Chapter 6

Determinants of Activity in the Erythropoietin Receptor Studied with Molecular Dynamics Simulations
Chapter 6

1 Introduction

In 2005 the American cyclist Lance Armstrong wrote sports history by winning the Tour the France for the seventh time in a row. One month after the historical victory with which Armstrong ended his career as a cyclist, a French doping laboratory declared that urine samples from Armstrong collected before the Tour de France of 1999 tested positive for the presence of a doping agent, EPO. Armstrong denied the allegations.

1.1 Erythropoietin (EPO)

EPO or erythropoietin is best known for its misuse as a performance enhancing agent in endurance sports, such as cycling and long-distance running. It is used as a form of blood doping (for a review see e.g. [1]) and leads to an increase in the oxygen uptake capacity by stimulating the growth of red blood cells (erythropoiesis). It is produced by the human body, which has made it difficult to detect whether somebody has taken additional EPO to boost performance. The reinvestigation mentioned of the 1999 urine samples was performed to test a new and more sensitive assay for EPO.

The history of EPO dates back to the 19th century, when it was discovered that the thin atmosphere and low oxygen concentrations encountered in high mountains, induced a rise in the hematocrit, the amount of red blood cells in the blood. The biological importance of such a mechanism was obvious, but the process was not understood. In 1906 Carnot and Deflandre performed a series of experiments in which blood plasma taken from rabbits with induced anaemia was found to induce haematopoiesis (increase of blood volume) in normal rabbits. They postulated that the blood plasma contained a humorous factor, haematopoiétin, responsible for the effect. It took several decades before their results were verified and it was not until 1953 that the factor, then termed erythropoietin, was identified [2]. In 1977 Miyake et al. collected a small fraction of purified EPO by high performance liquid chromatography (HPLC) [3], which allowed the determination of the amino acid composition [4].

EPO is a 34 kDa glycoprotein, consisting of 166 amino acids with a total mass of 18 kDa and four oligosaccharide chains the composition of which is species dependent. The main source of EPO in the human body is the kidney [5, 6]. During fetal development most of the EPO originates from
the liver[7], but in adults the contribution of the liver to the total amount of EPO is ~10%[8]. It is worth noting that the expression of EPO has also been demonstrated in the brain, where it acts as a neuroprotectant factor rather than stimulating erythropoiesis. For a review on the neuroprotective effects of EPO, the reader is referred to reference [9].

Recombinant EPO has been used as a therapeutic agent since 1989 under the name Epogen (Amgen) to treat anaemia of renal origin and specifically for treatment of anaemia associated with chemotherapy in cancer. In addition, the finding that EPO has a neuroprotective effect and is released after brain ischemia has raised further interest in EPO, as a possible agent to reduce the damage following stroke. In this regard, it is worth noting that EPO has been found capable of crossing the blood-brain barrier[10]. EPO is presently the worlds leading biopharmaceutical with world wide sales revenues over 2004 of more than 10 G$.

1.2 The erythropoietin receptor (EPOR)

Erythropoietin acts by binding to the EPO receptor, resulting in the proliferation and differentiation of stem cells into red blood cells (erythrocytes). It is involved in two stages during the process of erythropoiesis. In the first of these stages, proliferation of burst-forming unit erythroid cells (BFU-e) and differentiation into colony-forming unit erythroid cells (CFU-e). The role of EPO in this stage is redundant and can be replaced by other stimuli[11, 12]. A non-redundant role is played by EPO in the final stage of the process, namely the differentiation of CFU-e cells into normoblasts, which subsequently mature into reticulocytes and finally form erythrocytes[13, 14].

The EPO receptor is a dimeric receptor, related to the growth hormone (GH) receptor and to the receptor for the granulocyte macrophage colony-stimulating factor (GM-CSF). Together with a number of other receptors with similar features they form an important cytokine receptor superfamily, which is reviewed in [15]. The EPOR is characterized by an extracellular domain, consisting of two distinct subdomains, with two highly conserved cysteine bridges (See Figure 6.1A). In addition, there is a typical WSxWS domain, which is also highly conserved across the superfamily. The intracellular domain is associated with a Janus kinase (JAK2). In addition, the receptor contains a number of phosphorylation sites on its cytoplasmic domain. The biologically active dimeric receptor has a characteristic T-shape.

In recent years, the crystal structure of the EPOR has been solved by two independent groups, revealing the asymmetric receptor structure in a 2:1 complex with EPO. In addition, a crystal structure has been solved of the EPOR in the absence of EPO, supporting evidence for a self-associated unbound receptor dimer (see Figure 6.1B).

