Molecular Regions Controlling the Activity of CNG Channels

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ABSTRACT The α subunits of CNG channels of retinal photoreceptors (rod) and olfactory neurons (olf) are proteins that consist of a cytoplasmic NH_2 terminus, a transmembrane core region (including the segments S1–S6), and a cytoplasmic COOH terminus. The COOH terminus contains a cyclic nucleotide monophosphate binding domain NBD) that is linked by the C-linker (CL) to the core region. The binding of cyclic nucleotides to the NBD promotes channel opening by an allosteric mechanism. We examined why the sensitivity to cGMP is 22 times higher in olf than in rod by constructing chimeric channels and determining the [cGMP] causing half maximum channel activity (EC_{50}). The characteristic difference in the EC_{50} value between rod and olf was introduced by the NH_2 terminus and the core-CL region, whereas the NBD showed a paradoxical effect. The difference of the free energy difference $\Delta(\Delta G)$ was determined for each of these three regions with all possible combinations of the other two regions. For rod regions with respect to corresponding olf regions, the open channel conformation was destabilized by the NH_2 terminus ($\Delta(\Delta G) = -1.0$ to -2.0 RT) and the core-CL region ($\Delta(\Delta G) = -2.0$ to -2.9 RT), whereas it was stabilized by the NBD ($\Delta(\Delta G) = 0.3$ to 1.1 RT). The NH_2 terminus deletion mutants of rod and olf differed by $\Delta(\Delta G)$ of only 0.9 RT, whereas the wild-type channels differed by the much larger value of 3.1 RT. The results show that in rod and olf, the NH_2 terminus, the core-CL region, and the NBD differ by characteristic $\Delta(\Delta G)$ values that do not depend on the specific composition of the other two regions and that the NH_2 terminus generates the main portion of $\Delta(\Delta G)$ between the wild-type channels.

KEY WORDS: ion channel • ligand • cyclic nucleotide • gating

INTRODUCTION

Modulation of the activity of CNG channels generates the light-induced electrical response of rod photoreceptors and the odor-induced electrical response in olfactory cells. Native rod CNG channels are heteromultimers formed from α and β subunits (Chen et al., 1993, 1994; Körschen et al., 1995). Expression of the α subunit only is sufficient to produce functional homomultimeric channels (Varnum and Zagotta, 1996), whereas the β subunit does not form functional channels on its own but imparts properties onto the heteromultimer that are characteristic of native channels (Chen et al., 1993, 1994; Bucossi et al., 1997; Shapiro and Zagotta, 1998; Bönigk et al., 1999).

Opening of CNG channels is strongly promoted by the binding of cyclic nucleotides to a binding domain that is ∼120 residues long (Kaupp et al., 1989) and located near the COOH terminus of the channel protein (for review see Finn et al., 1996). This sequence is homologous to cyclic nucleotide monophosphate binding domains of other proteins (Shabb and Corbin, 1992). In the past years, it has been shown by several investigators that a sequence of ∼90 residues linking the cyclic nucleotide monophosphate binding domain to the S6 transmembrane segment (C-linker) plays a key role for the different sensitivity to cyclic nucleotides among the CNG channels (Gordon and Zagotta, 1995a,b; Broillet and Firestein, 1996; Gordon et al., 1997; Zong et al., 1998; Paoletti et al., 1999). Using chimeric constructs between the bovine rod and the rat olfactory channel, Gordon and Zagotta (1995b) observed that the great difference in the [cGMP] causing half maximum channel activity (EC_{50})* is determined by further regions: the NH_2 terminus, the S5 segment, and a large part of the core running from S2 to S6, including S5. Also, in rod, specific interactions between the NH_2 terminus and the C-linker were reported to be relevant for opening (Gordon et al., 1997), including the formation of a disulfide bond between C35 in the NH_2 terminus and C481 in the C-linker.

Despite growing insight into the role of the different channel regions, our present knowledge about the molecular events underlying the gating is only poor. Herein, we screen CNG channels for regions determining the EC_{50} by constructing chimeric channels between the α subunits of bovine retinal photoreceptors

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*Abbreviations used in this paper: CL, C-linker; cNMP, cyclic nucleotide monophosphate; EC_{50}, [cGMP] causing half maximum channel activity; NBD, cNMP-binding domain; NT, NH_2 terminus; olf, olfactory channel; rod, rod channel.

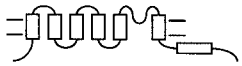
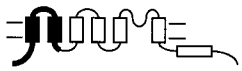
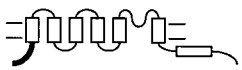
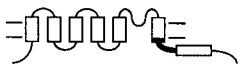
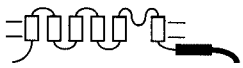
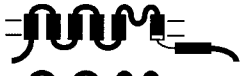
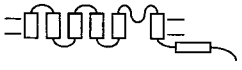
Name	Cartoon	Sequence		
rod		rM_1 - D_{690}		
olf		oM_1-P_{663}		
r3o		rM_1-Q_{224}	oG_{202}-P_{663}	
o3r5o		oM_1-Q_{201}	rG_{225}-I_{298}	oS_{276}-P_{663}
o5r		oM_1-I_{272}	rF_{296}-D_{690}	
r1o		rM_1-D_{157}	oP_{135}-P_{663}	
oLrBo		oM_1-I_{367}	rV_{391}-P_{500}	oG_{478}-P_{663}
oBr		oM_1-P_{477}	rG_{501}-D_{690}	
r1oBr		rM_1-D_{157}	oP_{135}-P_{477}	rG_{501}-D_{690}
o1r		oM_1-P_{134}	rP_{158}-D_{690}	
r1o3r		rM_1-D_{157}	oP_{135}-Q_{201}	rG_{225}-D_{690}
r3o5r		rM_1-Q_{224}	oG_{202}-I_{275}	rS_{299}-D_{690}
r5oLr		rM_1-I_{295}	oF_{273}-I_{367}	rV_{391}-D_{690}
rLo		rM_1-I_{390}	oV_{368}-P_{663}	
rLoBr		rM_1-I_{390}	oV_{368}-P_{477}	rG_{501}-D_{690}
rBo		rM_1-P_{500}	oG_{478}-P_{663}	
o1rBo		oM_1-D_{134}	rP_{168}-P_{500}	oG_{478}-P_{663}
-1r		rI_{156}-D_{690}		
-1o		oL_{133}-P_{663}		

