Supplementary Information

Determination of cyclic nucleotide content by RP-HPLC. HPLC equipment was from Waters GmbH (Eschborn, Germany). We used a LiChrosphere® 100 RP-18 column (5µm) (Merck, Darmstadt, Germany). The absorption was detected at a wavelength of 258 nm. Protein samples were denatured with 5 % (v/v) perchloric acid at 4°C for 30 minutes and centrifuged at 17,600 g at 4°C for 30 min. The supernatant was neutralized und passed over the column. A linear gradient was produced from solution A (5 mM KH_2PO_4, pH 5.0) and solution B (methanol, HPLC grade) at a flow rate of 1.2 ml/min.

The gradient for the cAMP calibration was:

Time (min)	Solution A (%)	Solution B (%)
0	100	0
4	85	15
9	30	70
10	100	0

For the calibration of 8-CPT-cGMP and mixtures of cyclic nucleotides, the gradient was:

Time (min)	Solution A (%)	Solution B (%)
0	100	0
4	85	15
9	30	70
12	0	100
14	0	100
15	100	0

Analysis of CD spectra. CD spectra were analyzed using the CDSSTR (Lobley et al, 2002; Whitmore & Wallace, 2004) software of the DichroWeb online CD analysis facility.

Analysis of ligand binding using fluorescence: We analyzed the fluorescence binding data in terms of a simple binding scheme:

$$R + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RL$$

K_D is given by:

$$K_D = \frac{k_{-1}}{k_1} = \frac{R \cdot L}{RL} = \frac{(R_t - RL)(L_t - RL)}{RL} \quad (1)$$

$$\rightarrow RL = \frac{1}{2}(R_t + L_t + K_D) - \sqrt{\frac{1}{4}(-R_t - L_t - K_D)^2 - R_t \cdot L_t} \quad (2)$$

$$\Delta F = RL \cdot x \quad (3)$$

wherein R, L, and RL refer to the free concentrations of the receptor, ligand, and receptor-ligand complex, respectively. R_t and L_t refer to the total concentrations of receptor and ligand, respectively. The normalization factor *x* relates the concentration of bound 8-NBD-cNMP to the fluorescence change (ΔF).

In competition experiments, binding of 8-NBD-cAMP to the protein was studied in the presence of various amounts of cAMP or cGMP:

$$K_{Df} = \frac{(R_t - RL_f - RL_n)(L_{tf} - RL_f)}{RL_f} \quad (4)$$

$$K_{Dn} = \frac{(R_t - RL_f - RL_n)(L_{tn} - RL_n)}{RL_n} \quad (5)$$

wherein K_{Df} and K_{Dn} denote the K_D of the fluorescent and non-fluorescent ligand; RL_f, the concentration of the complex between receptor and the fluorescent ligand; RL_n, the concentration of the non-fluorescent ligand complex; L_{tf} and L_{tn}, denote the total

