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Modelling and mutation studies on the histamine H_1-receptor agonist binding site reveal different binding modes for H_1-agonists: Asp^{116} (TM3) has a constitutive role in receptor stimulation

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Summary

A modelling study has been carried out, investigating the binding of histamine (Hist), 2-methylhistamine (2-MeHist) and 2-phenylhistamine (2-PhHist) at two postulated agonistic binding sites on transmembrane domain 5 (TM5) of the histamine H_1-receptor. For this purpose a conformational analysis study was performed on three particular residues of TM5, i.e., Lys^{200}, Thr^{203} and Asn^{207}, for which a functional role in binding has been proposed. The most favourable results were obtained for the interaction between Hist and the Lys^{200} / Asn^{207} pair. Therefore, Lys^{200} was subsequently mutated and converted to an alanine, resulting in a 50-fold decrease of H_1-receptor stimulation by histamine. Altogether, the data suggest that the Lys^{200} / Asn^{207} pair is important for activation of the H_1-receptor by histamine. In contrast, analogues of 2-PhHist seem to belong to a distinct subclass of histamine agonists and an alternative mode of binding is proposed in which the 2-phenyl ring binds to the same receptor location as one of the aromatic rings of classical histamine H_1-antagonists. Subsequently, the binding modes of the agonists Hist, 2-MeHist and 2-PhHist and the H_1-antagonist cyproheptadine were evaluated in three different seven-α-helical models of the H_1-receptor built in homology with bacteriorhodopsin, but using three different alignments. Our findings suggest that the position of the carboxylate group of Asp^{116} (TM3) within the receptor pocket depends on whether an agonist or an antagonist binds to the protein; a conformational change of this aspartate residue upon agonist binding is expected to play an essential role in receptor stimulation.

Introduction

Of the many histamine analogues that have been tested on H_1-receptors, histamine (**1**, Fig. 1) itself appears to be among the most potent and effective H_1-agonists [1]. Potent and highly selective histamine H_1-agonists belong to the class of 2-phenylhistamine analogues [2,3]: compounds **11b** and **11c** (Fig. 1) have H_1-agonistic activities of 129% and 112% relative to histamine, respectively [4].

In general, several five-membered rings (i.e., **2**, **3**, **8**–**10**, Fig. 1), and even six-membered rings such as in 2-pyridylethylamine (2-PEA, **4**) can replace the imidazole ring of histamine, while retaining H_1-agonist activity [1]. A proximal basic nitrogen (N^{π}) appears to be crucial for agonistic activity; it is found in all H_1-agonists with an activity >0.1% of that of histamine (compare **4** with **5** and **6**, Fig. 1). Moreover, this proximal nitrogen cannot be methylated without total loss of activity (compare **1** with **7**, Fig. 1). Therefore, the bioactive form of histamine at H_1-receptors is most likely the N^{τ}-H tautomer in which the N^{π} is freely accessible.

Substitution in the imidazole ring at the 2-position with alkyl groups higher than methyl (**8**) is highly unfavourable for H_1-receptor activity, probably due to steric

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Abbreviations: 2-MeHist, 2-methylhistamine; 2-PEA, 2-pyridyl-ethylamine; 2-PhHist, 2-phenylhistamine; CHO, Chinese hamster ovary; E_{int}, interaction energy; E_{str}, strain energy; GES, global energy structure; gpH_1R, guinea pig H_1-receptor; GPCR, G-protein coupled receptor; Hist, histamine; N^{π}, proximal nitrogen; N^{τ}, tele nitrogen; TM, transmembrane domain; WT, wild type.

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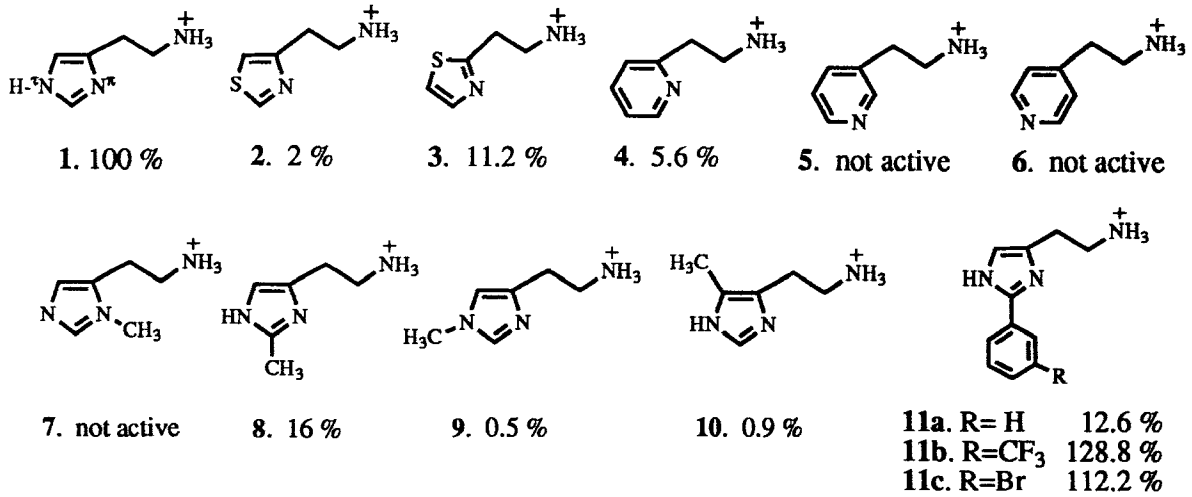

Fig. 1. A selection of compounds, illustrating some aspects of the structural properties necessary for H_1-receptor activation (see text). Agonist activities measured on the guinea pig ileum of compounds **2–10** relative to histamine (= 100%) were taken from Cooper et al. [1]. Relative activities of **11a–c** were taken from a study by Zingel and Schunack [4]. Compounds **1**, **3**, **4**, **8** and **11c** have recently been tested on two mutant H_1-receptors ($Thr^{203} \rightarrow Ala^{203}$ and $Asn^{207} \rightarrow Ala^{207}$ [5]). Histamine (**1**), 2-methylhistamine (**8**) and 2-phenylhistamine (**11a**) are investigated in the present theoretical study.

effects [5]. Therefore, at first sight, it is surprising that aromatic groups are tolerated at this position (i.e., **11a–c**). However, recent site-directed mutagenesis studies by our group have revealed that 2-phenylhistamine analogues form a distinct subclass of histamine agonists, since they appear to interact differently with Asn^{207} in TM5 [5].

The aspartic acid in transmembrane domain 3 (TM3), which is conserved in all aminergic receptors (Asp^{116} in the guinea pig H_1-receptor (gpH_1R) [6]), has been shown to be essential for agonist and antagonist binding at histamine H_1-receptors [7]. For G-protein coupled receptors (GPCRs) that bind aminergic ligands, it is generally accepted that this aspartate interacts with the positively charged basic group of the ligand. In contrast, receptor specificity appears to be defined by residues in other TM domains, especially those in TM5, as has been suggested by mutagenesis studies on the hamster β_2-adrenergic receptor (Ser^{204} and Ser^{207} [8]) and the canine H_2-histaminergic receptor (Asp^{186} and Thr^{190} [9]). Therefore we decided to identify the corresponding functional residues in TM5 of the H_1-receptor that are responsible for binding of the imidazole ring of histamine in its N^{τ}-H tautomeric form. Interaction between the imidazole ring and the H_1-receptor is likely to be optimal when both the N^{τ}-H and the N^{π} functional groups can form hydrogen bonds with protein residues. Possible hydrogen-bond donor and acceptor functionalities in TM5 are Asn^{207}, as an H-bond accepting moiety for the N^{τ}-proton, and either Thr^{203} or Lys^{200} as an H-bond donor for N^{π}. The presence of Lys^{200} in this region of TM5 is remarkable, since in all other aminergic GPCRs no charged residues are observed in the corresponding TM domain (Fig. 2).