1.3 EPOR activation

It was long thought that dimerization was the key event in the signalling of the EPO receptor, but it is now well established that the receptor resides on the cell surface as a preformed dimer. Stimulation by the ligand results in a repositioning of intracellular domains, causing transphosphorylation of the two associated JAK2 molecules. The activated JAK2 phosphorylates a number of tyrosine residues on the cytoplasmic domain of the receptor as well as tyrosine residues of various signalling molecules. The activated receptor in turn activates second messenger systems, such as shc, PI-3’ kinase, SHP1, SHP2 and Grb2, resulting in the activation of MAP kinase and STAT5, a signal transducer and activator of transcription. The latter migrates to the cell nucleus after activation, where it modulates the transcription of genes.
It is generally assumed that in the unliganded receptor dimer the two JAK2 molecules are oriented such that transphosphorylation can not occur[16]. Binding of EPO causes a reorientation of the JAK2 molecules, such that they can interact. Apparently, the reorientation of the extracellular domains by EPO is the event triggering the activity of the receptor. In an attempt to understand the linkage between the extracellular EPO binding domain and the intracellular domains, Seubert et al.[17] performed a study in which they replaced the extracellular domains with a coiled coil linker. By varying the number of residues in the spacer, they could control the relative rotational arrangement of the transmembrane and intracellular domains and investigate the activity associated with the different rotational relationships. Of the seven relative orientations of the transmembrane domains they investigated (Figure 6.2A), one was identified as the most active, and two alternative orientations were found to be partially active. The other four orientations were inactive.

Figure 6.1: Crystal structures of the erythropoietin receptor. A. EPOR in complex with EPO (shown in dark grey). EPOR consists of two distinct domains, D1 and D2. The receptor also contains a WSxWS motif, which is conserved throughout the haematopoietin receptor super family. B. The unliganded EPOR dimer, showing the scissor like arrangement. C. EPOR in complex with EMP1, D. EPOR in complex with EMP33.
Note that all seven orientations investigated in the study of Seubert et al. were symmetric. This is expected, considering the use of a symmetrical coiled coil linker, and the indistinguishability of the two intracellular domains. However, in the crystal structures of the EPO – EPOR complex, which is expected to correspond to the most active state of the receptor, there is a $120^\circ$ angle between the receptor subunits. Assuming that the linkers between the extracellular and the transmembrane domains in the two receptor subunits are indistinguishable, the relative orientation of the transmembrane domains should not depend on the receptor subunit chosen as reference. In this regard, the model of Seubert et al. is inconsistent with the available crystal structures. This is illustrated in Figure 6.2B. However, the inconsistency between the crystal structures and Seubert's model of the most active relative orientation of the transmembrane and juxtamembrane domains can be addressed if the symmetrical arrangement proposed by Seubert et al. is replaced by an asymmetrical one, which is invariant under a change in the domain used as reference. Two possible orientations, which are consistent with the crystal structures and are close to the most active relative orientation identified by Seubert et al., are shown in Figure 6.2B.

### 1.4 EPO mimetic peptides (EMPs)

With regards to its therapeutical use, EPO has the disadvantage that it can not be administered orally. Instead it needs to be injected, either subcutaneously or intravenously. For this reason, research groups have tried to develop small peptidic and non-peptidic molecules capable of modulating EPOR activity, with the aim of identifying orally active EPO mimetic compounds. This has led to the development of a series of EPO mimetic peptides (EMPs). These peptides consist of around 13 amino acids, show no resemblance with EPO and display activity for the EPO receptor both in vitro and in vivo[18]. Unfortunately, these peptides were not orally available, but they have contributed to understanding the functioning of the EPOR and design strategies for small peptide ligands to mimic the function of large protein ligands.

The crystal structures of the EPOR in conjunction with an agonistic EMP (EMP1) and in conjunction with an antagonistic EMP (EMP33) have been solved by Livnah and co-workers[18, 19]. From these structures, they concluded that activity is regulated by small differences in the relative orientation of the two extracellular domains. However, the difference between the structures is small and it is unlikely that this difference is enough to account for the difference between activity and inactivity. Regarding the work of Seubert et al., mentioned above, it is unlikely that the small difference between the crystal structures of the EMP1 and EMP33 bound EPOR can account for the required difference in the rotational relation between the transmembrane domains.

### 1.5 Aim of the Study

The aims of this study were to a. investigate the effect of the binding of EPO and a series of EPO mimetic peptides on the relative orientation of the two receptor subunits and b. investigate any correlation between the different relative orientations observed in the simulations and the experimentally known activity of the ligand.
Figure 6.2: Active and inactive relative orientations of the transmembrane and juxtamembrane domains of EPOR. A. Seven symmetric arrangements of the transmembrane / juxtamembrane domains as proposed by Seubert et al.[17] The relative activity of the model is indicated. This figure was modified from Seubert et al.[17] The models show the relative orientations expected of the transmembrane / juxtamembrane α-helices represented as helical wheels. Four key residues, which are essential for JAK2 activity, are shown in colour: Ser328 (red), Leu253 (yellow), Ile257 (green), and Trp258 (blue). For further details and explanation of the models, the reader is referred to the original article. B. Asymmetry in the EPO – EPOR complex. Two instances of the crystal structure of the EPO – EPOR complex (1CN4) were aligned using the two alternate receptor subunits (A and B). This shows that the relative orientation of A with respect to B is different from that of B with respect to A. C. Assuming that one of the relative orientations in the crystal structure (e.g. A with respect to B) corresponds to the most active relative orientation of the transmembrane / juxtamembrane domains suggested by Seubert et al.[17], the reverse relation (B with respect to A) yields a different relative arrangement. D. The inconsistency between the model proposed by Seubert et al.[17] and the crystal structure can be solved by choosing a slightly different, asymmetric, relative orientation of the transmembrane / juxtamembrane domains. This model is used as a reference in our study.
Results

