Fluorescence resonance energy transfer (FRET) is a powerful biophysical tool to determine the distances between a donor and an acceptor. This methodology can be applied to distinct positions within a ligand and receptor as they exist within a molecular complex to elucidate spatial constraints. By utilizing ligands with distinct biological properties, stabilizing the receptor into fully active, partially active, and inactive conformations, this approach can be useful for the elucidation of the conformational changes that are associated with receptor activation.

Recently, we utilized this technique to measure a series of intermolecular distances between a fixed point at the amino-terminal end of a CCK analogue that represents a full agonist and distinct points on the exposed surfaces of the CCK receptor (1). This was accomplished by the individual introduction of reactive Cys residues at positions thought likely to accommodate this modification without having negative functional implications based on structure-activity relationships among structurally related receptors. This was fully validated (3). Subsequently, cells expressing these receptor constructs were allowed to react with a cell-impermeant, fluorescent, thiol-reactive methanethiosulfonate (MTS) reagent that was specifically prepared (1, 3). The fluorescent donor was provided to the system in a highly specific way, taking advantage of the structural specificity of interaction between hormone and receptor, utilizing a fluorescent analogue of the natural peptide ligand of this receptor. A series of controls established that transfer only occurred between the receptor-bound ligand and the specific sites within the receptor (1). The data from the current approach utilizing FRET between ligand and receptor complements other experimentally derived constraints that have come from structure-activity studies of ligand and receptor, receptor mutagenesis, photoaffinity labeling studies, and studies of the fluorescence properties of ligand probes (1, 4–7). These have provided a workable model of the CCK-ligand-occupied type A CCK receptor as well as insights into the types of conformational changes that might occur upon receptor activation. The data in the current work provide the first quantitative measurements to be applied to these working models to help describe the details of the conformational changes that are associated with agonist-stimulated receptor activation and initiation of signaling.

### EXPERIMENTAL PROCEDURES

**Materials**—Synthetic CCK octapeptide (CCK-26–33, using the numbering convention based on the 33-residue CCK peptide first isolated from porcine duodenum) was purchased from Peninsula Laboratories (Belmont, CA). 2-Aminothioethyl-methanethiosulfonate hydrochloride was from Toronto Research Chemicals, (Ontario, Canada). Alexafluor-N-hydroxysuccinimide and Alexafluor-N-hydroxysuccinimide were from Molecular Probes (Eugene, OR). All other reagents were analytical grade.

**Design and Preparation of Fluorescence Donors and Acceptors for FRET Studies**—As we reported earlier in analogous agonist ligand studies of the same receptor, we chose Alexafluor incorporated into the ligand as donor and Alexafluor-derivatized sites within the receptor as
FIG. 1. Structure of fluorescent probes. Shown are the chemical structures of the fluorescent probes used in this study. The fluorescent indicator, Alexa, was situated at the amino terminus of the CCK peptide analogues with no spacer between the peptide and fluorescent moiety. Shown also are the sequences of the antagonist, Gly-[D-Trp31,Nle28,31]CCK-26–32; phenethyl ester, and partial agonist, Gly-[Nle60]CCK-26–32; phenethyl ester, peptides used as the base for these probes.

acceptors (1). For this pair of fluorophores, adequate spectral overlap exists between donor emission and acceptor excitation for the demonstration and quantitation of distances less than 62 Å (distance at which 50% energy is transferred). CCK peptides derivatized with Alexa488 were chosen for this study rather than other previously described fluorescent derivatives of this hormone that utilize fluorophores such as acrylodan and nitrobenzoxadiazole because the latter have been shown to be quenched by KI much less effectively than the Alexa488, suggesting that they might not be optimal for FRET studies (7).

Like the previously studied CCK agonist analogue, the fluorescence donor was positioned at the amino terminus of the probes in position 24, with the partial agonist representing Alexa488-Gly-[Nle60]CCK-26–32; phenethyl ester (Alexa488-partial agonist) and antagonist representing Alexa568-Gly-[D-Trp31,Nle28,31]CCK-26–32;phenethyl ester (Alexa568-antagonist) (Fig. 1). These peptides were synthesized and purified to homogeneity in our laboratory, as described previously (7). The parental peptides each have a single reactive amino group, which could be derivatized in solution with the N-hydroxysuccinimide ester of Alexa488. These fluorescent peptides were purified to homogeneity by reversed-phase HPLC using a C-18 column and were characterized by mass spectrometry. Previous structure-activity studies have shown that the amino terminus of these peptides can tolerate such a modification (7). The antagonist fluorescent probe was recently shown to bind specifically and saturably to the CCK receptor and to inhibit the CCK-stimulated increase in intracellular calcium (7).