In the primary amino acid sequence of the histamine H_1-receptor, a third hydrogen-bond donor residue (Thr^{212}) is observed in the proximity of Asn^{207} (Fig. 2). However, with respect to the above-mentioned residues Lys^{200}, Thr^{203} and Asn^{207}, residue Thr^{212} is found at the opposite site of the TM5 α-helix (i.e., $C^{\beta}(Thr^{203})$-$C^{\alpha}(Thr^{203})$-$C^{\alpha}(Thr^{212})$-$C^{\beta}(Thr^{212}) = 151°$). Since, based on sequence homology with the β-adrenergic receptor and the histamine H_2-receptor, Thr^{212} can be expected to be absent from the receptor binding pocket and to point towards the membrane environment, we did not further consider Thr^{212} in our modelling study.

A severe disadvantage of GPCR modelling studies using the cryomicroscopy structure of bacteriorhodopsin [10] as a template is the known low homology between the TM domains of GPCRs and bacteriorhodopsin (6–11% identity). This low homology has resulted in largely different alignments used by various research groups [11–18]. It is therefore evident that the choice of an alignment for GPCR homology building remains fairly arbitrary. As yet, there is no consensus on the relative orientation of the GPCR α-helices. Therefore, docking studies must be interpreted with caution, especially when the interaction between (usually small) agonists and residues in different TM domains is investigated.

In view of these drawbacks, we have chosen a simplified strategy and focussed in detail on the possible interactions of histaminergic ligands with only one TM domain (TM5). The influence of other domains than TM5 on agonistic action was temporarily neglected and TM5 was treated as a separate entity (an oligopeptide containing 10 residues). An extensive conformational analysis of the three candidate residues Lys^{200}, Thr^{203} and Asn^{207} was performed and their interaction with the imidazole ring of Hist (**1**), 2-MeHist (**8**) and 2-PhHist (**11a**) was studied.

Subsequently, stable agonist–oligopeptide complexes were selected for investigating agonist–TM5 interactions in complete 3D models of the H_1-receptor. Three different alignments [12,16,18] were evaluated. In each receptor model and for several TM5–agonist complexes the feasibility of the interaction between the protonated ethylamine side chain of the agonist and Asp^{116} in TM3 was investigated. For this purpose, the oligopeptide backbone atoms of these complexes were matched with the backbone atoms of the 10 corresponding residues in the 3D model of the protein, thus representing the binding mode of the agonist having previously determined optimal interactions with TM5.

In order to reveal whether distinct differences are present between the agonistic and antagonistic binding sites on the H_1-receptor, the H_1-antagonist cyproheptadine was docked into the three different receptor models. The bioactive conformation of this compound was obtained from a previous study on the H_1-antagonistic binding site in which a stereoselective pharmacophore was derived by superimposing seven (semi-)rigid and potent antihistamines [19]. This H_1-antagonist pharmacophore is represented by two aromatic rings and the positions of the C^{α} and C^{β} carbons of the aspartate from TM3 (Asp^{116}) to which the basic nitrogen of antagonists is bound (see Fig. 7A).

During the course of our study, it became apparent that the 2-phenyl ring of 2-PhHist is important for receptor affinity. Interestingly, the so-called 'cis' aromatic ring of H_1-antagonists plays a similar role [19]. Therefore, in our attempt to find an alternative binding mode for 2-PhHist analogues, the 2-phenyl ring of **11c** was matched with the 'cis' ring of the H_1-antagonist pharmacophore, which indeed led to the identification of an acceptable alternative agonist binding mode for 2-PhHist analogues.

Methods

Conformational analysis

The histamine H_1-receptor was constructed from the PDB structure of bacteriorhodopsin according to the alignment of Trumpp-Kallmeyer et al., and was minimised with Quanta/CHARMm v. 22.0 (Quanta 4.0, Molecular Simulations Inc., Burlington, MA) [12]. Subsequently, 10 residues from TM5 (Phe-Lys-Val-Met-Thr-Ala-Ile-Ile-Asn-Phe) were extracted as an α-helical oligopeptide with neutral N- and C-termini and used for further investigations.

In order to investigate all acceptable interactions between the residues in TM5 and an imidazole ring, it is neccesary to use adequate energy functions for hydrogen-bonded interactions. However, the available hydrogen-bonding functions of ChemX ($V_{hb} = C/r^A - D/r^B$) or AMBER (angle-independant (10,12) Lennard-Jones) do not account adequately for the directionality of hydrogen bonds. CHARMm does include directionality in its hydrogen-bonding function ($E_{hb} = [(A/r_{AD}^6) - (B/r_{AD}^4)] \times \cos^m$

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                          *     *     *
HIS1     194 Y N V T W F K V M T A I I N F Y L P T L L M L W F Y A K I 222
HIS2     177 Q V N L V Y G L V D G L V T F Y L P L L V M C I T Y Y R I 205
ADR-β1   219 V T N R A Y A I A S S V V S F Y V P L C I M A F V Y L R V 247
ADR-β2   194 F T N Q A Y A I A S S I V S F Y V P L V I M V F V Y S R V 222
ADR-β3   199 A S N M P Y V L L S S S V S F Y L P L L V M L F V Y A R V 227
ADR-α1a  207 T E E V G Y A I F S S V C S F Y L P M A V I V V M Y C R V 235
ADR-α2   191 N D Q K W Y V I S S C I G S F F A P C L I M I L V Y Y R I 217
DOP1     189 S L S R T Y A I S S S V I S F Y I P V A I M I V T Y T R I 219
DOP2     185 A N P A F V V Y S S I V S F Y V P F I V T L L V Y I K I Y 213
DOP4     188 E D R D Y V V Y S S V C S F F L P C P L M L L L Y W A T F 216
5HT1a    190 S K D H G Y T I Y S T F G A F Y I P L L L M L V L Y G R I 218
ACM2     181 F S N A A V T F G T A I A A F Y L P V I I M T V L Y W H I 209
ACM3     190 L S E P T I T F G T A I A A F Y M P V T I M T I L Y W R I 218
ACM4     226 L S N P A V T F G T A I A A F Y L P V V I M T V L Y I H I 254
ACM5     188 L S E P T I T F G T A I A A F Y I P V S V M T I L Y C R I 216
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Fig. 2. Multiple sequence alignment of TM5 for a representative set of aminergic GPCRs. Numbers refer to the first and last amino acid residue of the row. In all catecholaminergic receptors (ADR, DOP), conserved serine residues are found in TM5. Their positions correspond with Thr^{203} and Asn^{207} in the guinea pig histamine H_1-receptor and with Asp^{186} and Thr^{190} in the canine H_2-receptor. Lys^{200} is found in the histamine H_1-receptor in a region of the TM5 domain where no charged residues are observed in all other aminergic GPCRs. Histaminergic receptors: HIS_1 = PIR2:JX0287 (guinea pig), HIS_2 from Ref. 9 (canine). Adrenergic receptors (human): ADR-β_1 = PIR2:A39911, ADR-β_2 = PIR2:A28405, ADR-β_3 = PIR2:A41348, ADR-α_{1a} = PIR1:JH0447, ADR-α_2 = PIR2:A34169. Dopaminergic receptors (human): DOP_1 = PIR1:DYHUD1, DOP_2 = PIR1:DYHUD2, DOP_4 = PIR1:DYHUD4. Serotoninergic receptors (rat): $5HT_{1a}$ = PIR1:JH0315. Acetylcholinergic–muscarinergic receptors (human): ACM2 = PIR2:S10126, ACM3 = PIR2:S10127, ACM4 = PIR2:S10128, ACM5 = PIR2:JT0530.

