The pore structural changes associated with Cys-loop receptor gating are currently the subject of considerable interest. Several functional approaches have shown that surface exposure of pore-lining side chains does not change significantly during activation. However, a disulfide trapping study on α1T6Cβ1T6C γ-aminobutyric acid type A (GABA<sub>A</sub>) receptors (GABA<sub>A</sub>Rs), which showed that adjacent β subunits cross-link in the open state only, concluded that channel gating is mediated by β subunits contra-rotating through a summed angle of ~120°. Such a large rotation is not easily reconciled with other evidence. The present study initially sought to investigate an observation that appeared inconsistent with the rotation model: namely that α1T6Cβ1T6C GABA<sub>A</sub>Rs expressed in HEK293 cells form 6′ cysteine-mediated disulfide bonds in the closed state. On the basis of electrophysiological and Western blotting experiments, we conclude that adjacent βT6C subunits dimerize efficiently in the closed state via cross-links between their respective 6′ cysteines and that this locks the channels closed. This questions the β subunit contra-rotation model of activation and raises the question of how the closed state cross-links form. We propose that β subunit 6′ cysteines move into sufficiently close proximity for disulfide formation via relatively large amplitude random thermal motions that appear to be a unique feature of β subunits. Because dimerized channels are locked closed, we conclude either that the spontaneous β subunit movements or asymmetries in the movements of adjacent β subunits during activation are essential for pore opening. Our results identify a novel feature of GABA<sub>A</sub>R gating that may be important for understanding its activation mechanism.

Cys-loop receptors mediate fast neurotransmission in the nervous system. These receptors are pentameric homomers or heteromers, with each subunit comprising four α-helical transmembrane domains and a large extracellular amino-terminal domain that harbors the ligand binding sites and the signature Cys-loop (1–4). Each subunit contributes its second transmembrane domain (TM2)<sup>3</sup> to the lining of the axial channel pore. Neurotransmitter binding initiates a conformational change at the extracellular binding sites which is propagated throughout the receptor (5) culminating in the activation of the channel gate located in either the central region (6, 7) or the intracellular region (8, 9) of the pore. There is currently a great deal of interest in understanding how the TM2 domains move to mediate channel activation.

The original model, based on electron diffraction images of two-dimensional crystal arrays of Torpedo nicotinic acetylcholine receptors (nAChRs), concluded that the TM2 domains were kinked inwards to form a central pore constriction and that upon ligand binding they rotated about their long axes to open the pore (10). However, several functionally based approaches have since shown that the pattern of residue exposure in the pore does not change in a manner suggesting a significant rotation between the closed and open states (8, 9, 11). Thus, any rotational movement must be small.

However, cysteine trapping experiments on structurally related γ-aminobutyric acid type A receptors (GABA<sub>A</sub>Rs) incorporating introduced cysteines in the pore concluded that adjacent subunits experienced large contra-rotations during channel activation (12). The total rotational movement, which was proposed to be around 120°, is not easily reconciled with other studies. Specifically, Horenstein <i>et al.</i> (12) showed that αT6CβT6C GABA<sub>A</sub>Rs recombinantly expressed in *Xenopus* oocytes were locked in the open state by the application of an oxidising agent. Western blots of αT6CβT6C GABA<sub>A</sub>Rs expressed in HEK293 cells revealed that βT6C subunits were cross-linked in the open state but not in the closed state. The authors concluded that adjacent βT6C subunits underwent a contrarotation from the closed to the open states so that their T6′C side chains moved into close enough proximity to permit disulfide bond formation without blocking current flow.

In apparent contrast, an electrophysiological analysis of the effects of oxidizing and reducing agents on the same GABA<sub>A</sub>R subunits recombinantly expressed in HEK293 cells suggested that 6′ cysteines efficiently formed disulfide bonds in the closed state and in the desensitized state(13). This suggests that the functional properties of identical αT6CβT6C GABA<sub>A</sub>Rs vary dramatically between the *Xenopus* oocyte and the HEK293 cell expression systems. We initially postulated that the HEK293

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3 The abbreviations used are: TM, transmembrane domain; Cuphen, copper sulfate:phenanthroline; DTT, dithiothreitol; GABA<sub>A</sub>, γ-aminobutyric acid type A; GABA<sub>A</sub>R, GABA<sub>A</sub> receptor chloride channel; nAChR, nicotinic acetylcholine receptor cation channel; NEM, N-ethylmaleimide; PBS, phosphate-buffered saline.
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cell electrophysiology results could be reconciled with the HEK293 cell biochemical cross-linking experiments if closed state disulfide bond formation involves intrasubunit, rather than intersubunit, cross-inks. The aim of the present study is to investigate the molecular basis of closed state disulfide bond formation in HEK293-expressed α^TεCβ^TεC-GABA_A Rs. This may provide new insights into closed state structure, side chain orientation, and side chain mobility in this critical region of the pore.