The acceptor Alexa568 was covalently attached to specifically engineered Cys residues present within the 4th C-terminal CCK receptor. These were prepared by the reaction of a thiol-reactive fluorescent MTS reagent (Alexa560-MTS) with intact cells previously prepared to express a series of pseudo-wild type CCK receptor constructs with each having a single reactive Cys residue in a distinct receptor ectodomain (3). These monoreactive receptor constructs were previously characterized and validated as being fully biologically active (3). The thiol-reactive fluorescent reagent was prepared by acylation of the free amino group of 2-aminoethy1-methanethiosulfonate hydrochloride with Alexa560-N-hydroxysuccinimide ester, as we reported previously (1). Of note, these receptor constructs were also truncated to eliminate the first 30 residues of the amino-terminal tail region, known to have no functional influence on peptide ligand binding or biological activity (3) and believed to act as a cover of the peptide-binding cleft (8).

Cell Culture—Chinese hamster ovary (CHO) cell lines stably expressing each of the relevant CCK receptor constructs, representing the null Cys-reactive pseudo-wild type (C94S) and the series of monoreactive pseudo-wild type receptors, have been previously prepared and characterized (3). Cells were grown in tissue culture flasks containing Ham’s F-12 medium supplemented with 5% fetal clone-2 (HyClone Laboratories, Logan, UT) in a humidified environment containing 5% carbon dioxide. Cells were passaged approximately two times/week.

Fluorescent Labeling of Intact Cells—The procedure utilized for the fluorescent labeling of the receptor-bearing cells was identical to that reported previously (1). In brief, cells were detached from the flasks using non-enzymatic cell dissociation medium (Sigma). Intact cells were incubated with 1 μM Alexa488-MTS reagent for 20 min at room temperature in Krebs-Ringer-HEPES (KRH) medium containing 25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM KH2PO4, 1.2 mM MgSO4, and 0.01% soybean trypsin inhibitor. Unreacted Alexa488-MTS reagent was removed by centrifugation and repeated washing. Labeled cells were then incubated with 25 nM Alexa488-partial agonist or Alexa488-antagonist for 2 h at 4 °C in KRH buffer, pH 7.4, containing 0.2% bovine serum albumin. Temperature control was critical to ensure absence of internalization of receptor-bound ligand. Unbound ligand in the medium was subsequently removed by centrifugation and repeated washing with iced buffer, and the cells were suspended in ice-cold KRH medium for FRET studies.

Fluorescence Spectroscopy—Steady-state fluorescence was recorded using a SPEX Fluorolog spectrofluorophotometer (SPEX Industries, Edison, NJ) at 25 °C using intact cells in suspension in a 1-ml quartz cuvette. Multiple fluorescence spectra were accumulated after excitation at 482 nm, with the emission spectra collected from 490 to 700 nm using an integral of 0.3 s/hm. Unlabeled CHO cells not expressing this receptor were used to control for background fluorescence and light scattering, and these data were subtracted from those collected under experimental conditions.

Fluorescence Resonance Energy Transfer—FRET studies were carried out using the procedure that we validated previously (1). In brief, the critical distance (Rc) represents a constant that is characteristic of the donor-acceptor pair being utilized and corresponds to the distance at which transfer efficiency is 50% between the donor (Alexa488-ligands) and acceptor (Alexa560-CCK receptor). This was calculated using the Förster equation (2, 9).

$$R_c = \frac{9786 \times J_0 \times 4 \times Q_D^{1/6} \times A}{\lambda}$$ (Eq. 1)