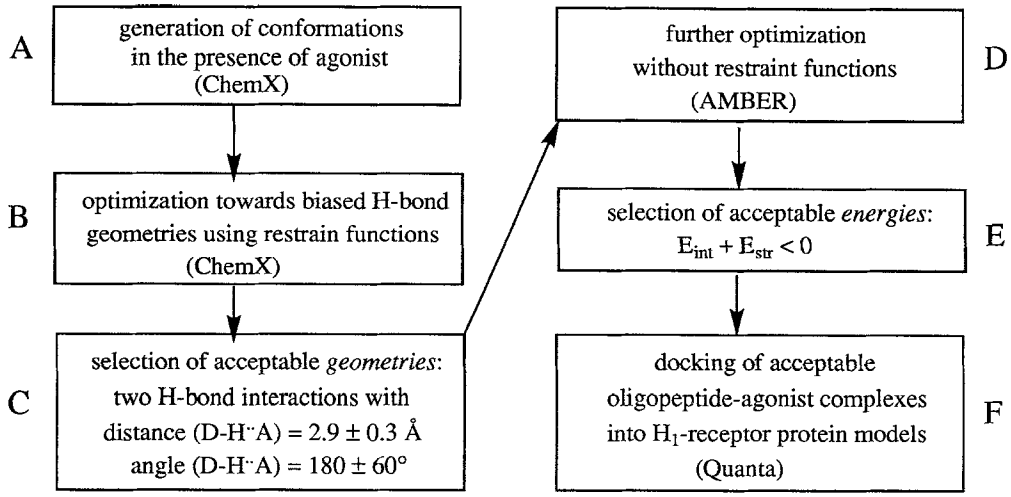

Fig. 3. Schematic representation of the methods used to obtain stable complexes of histamine analogues with residues in TM5 of the histamine H_1-receptor. (A) Conformations are generated for the oligopeptide as described in the text in the presence of agonist. (B) The orientation of the imidazole is optimized with respect to the oligopeptide (Fig. 4) using X-ray database results [20]. (C) Complexes are selected using predefined criteria for proper H-bonds, after which they are further optimized in AMBER (D) and selected based upon their final energy (E). The remaining complexes are incorporated into complete 3D protein models and the agonist binding sites are investigated (F) (see text for further details).

$(\Theta_{AHD}) \times \cos^n(\Theta_{AAAH}) \times sw\,(r^2_{AD}, r^2_{on}, r^2_{off}) \times sw\,(\cos^2(\Theta_{AHD}), \cos^2(\Theta_{on}), \cos^2(\Theta_{off}))$, but in our experience this function leads to geometries (i.e., $\Theta_{C=O\cdots H}$ being 180°) which are not in agreement with protein X-ray data [20]. In order to prevent a large number of geometries with improper hydrogen bonds resulting from the conformation analyses and the subsequent ChemX optimization on three residues of TM5 (Lys^{200}, Thr^{203} and Asn^{207}) in the presence of agonist, we biased the directionality of the proposed hydrogen bonds with restraint functions on predefined dummy atoms. For further optimization and calculation of interaction energies we used the AMBER force field (Kollman's united and all atom force field with additional parameters for organic functionality [21]), since it is especially parametrised for the simulation of proteins.

Overall, a strategy was developed in which the geometries of oligopeptide–agonist complexes were generated with ChemX (Figs. 3A,B) and those with proper hydrogen-bond angles and torsion angles were selected (Fig. 3C) for further optimization and calculation of the interaction

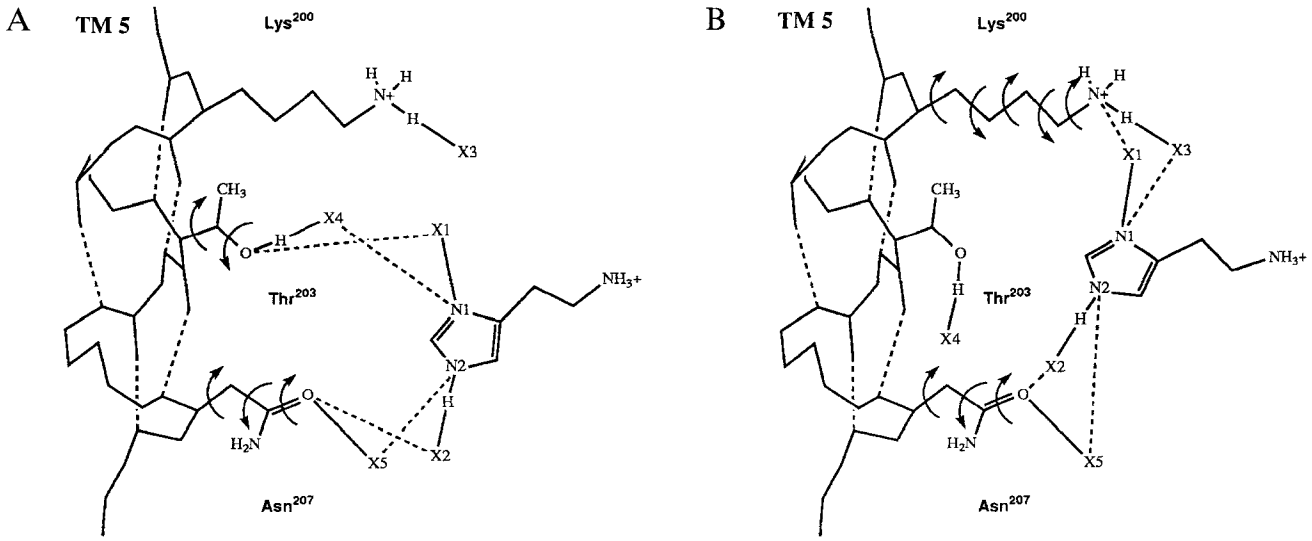

Fig. 4. In order to study all possible interactions with histamine, conformational analysis studies were performed on Lys^{200}, Thr^{203} and Asn^{207}, present in TM5 of the histamine H_1-receptor. In our hypothesis, either the Thr^{203}/Asn^{207} pair (A) or the Lys^{200}/Asn^{207} pair (B) can be expected to interact with the imidazole ring of histamine analogues. Different conformations were generated for the putative interacting residues by adequately chosen torsion angle increments along several rotatable bonds (indicated by arrows) (Fig. 3A). Appropriate X-ray hydrogen-bond geometries are mimicked by dummy atoms on the imidazole ring (X1 and X2) and the proposed interacting residues (X3–X5). In the minimization procedure (Fig. 3B), four restraint functions are applied on the distances between the dummy atoms and their counteratoms (dashed lines).