EXPERIMENTAL PROCEDURES

Mutagenesis of GABA_A R cDNAs—The rat GABA_A R α1 and γ2 subunit cDNAs were subcloned into the pcDNA3.1 plasmid vector and the rat β1 subunit cDNA was subcloned into the pIRE52-EGFP plasmid vector. Site-directed mutagenesis of these cDNAs was performed using the QuikChange II mutagenesis protocol to introduce single codon mutations (Stratagene, La Jolla, CA). Using the same technique, insertions encoding FLAG (DYKDDDDK) and myc (EQKLISEEDL) epitopes were incorporated at amino acid position 4 of the mature α1 and β1 subunit coding sequences, respectively. The successful incorporation of all mutations was confirmed by automated sequencing of the complete coding sequence.

Western Blotting—HEK293 cells were transfected with the appropriate cDNAs using Polyfect (Qiagen) according to the manufacturer’s protocol. The α and β subunit cDNAs were transfected in a 1:1 ratio. However, to generate γ subunit-containing GABA_A Rs, the α, β, and γ subunit cDNAs were transfected in a 1:1:3 ratio. At around 72 h after transfection, the cells were washed twice with phosphate-buffered saline and treated with a solution containing 100 µM copper sulfate and 400 µM phenanthroline (100:400 µM Cu:phen) in the presence or absence of 100 µM GABA. After 30-min incubation with gentle rocking at room temperature, the cells were again washed twice with PBS. They were then treated for 30 min with either PBS only or PBS plus 10 mM dithiothreitol (DTT). After a final wash with PBS, 0.5 ml of homogenization buffer at 4 °C was added to the dishes, and the cells were harvested with a rubber policeman. The homogenization buffer contained: 320 mM sucrose, 20 mM Tris-HCl (pH adjusted to 7.4 at 4 °C), 5 mM EDTA, 1 mM n-phenylmethanesulfonyl fluoride, and Complete Mini Protease Inhibitors EDTA-free (Roche Diagnostics GmbH, Germany).

After harvesting, the cells were homogenized on ice using a Dounce homogenizer (50 strokes) followed by several passages through a hypodermic syringe fitted with a 30-gauge needle. The resultant homogenate was centrifuged for 10 min at 1,500 × g (4 °C) to precipitate out nuclei and unbroken cells. This centrifugation procedure was repeated and the supernatant was separated from the pellet. The supernatant was next centrifuged for 10 min at 10,000 × g at 4 °C. The small resultant pellet contained the crude membrane fraction. Enrichment of solubilized receptor in this fraction was confirmed by Western analysis (data not shown). A 40-µl volume of solubilization buffer was added to this pellet, and the resulting suspension was incubated for 30 min at 4 °C to extract integral membrane proteins. The solubilization buffer contained: 1% Nonidet P-40, 1% Triton X-100, 0.1 M Tris-HCl (pH adjusted to 7.4 at 4 °C), 0.3 M NaCl, 10 mM EDTA, 1 mM n-phenylmethanesulfonyl fluoride, Complete Mini Protease Inhibitors EDTA-free, adjusted to pH 7.4 at 4 °C. Then 40 µl of 2 × SDS loading buffer (no bromphenol blue/no DTT) containing 10 M urea was added, and the sample was heated at 65 °C for 20 min and stored at −20 °C.