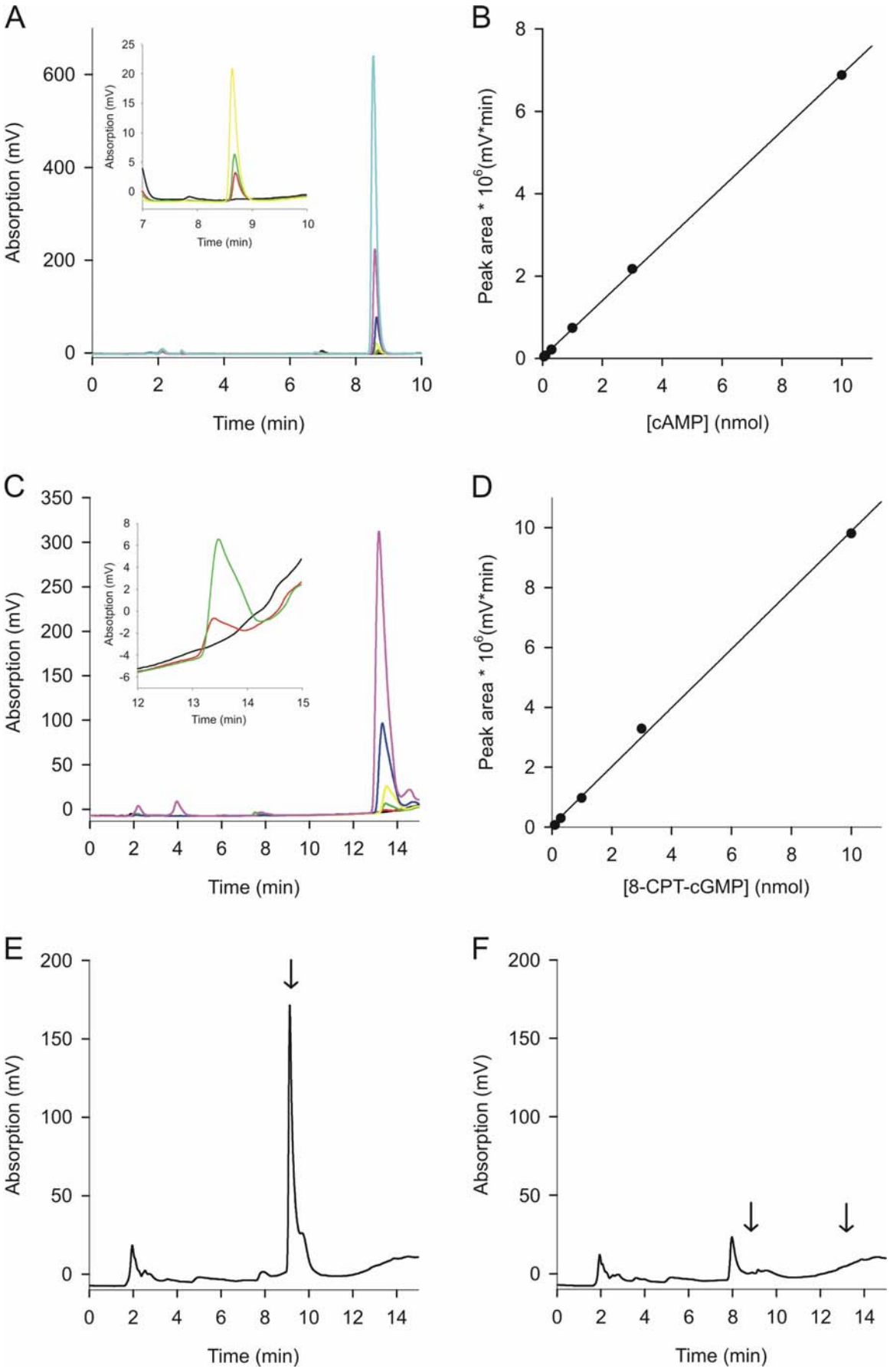
concentration of fluorescent and non-fluorescent ligand. Combining equations (4) and (5) and rearranging yields:

$$\left(1-\frac{K_{Dn}}{K_{Df}}\right)RL_f^{\,3}+\left(K_{Dn}-K_{Df}-R_t+R_t\frac{K_{Dn}}{K_{Df}}-L_{tf}+L_{tn}+2L_{tf}\frac{K_{Dn}}{K_{Df}}\right)RL_f^{\,2}+$$
$$\left(R_tL_{tf}-\frac{R_tL_{tf}K_{Dn}}{K_{Df}}-L_{tf}^{\,2}\frac{K_{Dn}}{K_{Df}}-L_{tn}L_{tf}-K_{Dn}L_{tf}-\frac{R_tL_{tf}K_{Dn}}{K_{Df}}\right)RL_f+ \tag{6}$$
$$\frac{R_tL_{tf}^{\,2}K_{Dn}}{K_{Df}}=0$$

