# Structural characterization of the parathyroid hormone receptor domains determinant for ligand binding

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## Abstract

**Over the years, the association of peptide ligands to Family B GPCRs (G-protein coupled receptors) has been characterized by a number of experimental and theoretical techniques. For the PTH (parathyroid hormone) ligand–receptor system, important insight has been provided by photoaffinity labelling experiments and the elucidation of direct contact points between ligand and receptor. Our research has focused on the structural elucidation of the receptor domains shown to be involved in the binding of PTH. Employing a combination of carefully designed receptor domains, solution-state NMR carried out in the presence of membrane mimetics and extensive computer simulations, we have obtained a well-resolved model of the ligand–receptor complex for PTH. Here, we review the development of this model and highlight some inherent limitations of the methods employed and their consequences on interpretation of the ligand–receptor model.**

## PTH (parathyroid hormone)

PTH is a major regulator of blood calcium concentration and bone homoeostasis [1,2]. PTH is secreted from the parathyroid glands as an 84-amino-acid protein (approx. molecular mass of 9300 Da). Cleavage in the liver and kidney produces N-terminal fragments of 34, 36 and 37 amino acids which constitute functional domains, retaining high binding affinity and activity. Indeed, PTH-(1–34) is often utilized as the prototype PTH analogue, retaining high affinity for the PTH1R (PTH type 1 receptor) and PTH bone-related activities. The biological role of the C-terminal portion of the hormone is an area of active study.

The related hormone, PTHrP (PTH-related protein), binds to and activates PTH1R and has been related to fetal development, as a regulator of placental calcium and formation of cartilage, as well as being associated with malignant tumours, leading to severe hypercalcaemia [3]. PTHrP-(1–34) and PTHrP-(1–36) are fully active, and similarly to PTH-(1–34) has been the major focus in studies aimed a elucidation of the association with PTH1R.

PTH1R is a member of the Family B family of the GPCRs (G-protein-coupled receptors), mediates the action of PTH and PTHrP. PTH1R is receiving increasing attention as a potential target for therapeutic treatments of disorders of mineral ion homoeostasis and the skeleton, including hyperparathyroidism, humoral hypercalcaemia of malignancy, and osteoporosis [4]. Progress towards the elucidation of the interaction between PTH and PTH1R at the molecular level will facilitate the design of specific drugs to treat PTH-related diseases.

**Key words:** Family B G-protein-coupled receptor (Family B GPCR), ligand-binding determinant, parathyroid hormone (PTH), peptide hormone, photoaffinity labelling, receptor domain.

**Abbreviations used:** Bpa, *p*-benzoylphenylalanine; CRF, cotricotropin-releasing factor; DPC, dodecylphosphocholine; EC, extracellular loop; GPCR, G-protein-coupled receptor; NOE, nuclear Overhauser effect; PTH, parathyroid hormone; PTH1R, PTH type 1 receptor; PTHrP, PTH-related protein; TM, transmembrane helix.

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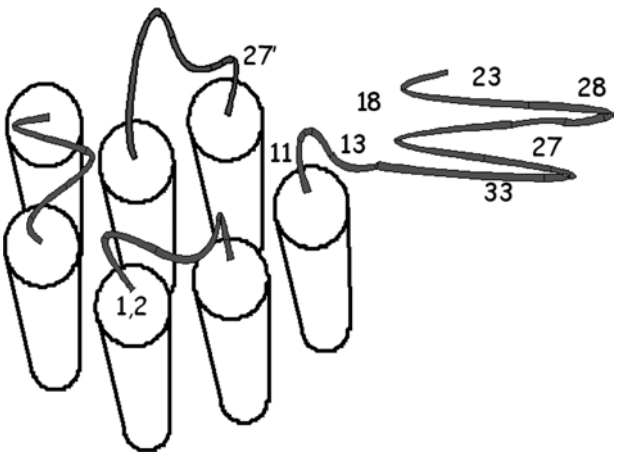
## Bimolecular association

From extensive mutagenesis and chimaera studies, targeting both PTH and PTH1R, functional roles have been ascribed to distinct domains of the ligand and receptor. Modifying the C-terminus of PTH was found to mainly alter the binding affinity and has been correlated with targeted modifications located in the large, extracellular, N-terminus of the receptor. In contrast, modifications of the N-terminus of PTH, including the simple removal of the N-terminus of the first amino acid, diminished activity accompanied by only a slight reduction in receptor binding affinity [5]. These data clearly defined a binding domain (the C-terminus of the ligand) and an activation domain (N-terminus of the ligand) and allowed for the development of a low-resolution model of ligand binding, in which the C-terminus of PTH bound to the N-terminus of PTH1R, providing the proper topological orientation for the N-terminus of PTH to associate with the central TM (transmembrane helix) bundle of the receptor, leading to activation [6].