TABLE 1
THEORETICAL RESULTS ON THE INTERACTION BETWEEN H_1-AGONISTS AND TRANSMEMBRANE DOMAIN 5 OF THE HISTAMINE H_1-RECEPTOR[a]

	Number of (remaining) conformations				E_{int}	E_{str}	ΔE (kcal/mol)
	N_{start}	N_{ChemX}	N_{AMBER}	N_{stable}			
Thr^{203}/Asn^{207}							
Hist	324	26	11	3	−8.03	6.68	−1.35
2-MeHist	324	19	2	0	−11.18	14.66	3.48
2-PhHist	324	9	1	0	−12.39	19.73	7.34
Lys^{200}/Asn^{207}							
Hist	8748	67	63	15	−11.08	4.70	−6.38
2-MeHist	8748	67	53	12	−14.11	6.54	−7.57
2-PhHist	8748	56	42	18	−15.46	7.67	−7.79

[a] Results of the conformational analyses on the Thr^{203}/Asn^{207} and Lys^{200}/Asn^{207} pairs in the presence of histamine (Hist, **1**), 2-methylhistamine (2-MeHist, **3**) or 2-phenylhistamine (2-PhHist, **11a**). N_{start} presents the number of conformations generated with ChemX in the presence of ligand (Fig. 3A). N_{ChemX} is the number of conformations with acceptable H-bond interactions (geometric criteria; see Fig. 3C) between the agonist imidazole and the protein residue pair (two H-bonds). These were obtained after ChemX optimization of the N_{start} conformations in the presence of agonist (Fig. 3B) and with penalty functions on the appropriate donor, acceptor and dummy atoms (as in Figs. 4A,B). N_{AMBER} indicates the number of conformations of the oligopeptide–ligand complex with acceptable H-bond interactions after AMBER optimization of the N_{ChemX} conformations. N_{stable} represents the peptide–agonist complexes which fulfil the criterium $\Delta E < 0$, where ΔE indicates the stability of the complex (see Eqs. 1–3). Only for the complex with the lowest ΔE value, the corresponding E_{int}, E_{str} and ΔE values are given.

energies with AMBER (Figs. 3D,E). These methods are described in more detail below.

The possible interaction sites of Hist (**1**), 2-MeHist (**8**) and 2-PhHist (**11**) with residues in TM5 were represented by either the Asn^{207}/Thr^{203} pair (Fig. 4A) or the Asn^{207}/Lys^{200} pair (Fig. 4B). These two interaction sites were investigated separately. For each of the two pairs a large number of conformations (324 and 8748, respectively; see Table 1) was generated with ChemX (January 1993 version) in the presence of agonist, while the atoms of the oligopeptide backbone and the remaining side chains were unchanged (Figs. 3A and 4). The stepwise increment for the torsion angles indicated in Fig. 4 was either 180° (two steps for sp^2-sp^2 atom bonds), 60° (six steps for sp^2-sp^3 atom bonds) or 120° (three steps for sp^3-sp^3 atom bonds), corresponding to the likely number of local minima for these bonds.

Dummy atoms were connected to the H-bond donor or acceptor functions of the imidazole ring of the agonists (X1 and X2) and the three amino acid residues (X3–X5) and put at those positions where the counter donor or acceptor atoms are expected according to a protein X-ray database search [20]. Subsequently, four penalty functions were used (restraint constant $k = 100$ kJ/Å^2) to minimize the distance between the dummies and their counter-atoms. These penalty functions are considered as restrain forces ($F_r = k(l - l_0)$) during optimization of the ChemX van der Waals energy (ChemX Reference Guide 1993 [22]). The ChemX vdW energy neglects bond and angle strain energy terms, and uses only torsion, nonbonded, electrostatic and hydrogen-bonding energy terms ($E_{vdW} = V_{tor} + V_{nb} + V_{el} + V_{hb}$). Thus, the orientation and the position of the agonist relative to the respective residue pair and the torsion angles of the two considered residues (Lys/Asn or Thr/Asn; indicated by arrows in Fig. 4) are optimized (Fig. 3B).

Low-energy TM5–ligand conformations (energies less than 50 kJ/mol from the lowest energy) were selected with two H-bonds between the imidazole ring and oligopeptide residues fulfilling the following criteria: distances (D-H··A) of 2.9±0.3 Å and angles (D-H··A) of 180±60° (Fig. 3C). The selected structures were further optimized with AMBER (derivative convergence < 0.005 kJ/Å mol) using the PR Conjugate Gradient (PRCG) method in BatchMin v. 4.5/MacroModel v. 2.5, releasing all restraints except those on the oligopeptide backbone. In these calculations, structures were optimized employing a constant dielectric ($\varepsilon = 1$), the standard united atom AMBER charge set and Kollman's 6,12 Lennard-Jones hydrogen-bonding treatment (Batchmin User Manual v. 4.0, Colombia University, New York, NY).

The AMBER global energy structure of the oligopeptide in the absence of agonist was obtained from a conformational analysis in MacroModel (v. 2.5) by rotation along the indicated oligopeptide bonds (see the arrows in Fig. 4) using torsional increments as before in the presence of agonist. The global energy structures of the agonists (Hist, 2-MeHist and 2-PhHist) were obtained using increments of 60° for the rotatable bonds of the ethylamine side chain.

Energy calculations

The AMBER interaction energy (E_{int}) of the optimized agonist–oligopeptide complex is calculated by:

$$E_{int} = E_{comp} - E_{olig} - E_{ago} \quad (1)$$

where E_{comp} is the energy of the optimized complex and

E_{olig} and E_{ago} are the energies of the separated oligopeptide and agonist in the complex, respectively.

The strain energy imposed on the residues of the oligopeptide while interacting with the ligand is calculated by:

$$E_{str} = E_{olig} - E_{olig,GES} \quad (2)$$

where $E_{olig,GES}$ is the energy of the global minimum structure of the oligopeptide in the absence of agonist. No strain energy is induced upon the agonist, since the ethylamine side chain is kept in a fixed extended local minimum conformation.

The interaction energy of the oligopeptide–ligand complex should at least compensate the strain energy imposed on the oligopeptide residues by the agonist. Therefore, 'stable' complexes are assumed to be represented by negative ΔE values:

$$\Delta E = E_{int} + E_{str} = E_{comp} - E_{ago} - E_{olig.GES} \quad (3)$$

Receptor docking

Three different H_1-receptor models, differing in the relative orientation of their seven TM helices, were built from the cryomicroscopy structure of bacteriorhodopsin (PDB code 1brd). Two models were built in analogy with the alignments of Trumpp-Kallmeyer et al. and Timms et al. [12,16], using the homology building options of Quanta v. 3.3. A third H_1-receptor model was obtained from the 7TM file server of the EMBL (Heidelberg) [18]. Prior to docking, these transmembrane seven-helical bundles were optimised with CHARMm v. 22.0. The oligopeptide backbone atoms of selected TM5–agonist complexes (vide supra) were fitted onto the backbone atoms of the 10 corresponding residues in TM5 using ChemX fit procedures; subsequently, the duplicate TM5 residues were removed.

In order to investigate the antagonist binding site in the H_1-receptor, the previously determined cyproheptadine–aspartate complex (Fig. 7A [19]) was docked into the protein such that the aspartate of the complex matched Asp^{116} of the model (by fitting C^{α} and C^{β} atoms). The superfluous aspartate was subsequently removed and Asp^{116} was rotated around its C^{α}-C^{β} bond in order to establish a favourable orientation for the coupled antagonist. The unique antagonist–protein model with minimal steric hindrance was energy optimized with CHARMm v. 22.0 (Fig. 7C).

Mutation studies

Recently, our group has performed a pharmacological characterization of the wild-type H_1-receptor and two mutants ($Thr^{203} \rightarrow Ala$ and $Asn^{207} \rightarrow Ala$) stably transfected into Chinese hamster ovary (CHO) cells [5]. In the present study new results are presented concerning the mutation of Lys^{200} (to alanine). This gpH_1-receptor mutant was successfully expressed in CHO cells and tested according to procedures described earlier [5].