A series of 13, 25 ns, simulations of the extracellular part of the erythropoietin (EPO) receptor (EPOR) as an unliganded dimer and in conjunction with each of a series of agonistic and antagonistic ligands have been performed. A summary of the simulations is listed in Table 1. Throughout this study, the assumption is made that the state of the receptor (active or inactive) is determined primarily by the configuration of the extracellular ligand binding domain (EBP), which is in turn determined by the binding of the ligand. In particular, the relative orientation of the D2 domains, which are attached to the transmembrane domains, is expected to be related to the activity of the intracellular domains. Here, we study the relative orientations of these domains in the simulations by looking at the distances and the rotational relationship, expressed as the Euler angles. Euler angles were calculated after reduction of the structures using the method described in Chapter 2.8.3. The reduction of the structures and determination of the Euler angles is shown in Figure 6.3.

2 Results

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2.1 The erythropoietin – erythropoietin receptor complex

The first step in this study was to characterize the configuration and dynamics of the EPO – receptor complex. As mentioned in the introduction, the configuration of the complex is assumed to correspond to the most active state of the receptor. There are two crystal structures available of the EPO – EPOR complex, solved by Syed et al. from two different crystal forms. These structures differ slightly. Although data will be presented from a simulation starting from one of these crystal structures (PDB ID 1EER), a control simulation starting from the other crystal form (1CN4) suggests

<table>
<thead>
<tr>
<th>System</th>
<th>Ligand</th>
<th>Ligand sequence (EMP)</th>
<th>IC50 (mM)</th>
<th>ED50 (mM)</th>
<th>Crystal Structure</th>
</tr>
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<tr>
<td>1</td>
<td>EPO</td>
<td></td>
<td>0.00018</td>
<td>0.00001</td>
<td>1EER</td>
</tr>
<tr>
<td>2</td>
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<td>3</td>
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</tr>
<tr>
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<td>GGTAS CHFGP LTWVC KPQGG</td>
<td>58</td>
<td>IA</td>
<td>1EBP</td>
</tr>
<tr>
<td>5</td>
<td>EMP7</td>
<td>GGTTS CHFGP LTWVC KPQGG</td>
<td>26</td>
<td>&gt;10</td>
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</tr>
<tr>
<td>6</td>
<td>EMP8</td>
<td>GGTFS CHFGP LTWVC KPQGG</td>
<td>1.5</td>
<td>&gt;10</td>
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</tr>
<tr>
<td>7</td>
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<td>0.1</td>
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</tr>
<tr>
<td>8</td>
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<td>GGTYS CHFGP LTWVC KPQ</td>
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<td>0.0115</td>
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</tr>
<tr>
<td>9</td>
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<td>TYS CHFGP LTWVC KPQGG</td>
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<td>0.3</td>
<td>1EBP</td>
</tr>
<tr>
<td>10</td>
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<td>&gt;10</td>
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<tr>
<td>11</td>
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</tr>
<tr>
<td>12</td>
<td>EMP33e</td>
<td>GGTXS CHFGP LTWVC KPQGG</td>
<td>15</td>
<td>IA</td>
<td>1EBA</td>
</tr>
</tbody>
</table>

a) Binding data (IC50) were taken from reference [38](EPO) and from reference [18](EPO mimetic peptides).
b) ED50 is the concentration of ligand which corresponds to 50% of the maximal EPO stimulation; IA is inactive. Data were taken from reference [38](EPO) and from reference [18](EPO mimetic peptides).
c) The starting structure for the simulation system was based on the crystal structure of the EMP1 – EPOR complex. The EMP1 structure was modified to correspond to the sequence given.
d) In EMP28 X = D-Tyr

e) In EMP33 X = 3,5-dibromotyrosine
that the results of these simulations converge and that the same region of conformational space is sampled after relaxation of the structures (data not shown).

The results from the simulation of the EPO – EPOR complex are shown in Figure 6.4 (page 145). Figure 6.4A shows an overlay of the two crystal structures with the final structure obtained from the simulation. The structures were aligned by performing a least squares fit on the backbone atoms of one of the D2 domains. In Figure 6.4B, C and D, scatterplots of the different combinations of Euler angles are shown, which characterize the relative orientations of the D2 domains.

It can be seen from Figure 6.4A that there are small differences between the two crystal forms, and between both crystal structures and the structure obtained from the simulation. Nonetheless, the effect on the Euler angles obtained is substantial. In particular the $\phi$ and $\psi$ angles obtained from the simulation are quite different from those obtained from the crystal structures. Inspection of the time evolution of the three Euler angles revealed that the change occurs in the first few nanoseconds of the simulation (data not shown).