Further elucidation of the bimolecular interaction of PTH and PTH1R has been provided by photoaffinity labelling, and the direct contact points between the ligand carrying a photolabile moiety [e.g. Bpa (*p*-benzoylphenylalanine)] and the receptor [7]. Photoaffinity scanning studies incorporating Bpa in the C-terminus of PTH (e.g. positions 22, 27, 28 or 33) identified interactions sites with the extracellular N-terminus of PTH1R [8], while PTH analogues with individual Bpa substitutions within the mid-region (residues 11, 15, 18 or 21) formed interactions with the N-terminus adjacent to TM1 [9].

**Figure 1 | Mapping PTH binding to PTH1R**

The contact sites of PTH by photoaffinity labelling experiments mapped out on to a schematic diagram of PTH1R, based on the structure of rhodopsin. The site at which the Bpa was incorporated into PTH is indicated. The data provide the relative orientation of the ligand while bound to the receptor.



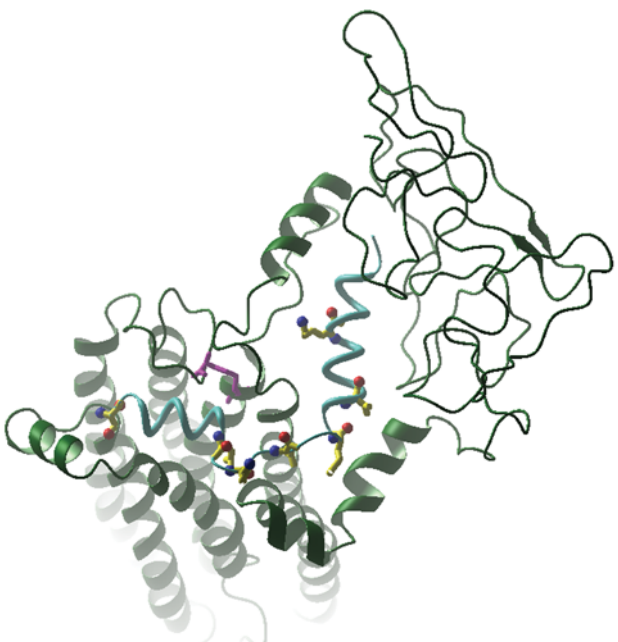

The N-terminus of PTH (residues 1 and 2) were found to interact with $Met^{425}$, located at the extracellular end of TM6 and beginning of EC3 (third extracellular loop) [10]. A schematic diagram of these sites of interaction are illustrated in Figure 1.

## Structural characterization of PTH and domains of PTH1R

During the last decade we have used high-resolution NMR coupled with extensive computer simulations to provide a structural basis for the interpretation of the results from photoaffinity labelling [11]. The general approach is to treat the GPCR as a multi-domain protein and structurally examine each domain individually, a method commonly employed for soluble proteins [e.g. the structures of the SH (Src homology) 2 and 3, and kinase domains of Src]. Here, the TMs are treated as the linkers between the domains (e.g. EC3 is subtended by the 'linkers' TM5 and TM6). In contrast with soluble proteins where the linkers are structurally undefined, with GPCRs the linkers can be assumed to be well defined $\alpha$-helices, with kinks introduced from proline residues. In the design of the receptor domains, we include large segments of the TMs that will incorporate into the micellular solution used in the spectroscopic studies and thereby better mimic attachment to the intact TM in the membrane-embedded receptor. Additionally, retaining a portion of the TM facilitates the incorporation of the structural features of the domain into the model of the receptor. For example, in the examination of EC1 of PTH1R, the structural features were incorporated into the model of the receptor employing the overlapping sequences of the extracellular ends of TM2 and TM3 [12]. Using this approach, we have determined the structure of the proximal N-terminus PTH1R-(168–198), the EC1 PTH1R-(241–285) and the EC3 PTH1R-(420–450) using high-resolution NMR

**Figure 2 | PTH–PTH1R complex**

The NMR-based structural features of the proximal N-terminus, EC1 and EC3 incorporated into the molecular model of PTH1R (green, the Cys–Cys bond is purple as a point of reference). The ligand (blue) has been placed into the receptor based on the photoaffinity labelling contact points; the amino acids used in these studies are shown (yellow). Outside the proximal region PTH1R-(168–198) solved by NMR and maintenance of the three disulfide bonds, no structural features for the N-terminus have been assumed in this model.



in the membrane mimetic of zwitterionic micelles of DPC (dodecylphosphocholine) [12,13].