Results and Discussion

Interactions of agonists with the Thr^{203}/Asn^{207} pair

The conformational analysis of the Thr^{203}/Asn^{207} pair yielded 324 conformations (N_{start}; see Table 1) in the presence of either agonist Hist (Fig. 4A), 2-MeHist or 2-PhHist. Optimization with ChemX resulted in respectively 26, 19 and 9 conformations with acceptable H-bond geometries (N_{ChemX}; see Table 1). After further minimization with AMBER, with restrain functions only on the backbone, 11, 2 and 1 conformations with satisfactory hydrogen-bond interactions (Thr-O-H··N^{π}/N^{τ}-H··O=Asn) remained, respectively (N_{AMBER}). Hist was the only agonist for which three 'stable' ($\Delta E < 0$) complexes could be formed (N_{stable}; see Table 1), although the energy gain from these interactions is low ($\Delta E > -1.35$ kcal/mol; see Table 1). Moreover, the strain energies imposed on the side chains in order to establish these interactions are probably partly due to a loss of the internal H-bond between Thr^{203} and the protein backbone; they are generally high ($E_{str} \approx 7$–20 kcal/mol; see Table 1). For these reasons a strong and also selective binding of the agonist imidazole ring by Thr^{203} and Asn^{207} seems unlikely.

Interactions of agonists with the Lys^{200}/Asn^{207} pair

Formation of several stable complexes resulted from the conformational analysis study on the Lys^{200}/Asn^{207} pair in the presence of each investigated agonist (Fig. 4B). For this pair of residues a large number of conformations was generated ($N_{start} = 8748$; Table 1), merely due to the many rotatable bonds in Lys^{200}. After optimization with AMBER, a large number of complexes was observed with satisfactory H-bonds for all three agonists (63, 53 and 42 for Hist, 2-MeHist and 2-PhHist, respectively; Table 1). All complexes were stabilised by two presumably essential H-bonds (Lys-ε-N^{+}-H··N^{π} and N^{τ}-H··O=Asn). A large number of these complexes (15, 12 and 18, respectively; Table 1) were found to be 'stable'.

Interestingly, in all 'stable' complexes, the imidazole ring of the agonist is found to bind to a conformation of TM5 which is very similar to the GES of the oligopeptide in the absence of agonist. The GES is a structure in which the Thr^{203} side chain is hydrogen bonded to the backbone carbonyl of Lys^{200}, while the conformation of the Lys^{200} side chain is mainly determined by two H-bonds, one to the hydroxyl oxygen of Thr^{203} and one to its own backbone carbonyl (see Fig. 5A). The amine of Asn^{207} also interacts via an H-bond with its own backbone carbonyl. Since the charged amino group of Lys^{200} is bent in the direction of Asn^{207}, these residues constitute a perfect binding site for the imidazole ring (Fig. 5). The strain energy which is imposed upon the residues upon

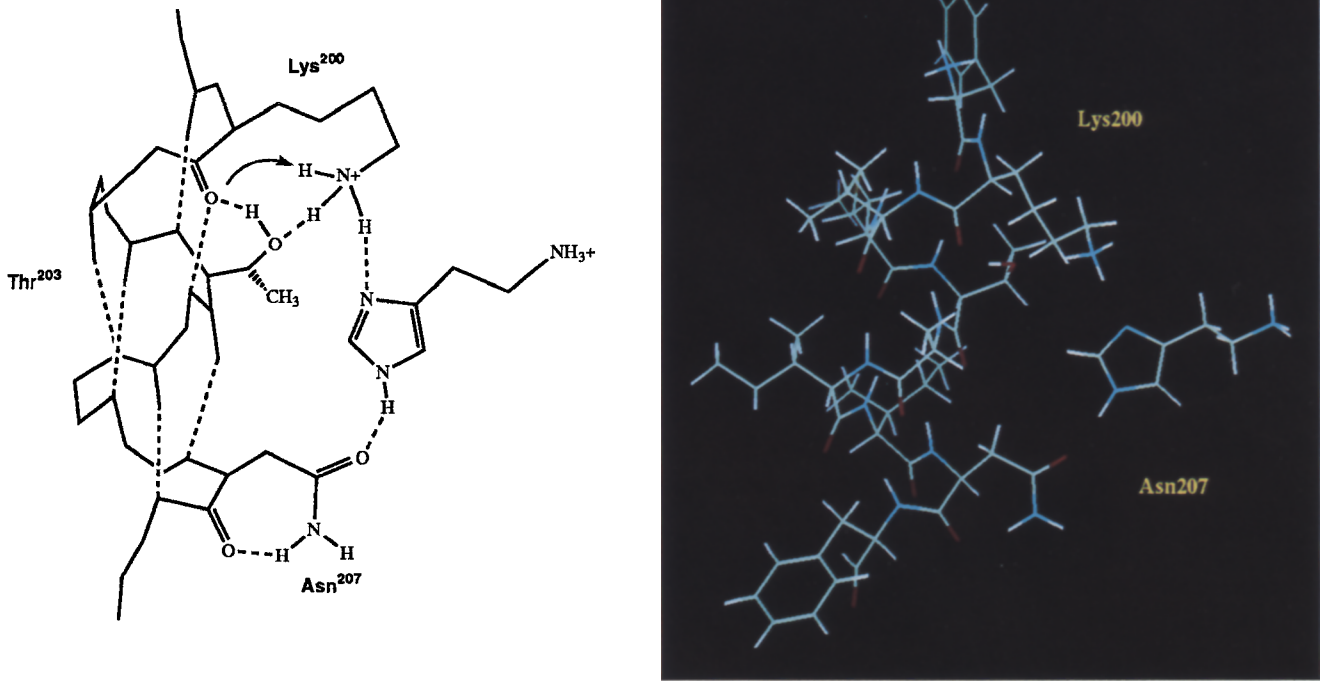

Fig. 5. Schematic (left) and computer graphics (right) representation of the interaction of histamine with Lys^{200} and Asn^{207} in TM5. The oligopeptide remains close to its GES; the hydrogen bond between the ε-amino group of Lys^{200} and its backbone carbonyl is broken (indicated by an arrow) and two hydrogen bonds with the imidazole ring are established (one with Lys^{200} and one with the carbonyl of Asn^{207}).

agonist binding largely results from breaking of an internal H-bond (Lys^{200}). This energy is different for Hist (4.70), 2-MeHist (6.54) and 2-PhHist (7.67) (Table 1). However, in all cases the strain energy is greatly surpassed by the H-bond interaction energy of the complexes, as demonstrated by negative energy balances ($\Delta E = -6.38$, -7.57 and -7.79 kcal/mol, respectively; Table 1).

It is worthwhile mentioning that some important differences in the geometries of these three complexes were observed, although in each case two hydrogen bonds could be established between the agonist and the Lys/Asn pair. Histamine and 2-MeHist appear to interact in an orientation perpendicular to the α-helix backbone of TM5 (Fig. 5B), thus preventing that large substituent groups at the 2-position can be accommodated in this binding mode. As a consequence, 2-PhHist is pushed away from the backbone, resulting in a sideways orientation of the imidazole ring (in parallel with the backbone) and the phenyl ring binding in the free space close to Ala^{205} (see also the section on ligand docking of 2-PhHist).

The results described above indicate that for the three agonists studied, the Lys/Asn pair constitutes a more likely binding site than the Thr/Asn pair.

Interpretation of binding and functionality of wild-type and mutant H_1-receptors

Previously, we have reported pharmacological data on the wild-type H_1-receptor and two mutants with alterations in TM5 ($Thr^{203} \rightarrow Ala$ and $Asn^{207} \rightarrow Ala$) [5]. Whereas the Thr^{203} mutation had almost no effect on agonist action, the Asn^{207} mutation resulted in a severe loss of affinity and functional activity in the case of Hist (**1**) and 2-MeHist (**8**) (Table 2). Since the Asn^{207} mutation had only a small effect on the affinity of agonists that lack an N^{τ}-atom (**3** and **4**), it was concluded that the N^{τ}-proton interacts with Asn^{207} [5].