As mentioned in the introduction, it is expected that the EPO induced configuration of the EPOR, and notably the resulting relative orientation of the D2 domains, corresponds to the most active state of the receptor. Under the assumption that the configuration obtained at the end of the simulation of the EPO – EPOR complex reflects the physiological state, the average Euler angles and shift obtained from the last 5 ns of simulation were used to refine the model proposed in Figure 6.2B. Recall that this model was based on the 120° angle between the receptor subunits observed in the crystal structures. In Figure 6.5 the new model is shown, which is used as the reference for the average relative orientations obtained from the other simulations.

![Figure 6.3: Structure reduction and definition of orientational relationships using Euler angles](image)

A. For each structure, the D2 domains were reduced by replacing them with the three eigenvectors, obtained from the 3×3 positional covariance matrix of the atoms. The eigenvectors of one of the domains, in this example the orange one, was aligned with the coordinate system. B. X-convention Euler angles ($zxz$-rotation). To determine the rotational relationship between the two D2 domains, one of the domains was aligned along the z-axis and the rotations around the z-axis ($\phi$), the x-axis ($\theta$) and the z-axis ($\psi$), required to obtain the orientation of the second domain were determined.
2.2 The unliganded erythropoietin receptor

The crystal structure of the unliganded EPOR reveals a symmetrical arrangement, characterized by a scissor-like configuration and a large distance between the C-termini of 4.4 nm[16]. Using this crystal structure as a starting point a single simulation was performed. Results obtained from this simulation are summarized in Figure 6.6. Although the structure overall did not change significantly during the simulation, there was some loss of symmetry. In Figure 6.6A an overlay of the crystal structure and the structure obtained at the end of the simulation is shown. These structures were aligned by performing a least squares fit on the backbone atoms of one of the D2 domains. Note that for the structure obtained from the simulation the two equivalent orientations are shown, obtained by fitting on each of the D2 domains. In Figure 6.6B and C the distributions of the different combinations of the Euler angles are shown. From these figures it is clear that there is little change in the relative orientation of the D2 domains. The difference between the orientation in the crystal structure and the average orientation from the last 5 ns of simulation is given by the differences in the Euler angles \((\Delta \phi \ \Delta \theta \ \Delta \psi) = (16^\circ \ -16^\circ \ 24^\circ)\). When relating the results obtained from the simulations, as well as the crystal structure to the model proposed in Figure 6.5, it is clear that both the crystal structure and the simulation reveal an orientation which is quite different from the configuration observed for the EPO – EPOR complex. This is shown in Figure 6.6D.

2.3 The erythropoietin receptor in complex with EPO mimetic peptides

Two crystal structures of the EPOR in complex with an EPO mimetic peptide have been made available by Livnah et al[18, 19]. These are the active EMP1 – EPOR complex[19] and the inactive EMP33 – EPOR complex[18]. Both of these crystal structures were used as the starting point for a simulation. In addition, the structure of the EMP1 – EPOR complex was used as a template to model a number of other EMP – receptor complexes (see Table 1)

In Figure 6.7 the results obtained from simulations of the EMP bound EPO receptors are shown as scatterplots of the \(\phi\) and \(\psi\) angles. In addition, this figure shows the average relative orientations obtained from the last 5 ns of the simulations according to the model presented above. From the plots of the \(\phi\) and \(\psi\) angles it can be seen that for most systems there is little deviation from the orientation in the starting structures. This is also reflected in the \(\theta\) angles, which show no significant changes for most of the EMP – EPOR complexes (data not shown). The region of the \(\phi\) and \(\psi\) angles sampled in these simulations is distinct from the region sampled in the simulation of the EPO – EPOR complex,
as well as from the region sampled in the simulation of the unliganded EPOR. Note that for most simulations the sampled regions lie above the band running from the EPO – EPOR complex to the unliganded EPOR. The only exception is EMP33, which changes its orientation significantly during the simulation. Interestingly, the average configuration obtained from the last 5 ns of simulation of this complex is closer to that obtained for the unliganded receptor dimer than to that obtained for the EPO bound receptor. For the other EPO mimetic peptides the average configurations obtained from the last 5 ns of simulation are different from each other, but do not reveal trends consistent with the differences in activity.

3 Discussion

To try to understand the mechanisms underlying activity and inactivity in the erythropoietin receptor, a series of simulations was performed of the EPO receptor as an unliganded dimer, in complex with its endogenous ligand (EPO) and in complex with a series of synthetic EPO mimetic peptides. The analysis focused on the relative orientations of the membrane bound (C-terminal) D2 domains. These domains are directly connected to the transmembrane helices. Their relative orientation in terms of the distance and rotational configuration is expected to be directly linked to the activity of the intracellular domains. The activity presumably depends on the relative orientation of the two intracellular JAK2 domains, associated with the receptor intracellular domain.

3.1 The erythropoietin – erythropoietin receptor complex

In the simulation of the EPO – EPOR complex, the system rapidly relaxes to a well defined configuration. This configuration was different from both of the available crystal structures of the EPO – EPOR complex, which originate from two different crystal forms. The data presented here was generated from the structure 1EER. However, a short control simulation started from the other crystal structure (1CN4) suggests that the same final configuration is obtained. Considering the irregular shape of the EPO – EPOR complex, it is possible that the structure in the crystal is influenced by crystal packing forces. The fact that two different crystal forms yield different structures supports this possibility. The configuration obtained in the simulation appears well defined and the two alternate crystal forms appear to converge.