Equation 6 was solved for RL_f using Cardano's method. Furthermore,

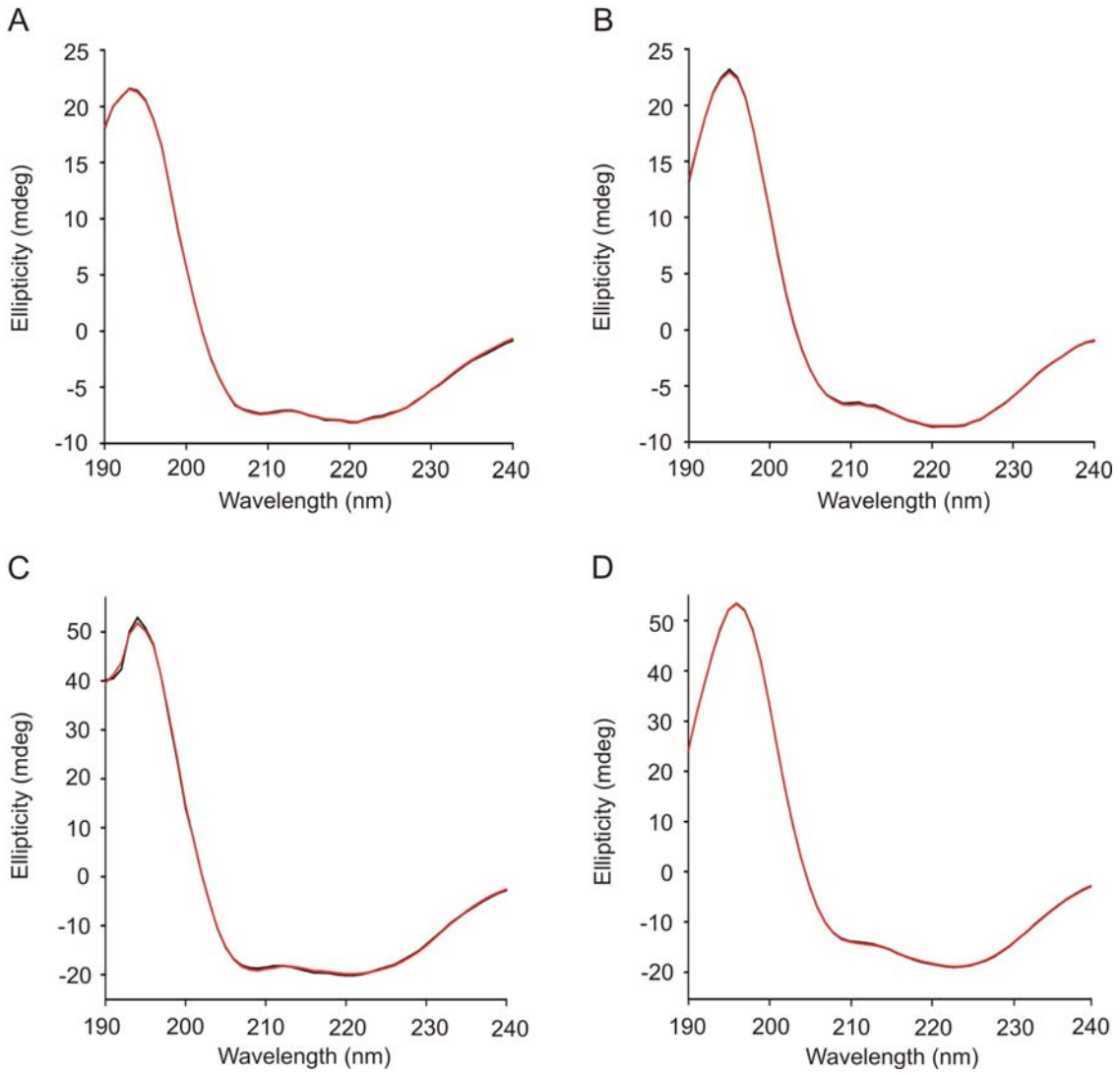
$$\Delta F = RL_f \cdot x \tag{7}$$

The data was fitted with a least-squares routine to equation 7.


A
Absorption (mV)
Time (min)
B
Peak area * 10^6(mV*min)
[cAMP] (nmol)
C
Absorption (mV)
Time (min)
D
Peak area * 10^6(mV*min)
[8-CPT-cGMP] (nmol)
E
Absorption (mV)
Time (min)
F
Absorption (mV)
Time (min)

Supplementary Figure 1: Determination of the amount of cyclic nucleotides in the protein sample by RP-HPLC. (A**)** Calibration of the column with cAMP. The different traces show various amounts of cAMP passed over the column (in nmol): 0 (black), 0.06 (red), 0.1 (green), 0.3 (yellow), 1 (dark blue), 3 (purple) and 10 (light blue). The inset shows the traces ≤ 0.3 nmol cAMP on an expanded scale. cAMP elutes at a retention time of 8.7 min. The average retention time was 8.8 ± 0.2 min (38 experiments). **(**B**)** Linear regression of the calibration shown in (A). The peak area was plotted against the cAMP amount. The regression line ($y = a\ x + y_0$; with y = peak area and x = cAMP amount) was fitted with the parameters: a = 686,996 mV min $nmol^{-1}$and y_0 = 30,578 mV min. **(**C**)** Calibration of the column with 8-CPT-cGMP. The traces show various amounts of 8-CPT-cGMP passed over the column (in nmol): 0 (black), 0.1 (red), 0.3 (green), 1 (yellow), 3 (dark blue), and 10 (purple). The inset shows traces ≤ 0.3 nmol on an expanded scale. 8-CPT-cGMP elutes at a retention time between 13.2 – 15.5 min depending on the concentration. The average retention time was 13.2 ± 0.4 min (17 experiments). **(**D**)** Linear regression of the calibration shown in (C). The peak area was plotted against the 8-CPT-cGMP amount. Fit parameters: a = 983,094 mV min $nmol^{-1}$ and y_0 = 53,795 mV min. **(**E**)** Analysis of the supernatant of 4.9 nmol denatured mlCNG protein. cAMP (arrow) eluted at a retention time of 9.1 min. The peak area corresponds to 3.7 nmol cAMP, i.e. 75.5% of the binding sites are occupied by cAMP. **(**F**)** Analysis of the supernatant of 2.5 nmol denatured mlCNG protein after removal of cAMP by the 8-CPT-cGMP wash procedure. cAMP (left arrow) as well as 8-CPT-cGMP (right arrow) are below the detection limit.