However, the question concerning the amino acid serving as an H-bond-donating residue to the N^{π}-atom present in all potent H_1-agonists remained unsolved. The results of Leurs et al. showed that Thr^{203} is unlikely to be involved in binding the N^{π}-atom of histamine [5]. In the present modelling study, we confirm that a strong and simultaneous binding of Thr^{203} and Asn^{207} to the imidazole ring is indeed unlikely, as the highest observed energy yield for such an interaction is only -1.35 kcal/mol (in the case of histamine; Table 1).

On the basis of our current results, the strong H-bond-donating residue Lys^{200} was mutated to an alanine, causing a fivefold decrease in histamine affinity and an even higher decrease in the functional EC_{50} value (50-fold; Table 2) [30]. These observations are in full agreement with our modelling investigations, which reveal that the Lys^{200}/Asn^{207} residue pair in TM5 is especially suited to bind an imidazole ring (energy gain: 6–8 kcal/mol; see

TABLE 2
AFFINITY AND ACTIVITY OF H_1-AGONISTS FOR WILD-TYPE AND MUTANT H_1-RECEPTORS

	K_i (μM)					EC_{50} (μM)				
	Wild type		Thr^{203} → Ala[a]	Asn^{207} → Ala[a]	Lys^{200} → Ala[b]	Wild type		Thr^{203} → Ala[a]	Asn^{207} → Ala[a]	Lys^{200} → Ala[b]
	A[a]	B[b]				A[a]	B[b]			
Hist (**1**)	12	30	26	>1000	150	3.9	1.2	7.5	>1000	63
2-PEA (**4**)	38	69	44	129	86	–	23	–	–	340
2-MeHist (**8**)	42	98	25	>1000	60	–	8.5	–	–	16
m-Br-2-PhHist (**11c**)	0.67	0.87	0.44	4.3	2.2	0.56	0.59	0.53	6.6	0.56

[a] Earlier pharmacological data of four histamine H_1-agonists measured on wild-type and two mutant H_1-receptors (Thr^{203} → Ala and Asn^{207} → Ala), reported by Leurs et al. [5].
[b] New pharmacological data on wild-type and the Lys^{200} → Ala mutant H_1-receptor [30].
Binding constants (K_i; displacement of 1.5 nM [^{3}H]-mepyramine) were measured on stably transfected H_1-receptors in CHO cells [5] or transiently in HEK-293 cells [30]. Functional EC_{50} values were obtained by measuring [^{3}H]IP_x production in CHO cells. Bold numbers refer to the structural formulas in Fig. 1.

Table 1 and Fig. 5). We therefore conclude that Lys^{200} interacts specifically with the N^{π}-atom of histamine. This is further supported by a 15-fold decrease in the EC_{50} value of the agonist 2-pyridyl-ethylamine (2-PEA (**4**); Fig. 1) upon mutation of Lys^{200} (Table 2).

Close inspection of the agonist–oligopeptide complexes reveals that the orientation of 2-MeHist in the binding pocket is similar to that of Hist, including two hydrogen bonds with the Lys^{200}/Asn^{207} pair (ΔE values are −6.38 and −7.57 kcal/mol for Hist and 2-MeHist, respectively; see Table 1). This interaction is supported by the strong effect of the Asn^{207} mutation on the agonist binding of 2-MeHist. However, the effect of the Lys^{200} mutation is marginal, which is rather unexpected (EC_{50} changes from 8.5 to 16 μM). It is likely that 2-MeHist is oriented differently from Hist, in such a way that the H-bond with Asn^{207} is still possible, but the H-bond with Lys^{200} is lost. Both the oligopeptide model system and the complete H_1-receptor models (see below) were unable to reveal any cause for this reorientation. Possibly, the position of Asp^{116}, which interacts with the ethylamine side chain, is not 'correct' in the used protein models due to deficiencies in homology building based on bacteriorhodopsin (see below).

Although 2-PhHist can be accommodated in the Lys/Asn pocket via two hydrogen bonds, the mutation data (Table 2, **11c**) indicate this orientation to be wrong, since both the lysine and the asparagine mutation have negligible effects. Therefore, in the complete protein models (see below), a different binding mode for 2-PhHist analogues was sought, outside the Lys/Asn and Thr/Asn sites.

Ligand docking in H_1-receptor models

Complete receptor models were constructed in order to further identify the possible binding modes of the three agonists (**1**, **8**, **11a**) and also to answer the question whether an ionic H-bond could be established with the third interacting H-bond-accepting residue Asp^{116} in TM3. Three different alignments with bacteriorhodopsin were used [12,16,18], yielding three H_1-receptor models in which the oligopeptide–agonist complexes were docked (see Methods). Based on these results, the alignment of Trumpp-Kallmeyer et al. [12] was rejected, since the agonists were penetrating the backbone of TM6 and/or pointing to the apparent membrane environment. In the two remaining models the distance between the carboxylate oxygen of Asp^{116} and the position of the basic nitrogen of the agonist appeared to be relatively large: 4–5 Å in Oliveira's model (Figs. 6A,B) and 6–7 Å in Timms' model (not shown), whereas the optimal distance for an ionic hydrogen bond (N^+-O^-) is about 2.9 Å [20]. These results indicate a probable incorrectness of GPCR modelling based on a light-induced non-GPCR (see also the Introduction). The distance between TM3 and TM5, and possibly also their relative orientation, deviates from the structural features of bacteriorhodopsin. This observation is supported by recent findings in the electron microscopy structure of the GPCR bovine rhodopsin, in which the relative orientations of the TM domains deviated slightly from those in bacteriorhodopsin [23]. Since all three agonists could be accommodated in the two protein models, with interactions as found in the oligopeptide model system, the results of the mutation studies with 2-MeHist (Lys^{200} mutation; see above) can only be explained by considering possible deficiencies in the relative orientations of the TM domains.

The binding mode of 2-PhHist, derived from the oligopeptide model system, is also feasible within the two protein models. However, its side chain approaches Asp^{116} from an angle that is quite different (Fig. 6B) from the one of 2-MeHist or Hist (Fig. 6A), which influences the orientation and position of the carboxylate. Since the mutation data are not consistent with the binding mode of 2-PhHist as presented in Fig. 6B, we conclude that the presented orientation of the basic nitrogen and Asp^{116} might be unfavourable for agonist action. Therefore, another binding mode was searched for, in which binding

of Asp^{116} to 2-PhHist occurs in a similar way to that of (2-Me)Hist. Inevitably, this must lead to a binding mode in which the imidazole ring of 2-PhHist occupies a different receptor site than those of Hist and 2-MeHist. Furthermore, the 2-phenyl ring should preferably be positioned at a receptor site which explains the enhanced affinity of 2-PhHist compared to Hist in the wild-type receptor.

Considering the above data, we anticipated the possibility that the binding site for 2-PhHist is similar to the binding site of H_1-antagonists. Recently, the H_1-antagonist pharmacophore was derived [19], consisting of two aromatic ring systems, the so-called 'cis' and 'trans' rings, and the C^{α} and C^{β} atoms of an aspartic acid (Asp^{116}). The ring system is fixed with respect to the aspartate (Fig. 7A). The 'cis' ring should preferably be aromatic, while a small lipophilic para-substituent enhances affinity. Remarkably, in the case of 2-PhHist a small lipophilic (meta)substituent (**11b,c**) also increases affinity. To investigate whether the H_1-agonist 2-PhHist could possibly occupy the H_1-antagonist binding site, the agonist was matched with the H_1-antagonist cyproheptadine coupled to an aspartate (Fig. 7A). The two aromatic ring systems and lipophilic substituents important for affinity were superimposed. While restraining the 2-phenyl ring at the position of the H_1-antagonist 'cis' ring, the GES of **11c** was optimized with AMBER with respect to the fixed aspartate. Following this procedure, an unexpected result was obtained. It appeared that the 2-PhHist analogue **11c** interacts with Asp^{116} in an acceptable 'folded' conformation, in which the ethylamine side chain forms an internal H-bond with the imidazole ring (Fig. 7B). The geometry of **11c** in this conformation is almost identical to that of the GES (also a 'folded' conformation), with an intramolecular energy of 0.82 kcal/mol above the GES. Remarkably, the conformation of Asp^{116} interacting with 2-PhHist (Fig. 7B) is quite different from its conformation when binding with the H_1-antagonist cyproheptadine (Fig. 7A). This suggests that 2-PhHist analogues stabilize a different conformation of Asp^{116} than do H_1-antagonists (see also below).