Sample concentrations were analyzed from 10-µl aliquots using the BCA Protein Assay kit (Pierce). The remaining sample was diluted to 100 µl total volume to yield final concentrations of 5 M urea, 2% SDS, bromphenol blue and, where appropriate, 10 mM DTT. The samples were again heated at 65 °C for 15 min immediately prior to SDS-PAGE separation. Then 100 µg of total protein from each sample was loaded on a 10% acrylamide gel. Electrophoresis was carried out for 1 h using the Bio-Rad Mini-PROTEAN 3 Cell. Protein bands were subsequently transferred to PVDF membrane by electrotransfer for 1 h using a Bio-Rad mini Trans-Blot electrophoretic transfer cell. PVDF membranes were blocked for 1 h with 3.5% skimmed milk in Tris-buffered saline containing 0.05% Tween 20 and processed with either anti-FLAG M2 monoclonal antibody (Sigma) or anti-myc polyclonal antibody (Cell Signaling) and subsequently with anti-mouse horseradish peroxidase-conjugated secondary antibody (goat; Santa Cruz Biotechnology) and anti-rabbit horseradish peroxidase-conjugated secondary antibody (goat; Santa Cruz Biotechnology), respectively. PVDF membranes were developed using Super Signal West Pico (Pierce).

An Odyssey infrared imaging system (Li-Cor Biotech, Lincoln, NE) was used for direct quantitation of Western blots. For this assay, the Western blots were developed essentially as described previously but with the following exceptions. The protein bands were transferred to nitrocellulose membrane (BioTrace NT; Pall Corp.), and the membrane was subsequently blocked with Odyssey Blocking Buffer. All antibodies were diluted into Odyssey Blocking Buffer containing 0.1% Tween 20 for use in the incubation step. IRDye 880 CW-conjugated goat anti-rabbit IgG and IRDye 680-conjugated goat anti-mouse were used as secondary antibodies. The two epitopes were simultaneously observed using two-color detection, and images of the membrane were analyzed using Odyssey software.

Electrophysiology—HEK293 cells were transfected with wild type (WT) or mutant α, β and γ subunit cDNAs using a calcium phosphate precipitation protocol. When co-transfecting α and β subunits, their respective cDNAs were combined in a ratio of 1:1, although α, β and γ subunits were transfected in a ratio of 1:1:3. After exposure to transfection solution for 24 h, cells were washed twice using the culture medium and used for recording over the following 24–72 h. The cells were observed using an inverted fluorescent microscope and currents were measured using the whole cell patch clamp configuration. Cells were perfused by a control solution that contained (in mM): 140 NaCl, 5 KCl, 2 CaCl_2, 1 MgCl_2, 10 HEPES, 10 glucose, with the pH adjusted to 7.4 with NaOH. Patch pipettes were fabricated from borosilicate hematocrit tubing (Vitrex, Modulohm, Denmark) and heat-polished. Pipettes had a tip resistance of ~1.5 MΩ when filled with the standard pipette solution which contained (in mM): 145 CsCl, 2 CaCl_2, 2 MgCl_2, 10 HEPES, 10 EGTA, with the pH adjusted to 7.4 with NaOH. After establishment of the whole cell configu-
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**RESULTS**

**Electrophysiological Properties of αβ GABA\textsubscript{A}Rs—** Sample GABA dose-response relationships for the WT and mutant GABA\textsubscript{A}Rs subunit combinations investigated in this study are shown in Fig. 1A. The averaged dose-response curves are presented in Fig. 1B, with mean parameters of best fit displayed in Table 1. There was no evidence for tonic activity in any of the mutant GABA\textsubscript{A}Rs investigated. There was no systematic variation in resting whole-cell resistance in cells expressing any of these receptors, and 10 μM picrotoxin had no significant effect on base-line currents in cells expressing robust GABA-activated currents (n = 5 cells expressing each subunit combination). In all electrophysiological and biochemical experiments undertaken in the present study, GABA\textsubscript{A}Rs were activated by a 100 μM concentration of GABA, which was saturating at all receptors.

It is also apparent in Fig. 1A that GABA\textsubscript{A}Rs incorporating T6\textsuperscript{C} mutations exhibit an increased desensitization rate. The mean time constants of desensitization in the presence of 100 μM GABA for the three relevant receptors were as follows: α\textsuperscript{T6\textsuperscript{C}}β\textsuperscript{T6\textsuperscript{C}}, 133 ± 9 ms; α\textsuperscript{WT}β\textsuperscript{T6\textsuperscript{C}}, 107 ± 12 ms; α\textsuperscript{T6\textsuperscript{C}}β\textsuperscript{WT}, 421 ± 33 ms (all averaged from five cells). Such rapid desensitization means that it is difficult to apply oxidizing or reducing reagents selectively to the open state in either biochemical or electrophysiological experiments.