As mentioned in the introduction, Seubert et al. investigated seven possible symmetric relative orientations of the transmembrane and cytosolic domains. They successfully identified one orientation which was more active than any of the other six, and two alternative orientations which were partially active (see Figure 6.1A)[17]. Unfortunately, in their study they did not consider the asymmetry imposed on the receptor by EPO. In fact, their model is inconsistent with the relative orientations found in our study, as well as with the relationships between the D2 domains observed in the crystal structures. To illustrate this, consider the two sets of Euler angles and shifts obtained from the last 5 ns of simulation of the EPO – EPOR complex. Denoting the two D2 domains A and B, the first set corresponds to the orientation of A relative to B, whereas the second set corresponds to the orientation of B relative to A. If one of these sets (say the A to B relationship) would correspond to the relative orientation proposed by Seubert et al., the alternative orientation (B to A) would lead to a different arrangement (see Figure 6.5). However, given that A and B are indistinguishable, both relative orientations are necessarily equivalent in terms of the established orientation of the transmembrane domains. Based on this, we tried to modify the model proposed by Seubert et al.
using the relationships obtained from the simulation. We found one orientation which was close to their model, but invariant under the choice of the reference (A or B). This model, and the consistency thereof with the data obtained from the simulation, is shown in Figure 6.5B.

3.2 The unliganded erythropoietin receptor

The unliganded receptor dimer underwent only minor rearrangements during the 25 ns simulation, but there was some loss of the symmetry and slight changes in the $\phi$ and $\psi$ angles. The primary differences between the EPO bound configuration and the unliganded receptor dimer are maintained during the simulation. Most notably, the orientation of the D2 domains as reflected in the $\phi$ and $\psi$ angles is different.

Previously, it has been suggested that the main factor for maintaining an inactive state in the unliganded dimer was the distance of 4.4 nm between the C-termini[16]. However, our simulations suggest another factor that could contribute. If the orientational relationships obtained from the simulation of the unliganded receptor dimer are applied to the model proposed in the previous section, this leads to an alternative relative orientation, which is shown in Figure 6.6D. The arrangement of the transmembrane and juxtamembrane domains expected from these results resembles the relative orientations proposed for the \textit{cc}-EpoR-I and \textit{cc}-EpoR-V models from Seubert \textit{et al.} (see Figure 6.1A), which were both found to be inactive.

3.3 The EMP1 – EPOR complex and the EMP33 – EPOR complex

The crystal structures of the EMP1 – EPOR and the EMP33 – EPOR complexes are only marginally different from each other. In particular, the relative orientation of the D2 domains is almost identical in both structures. The main difference between the structures is in the relative orientations of the D1 domains, but given that these domains are not directly connected to the transmembrane domains, it is the orientation of the D2 domains which is expected to be the main determinant of the activation of the intracellular domains. In this regard, the crystal structures are unlikely to properly reflect the active and inactive states of the receptor. In particular, the conclusions drawn from the two crystal structures, i.e. that the relative orientation of the D1 domains could account for the difference and that the difference between activity and inactivity may result from very subtle differences in the membrane bound domains[18], are questionable.

The simulation of the EMP1 – EPOR complex shows little change in the relative orientation of the membrane bound D2 domains, as reflected in the interdomain distance and the $\phi$, $\theta$ and $\psi$ angles, although there are changes in the overall conformation. However, in the simulation of the EMP33 – EPOR complex the relative orientation of the D2 domains changes significantly. In terms of the $\phi$ and $\psi$ angles, the simulation of this complex shows a shift towards the region sampled by the unliganded receptor dimer. Furthermore, if the relationships between the domains, obtained from these simulations, are applied to the model presented in Figure 6.5, the resulting relative orientations are clearly different (Figure 6.7). The differences observed in the simulations provide a possible explanation for the difference in activity of the EMP1 and the EMP33 bound EPOR.

Taken together, these simulations allow us to distinguish between the configuration of EPOR when bound by EMP1 and EMP33 and provide a possible explanation for the difference in activity of these ligands. The different ligands result in different orientations of the D2 domains once crystal packing forces are removed.
3.4 The erythropoietin receptor in complex with other EPO mimetic peptides

In addition to the simulations starting from the crystal structures, a series of simulations was performed of the receptor complexed with a number of other EPO mimetic peptides (see Table 1). These were modelled using the EMP1 – EPOR complex as a template. The activity of these EPO mimetic peptides, measured as the ED50, i.e. the effective dose corresponding to 50% of the maximal EPO response, ranges from 0.0115 µM (EMP16) to inactive (EMP6)[18]. The majority of these peptides fall in two broad classes: those with an ED50 of 0.1-0.3 µM (EMP11, EMP14, EMP17) and those with an ED50 higher than 10 µM (EMP7, EMP8, EMP19, EMP28).