FIGURE 1. Structure of the chimeras between rod and olf. As shown in the cartoons, each channel/chimera consists of an NH_2 terminus (left), six transmembrane helices (S1–S6; vertical boxes) linked by extramembrane linkers including the pore region (lines), the cyclic nucleotide monophosphate binding domain (NBD; horizontal box plus adjacent line at the right), and the C-linker (CL; line between the sixth helix and the NBD). Deletion mutants (−1r, −1o) lack an NH_2 terminus. Black boxes and fat lines indicate rod sequences, whereas white boxes and thin lines indicate olf sequences. At the right, the exact splice sites of the rod (r) and olf (o) sequences are given by the terminal amino acids. In the names, reading from the left roughly indicates the sequence of rod "r" or olf "o" regions in the direction from the NH_2- to the COOH terminus. "1...6," "L," and "B" indicate the six transmembrane helices, the C-linker, and the NBD, respectively. For further explanation see text.

(rod) and olfactory neurons (olf). We show that multiple regions along the sequence between NH_2 terminus and C-linker determine the characteristic difference in the EC_{50}, whereas the cyclic nucleotide monophosphate binding domain has a surprising paradoxical effect. Systematic swap of the NH_2 terminus, the core region including the C-linker, and the cyclic nucleotide binding domain shows that the difference of the free energy difference $\Delta(\Delta G)$ between corresponding rod and olf regions is characteristic, independent of the composition of the channel background.

MATERIALS AND METHODS

Composition of the Chimeras

The α subunits of bovine rod and olfactory channels (accession No. 51604 and 55010, respectively) and most of the chimeras between these channels were provided by U.B. Kaupp (Institut für Biologische Informationsverarbeitung, Forschungszentrum Jülich, Germany). Fig. 1 summarizes the name, cartoon, and splice sites of all chimeras used. Chimera r1o3r was constructed by a recombinant PCR approach. For this purpose, plasmids encoding r1o and rod were used as templates for the primary amplification of the desired DNA fragments. PCR products were excised from an agarose gel and coupled in frame in a second PCR reaction. The resulting

product was subcloned into the SalI/AsuII sites of the plasmid encoding chimera o5r. For the construction of r5oLr, a BstXI site was introduced at the olf/rod splice site of a chimera oLr (the amino acids M_1-I_{367} of olf and V_{391}-D_{690} of rod; not shown in Fig. 1), which was finally used to combine respective fragments of a chimera r5o (amino acids M_1-I_{295} of rod and F_{273}-P_{663} of olf; not shown in Fig. 1) and the modified sequence of oLr. The successful exchange of nucleotide sequences was verified by DNA sequencing.

The name of the chimeras was specified in the following way: "o" indicates an olf sequence and "r" indicates a rod sequence. The transmembrane helices, the C-linker, and the NBD are indicated by the abbreviations "1...6," "L," and "B," respectively. Reading from the left roughly indicates the composition of the chimeras in the direction from the N- to the COOH terminus. One of the abbreviations "1...6," "L," or "B" before an "r" or "o" indicates that the splice site is located before the respective rod or olf region. For example, "o1rBo" means that the chimera contains an NH_2 terminus from olf, the transmembrane segments S1–S6 plus the C-linker from rod, and a nucleotide binding domain from olf.

Oocyte Preparation

Oocytes of *Xenopus laevis* were prepared as described previously (Benndorf et al., 1999). In brief, ovarian lobes were obtained under anesthesia (0.3% 3-aminobenzoic acid ethyl ester) and transferred to a Petri dish containing the following Barth medium (in mM): 84 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.82 $MgSO_4$, 0.33 $Ca(NO_3)_2$, 0.41 $CaCl_2$, and 7.5 Tris, pH 7.4 (HCl). Oocytes in stages V and VI were prepared by incubation for 20–30 min in a Ca^{2+}-free Barth medium containing either 1 or 2 mg/ml collagenase. Within 2–7 h after isolation and defolliculation, cRNA specific for the respective channel was injected into the oocytes through glass micropipettes. Oocytes were further incubated at 18°C for 2–7 d until experimental use. Before patching, the vitelline membrane of the oocytes was removed after exposing the cells to the following hypertonic "skinning" solution (in mM): 200 aspartate, 20 KCl, 1 $MgCl_2$, 5 EGTA, and 10 HEPES, pH 7.4 (KOH).

Recording Technique

The oocytes were transferred to the experimental chamber that was mounted on the stage of an inverted microscope. The patch pipettes were pulled from borosilicate glass tubing. The glass tubing had an outer diameter of 2.0 mm and an inner diameter of 1.0 mm. The pipette resistance after fire polishing was 1–3 MΩ. The bath solution contained the following (in mM): 140 KCl, 10 NaCl, 1 EGTA, and 5 HEPES, pH 7.4 (KOH). The pipette solution contained the following (in mM): 145 NaCl, 5 KCl, 1 EGTA, and 5 HEPES, pH 7.4 (NaOH). The currents were recorded in inside-out patches with a conventional patch-clamp technique (Hamill et al., 1981). The CNG channels were activated by replacing the bath solution with a respective solution containing cGMP. Each excised patch was first exposed to a saturating [cGMP] to determine the maximum current.

