receptor experiments the Cu:phen concentration was over 10 times higher and the temperature was 12–15 degrees higher (37 °C and 25 °C for the chemosensory and GABAβ receptors, respectively). Extrapolating from the results of Careaga and Falke (28), the collision rate between the engineered 2β0' Cys must be at least 108 s−1 and is probably higher, because their experiments were performed under significantly more oxidizing conditions than ours. In the bacterial mechanosensitive channel MacL state-dependent disulfide bond formation occurs between engineered Cys residues that are separated by >10 Å in the crystal structure. Disulfide bond formation between the MacL V15C required 1 mM Cu:phen and took about 30 min to go to completion (63). This suggests a lower collision frequency in MacL V15C than we observed for the GABAα receptor 2β' Cys in the present study.

Alternative Interpretations—Although we believe that the above interpretation of our data is most likely, it rests on the structural foundation provided by the 4 Å resolution ACh receptor structure. An alternative interpretation that we believe is unlikely is that we cannot exclude is that our data suggest that the closed-state structure of the GABAβ receptor is different from the published ACh receptor structure (13). The ability to form disulfide bonds and a high affinity Zn2+-binding site only between the β20'Cys may indicate there is a significant structural asymmetry at the 20' level such that the average separation of the β20' residues is smaller than the α20' residues.

CONCLUSION

We have shown that at the 20' level in the GABAβ receptor M2 segments, a disulfide bond or a high affinity Zn2+-binding site can form between engineered Cys residues in the β subunits. In contrast, an engineered Cys at the aligned position in the α subunits forms neither. Based on the roughly symmetric positions of the aligned residues relative to the channel axis in the 4 Å ACh receptor structure, we infer that the extracellular ends of the β M2 segments are more mobile than the α M2 segments. The increased mobility is likely due to looser protein packing around the β M2 segments. In the ACh receptor structure the α carbon atoms of the non-adjacent 20' residues are separated by ~19 Å. Thus, together the two β20'Cys must move ~14 Å to form a disulfide bond or each must move about 7 Å toward the central axis. A similar amount of translational movement would be necessary to bring the two Cys into close proximity to form a Zn2+-binding site. Given the high affinity with which Zn2+ was bound by the two β20'Cys, we infer that there must be a low energy barrier to this movement. This suggests that there is a relatively flat potential energy surface for the movement of the β M2 segments. These experiments begin to provide information on the dynamic movement of the channel-lining M2 segments and complement the static picture of the channel structure that is obtained from the cryo-EM structure of the homologous ACh receptor.

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