Supplementary Figure 2



Supplementary Figure 2: CD spectra of the purified CNBD protein (black). The traces in red have been computed by the program CDSSTR (Lobley et al, 2002; Whitmore et al, 2004). (A) Refolded CNBD (8 µM) in the absence of cAMP. (B) Refolded CNBD (8 µM) in the presence of cAMP. (C) Mutant R348A (11 µM). (D) CNBD after purification (9 µM). Spectra were measured in 0.1 cm cuvettes (A, B) or in 0.2 cm cuvettes (C, D).

Supplementary Table 1: Composition of secondary structure elements (SSE) of CNBD proteins computed by the algorithm CDSSTR (Lobley et al, 2002; Whitmore et al, 2004) for the CD spectra shown in Supplementary Figure 2 (data given in %).

	α_R	α_D	β_1	β_2	T	UO
Refolded CNBD	26	16	12	6	14	26
Refolded CNBD + cAMP	28	20	10	4	17	21
CNBD (R348A)	23	17	12	7	18	23
Native CNBD	26	18	9	7	19	21

α_R, regular helix; α_D, distorted helix; β_1, regular strand; β_2, distorted strand;
T, turns; UO, unordered.

Supplementary Table 2: Average SSE derived from the CD spectra of the CNBD proteins from 2-4 experiments (data given in %).

	α_R	α_D	β_1	β_2	T	UO
Refolded CNBD	25.8	17.5	11.0	6.5	16.5	22.8
Refolded CNBD + cAMP	26.8	18.8	10.5	6.3	16.5	20.8
CNBD (R348A)	23.5	16.5	11.5	6.5	15.0	26.5
Native CNBD	26.0	17.3	9.8	6.3	16.8	23.8

The fractions of helix α and sheet β given in the text were obtained by adding the corresponding regular and distorted fractions, $\alpha = \alpha_R + \alpha_D$; $\beta = \beta_1 + \beta_2$.

Supplementary Table 3: Residues contributing to the SSE determined from the crystal structures of the liganded CNBD protein and the R348A mutant (Kabasch & Sander, 1983).

The R348A mutant contains 30 % helices and 26 % sheets. The ligand-bound CNBD protein contains 36 % helices and 24 % sheets. The PDB entries for the liganded CNBD and the R348A mutant are 1VP6 and 1U12 (Clayton et al, 2004), respectively. The total number of residues of the CNBD protein is 142 amino acids.

R348A			Liganded CNBD		
residue number	SSE	residues	residue number	SSE	residues
221-231	H	11	220-230	H	11
241-248	H	8	241-250	H	9
252-256	S	5	252-256	S	4
261-265	S	5	261-263	S	3
271-277	S	7	271-276	S	6
280-283	S	4	279-284	S	5
288-291	S	3	288-291	S	3
294-297	S	3	294-297	S	3
310-313	S	4	298-303	H	6
317-323	S	6	311-313	S	3
324-333	H	10	317-323	S	7
336-348	H	13	324-333	H	11
			335-348	H	14

H, helix; S, sheet

REFERENCES

1. Clayton GM, Silverman WR, Heginbotham L, Morais-Cabral JH (2004) Structural basis of ligand activation in a cyclic nucleotide regulated potassium channel. *Cell,* **119**: 615-627

2. Kabasch W, Sander C (1983) Dictionary of protein secondary structure: Pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers,* **22**: 2577-2637

3. Lobley A, Whitmore L, Wallace BA (2002) DICHROWEB: an interactive website for the analysis of protein secondary structure from circular dichroism spectra. *Bioinformatics,* **18**: 211-212

4. Whitmore L, Wallace BA (2004) DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data. *Nucleic Acids Res,* **32**: 668-673