Recording was performed with an amplifier (model Axopatch 200A; Axon Instruments). The currents were filtered at a cut-off frequency of 10 kHz. The holding voltage was generally 0 mV. The membrane voltage was first stepped to −100 mV and then to +100 mV. The pulse duration and the repetition rate of the pulses were chosen such that, at the end of the pulses, the current amplitude was constant. All measurements were performed at room temperature (22–24°C).

Data Acquisition and Analysis

Recording and analysis of the data was performed on a Pentium PC with the ISO2 software (MFK Niedernhausen). All currents were corrected for the capacitive and the very small leak components by subtracting respective currents in the absence of cGMP in the bath solution. The currents considered herein are averages of 5–30 consecutive recordings. The sampling rate was 2–10 kHz. Dose–response relationships were determined from the steady-state current at +100 mV by normalizing the current I at the actual [cGMP] with respect to the current I_{max} at saturating [cGMP] and fitting the data points with a Hill equation of the form

$$I/I_{max} = [cGMP]^H/([cGMP]^H + EC_{50}^H), \quad (1)$$

yielding values for EC_{50} and H, the [cGMP] of half maximum activity of the channels and the Hill coefficient, respectively. The curves were fitted to the data with appropriate nonlinear approximation algorithms. The Hill coefficients varied between 2.0 and 3.3. In the present report, they were not further considered because we did not detect a systematic dependency.

Statistics

Statistical data are given as mean ± SEM. SEM of Δ(ΔG) (see below) was calculated according to the error propagation law. *t* test with a significance level $P < 0.05$ was used to detect significant differences between data.

RESULTS

The Different EC_{50} Value in rod and olf Is Determined by Multiple Regions between NH_2 Terminus and C-linker, Whereas the Cyclic Nucleotide Binding Domain Has a Paradoxical Effect

Fig. 2 shows dose–response relationships and a plot of the resulting EC_{50} values obtained from chimeras containing rod regions in an olf background. The chimeras r3o, o3r5o, and o5r were designed to probe the influence of the regions S1–S2 (plus the respective NH_2 terminus; NT), S3–S4, and S5-P-S6 (plus the respective C-linker [CL], cyclic nucleotide monophosphate [cNMP] binding site, and the adjacent COOH-terminal sequence). Herein the combination of the cNMP-binding site and the respective COOH-terminal sequence is lumped to the cNMP-binding domain (NBD). Transplanting the rod NT-S1-S2 region (chimera r3o) to olf also transfers a large portion of the higher EC_{50} of rod. This result agrees with findings for a similar chimera between catfish olf and bovine rod (Tibbs et al., 1997). A smaller portion of the higher EC_{50} was also transferred with the S5-P-S6-CL-NBD region (chimera o5r). The S3–S4 region of rod did not transfer the higher EC_{50} value of rod to olf (chimera o3r5o). Transfer of the rod NH_2 terminus alone (chimera r1o) caused only a 4-fold increase of the EC_{50} compared with a 14-fold increase in chimera r3o. This result shows that both the NH_2 terminus and the S1–S2 region contribute to the different EC_{50} in rod and olf. We further attempted to differentiate the influence of the C-linker and the NBD. Transfer of the C-linker alone (chimera oLrBo) produced a similar shift of the EC_{50} as observed in chimera o5r. Therefore, the C-linker is a third region that