As shown previously (13), the α\textsuperscript{WT}β\textsuperscript{WT} GABA\textsubscript{A}R sustains a constant current magnitude when stimulated repeatedly with saturating GABA pulses. Although 100:400 μM Cu:phen applied in the closed or open states had no effect on current magnitude, a 2-min application of 10 mM DTT in the closed state caused a weak transient potentiation that is most likely a pharmacological action (13). In contrast, the α\textsuperscript{T6\textsuperscript{C}}β\textsuperscript{T6\textsuperscript{C}} GABA\textsubscript{A}R exhibited a spontaneous decline in current magnitude over time that was accelerated by the application of Cu:phen (Fig. 2A). In all experiments described in Fig. 2, the displayed recordings commenced directly following a 1-min application of 10 mM DTT. This reduction in current magnitude did not reverse spontaneously but was completely

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**TABLE 1**

| GABA\textsubscript{A}R | GABA EC\textsubscript{50} (μM) | n | I\textsubscript{max} (μA) |
|-------------------------|--------------------------|---|------------------|
| α\textsuperscript{WT}β\textsuperscript{WT} | 3.8 ± 0.5 | 1.3 ± 0.2 | 8.4 ± 1.7 |
| α\textsuperscript{T6\textsuperscript{C}}β\textsuperscript{T6\textsuperscript{C}} | 10.4 ± 2.3 | 1.0 ± 0.2 | 5.4 ± 1.9 |
| α\textsuperscript{T6\textsuperscript{C}}β\textsuperscript{WT} | 2.8 ± 0.6 | 0.8 ± 0.1 | 2.6 ± 0.4 |
| α\textsuperscript{WT}β\textsuperscript{T6\textsuperscript{C}} | 0.7 ± 0.3 | 0.6 ± 0.1 | 6.1 ± 2.7 |
| α\textsuperscript{T6\textsuperscript{C}}β\textsuperscript{T6\textsuperscript{C}} | 3.7 ± 0.5 | 1.4 ± 0.2 | 5.8 ± 1.6 |
| α\textsuperscript{T6\textsuperscript{C}}β\textsuperscript{T6\textsuperscript{C}} | 5.3 ± 0.4 | 1.8 ± 0.2 | 21.0 ± 2.4 |
| α\textsuperscript{T6\textsuperscript{C}}β\textsuperscript{WT} | 11.6 ± 1.6 | 1.5 ± 0.2 | 10.1 ± 1.9 |
| α\textsuperscript{WT}β\textsuperscript{T6\textsuperscript{C}} | 6.3 ± 0.6 | 1.8 ± 0.1 | 13.4 ± 0.8 |

**Data Analysis—** Results are expressed as means ± S.E. of the mean of three or more independent experiments. The Hill equation, fitted using a non-linear least squares algorithm (Sigmaplot 9.0, Jandel Scientific, San Rafael, CA), was used to calculate the saturating current magnitude (I\textsubscript{max}), half-maximal concentration (EC\textsubscript{50}) and Hill coefficient (n\textsubscript{H}), values for GABA activation. Statistical significance was determined by paired or unpaired Student's t test, as appropriate, with p < 0.05 representing significance. All Western blots are representative of at least three independent experiments.
Cross-linking of Adjacent GABA<sub>A</sub> Receptor β1 Subunits

A

![Figure 2. Effects of oxidation and reduction of β6 substituted cysteines on GABA-activated currents in αβ GABA<sub>A</sub>Rs.](image)

B

![Figure 2. Effects of oxidation and reduction of β6 substituted cysteines on GABA-activated currents in αβ GABA<sub>A</sub>Rs.](image)

We hypothesized that the results of Fig. 2 might be explained by inrasubunit disulfide formation in the closed state. The rat α1 subunit contains endogenous cysteines at position 233 in TM1 and at 292 in TM3, whereas the rat β1 subunit contains an endogenous cysteine at position 288 in TM3. To test for the involvement of the endogenous α subunit cysteines in disulfide bond formation, we created the triple mutant α<sup>TeC</sup>C<sub>233S</sub>, C292S subunit. The α<sup>T6C</sup>C<sub>233S</sub>C292S β<sup>WT</sup> GABA<sub>A</sub>R was found to be completely insensitive to both Cu:phen and DTT (Fig. 2B). Since the α<sup>WT</sup>β<sup>WT</sup> GABA<sub>A</sub>R was also insensitive to these compounds, we cannot exclude the possibility that one or both of the endogenous cysteines forms disulfide bonds with T6C in the α subunit. However, the large spatial separation between T6' and the endogenous cysteines renders such an interaction unlikely in correctly folded, functional GABA<sub>A</sub>Rs (14). Of course, the results could also be explained by α<sup>T6C</sup> subunits cross-linking with each other via their 6' cysteines or with the β subunit via its endogenous C288 residue. Western blotting (see below) was used to investigate this possibility further.