A value of 1.4 was used for n, the refractive index of aqueous medium. $J_0$ is a geometric factor describing the relative orientation of the transition dipole of the donor and acceptor fluorophores. If donor and/or acceptor are able to exhibit isotropic, dynamic orientation within the time scale of the fluorescence lifetime of the probes, then this orientation factor converges toward a value of ½. Support for this assignment comes from our previously published reports of evidence for rotational freedom of the receptor-bound agonist and antagonist probes (1, 7) and from our previously published data on the steady-state fluorescence anisotropy of the receptor-bound probes exhibited steady-state fluorescence anisotropy under the conditions of the FRET assay (room temperature) of 0.04, 0.05, and 0.12 for the agonist, partial agonist, and antagonist probes, respectively. Additional support for this comes from rotational correlation times ($\phi$) for the receptor-bound agonist (fast component 0.36 ns, slow component 34 ns), partial agonist (0.32 and 49.1 ns, respectively), and antagonist (0.90 and 55.6 ns, respectively). $Q_D$ is the quantum yield for fluorescence of the donor in the absence of acceptor. Quantum yields were measured for the antagonist and partial agonist probes in solution by comparing their emission intensities with those of sodium fluorescein in 0.1 N NaOH, where it has a standard quantum yield value of 0.92. Values for quantum yield of both Alexa488-ligands in solution were 0.60. Direct measurement of the quantum yields of the receptor-bound probes was also performed. For this, a plasma membrane-enriched particulate fraction of the CHO-CCK cells was utilized. Membranes (50 μg) were incubated with a 10 nm fluorescent probe for 20 min at 25 °C. At that point, free probe was removed from the membrane-bound probe by centrifugation at 20,000 × g at 4 °C and washing with iced KRH buffer. Fluorescence emission spectra between 500 and 600 nm were collected after excitation at 482 nm. In a second identical tube, probe-bound membranes were incubated at 37 °C for 10 min with 1 μM non-fluorescent peptide, and the fluorescence spectra were similarly acquired. To be certain that this incubation with excess non-fluorescent ligand was able to dissociate the fluorescent ligand from the receptor, another identical tube was similarly treated, but rather than acquire the fluorescence spectra of the entire contents, the membranes and supernatant were separated by centrifugation, and both were separately evaluated. Indeed, quantitation of the fluorescence in the membranes represented only 4–5% of the membrane-bound fluorescence at the start of the competition. Similarly, the supernatant was shown to contain 90–91% of the membrane-bound fluorescence at the start of the competition. This established the validity of the quantum yield yields of the receptor-bound probe relative to that of the dissociated probe free in solution. The quantum yields of the receptor-bound partial agonist and antagonist probes were 95 and 96% of the values measured in solution. Thus, the quantum yields were 0.58 for both receptor-bound probes. These values were utilized for calculations.

$J$ is the integral of the spectral overlap between donor emission ($F(\lambda)$)
and acceptor absorbance (ε(λ)) spectra. It was calculated by the following equation,

\[ J = \frac{F(\lambda)}{F(\lambda + \lambda A)} \frac{\lambda A}{\lambda} \frac{F(\lambda)}{F(\lambda + \lambda A)} \]  

(Eq. 2)

where λ is the wavelength in cm, F(λ) is the normalized and integrated fluorescence of the donor at wavelength λ, and ε(λ) is acceptor absorption in absorbance units at wavelength λ for the overlapping area.

The efficiency of fluorescence energy transfer (E) was calculated by the fractional decrease in the fluorescence intensity of the donor (D) in approximation with the acceptor (A),

\[ E = 1 - \frac{F_{D}}{F_{A}} \]  

(Eq. 3)

where \( F_{D} \) and \( F_{A} \) are the fluorescence intensities of the donor (Alexa488-ligands) in the absence and presence of the acceptor (Alexa568-CCK receptor). A series of controls was used. These included the addition of 100-fold molar excess of non-fluorescent CCK competitor, the use of cell lines expressing a null-reactive CCK receptor, and the use of the parental CHO cell line that does not express the CCK receptor at all. These were exposed to the standard labeling procedure using the fluorescent reagent, and they were subsequently incubated with the fluorescent donor ligand.

The efficiency of FRET is dependent on the inverse sixth power of the distance between donor and acceptor (2). Using the calculated efficiency of transfer, E, the average proximal distance, \( R_{A} \) between donor and acceptor was calculated by the equation,

\[ R_{A} = \frac{\lambda A}{\lambda} \frac{F(\lambda)}{F(\lambda + \lambda A)} \]  

(Eq. 4)

where \( R_{A} \) is the distance between donor and acceptor with an efficiency of energy transfer of 50%.

RESULTS

Development and Characterization of Receptor Probes—The fluorescent partial agonist probe, Alexa488-Gly-(Nle28,31)CCK-26–32]phenethyl ester, was synthesized and purified to homogeneity using reversed-phase HPLC. Its structure was confirmed by mass spectrometry. The analogous fluorescent antagonist and agonist probes have previously been prepared and characterized (7).

This partial agonist probe was found to bind to the CCK receptor specifically and saturably (K_i value 73 ± 10 nM), with affinity approximately an order of magnitude lower than its non-fluorescent analogue (K_i value 3.4 ± 0.2 nM) (Fig. 2). The ability of this probe to stimulate intracellular calcium was monitored as an indication of its predicted partial agonist status. As expected, it was able to stimulate a submaximal intracellular calcium response in CCK receptor-bearing CHO-CCKR cells that was similar to its non-fluorescent analogue (even though the highest concentrations could not be achieved with the fluorescent probe) (Fig. 2).