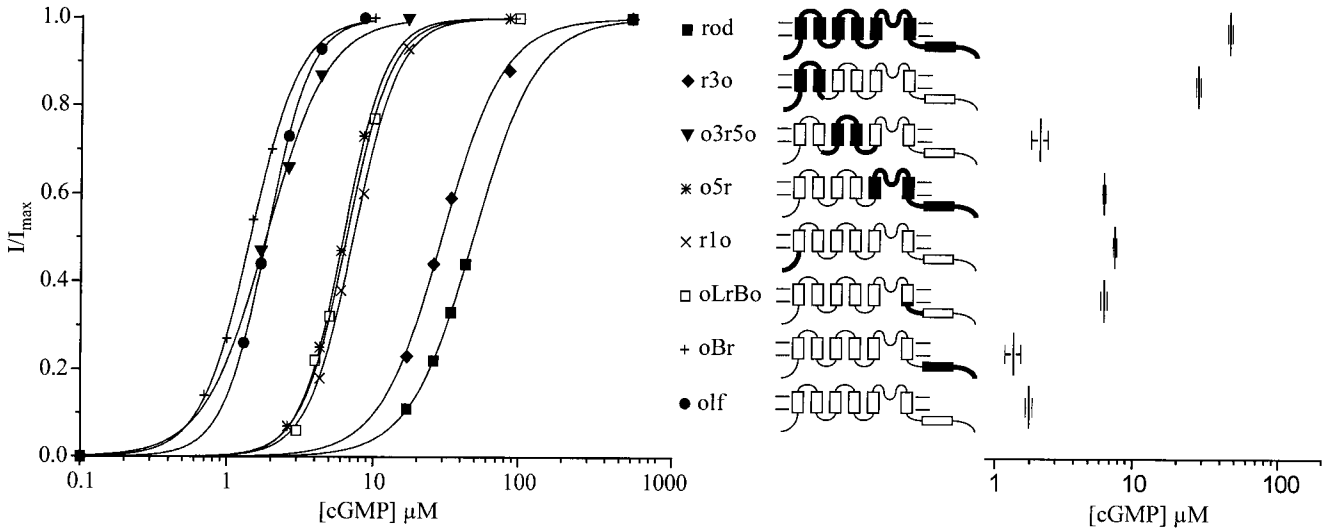


FIGURE 2. Dose–response relationship for the activation of chimeric channels constructed by transferring rod segments into an olf environment. The names and the cartoons of the channels/chimeras are listed in the middle. Fat lines and black boxes indicate extramembrane and transmembrane segments of rod, respectively. Thin lines and white boxes indicate the respective segments of olf. The symbols in the left diagram correspond to those left to the names. The data points of the dose–response relationships stem from representative experiments. The curves are best fits to Eq. 1. The following parameters were obtained: for rod, EC_{50} = 45.0 μM, H = 2.3; for r3o, EC_{50} = 27.4 μM, H = 2.7; for o3r5o, EC_{50} = 2.3 μM, H = 2.1; for o5r, EC_{50} = 6.3 μM, H = 3.3; for r1o, EC_{50} = 7.5 μM, H = 3.0; for oLrBo, EC_{50} = 6.4 μM, H = 3.0; for oBr, EC_{50} = 1.54 μM, H = 3.6; and for olf, EC_{50} = 1.97 μM, H = 2.8. In the right diagram the values of EC_{50} are graphically illustrated by their mean ± SEM. The means were calculated from 4 to 16 patches. The systematic transfer of the rod segments into an olf background identifies the NH_2 terminus, the S1–S2 region, and the C-linker as critical regions determining the higher EC_{50} value in rod compared with olf. The transfer of the rod NBD into an olf background caused a lower EC_{50} than in olf itself.

determines the EC_{50} values. Quite surprisingly, transfer of the rod NBD (chimera oBr) produced a lower EC_{50} value than observed in olf.

In Fig. 3, the first six chimeras illustrate effects of olf regions in a rod background. The first four of these chimeras probe the contributions of the NH_2 terminus (chimera o1r), S3–S4 region (chimera r3o5r), S5-P-S6 region (chimera r5oLr), and CL-NBD region (chimera rLo). The S1–S2 region is not included because the respective chimera r1o3r did not produce functional channels. Along with the olf NH_2 terminus (chimera o1r), a considerable portion of the lower EC_{50} is transferred from olf to rod. Smaller, but significant effects were generated by the transfer of the CL-NBD region (chimera rLo) and the S5-P-S6 region (chimera r5oLr). As in the reverse chimera, the transfer of the S3–S4 region was without effect (chimera r3o5r). We also tested for the individual effects of the C-linker (chimera rLoBr) and the NBD (chimera rBo). The transfer of the C-linker (chimera rLoBr) lowered the EC_{50} value much more than the transfer of the larger CL-NBD region (chimera rLo). With respect to the NBD (chimera rBo), we again observed a paradoxical finding: the chimera rBo had a larger EC_{50} value than rod.

It has been suggested that the NH_2- and COOH-terminal regions in both rod and olf undergo an intramolecular interaction that modulates the ease by which channels are opened by cyclic nucleotides (Gordon et al., 1997; Varnum and Zagotta, 1997). To study the functional consequences of these interactions, we also constructed double chimeras that contained both the rod NH_2 terminus and NBD in an olf background (o1rBo) and, vice versa, the olf NH_2 terminus and NBD in a rod background (r1oBr). Both double chimeras produced EC_{50} values intermediate between the EC_{50} values from rod and olf (Fig. 3).

NH_2 Terminus, NBD, and Core-CL Region Produce Characteristic Shifts of the EC_{50} Value Independent of the Channel Background

Fig. 4 illustrates schemes of channels composed either of the rod (black boxes) or olf (white boxes) NH_2 terminus, core region (S1–S6) plus the C-linker (core-CL), and NBD. The eight chimeras and wild-type channels are ordered from top to bottom by increasing EC_{50} values (Figs. 1 and 2). To quantify the energetic effects of corresponding rod and olf regions, we calculated differences in the free energy difference ($\Delta(\Delta G)$) between the channels/chimeras according to Eq. 2:

$$\Delta(\Delta G) = -RT \ln(EC_{50,1}/EC_{50,2}), \qquad (2)$$

where R is the molar gas constant and T is the temperature in K. $EC_{50,1}$ is the EC_{50} value of the channel/chimera with the rod region, and $EC_{50,2}$ is the EC_{50} value