**Western Blotting of αβ GABA<sub>A</sub>Rs—**

Western blotting experiments using anti-myc epitope antibody to identify tagged β subunits revealed that the α<sup>WT</sup>β<sup>T6C</sup> and α<sup>T6C</sup>β<sup>WT</sup> GABA<sub>A</sub>Rs efficiently formed dimers in both the absence and presence of GABA, whereas the α<sup>WT</sup>β<sup>WT</sup> and α<sup>T6C</sup>β<sup>WT</sup> GABA<sub>A</sub>Rs did not form dimers under either condition (Fig. 3A). The blot in Fig. 3A was generated in the absence of NEM, which is often used to alkylate free sulfhydrys to prevent disulfide formation following protein structural disruption during the protein purification procedure. However, as seen in Fig. 3B, β subunit dimer bands were also seen in the closed state in the presence of 10 mM NEM. These dimers were not observed, however, when DTT treatment was included following oxidation and during SDS-PAGE sample preparation (Fig. 3C). Indeed, while dimer bands were observed in all samples containing β<sup>T6C</sup> subunits in the absence of DTT, the intensity of dimer bands was substantially decreased in all samples following treatment with DTT (Fig. 3D). The disappearance of dimer band in the presence of DTT is consistent with the reduction of intersubunit disulfide bonds. To quantify these changes, samples were transferred to nitrocellulose membrane for direct quantification of band intensity using the Odyssey Infrared Imaging System. In two separate experiments, an approximate 6-fold difference in the intensity of the dimer band was observed between samples treated with and without DTT (Fig. 4). A slight increase in the intensity of the monomer band in the presence of DTT was also reversed by a 1 min application of DTT (Fig. 2, A and B). We thus conclude that T6C residues participate in disulfide bonds that lock the channel closed. To isolate the subunit involved in this cross-linking, we investigated the effects of Cu:phen and DTT on the α<sup>WT</sup>β<sup>T6C</sup> and α<sup>T6C</sup>β<sup>WT</sup> GABA<sub>A</sub>Rs. As shown in the sample traces in Fig. 2A and summarized in Fig. 2B, the effect of Cu:phen and DTT on current magnitude in the α<sup>WT</sup>β<sup>T6C</sup> GABA<sub>A</sub>R was not significantly different to that observed in the α<sup>T6C</sup>β<sup>T6C</sup> GABA<sub>A</sub>R. This strongly suggests that β subunit 6' cysteines are major contributors to disulfide bond formation in the closed state. The magnitude of the Cu:phen-induced inhibition of α<sup>T6C</sup>β<sup>WT</sup> GABA<sub>A</sub>Rs was substantially diminished, although it remained statistically significant (Fig. 2A and B). This suggests that α<sup>T6C</sup> subunits may also be incorporated into spontaneously formed or Cu:phen-mediated disulfide bonds.

We hypothesized that the results of Fig. 2 might be explained by intrasubunit disulfide formation in the closed state. The rat α1 subunit contains endogenous cysteines at position 233 in TM1 and at 292 in TM3, whereas the rat β1 subunit contains 3A was generated in the absence of NEM, which is often used to alkylate free sulfhydrys to prevent disulfide formation following protein structural disruption during the protein purification procedure. However, as seen in Fig. 3B, β subunit dimer bands were also seen in the closed state in the presence of 10 mM NEM. These dimers were not observed, however, when DTT treatment was included following oxidation and during SDS-PAGE sample preparation (Fig. 3C). Indeed, while dimer bands were observed in all samples containing β<sup>T6C</sup> subunits in the absence of DTT, the intensity of dimer bands was substantially decreased in all samples following treatment with DTT (Fig. 3D). The disappearance of dimer band in the presence of DTT is consistent with the reduction of intersubunit disulfide bonds. To quantify these changes, samples were transferred to nitrocellulose membrane for direct quantification of band intensity using the Odyssey Infrared Imaging System. In two separate experiments, an approximate 6-fold difference in the intensity of the dimer band was observed between samples treated with and without DTT (Fig. 4). A slight increase in the intensity of the monomer band in the presence of DTT was also...
observed. Since variations in GABA\textsubscript{A}R protein concentrations between individual samples are to be expected, a more reliable measure is the ratio of dimer and monomer band intensities in each sample. Thus, while there is 10-fold more dimer in the absence of DTT, there is more monomer than dimer after reduction of the intersubunit disulfide cross-links with DTT. Taken together with the electrophysiology results, these observations strongly suggest that $\beta^{T6C}$ subunits cross-link in the closed state via their 6 cysteines and that this results in either channel block or in an inability of the receptors to open. The blot shown in Fig. 4 was also probed with an anti-FLAG epitope antibody to identify the $\alpha$ subunit. No discernible difference was observed between samples treated with DTT and those not treated with DTT. Direct quantification revealed no DTT-sensitive dimer bands indicating that a subunit 6 cysteines do not participate in subunit dimerization. Hence, our results show that subunit dimerization occurs efficiently between $\beta$ subunits via their 6 cysteines and that a subunit 6 cysteines do not contribute to subunit dimerization. These results are in accordance with the electrophysiological properties of the same receptors expressed in the same expression system (Fig. 2).