In addition to the membrane domains, an intensive study of PTH itself under a number of different conditions, including that provide by DPC micelles, was undertaken [14]. The data from this study clearly indicate that the hormone adopts two $\alpha$-helices when in the presence of zwitterionic micelles: one within the N-terminal activation domain and one in the C-terminal binding domain; the structural features are far less defined in aqueous solution. The incorporation of these experimental data into the molecular model of PTH1R, built upon the X-ray structure of rhodopsin [15], is illustrated in Figure 2. The model illustrated in Figure 2, incorporating the structural features of the receptor domains examined in isolation, recapitulates all of the photoaffinity labelling data for the PTH system.

Interestingly, all attempts to monitor ligand interaction with the receptor domains by NMR failed. For a number of Family A GPCRs (e.g. cholecystokinin, substance P/neurokinin-1), we were successful in measuring a number of intermolecular NOEs (nuclear Overhauser effects) between the ligand and receptor domain [16–19]. The solution of the stabilized receptor domain was titrated with the ligand, and distinct chemical shift perturbations could be observed

(and measured to obtain binding affinities) [16,17]. These experimental data provide additional experimentally based ligand–receptor contact sites involving the native ligand sequence and therefore complement the photoaffinity labelling results. We attribute the lack of success for the PTH system to the inherent flexibility of the ligand (the flexible domain between the N- and C-terminal helices of PTH) as well as to the larger size of the ligand interacting with distinct regions of the receptor. In contrast, cholecystokinin and substance P are 8 and 11 amino acids respectively, and adopt a conformation containing a single helical turn [17,19,20].

During the computer-based molecular modelling of the ligand–receptor system, we found it necessary to incorporate a relatively large distance [12–14 Å (1 Å = 0.1 nm)] between the C$\beta$ atoms of the ligand and receptor [9,21]. This distance is well in excess of the reactive sphere of the Bpa itself, reported to be 3.1 Å. The necessity of a loose distance restraint can be partially attributed to the fact that, during the simulations, the wild-type ligand (i.e. replacing the Bpa with the natural occurring amino acid) was used. Indeed, the C$\beta$ atoms were chosen as the points to apply the distance restraints to maximize the conformational freedom of the amino acids at the site of ligand–receptor interaction. Another aspect that cannot be ignored is the flexibility of the Bpa itself, with rotation about the side-chain $\chi_1$ dihedral angle producing a variation of approx. 8 Å itself. Attempts to decrease the distance restraints between the contact points resulted in physically unrealistic structures, with distortion of the ligand and/or receptor. A more detailed analysis of these efforts is in preparation (A. Wittelsberger, M. Rosenblatt and D.F. Mierke, unpublished work).

## General consensus for Family B receptors?

The model developed here for the PTH system provided in Figure 2 is in general agreement with results reported for other Family B GPCRs. The Family B receptor for secretin, for which a large number of photoaffinity labelling sites have been reported [22], provides the best comparison. The sites identified to date for the secretin system are in general agreement with the model presented here.

Importantly, the model presented in Figure 2 is consistent with a membrane-associated pathway, in which the ligand initially interacts with the membrane and then, via a two-dimensional diffusion process, binds to the receptor. Such a pathway has been clearly demonstrated using lipid-tethered peptide analogues for the cholecystokinin Family A GPCR [23]. From an eloquent transferred NOE experiment using pituitary adenylate cyclase-activating polypeptide, a Family B hormone system, only minor structural changes are observed for the ligand upon going from membrane-associated to receptor-bound [24], supporting the membrane-associated pathway for Family B ligands.

The structure of the central portion of the N-terminus of CRF (corticotropin-releasing factor), a Family B receptor, has been reported both alone and while associated with its ligand [25,26], representing a major advance in the understanding of the interactions of the C-terminus of the hormone with the receptor. Through homology with other Family B receptors, including PTH1R, this high-resolution structure has provided key insights into a number of ligand–receptor interactions identified previously through mutagenesis and photoaffinity labelling. Particularly relevant for the PTH system is that the region between the CRF-based structure and TM1 has been solved [13], clearly indicating two $\alpha$-helices on the membrane surface. This should allow for the determination of the relative topological arrangement of this important binding region with the receptor core and transmembrane bundle. Efforts along these lines of research are currently underway in our laboratory.

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