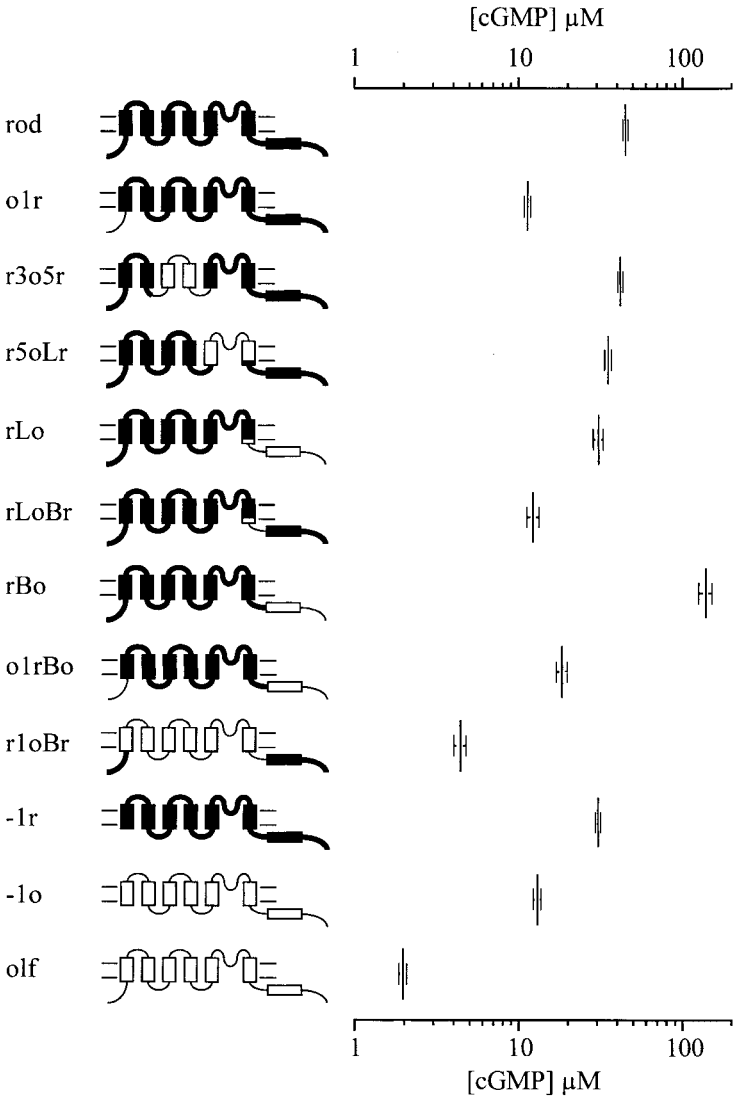


FIGURE 3. EC_{50} for the activation of chimeric channels constructed by transferring single olf regions into a rod environment (top six chimeras), both termini together in the respective other environment (o1rBo, r1oBr; chimera seven and eight from top), and by deleting the NH_2 terminus (bottom two chimeras). The EC_{50} values were obtained as described in Fig. 2. The systematic transfer of the olf segments into a rod background shows that the NH_2 terminus and the C-linker are important regions determining the lower EC_{50} value in olf compared with rod. A small effect on the EC_{50} was also observed for the S5-P-S6 region. The means were calculated from 4 to 28 patches each.

of the channel/chimera with the olf region. Hence, a negative $\Delta(\Delta G)$ value means that a rod region destabilizes the open channel conformation with respect to the corresponding olf region, whereas a positive $\Delta(\Delta G)$ value means that a rod region stabilizes the open channel conformation with respect to the corresponding olf region. The brackets to the right of the channel schemes and the corresponding bar graphs at the bottom of Fig. 4 indicate the $\Delta(\Delta G)$ values for the twelve possible comparisons.

The three regions tested produced consistent effects on $\Delta(\Delta G)$ with all possible combinations of the other two regions. The rod NH_2 terminus produced a $\Delta(\Delta G)$ of -1.0 to -2.0 RT with respect to the olf NH_2 terminus and the rod core-CL region produced a $\Delta(\Delta G)$ of -2.0 to -2.9 RT with respect to the olf core-CL region. These effects correspond to the higher EC_{50} in rod than in olf. In contrast, the rod NBD caused a $\Delta(\Delta G)$ of 0.3 to 1.1 RT with respect to the olf NBD. This effect corresponds to the paradoxical higher EC_{50} for channels with an olf NBD compared with otherwise equal channels with a rod NBD. The latter result is also substantiated by the 2.5 times larger EC_{50} in chimera rLo compared with chimera rLoBr (Fig. 3). These results show that each of the three regions in rod and olf causes a characteristic $\Delta(\Delta G)$ value that is largely independent of the combination of the other two channel regions. Therefore, it is suggested that the molecular interactions of these three channel regions are similar in rod and olf and largely independent of the specific composition of the channel background.

Properties of the rod and olf NH_2 Terminus Deletion Mutants

CNG channels also open with deleted NH_2 terminus (Gordon and Zagotta, 1995b; Brown et al., 1998). In rod, deletion of the NH_2 terminus caused a small but significant reduction of the EC_{50} value (30.5 ± 1.1 μM for −1r compared with 45.0 ± 1.6 μM for rod; Fig. 3). In olf, deletion of the NH_2 terminus (−1o) consider-