Fig. 3E shows that $\alpha^{T6C}$-containing receptors migrate at a slightly faster rate than $\alpha^{WT}$-containing receptors. This faster migration rate was abolished in $\alpha^{T6C}$, $\beta_{2SS}, \alpha^{WT}$ GABA\textsubscript{A}Rs and by DTT treatment (data not shown). This result provides some support for the conclusion from electrophysiological data (Fig. 2, C and D) that a subunit 6 cysteines may form intrasubunit disulfides with an endogenous $\alpha$ subunit cysteine.

**Cross-linking of Adjacent GABA\textsubscript{A} Receptor $\beta$1 Subunits**

**Electrophysiology and Western Blotting of $\alpha\beta$ GABA\textsubscript{A}Rs—** As $\alpha\beta$ GABA\textsubscript{A}Rs exist predominantly in a $2\alpha\beta$ stoichiometry (15), $\beta$-$\beta$ subunit dimers are theoretically possible between either adjacent or non-adjacent subunits. Because the present study shows that $\beta$ subunit dimerization results in pore closure or block, the possibility of disulfide formation between non-adjacent $\beta$ subunits cannot be ruled out. To determine whether non-adjacent $\beta$ subunits are able to form disulfides via their 6 cysteines, we investigated intersubunit disulfide bond formation in $\alpha\beta$ GABA\textsubscript{A}Rs, which have no adjacent $\beta$ subunits (15). Examples of the effects of 100-400 $\mu M$ Cu:phen and $\alpha^{WT}$ $\beta^{T6C}$ $\gamma^{WT}$ GABA\textsubscript{A}Rs are presented in the left panel of Fig. 5A. Although a 60 s application of Cu:phen transiently decreased current magnitude in both receptors, it had no irreversible effect. Results of similar experiments averaged from five cells expressing each GABA\textsubscript{A}R are summarized in Fig. 5A (right panel). These plots, which compare the magnitudes of currents measured 2 min after Cu:phen treatment relative to those of control currents, provide strong evidence that disulfide bond formation does not occur in $\gamma$ subunit-containing GABA\textsubscript{A}Rs. The expression of the $\gamma$ subunit was confirmed in two ways. First, because incorporation of $\gamma$ subunits confers diazepam sensitivity to recombinant GABA\textsubscript{A}Rs (15, 16), we quantitated the effect of a 1 $\mu M$ diazepam concentration on currents activated by 1 $\mu M$ GABA in the in the $\alpha^{WT}$ $\beta^{WT}$ $\gamma^{WT}$ GABA\textsubscript{A}R. A sample result is presented in Fig. 5C (left). The results averaged from 3 cells (Fig. 5C, right) confirm that $\gamma$ sub-
unit expression confers diazepam sensitivity onto recombinant 
GABA_\(_A\)_Rs. The same diazepam concentration had no signifi-
cant effect in five cells expressing \(\alpha^{\text{WT}}\beta^{\text{WT}}\) GABA_\(_A\)_Rs. We
could readily infer the presence of high molecular weights, these were not DTT-sensitive and were also observed in untransfected samples and are therefore nonspecific.