Preparation of Donor and Acceptor for FRET Studies—Key for the success of this experimental strategy is the lack of interference by non-receptor-bound fluorescence donor and fluorescence acceptor that might be attached to membrane proteins other than the receptor of interest. Critical to the success of this type of experiment is the spatial approximation between the fluorophore within the receptor-bound ligand and the fluorophore labeling of the receptor itself, while other positions of potential donor and acceptor molecules would be too far away from each other to induce significant energy transfer. Controls for each of these conditions were performed previously (1) and were once again included here.

Shown in Fig. 3 are the fluorescence emission spectra of the donor and acceptor. The fluorescent donor peptide demonstrated peak emission at 518 nm after excitation at 482 nm. The fluorescent acceptor demonstrated peak emission at 603 nm after excitation at 578 nm with minimal emission at this wavelength after it was excited at 482 nm. When these agents were in appropriate spatial approximation with each other (50% energy transfer at 62 Å) in the receptor-bearing cellular system, energy transfer from donor to acceptor could be observed (Fig. 4). Here, emission is demonstrated at 603 nm after excitation at 482 nm. No such energy transfer was observed when receptor occupation with the fluorescent ligand was blocked by saturation of the normal ligand-binding site with non-fluorescent CCK (Fig. 4, left panel) or when the experiment was performed with non-receptor-bearing parental CHO cells or with the null Cys-reactive pseudo-wild type CCK receptor-bearing CHO cell line (C94S) (Fig. 4, right panel). A significant fluorescent energy transfer signal was observed between Alexa488-labeled antagonist and partial agonist when bound to the monoreactive Cys mutant CCK receptor constructs that had been derivatized with Alexa568-MTS. These include CCK receptor constructs that had been labeled at position 94 within the second transmembrane segment, position 102 (N102C) within the first extracellular loop, position 204 (A204C) within the second extracellular loop, and position 341 (T341C) within the third extracellular loop. The profile of fluorescence emission shown in the left panel of Fig. 4 is representative of experiments with each of these constructs and with each of these probes.

Fluorescence Resonance Energy Transfer—The extent of spectral overlap was calculated for each of the monoreactive CCK receptor constructs, which are listed in Table 1. For the fluorescent antagonist the highest degree of spectral overlap was observed for position 204 within the CCK receptor, whereas for the fluorescent partial agonist the highest degree
of spectral overlap was observed for positions 102 and 341 within the receptor. The efficiency of energy transfer and the corrected distances were calculated for both antagonist and partial agonist probes and are illustrated in Table II.

**DISCUSSION**

Current understanding of the active and inactive conformations of members of the G protein-coupled receptor superfamily is quite limited. Our best insights have come from rhodopsin, for which a high-resolution crystal structure is available for the inactive state (10). Exposure to light cracks those crystals as a result of a conformational change, but the details of that conformation have not been clearly defined. Other biophysical studies with the same receptor and with the β2-adrenergic receptor, including electron paramagnetic resonance spectroscopy, fluorescence spectroscopy, alterations in cysteine accessibility, and use of engineered zinc-binding sites, have provided insights into the types of conformational changes believed to correlate with receptor activation (11–13). These have suggested that there is movement of the third and sixth transmembrane segments, with the cytoplasmic ends of these two helices close together in the inactive state and released to move apart in the active state. A counterclockwise rotation of the sixth transmembrane segment (when viewed from the extracellular side of the membrane) has also been predicted (14).

**TABLE II**

*FRET Analysis of CCK Receptor States*

| CCK receptor mutant | Efficiency | R  |
|---------------------|------------|----|
| Fluorescent partial agonist donor | C94 | 55 ± 4 | 21 ± 2 |
| N102C | 53 ± 1 | 18 ± 0.4 |
| A204C | 57 ± 2 | 25 ± 1 |
| T341C | 50 ± 4 | 17 ± 1 |
| Fluorescent antagonist donor | C94 | 57 ± 4 | 21 ± 2 |
| N102C | 55 ± 2 | 28 ± 2 |
| A204C | 74 ± 1 | 15 ± 1 |
| T341C | 55 ± 2 | 21 ± 1 |

The rhodopsin structure has provided a good template for the confluence of helices for Class I G protein-coupled receptors. However, there are strong data to suggest that even this is not relevant to the Class II G protein-coupled receptors (15) and that the loop and tail domains of rhodopsin are not relevant to other members of the Class I family (6). The helical bundle pattern of rhodopsin has provided particularly useful insights into the structure of biogenic amine receptors in the Class I family (10). Complementary studies with photoaffinity labeling and mutagenesis have supported such structural predictions.