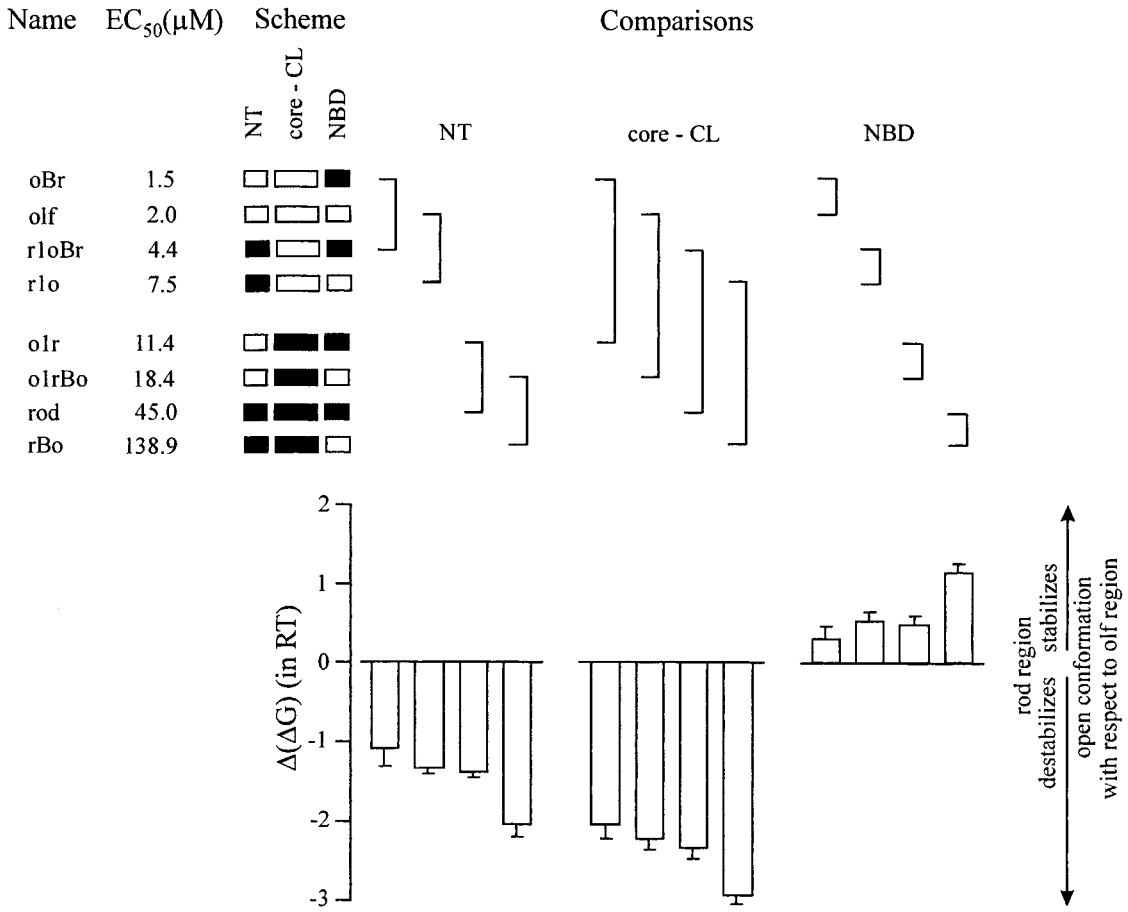


FIGURE 4. Differences in the free energy difference ($\Delta(\Delta G)$) caused by the three components NH_2 terminus (NT), core plus C-linker (core-CL), and cyclic nucleotide monophosphate binding domain (NBD). In the channel schemes a black box indicates the rod origin whereas a white box indicates the olf origin. The eight chimeras and channels are ordered from top to bottom according to an increasing EC_{50} (for SEM see Figs. 1 and 2). The brackets (right of the channel schemes) indicate pairs of channels/chimeras in which only one of the three components differs. The corresponding $\Delta(\Delta G)$ values (rod component with respect to olf component) were calculated according to Eq. 2. They are shown below each bracket in multiples of RT as bar graphs. A negative $\Delta(\Delta G)$ value indicates that a rod region destabilizes the open channel conformation with respect to the corresponding olf region, and a positive $\Delta(\Delta G)$ value indicates that a rod region stabilizes the open channel conformation with respect to the corresponding olf region.

ably increased the EC_{50} value (13.0 ± 0.7 μM for −1o compared with 2.0 ± 0.1 μM for olf; Fig. 3). Using Eq. 2, $\Delta(\Delta G)$ for −1r ($EC_{50,1}$) with respect to −1o ($EC_{50,2}$) results in only −0.9 RT, which is much less negative than −3.1 RT for rod with respect to olf (Fig. 5). Thus, the main portion of $\Delta(\Delta G)$ between the wild-type channels is introduced by the NH_2 termini.

Fig. 5 also illustrates the comparison of the $\Delta(\Delta G)$ values of channels/chimeras containing an NH_2 terminus with respect to the corresponding deletion mutant containing no NH_2 terminus. The main results are as follows. First, both the rod and the olf NH_2 terminus increase $\Delta(\Delta G)$ with respect to −1o. In contrast, with respect to −1r, only the olf NH_2 terminus increases $\Delta(\Delta G)$, whereas the rod NH_2 terminus decreases $\Delta(\Delta G)$. This means, that the rod NH_2 terminus stabilizes the open state only in the olf background, whereas in its natural rod background it slightly destabilizes the open state. Second, beside this opposite effect of the rod NH_2 terminus in the rod and olf background, $\Delta(\Delta G)$ between rod and o1r (1.4 RT) is indistinguishable from that between r1o and olf (1.3 RT). This result implies that the free energy for channel opening provided by the NH_2 terminus simply adds to the free energy of the channel background.

DISCUSSION

Effects of the NH_2 Terminus and NBD on the Sensitivity of rod and olf to cGMP

Our data are in line with previous results showing that the NH_2 terminus is one of the determinants of the characteristic EC_{50} of CNG channels (Goulding et al., 1994; Gordon and Zagotta, 1995b). In rod, Gordon et al. (1997) reported that the formation of a disulfide bond between the NH_2 terminus (C35) and the C-linker