No correlation was observed between the activity of these ligands and the relative orientations of the D2 domains in the simulations, as measured by the Euler angles of the D2 domains and the distances between the domains. It is possible that the use of the EMP1 – EPOR complex as a template biases the simulations, and that this bias could not be overcome during 25 ns of simulations. However, the stability of the structures during the last five nanoseconds in each of these simulations does suggest that a local equilibrium is reached. Clearly, to determine the influence of the starting structure, a series of simulations should be performed, using alternative structures such as the structure of the EMP33 – EPOR complex as a template.

Despite the lack of correlation between the activity of the EPO mimetic peptides and the results obtained from simulations, the simulations do provide a possible explanation of the inefficiency of the agonistic EPO mimetic peptides in comparison with EPO itself. Note that the most active EPO mimetic peptide included in this study, EMP16, has a thousand fold higher ED50 (0.0115 µM) than EPO itself (10 pM). This can be explained by a suboptimal orientation of the D2 domains, as reflected in the φ and ψ angles and the orientations shown in Figure 6.8.

Note that full thermodynamic equilibrium was not reached in these simulations. This would require sampling states with the ligand both bound and unbound and is beyond the reach of atomistic simulations at present. In this regard, it should be noted that the inactive ligand EMP6 was previously reported incapable of dimerization of the receptor. The inactivity of this ligand is probably better explained by the inability to bind the receptor than by the configuration established in the simulation. The lower affinity may also play a role in the decreased activity of the EPO mimetic peptides 7, 8, 19 and 28, with respect to the activity observed for EPO mimetic peptides 1, 11, 14, 16 and 17.

4 Conclusions

Molecular dynamics simulations were used to study the effect of the ligand on the configuration of the EPO receptor, notably on the relative orientations of the D2 domains. Under physiological conditions, these domains are connected to the transmembrane domains and their relative orientation is expected to be primarily involved in discriminating between the active and inactive states of the receptor. This study shows that the EPO bound receptor adopts a distinct configuration, which is different from the configurations adopted by the receptor in the absence of ligand or in the presence of any of the EPO mimetic peptides included in the simulations. Notably, the φ and ψ Euler angles (X-convention) could be used to discriminate between the different bound forms of the receptor. Interestingly, the φ and ψ angles obtained from the simulation also indicate substantial changes with respect to either of the crystal forms of the EPO – EPOR complex. The unliganded receptor dimer, on the other hand, is shown to adopt a configuration which is distinct from any of the other structures.
This study also provides a possible explanation for the inactive state of the EMP33 bound receptor as opposed to the EMP1 bound receptor, or the receptor in complex with any of the other (stronger or weaker) agonistic EPO mimetic peptides. In the simulation the configuration of the EMP33 bound receptor changes significantly and adopts an arrangement closer to the inactive unbound receptor dimer than any of the other systems included in the study. Next to this, the results obtained from the simulations can account for the orders of magnitude difference in the activity of EPO and the EPO mimetic peptides. Based on the simulations, we propose that this difference is due to the non-optimal relative orientation of the transmembrane domains, effected by the relative orientations of the D2 domains. Finally, except for EMP33, the simulations did not reveal apparent correlation between the activity of EPO mimetic peptides and the configuration of the receptor obtained.

5 Methodology

5.1 Simulations

5.1.1 General

Missing residues and atoms in each structure were modelled according to the configuration in the other structures where possible. Residues and side-chains not resolved in either of the structures were modelled using PyMol[20] according to the most likely geometry, in such a way that no overlaps between atoms were introduced. Simulations of the receptor – ligand complex were performed in an NDLP box[21], determined according to the method in Chapter 3, with a minimal distance between periodic images of 2.5 nm.

Simulations were performed with the Gromacs package for molecular simulations version 3.2.1[22-24] in conjunction with the GROMOS 43a2 united atom force field[25, 26]. Water molecules were modelled explicitly using the Simple Point Charge (SPC) model[27]. The protonation of ionizable groups was chosen appropriate for pH 7.0. Counterions were added to neutralize the net charge of the system and to reach a physiological salt concentration (0.15 M). Non bonded interactions were evaluated using a twin range cut off of 0.9 and 1.4 nm. Interactions within the shorter range cut off were evaluated at every step whereas interactions within the longer range cut off were evaluated every 10 steps. To correct for the neglect of electrostatic interactions beyond the longer range cut off, a Reaction Field (RF) correction[28] was used with $\varepsilon_{RF} = 78.0$. In all simulations the system was kept at a constant temperature of 300K by applying a Berendsen thermostat[29]. Protein and solvent molecules were independently coupled to the heat bath with a coupling time of 0.1 ps. Simulations were performed at constant volume. The time step used for the integration of the equations of motion was 0.002 ps. The bond lengths and angle of the water molecules were constrained using the SETTLE algorithm[30]. Bond lengths within the protein were constrained using the SHAKE algorithm[31]. Rotational and translation constraints were applied according to the method of Amadei et al.[32], to prevent periodic images from interacting directly. Starting velocities were randomly assigned from a Maxwellian distribution with different random seeds for each of the simulations. For all systems energy minimization in vacuum was performed to remove bad contacts within the protein. After solvation energy minimization was performed again to remove bad contact situation between the solvent and the protein. Prior to the production runs, short (10 ps) were performed with position restraints on all heavy atoms, followed by 10 ps equilibration of unrestrained molecular dynamics. The production runs of the unliganded receptor dimer and the receptor in conjunction with ligand had a length of 25 ns each.
Figure 6.4: Summary of simulation results from the EPO – EPOR complex. A. Overlay of the crystal structures 1EER (green) and 1CN4 (white) with the final structure obtained from the simulation (magenta). Structures were aligned by performing a least squares fit on one of the D2 domains. EPO was removed for clarity. B. Scatterplot of the Euler angles $\theta$ and $\phi$. Grey dots indicate the combined results of all simulations. Green and blue dots show the results of the first 20 ns of simulation and orange and red dots show the results of the last 5 ns of simulation. In green and orange the results obtained from fitting the D2 domain of the first chain are shown, whereas in blue and red the results obtained from fitting the other D2 domain on the reference domain are shown. The same colour scheme is used for the other scatterplots. C. Scatterplot of the Euler angles $\psi$ and $\phi$.