We have been particularly interested in the CCK receptor that represents a peptide receptor within the Class I family of G protein-coupled receptors. Extensive structure-activity insights for both CCK and its receptor, photoaffinity labeling data using probes throughout the pharmacophoric domain, and fluorescence studies have all resulted in a molecular model of the agonist-bound active conformation of this receptor that has a helical bundle region quite analogous to that of rhodopsin (1, 4–7, 10). Indeed, the root mean square deviation of the backbone of residues within the helices was found to be only 2.5 Å (6). Clearly, the least well defined portions of this receptor represent the loop and tail domains.

The first real insights into the conformations of the external loop and tail domains of the CCK receptor came from our recent work that utilized FRET of the active conformation of this receptor occupied by a full agonist ligand (1). The current report extends those insights quite substantially by including the partial agonist- and antagonist-occupied CCK receptor that, for purposes of functional coupling with heterotrimeric G proteins, must be in distinct conformations.

For purposes of interpretation of the results with these additional probes that possess distinct biological activities, it is important to understand the degree of similarity or difference in their position of docking to the CCK receptor. These probes have similar primary structures, sharing the critical amino acid functional groups that have been shown to be responsible for the specificity of CCK receptor binding, pointing toward similarity in the position of their docking to this receptor. Additional support for similar docking comes from photoaffinity labeling studies in which the same segment of the CCK receptor was covalently labeled by each of three analogous photolabile probes, with sites of covalent attachment through a benzoyl phenylalanine at position 24, the same position as that of the fluorescence indicator in the probes used in the current work (16). For still further validation of the similarity of the docking of these probes and for purposes of registration, in the current work all three of these structurally similar ligands resulted in a fixed distance measured between the fluorophore...
at the amino terminus of the probes at position 24 and receptor residue 94 within the intramembranous region of helix two.

It is quite interesting that the partial agonist-occupied receptor resulted in FRET distances that were not different from those of the full agonist probe except for a slightly longer distance to the second loop residue. This suggests that there were, indeed, subtle differences from the fully active to the partially active conformation of this receptor. This could certainly result in different coupling to a distinct G protein or different kinetics of coupling with the same G protein. Either of these approaches could provide enough of a difference to explain the distinct biology of this partial agonist ligand.

There were very significant differences in the distances to the first and second loop residues measured for the antagonist-occupied inactive conformation of the CCK receptor. The change in the distance to the first loop might be consistent with the movement of the third transmembrane segment that has been proposed previously (11–13), with this segment serving as one of the anchors of this loop. The movement of the sixth transmembrane segment could be expected to affect the third loop distance, and although this was indeed different in the active and inactive states, it was not a marked difference. The major change in the second loop distance is perhaps the most interesting and hardest to explain at the current time. Movements in the transmembrane segments (four and five) that anchor this loop have not been prominently described in previous studies. Also, this loop is the longest of the three extracellular loops in the CCK receptor (lengths of the first, second, and third extracellular loops of the CCK receptor are predicted to be ~15, 28, and 12 residues, respectively). Therefore, it would not be expected to have its conformation markedly affected by movement of those helices, and conversely, its movement might not be expected to easily exert tension upon the helical segments.

We recently studied the fluorescence properties of a series of fluorescent agonist probes of the CCK receptor as bound to this receptor (7). Using manipulations of the G protein-coupling interface, we were able to demonstrate that receptor activation results in the fluorophore moving into a more exposed, hydrophilic environment that is more amenable to quenching by iodide and also shows a lower anisotropy and a shorter lifetime (7). This movement was confirmed in analogous studies using a fluorescent antagonist probe (7).

Despite the design of the current studies, utilizing probes with distinct biological characteristics representing full agonist, partial agonist, and antagonist, with shared functionalities for similar receptor docking and sharing the positions of the fluorescence donor (position 24), the possibility (and even the likelihood) of distinct conformations of the probes adds a variable to the interpretation of the results. We know from a very recent report from this laboratory (17) that the established spatial approximation between the tyrosine sulfate in position 27 of the agonist ligand and receptor residue Arg197 (within the second extracellular loop) is not present in the inactive complex occupied by the peptide antagonist. Like the FRET distances reported here, that could reflect either (or both) changes in ligand conformation or changes in receptor conformation. We expect that both are relevant. Because of the degrees of freedom contributed by ligand and receptor conformation, we have not proposed a distinct conformational model for the inactive antagonist-occupied complex. A meaningful model for such a complex will require additional experimentally derived constraints. However, the distances provided in the current report clearly help move closer to this goal.

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