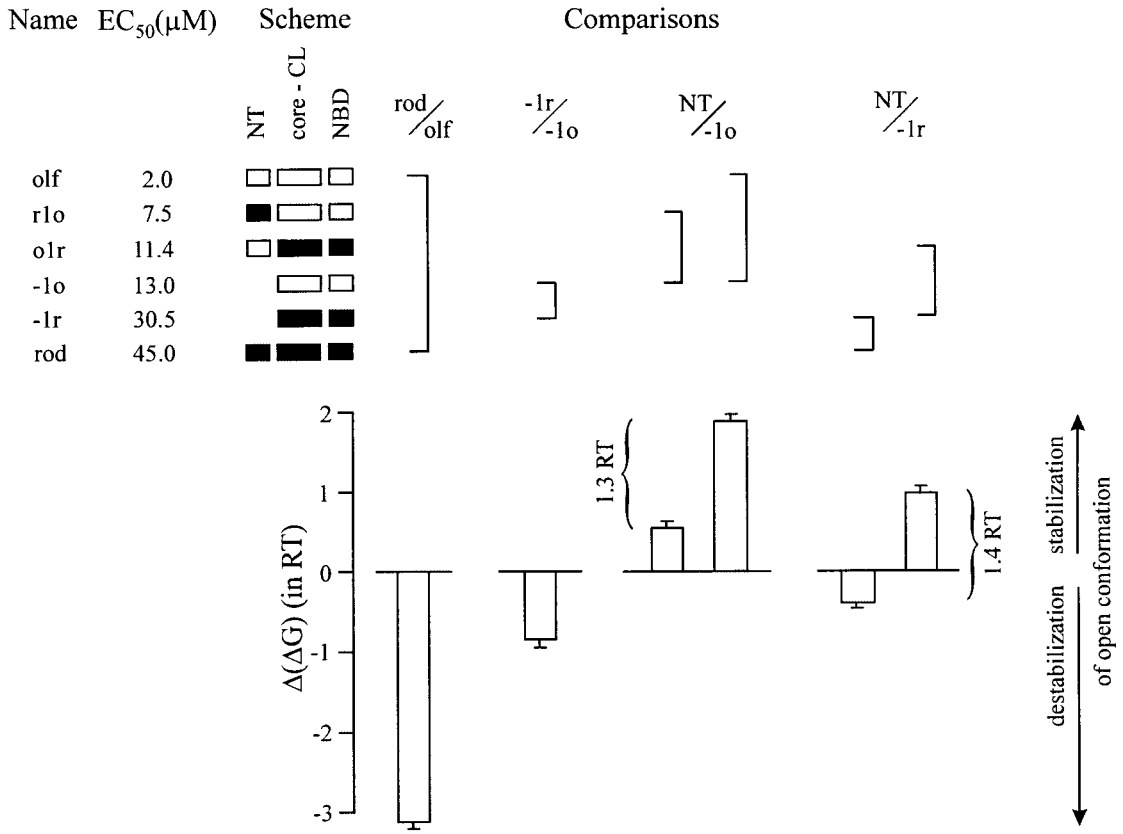


FIGURE 5. Differences in the free energy difference ($\Delta(\Delta G)$) of the NH_2 terminus deletion mutants (−1o, −1r) and the respective channels/chimeras including an NH_2 terminus. The comparisons are demonstrated in a fashion analogous to Fig. 4. The channels, chimeras, and deletion mutants are ordered from top to bottom according to an increasing EC_{50} (for SEM see Figs. 1 and 2). A negative $\Delta(\Delta G)$ value indicates that a channel or channel region destabilizes the open channel conformation with respect to another channel or channel region, whereas a positive $\Delta(\Delta G)$ value indicates that a channel or channel region stabilizes the open channel conformation with respect to another channel or channel region. Compared are the $\Delta(\Delta G)$ values of rod with respect to olf (rod/olf), of −1r with respect to −1o (−1r/−1o), of both NH_2 termini with respect to −1o (NT/−1o), and of both NH_2 termini with respect to −1r (NT/−1r). Rod with respect to olf is energetically more different ($\Delta(\Delta G) = -3.1$ RT) than −1r with respect to −1o ($\Delta(\Delta G) = -0.9$ RT). The amount of $\Delta(\Delta G)$ between channels with rod and olf NH_2 terminus with a rod background is similar to the respective amount of $\Delta(\Delta G)$ with an olf background (1.4 vs. 1.3 RT).

(C481) stabilizes the open state by a decrease of both the EC_{50} and the slope of the dose–response relationship. In our experiments, the olf NH_2 terminus decreased the EC_{50} (stabilized the open state) compared with the rod NH_2 terminus with all possible combinations of the core-CL region and the NBD (Fig. 4). Based on the fact that the olf NH_2 terminus does not contain the respective cysteine, it is concluded that the olf NH_2 terminus stabilizes the open state by a mechanism that does not involve the formation of a disulfide bond.

We also observed a stabilization of the open state by the olf NH_2 terminus with respect to the NH_2 terminus deletion mutants of both rod and olf: In olf, the $\Delta(\Delta G)$ value with respect to −1o was 1.9 RT, and in the chimera o1r the $\Delta(\Delta G)$ value with respect to −1r was 1.0 RT (Fig. 5). In contrast, the effect of the rod NH_2 terminus was opposite with respect to the two NH_2 terminus deletion mutants: in rod the $\Delta(\Delta G)$ value with respect to −1r was -0.4 RT, whereas in the chimera r1o the $\Delta(\Delta G)$ value with respect to −1o was 0.6 (Fig. 5). Despite this qualitatively different influence on the $\Delta(\Delta G)$ value by the rod NH_2 terminus, $\Delta(\Delta G)$ between the rod and olf NH_2 terminus was independent of having either a rod or an olf background, suggesting that the interactions of the NH_2 terminus with the rod or olf background are similar.

The finding that in rod channels deletion of the NH_2 terminus caused a small but significant reduction of the EC_{50} (30.5 ± 1.1 μM compared with 45.0 ± 1.6 μM) does not agree with the result of Brown et al. (1998) who did not observe a significant change in the respective EC_{50} value. One explanation for this discrepancy might be that the deletion mutant of Brown et al. (1998) contained eight amino acids of the olfactory channel just before the putative first transmembrane helix that our deletion mutant –1r did not contain.