Figure 6.6: Summary of results obtained from the simulations of the unliganded EPOR. A. Stereoscopic image (cross-eyed) of an overlay of the crystal structure of the unliganded EPOR dimer 1ERN (white) and the final structure obtained from the simulation. Structures were aligned by performing a least squares fit on the D2 domain of one chain. The asymmetry in the EPOR observed in the simulation is demonstrated by fitting the structure obtained from the simulation using the D2 domain of either chain (green and magenta structures). B. Scatterplot of the Euler angles $\theta$ and $\phi$. Grey dots indicate the combined results of all simulations. Green and blue dots show the results of the first 20 ns of simulation and orange and red dots show the results of the last 5 ns of simulation. In green and orange the results obtained from fitting the D2 domain of the first chain are shown, whereas in blue and red the results obtained from fitting the other D2 domain on the reference domain are shown. The same colour scheme is used for the other scatterplots. C. Scatterplot of the Euler angles $\psi$ and $\phi$. D. Average relative orientations obtained from the last 5 ns of simulation according to the model proposed in Figure 6.5.
Figure 6.7: Summary and comparison of results obtained from the simulations of the EMP1 – EPOR complex and the EMP33 – EPOR complex. A. Overlay of the crystal structures of the EMP1 – EPOR complex 1EBP (white) and the final structure obtained from the simulation of the EMP1 – EPOR complex (green). The two orientations arise from the difference in the relative orientation of chain A with respect to chain B and the relative orientation of chain B with respect to chain A. Structures were aligned by performing a least squares fit on the D2 domain of one chain. For each structure D2 domains of either chain were used for fitting to illustrate the asymmetries. Structures are seen down the first principal axis of the reference D2 domain. The view in this image is equal to that used in panel D, to allow a comparison of the resulting structures. B. Overlay of the EMP33 – EPOR complex 1EBA (grey) and the final structure obtained from the simulation of the EMP33 – EPOR complex (purple). C. Scatterplot of the Euler angles θ and φ obtained from the simulation of the EMP33 – EPOR complex (colouring as in Figure 6.4B). D. Scatterplot of the Euler angles ψ and φ obtained from the simulation of the EMP1 – EPOR complex. E. Average relative orientations obtained from the last 5 ns of the simulation of the EMP1 – EPOR complex according to the model proposed in Figure 6.5. F. Scatterplot of the Euler angles θ and φ obtained from the simulation of the EMP33 – EPOR complex. G. Scatterplot of the Euler angles ψ and φ obtained from the simulation of the EMP33 – EPOR complex. H. Average relative orientations obtained from the last 5 ns of the simulation of the EMP33 – EPOR complex according to the model proposed in Figure 6.5.
5.1.2 The EPO – EPOR complex

For the simulation of the EPO – EPOR complex, one of the crystal structures solved by Syed et al. was used. This structure has Protein Data Bank accession code 1EER and contains a complex of two EPOR subunits asymmetrically bound to one EPO molecule. The NDLP box used for the simulation had a volume of 627 nm$^3$ and after solvation it contained approximately 59k atoms.

5.1.3 The unbound EPO receptor

The simulation of the unbound receptor dimer was started from the crystal structure with Protein Data Bank accession code 1ERN[16]. This structure contains a symmetric arrangement of two EPOR subunits in a typical scissor-like conformation. The simulation was performed in a hexagonal prism, constructed such that the receptor had full rotational freedom around the symmetry axis, which corresponds to the axis perpendicular to the membrane, with a minimal distance between any two periodic images of 2 nm. The C-termini were constrained to a plane to mimic attachment to a cell membrane. The unit cell had a volume of 967 nm$^3$ and, after solvation it contained approximately 93k atoms.