As a second method of confirmation, we probed cells express-
ing putative \(\alpha^{\text{WT}}\beta^{\text{WT}}\) GABA_\(_A\)_Rs with an antibody for the \(\gamma\) subunit. As shown in Fig. 5D, antibody labeling was spe-
cific for the receptor incorporating the \(\gamma\) subunit. Finally,
Western blotting confirmed the absence of dimer bands in
\(\alpha^{\text{WT}}\beta^{\text{WT}}\gamma^{\text{WT}}\) GABA_\(_A\)_Rs (Fig. 5E).

**DISCUSSION**

**Summary of Results**—The main conclusion of this study is that adjacent \(\beta\) subunits dimerize efficiently in the closed state
via disulfide bond formation between their respective 6‘ cyste-
ine. This effectively locks the receptors into the closed state.
Because \(\alpha^{\text{T6C}}\beta^{\text{T6C}}\) and \(\alpha^{\text{WT}}\beta^{\text{T6C}}\) GABA_\(_A\)_Rs desensitize rapid-
ly (with time constants in the order of 100 ms) it was not possible to reliably apply oxidising or reducing agents in the open state. Hence it was not possible to establish whether disul-
fide bond formation could also occur in the open state. How-
ever, from previous electrophysiology results (13) and the pres-
ent Western blot data, we conclude that dimers form in the
desensitized state.

Our results also suggest that endogenous transmembrane
domain cysteines in \(\alpha^{\text{T6C}}\) subunits may also contribute to
disulfide formation. As \(\alpha^{\text{T6C}}\) subunits do not dimerise with
each other or with \(\beta\) subunits, such cross-links are most likely
formed within individual \(\alpha\) subunits. We did not investigate
their molecular basis in this study.

**Comparison with Previous Results**—A previous study also
presented evidence for dimerization of adjacent \(\beta\) subunits
(12). However, contrary to the present study, they concluded
that dimerization resulted in the irreversible opening of the
channel. The electrophysiological evidence for irreversible activ-
ation of \(\alpha^{\text{T6C}}\beta^{\text{T6C}}\) GABA_\(_A\)_Rs by Cu:phen was recorded from
receptors recombinantly expressed in *Xenopus* oocytes. The dras-
tic difference with the present results may be due to the different
expression systems. Structures of transmembrane proteins may
vary between HEK293 cell and *Xenopus* oocyte expression systems
due to variations in the membrane lipid composition or the
expression of cytoplasmic enzymes that control post-transla-
tional modifications, subunit folding or assembly. However,
both studies performed Western blotting on the same receptors
expressed in HEK293 cells. We have no explanation for the
different results. The present study makes a case for closed state
Cross-linking of Adjacent GABA<sub>A</sub> Receptor β1 Subunits

A recent study demonstrated β subunit dimerization via 6' cysteines in the absence of GABA in homomeric β3 GABA<sub>A</sub>Rs (17). Because these receptors gate spontaneously and the consequences of dimerization on receptor electrophysiological properties were not investigated, it was not possible to conclude whether dimerization occurred in the closed or open states or both. Nevertheless the result is illuminating as it shows that β subunit dimerization can occur in homomeric GABA<sub>A</sub>Rs. It has previously been thought that receptor subunit asymmetry was required to bring adjacent β subunit 6' cysteines into sufficiently close proximity to permit dimerization (12).

Implications for Pore Structure and Gating—There are currently three structural models to explain how TM2 domains move to mediate Cys-loop receptor activation. Based on differences in electron density images of Torpedo nAChRs in the closed and open states, it was originally suggested that channel opening was mediated by a rotation of the TM2 domains around their long axes (10). The most recent version of this model proposes a rotation of ~15°, which is too small to significantly alter side chain exposure to the pore (18). A second study, which quantitatively substantiated the reaction rates of protons with histidine side chains substituted sequentially for each TM2 residue in the muscle nAChR (11), concluded that the relative orientation of each residue with respect to the pore lumen did not change significantly as the channel opened and closed. This implies a parallel widening of the pore. A third study, based on the state dependence of zinc coordination by histidines substituted sequentially for nAChR pore-lining residues, concluded that channel opening was mediated by a rigid tilting motion of the TM2 domains around an axis parallel to the cell membrane that passes through the 6' residue (9). This model proposes no movement at the 6' residue but a narrowing of the outer part of the pore and a widening of the inner part of the pore. A consistent feature of all three models is that activation involves no substantial change in the orientation of pore-lining residues.