In order to further investigate the binding modes of cyproheptadine (representing the antagonist binding mode) and the agonist 2-PhHist within the protein, the H_1-antagonist (Fig. 7A) was docked into Oliveira's protein model in a unique orientation (Fig. 7C; see Methods). Subsequently, 2-PhHist was docked according to the fit onto the H_1-antagonist pharmacophore (Fig. 7D). The aromatic 'cis' ring is now found in the proximity of Trp^{161} (TM4), which is conserved within the cationic neurotransmitter GPCR subfamily and could possibly bind the aromatic ring by means of aromatic–aromatic interactions [24].

Figure 7C shows that the relatively large bulk volume of the antagonist can only be accommodated within the receptor pocket when Asp^{116} points in the direction of TM6. In contrast, upon binding of 2-PhHist (Fig. 7D), Asp^{116} attains a different conformation and is oriented in the direction of TM5. This latter finding is in agreement with the earlier proposed binding modes for the agonists Hist and 2-MeHist in Fig. 6A, in which the interactions between the agonists and the three residues Asp^{116}, Lys^{200} and Asn^{207} can only occur when Asp^{116} of TM3 assumes a conformation in which it points in the direction of TM5 (Fig. 6A). Therefore, we conclude that the orientation of the agonist 2-PhHist, as suggested by the fit onto the antagonist pharmacophore (Fig. 7A,B), is possible in the evaluated 3D receptor model. In addition, our findings suggest that the conformation of Asp^{116} is different for binding to agonists or antagonists. This observation may have important implications for the unraveling of activation mechanisms in GPCR models (see below).

The role of Asp^{116} in the activation mechanism of aminergic GPCRs

Asp^{116}, which is highly conserved in aminergic GPCRs, is generally seen as the main anchoring point for agonist and antagonist binding. Residues in other TM domains (especially TM5) appear to be involved in determining the selectivity of these receptors for their endogenous agonists, e.g., in adrenergic and/or dopaminergic receptors two serines interact with the agonist catechol moiety [8], and in the present study it was found that an aspartate and a threonine residue in the histamine H_2-receptor [9], and a lysine and an asparagine residue in the H_1-receptor, interact with the agonist imidazole ring. The question can be raised, however, whether activation of aminergic receptors is induced by the amine side chain or by the ring system of the agonist. It is remarkable that all aminergic agonists are relatively small molecules with short side chains and that all postulated binding modes for agonists like histamine and catecholamines suggest that they have some kind of bridging function between TM3 and TM5. Considering the relatively large gap between TM3 and TM5, it seems likely that the agonist interactions described above can only be established if Asp^{116} attains a conformation in which it points in the direction of TM5 (as in Fig. 6A). Basic H_1-antagonists, such as cyproheptadine, are likely to interact with the same aspartic acid in TM3 [7], but it appears that they stabilize a different conformation of Asp^{116} (Fig. 7C).

Since it is not possible to obtain a general mechanism for receptor stimulation via the variable part of aminergic ligands (e.g., histamine, (nor-)adrenaline, dopamine, serotonin, acetylcholine, muscarine), it makes sense to assign a key role to the conserved Asp^{116} residue in TM3. Our findings suggest that the induction of a conformational change in Asp^{116} by an agonist could be a general and crucial step in aminergic receptor stimulation. As a prerequisite for an activated receptor state, rearrangement of

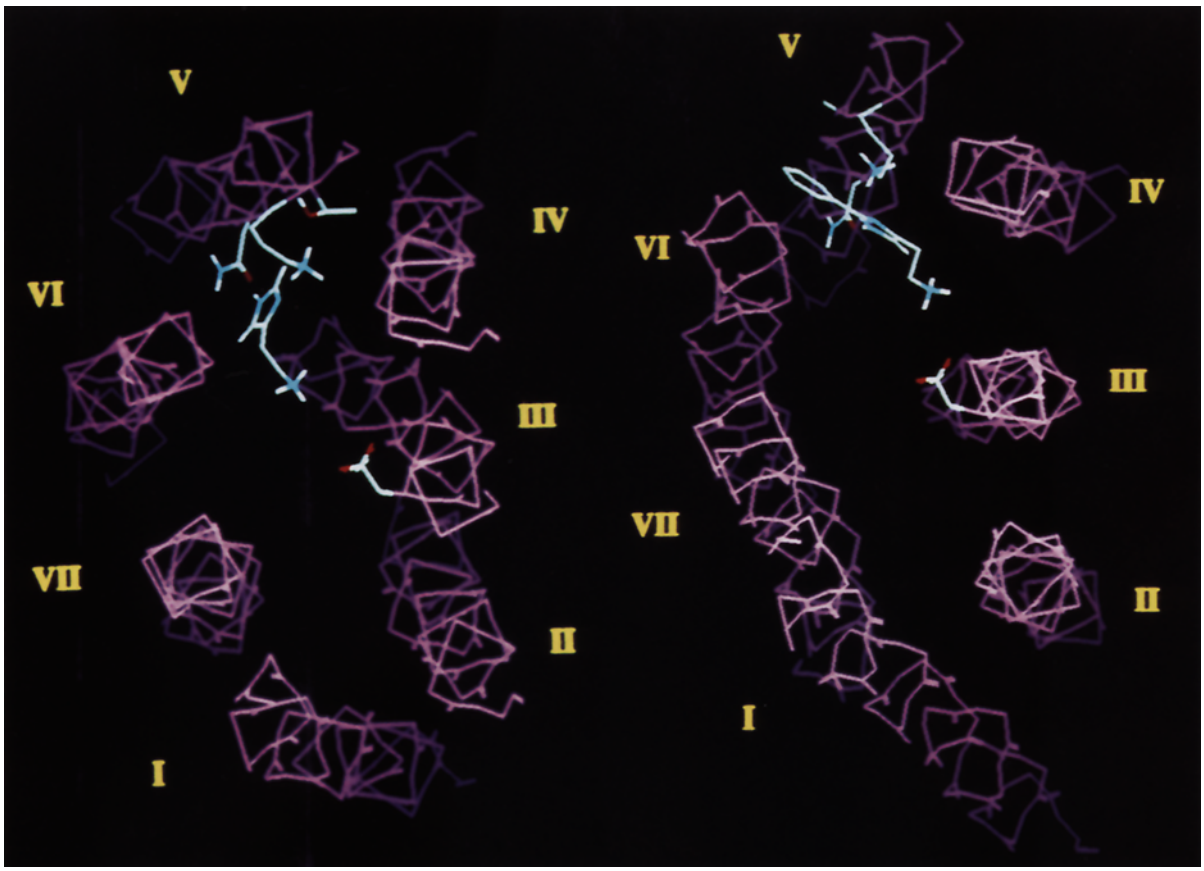

Fig. 6. Computer graphics representations of histamine (A) and 2-phenylhistamine (B) docked into the histamine H_1-receptor model of Oliveira et al. [18]. The orientations of Hist and 2-PhHist with respect to TM5 are a direct result of the AMBER optimisation. The imidazole ring of 2-PhHist (B) lies in parallel with the backbone of TM5 and its ethylamine side chain approaches Asp^{116} from a different angle than in the case of Hist (A). This mode of binding for 2-PhHist (B) is rejected based on mutation data (see text and Fig. 7 for a further explanation).