The paradoxical effect of the NBD on the EC_{50} (and thus on $\Delta(\Delta G)$) is new and surprising. Although the absolute values of $\Delta(\Delta G)$ between the rod and olf NBD are typically smaller (0.3–1.1 RT; Fig. 4) than those be-

tween rod and olf NH_2 terminus (1.1–2.0 RT; Fig. 4), the effects of the NBD are consistent with all combinations of the NH_2 terminus and the core-CL region. This consistency supports the idea that also this region interacts with well preserved parts of the protein.

Regions within the Core-CL Region Determining the Sensitivity of rod and olf to cGMP

Within the core-CL region, we observed that besides the S1–S2 region and the C-linker, also the S5-P-S6 region determines the larger EC_{50} value in rod compared with olf. This conclusion has been derived from two results: (1) the S5-P-S6 region of olf in rod (chimera r5oLr) caused a small but significant reduction of the EC_{50} value compared with rod (Fig. 3); and (2) taking into account the fact that the NBD of olf paradoxically increases the EC_{50} value, the similarity of the EC_{50} values of chimera o5r (S5-NBD region of rod in an olf background) and chimera oLrBo (C-linker of rod in an olf background) may be explained only by a decreasing effect of the S5-P-S6 region on the EC_{50}, balancing the effect of the olf NBD.

The S1–S2 region has been reported first by Goulding et al. (1994) to contribute to the EC_{50}. These investigators studied a chimera in which the S1–S2 region (including the S2–S3 linker) of the catfish olf was inserted in a bovine rod background. In our experiments, transfer of the olf S1–S2 region (without the S2–S3 linker) into a rod background did not produce functional channels. This negative result suggests either that the S1–S2 region requires the right S2–S3 linker for normal channel function or that the bovine olf S1–S2 region does not work properly in a bovine rod background, whereas the catfish olf S1–S2 region does. Nevertheless, our results also confirm a relevant role of the S1–S2 region because we observed a 14-fold increase of the EC_{50} value when inserting the rod NT-S1-S2 region into an olf background (chimera r3o) and only a 4-fold increase of the EC_{50} when inserting the rod NH_2 terminus alone (chimera r1o).

The C-linker has been shown repeatedly and with different approaches to contribute to the characteristic EC_{50} values of CNG channels. These approaches include studies on chimeric channels between catfish olf and bovine rod (Goulding et al., 1994), between *Caenorhabditis elegans* tax-4, catfish olf and bovine rod (Paoletti et al., 1999), and between rat olf and bovine rod (Gordon and Zagotta, 1995b). Other approaches include point mutations within the C-linker (Zong et al., 1998), modification of sulfhydryl groups within the C-linker (Brown et al., 1998), and potentiation of rod by the binding of transition metal divalent ions to H420 (Gordon and Zagotta, 1995b). Our data obtained in chimeric channels of bovine rod and bovine olf further confirm a relevant role of the C-linker for the EC_{50}. The only sequence within the large core-CL region not contributing to the determination of the EC_{50} is obviously the S3–S4 region because no effect was found in both respective chimeras (o3r5o, Fig. 2; r3o5r, Fig. 3).

Implications for the Interactions of the Channel Regions in the Gating Process

The experimental results summarized in Fig. 4 can be explained by assuming that the open probability of rod and olf CNG channels is governed by the balance of the action of three relatively independent regions: the NH_2 terminus, the NBD, and the core-CL region. Corresponding regions in rod and olf show characteristically different actions. These actions may be summarized as follows. First, the NBD exerts the leading effect on channel opening. This effect is strongly promoted by increasing the [cGMP]. The NBD of rod has a slightly larger opening effect than the NBD of olf at all [cGMP]. Second, the core-CL region has a much weaker and cGMP-independent effect on the channel opening compared with the NBD. The core-CL region of olf favors opening more than the core-CL region of rod. Finally, the NH_2 termini have differential effects on channel opening: the olf NH_2 terminus promotes channel opening with both olf and rod core-CL region. In contrast, the rod NH_2 terminus promotes opening only with the olf core-CL region, whereas it promotes closure with the rod core-CL region. All effects of the NH_2 terminus are weak compared with the leading effect of the NBD.

Now consider the two extreme cases: at zero cGMP, the open probability is extremely low. Typical reported values are 2.25×10^{-3} for catfish olf and 1.25×10^{-4} for RO133, a rod chimera with the catfish olf pore (Tibbs et al., 1997). The larger spontaneous activity in olf than in rod could be explained by the larger opening effects of the NH_2 terminus and the core-CL region. At saturating [cGMP], the NBD generates maximum open probability with all compositions of the NH_2 terminus and core-CL region. The observation that olf attains the maximum open probability at much lower [cGMP] than rod could be explained by the larger opening effects of the NH_2 terminus and the core-CL region.

The assumption of an independent action of the three regions can also explain the effects of the NH_2 termini in complete channels/chimeras with respect to the corresponding NH_2 terminus deletion mutants. Consider first the NH_2 terminus–deleted rod (−1r): the EC_{50} would be lower than in rod because of the lacking closing force of the rod NH_2 terminus, but it would be higher than in chimera o1r (olf NH_2 terminus in a rod background) because of the lacking opening force of the olf NH_2 terminus. In the NH_2 terminus–deleted olf (−1o), the EC_{50} would be much higher than in olf because of the lacking opening force of the olf NH_2 terminus, and it would be only slightly higher than in chi-

mera r1o (rod NH_2 terminus in an olf background) because of the lacking smaller opening force of the rod NH_2 terminus (Fig. 5). An independent action of the regions also explains why the $\Delta(\Delta G)$ between rod and o1r is similar to that between r1o and olf.

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