FIGURE 5. Effects of γ subunit incorporation on β<sup>6'C</sup> cross-link formation. A and B, the displayed recordings were commenced immediately after a 1-min application of 10 mM DTT. Repeated applications of 100 µM Cu:phen (unfilled bars) reveal no spontaneous rundown in GABA current magnitude and no irreversible effect of 100-400 µM Cu:phen (black bars) on current magnitude in either α<sup>WT</sup>β<sup>WT</sup>γ<sup>WT</sup> or α<sup>WT</sup>β<sup>REC</sup>γ<sup>WT</sup> GABA<sub>A</sub>Rs. The right panels summarize the results averaged from five similar experiments. C, pharmacological evidence for the functional incorporation of γ subunits into GABA<sub>A</sub>Rs. The right panel summarizes the results from three experiments (*, p < 0.05, using Student's paired t test). D, Western analysis with anti-γ2 antibody demonstrates incorporation of γ2 subunit in detergent soluble surface expressed GABA<sub>A</sub>Rs. Lower arrow, solubilized γ2 subunits. Upper arrow, γ2 aggregates. E, Western analysis with anti-myc antibody against the tagged β subunit shows that intermolecular disulfide bonds are formed between adjacent β6'C residues in αβ GABA<sub>A</sub>Rs following oxidation with Cu:phen. In contrast, no disulfide bonds are observed between non-adjacent β6'C residues in αβγ GABA<sub>A</sub>Rs following oxidation with Cu:phen. Arrow, β subunit dimers.
Cross-linking of Adjacent GABA<sub>A</sub> Receptor β1 Subunits

the closed state. Since the present study investigated hetero-
meric GABA<sub>A</sub>Rs, our results can be explained by proposing that adjacent β subunits are oriented asymmetrically with their 6′ cysteines facing toward each other. This explanation is supported by evidence from the structurally related α1 homomeric GlyR. This receptor, which presumably has a symmetrical closed state structure by virtue of its homomeric subunit composition, does not cross-link via 6′ cysteines in either the closed or open states (13). However, the observation of β subunit dimer formation in homomeric β3 GABA<sub>A</sub>Rs is difficult to reconcile with this conclusion. All three studies can be reconciled by proposing that α1 GABA<sub>A</sub>R subunits (and α1 GlyR subunits) are incapable of participating in 6′C-mediated cross-links with either themselves or with β GABA<sub>A</sub>R subunits.

We propose that β subunits achieve cross-linking due to an unusually high thermal mobility rather than by either an asymmetry in the relative positioning of subunits in the closed state or by differences in modes of α and β subunit displacement. In support of this, evidence has previously been presented that the outer half of the GABA<sub>A</sub>R β subunit TM2 is more mobile than that of the α subunit (19, 20). We note, however, that the region around 6′ is thought to be less mobile than the extracellular half of the β subunit TM2. Despite this, evidence has been presented for random thermal motion of the GABA<sub>A</sub>R β subunit 6′ residue in the closed state (20). We propose that a similar motion may result in the juxtapositioning of β subunit T6′C residues from adjacent subunits. While the results of the present study are consistent with the previous conclusion that β subunits are able to undergo large contrarotations (12), they do not lend support to the conclusion that these movements are associated exclusively with pore transitions to the open state. Nevertheless, because cross-linking of adjacent β subunits prevents the channels from opening, a movement of adjacent subunits relative to one another must be essential for channel gating. These may take the form of asymmetric movements (such as contrarotations of adjacent β subunits) or as asynchronies in the movement of adjacent subunits during gating. Asymmetries in collective subunit motions have previously been observed for 9′ residues during activation of the nAChR (21).

Conclusion—In conclusion, we show that adjacent β subunits in α1β1 GABA<sub>A</sub>Rs dimerise efficiently in the closed state via disulfide bond formation between their respective 6′ introduced cysteines. The dimerized receptors are thereby locked into the closed state. We propose that the 6′ cysteines are able to move into sufficiently close proximity for disulfide formation via relatively large amplitude thermal motions that are characteristic only of β subunits. As cross-links occur in both the absence and presence of GABA, we conclude that similar motions of β subunit TM2 segments occur in the closed and desensitized states, although the channel structures may be different. Because disulfide formation precludes channel activation, we conclude either that spontaneous β subunit movements or asymmetric movements of adjacent β subunits during activation are essential for channel activation. These results identify a novel feature of GABA<sub>A</sub>R gating that may be important for understanding the activation mechanism of these receptors.

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