Asp^{116} could fit in any of the various concepts of GPCR stimulation that have been proposed in the literature: conformational changes in the intracellular loops due to rearrangement of the seven-helix bundle [11,25], the so-called 'arginine switch' mechanism [26], or ligand-mediated proton transfer mechanisms [27–29]. The binding modes of H_1-receptor agonists and antagonists as indicated in our model mean that in attempts to unravel the activation mechanism of GPCRs, a crucial role should be considered for Asp^{116}.

Conclusions

In the present study we have combined results from site-specific mutagenesis studies on the histamine H_1-receptor with findings from different molecular modelling approaches such as conformational analyses, pharmacophore fitting and receptor docking studies. From our findings the following conclusions emerge:

(1) The conformational analyses on specific residues of TM5 of the histamine H_1-receptor demonstrate that residues Lys^{200} and Asn^{207} constitute a selective and energetically favourable binding site for the imidazole ring of histamine. This conclusion is supported by the severe decrease in activity of histamine for the $Lys^{200} \rightarrow$ Ala and $Asn^{207} \rightarrow$ Ala H_1-receptor mutants.

(2) In all three investigated H_1-receptor models, TM3 and TM5 are at least 1 Å too far apart to optimally establish the three proposed H-bond interactions between residues Asp^{116}, Lys^{200}, Asn^{207} and the agonist histamine (or 2-MeHist). This may either be due to the use of bacteriorhodopsin (not a GPCR) as a template molecule or to the chosen alignment which positions TM3 in a certain orientation with respect to TM5. With regard to this latter point, TM3 and TM5 have the best relative orientation in the H_1-receptor model based on the alignment of Oliveira et al. [18].

(3) The imidazole ring of 2-phenylhistamine seems to play a minor role in agonist binding. Based on the fit of 2-phenylhistamine onto an H_1-*antagonist* pharmacophore, the 2-phenylring of 2-PhHist interacts with the same receptor site as the so-called 'cis' ring of H_1-antagonists; an internal hydrogen bond between the amine function and the proximal nitrogen atom is essential for stabilizing this particular binding mode of 2-PhHist.

(4) The different conformations of Asp^{116} in TM3 ob-

served in our modelling studies upon H_1-agonist or H_1-antagonist binding indicate that this aspartate may play a key role in receptor stimulation. To accommodate H_1-antagonists, Asp^{116} attains a conformation in which it points in the direction of TM6 (possibly representing the resting state of the aminergic GPCR). Upon binding of small agonists such as histamine, Asp^{116} changes its conformation and points in the direction of TM5, which

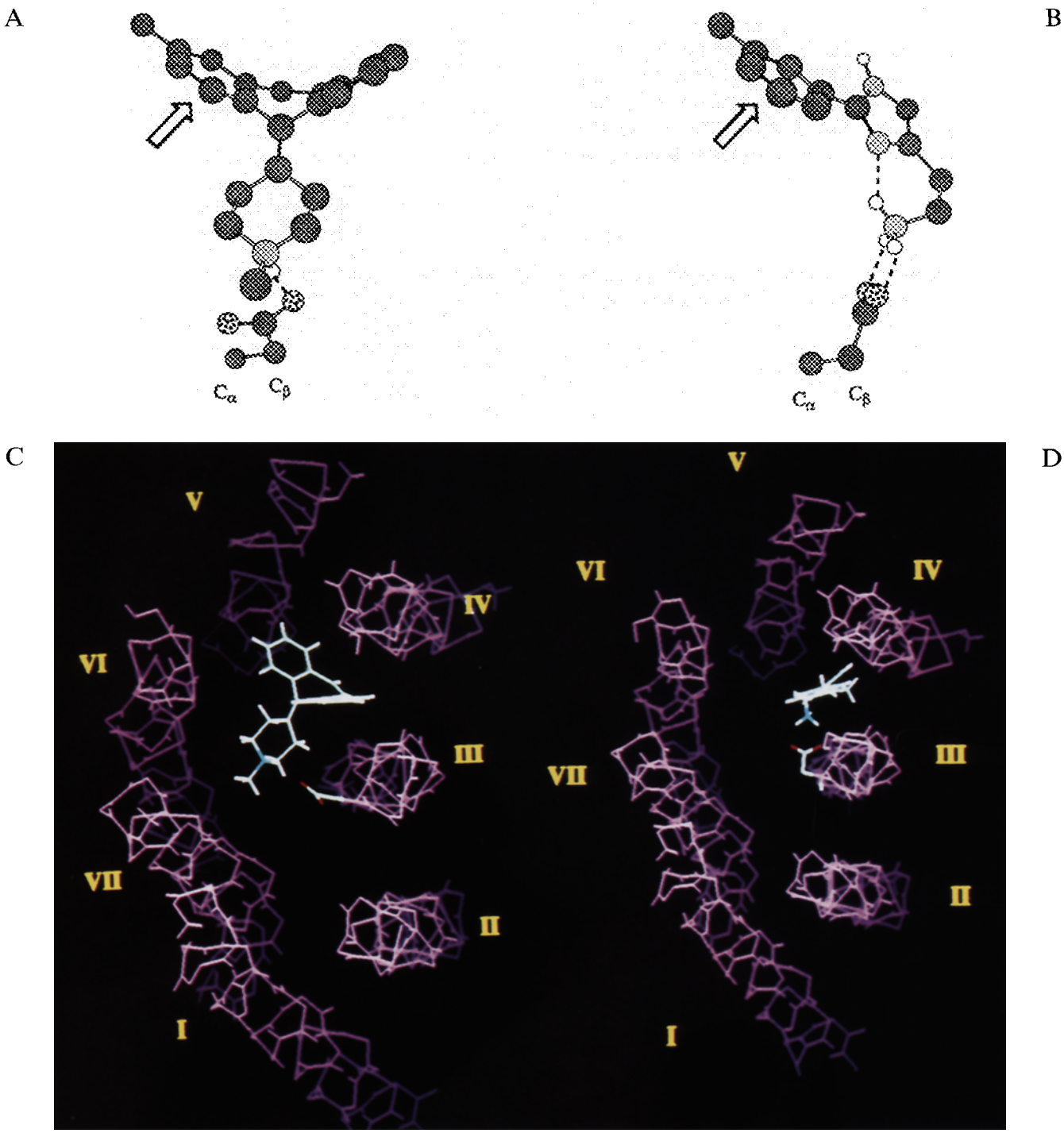

Fig. 7. (A) Speculative fit of a 2-phenylhistamine analogue (**11c**) onto the H_1-antagonist pharmacophore [19]. The 2-phenyl ring of **11c** is fitted onto the so-called 'cis' ring of the antagonist pharmacophore (indicated by an arrow). For the 'cis' ring, a small lipophilic para-substituent (i.e., CH_3 or Cl) is favourable for affinity; consequently, this group is matched with the favourable *meta*-Br substituent of **11c**. Optimization of **11c** with respect to the aspartate in structure 7A (fixed C^{α} and C^{β} atoms) results in the formation of an internal hydrogen bond in **11c** and a conformational change of the aspartate (B). Both the antagonist pharmacophore (cyproheptadine, A) and 2-PhHist (B) were docked into the H_1-receptor model based upon the alignment of Oliveira et al. [18] (C and D, respectively).

contains residues responsible for the observed selectivity, i.e., Lys^{200} and Asn^{207} (yielding the activated state of the GPCR). In this way, Asp^{116} is assigned a crucial function in triggering GPCR stimulation.

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