5.1.4 The EMP1 – EPOR complex

The 2:2 structure of the EPO receptor in complex with EPO mimetic peptide 1 (EMP1) was taken from the structure solved by Livnah et al. (PDB accession code 1EBP)[19]. The NDLP box used for the simulation had a volume of 485 nm$^3$ and after solvation it contained approximately 46k atoms.

5.1.5 The EMP33 – EPOR complex

The 2:2 structure of the EPO receptor in complex with EPO mimetic peptide 33 (EMP33) was taken from the structure solved by Livnah et al. (PDB accession code 1EBA)[18]. The NDLP box used for the simulation had a volume of 494 nm$^3$ and after solvation it contained approximately 46k atoms. EMP33 is characterized by the substitution of a tyrosine with a 3,5-dibromo-tyrosine. This modified residue is not present in the force field used. Partial charges on the atoms were obtained from the Dipole Preserving Charge (DPC) analysis due to Thole and van Duijnen[33]. Briefly, the quantum-chemical charge distribution obtained from a Hartree-Fock wave-function calculation of the molecule (3,5-dibromo-p-cresol) using GAMESS-UK, version 6.2[34], is represented by partial charges at the atomic sites in such a manner that (i) the overall dipole moment is reproduced and that (ii) a best representation of the local dipole moments is ensured. The method does not require calculation of the potential on a grid, as in the popular RESP fitting procedure[35], but DPCs are usually similar to RESP charges.
5.1.6 The EMPx – EPOR complexes

Starting structures for other EPO mimetic peptides in complex with the EPO receptor were modelled using the EMP1 – EPOR starting structure as a template. Modifications to the EMP1 structure were made using Pymol[20] and usually involved deletions of residues (EMP16, EMP17, EMP19 and EMP20) or of (parts of) side chains (EMP6, EMP8 and EMP11). In two cases a side chain entirely replaced (EMP7 and EMP14). Finally, EMP28 was formed by inverting chirality of the tyrosine residue, both in the structure and in the force field.

The simulation unit cell definition of the EMP1 – EPOR complex was used. After solvation the systems consisted of approximately 46k atoms.

5.2 Analysis

5.2.1 Structure Reduction

Prior to analysis, the reference structure, taken from the crystal structure 1EBP, was aligned such that the centre of geometry of the D2 domain of chain A coincided with the origin and the first principal axis of that domain was aligned with the z-axis. The reference structure was rotated such that the projection of the difference vector between the centres of geometry of the two D2 domains on the xy-plane coincided with the y-axis.

For each simulation all frames were aligned with the reference structure, by performing a least squares fit on the D2 domain. Both D2 domains were used for the fit, to account for both relative orientations in the asymmetric complexes, yielding two sets of aligned structures for each simulation. These aligned structures were processed using the method described in Chapter 2.8.3 to reduce the description of the domains to principal coordinates. The domains included in the investigation were the two D2 domains, defined by the residues Ile121-Thr220. The structure used to determine the principal axes in the first stage consisted of two D2 domains positioned at the origin, similar to the D2 domain of chain A of the reference structure. This was done to ensure that the principal axes of both D2 domains had equal definitions.

5.2.2 Calculation of Euler angles

Euler angles were calculated according to the so-called X convention. This means that the first rotation is over an angle $\phi$ around the z-axis, the second rotation is over an angle $\theta$ around the x-axis and the third rotation over an angle $\psi$ around the z-axis again. The angles were calculated from the rotation matrix, relating the D2 domain placed at the origin with the second D2 domain. The rotation matrix $R$ is given by

$$ R = BA^{-1} $$  \hspace{1cm} (6.1)

where $B$ is the matrix formed by the principal vectors of the second D2 domain and $A$ is the matrix formed by the principal vectors of the first D2 domain.

The calculation of the Euler angles was performed in R, a language and environment for statistical computing (http://www.r-project.org/)[36]. Calculation of average Euler angles was done using the orientlib package[37] for R.
Figure 6.8: Orientational relationships in the EPO receptor induced by EPO mimetic ligands. A. $\psi$ and $\phi$ angles (1) and average relative orientations according to the model in Figure 4 from the last 5 ns of simulation (2) obtained from EMP14 bound EPOR. B. $\psi$ and $\phi$ angles (1) and average relative orientations according to the model in Figure 4 from the last 5 ns of simulation (2) obtained from EMP16 bound EPOR. C. $\psi$ and $\phi$ angles (1) and average relative orientations according to the model in Figure 4 from the last 5 ns of simulation (2) obtained from EMP17 bound EPOR. D. $\psi$ and $\phi$ angles (1) and average relative orientations according to the model in Figure 4 from the last 5 ns of simulation (2) obtained from EMP6 bound EPOR. E. $\psi$ and $\phi$ angles (1) and average relative orientations according to the model in Figure 4 from the last 5 ns of simulation (2) obtained from EMP7 bound EPOR. F. $\psi$ and $\phi$ angles (1) and average relative orientations according to the model in Figure 4 from the last 5 ns of simulation (2) obtained from EMP19 bound EPOR. G. $\psi$ and $\phi$ angles (1) and average relative orientations according to the model in Figure 4 from the last 5 ns of simulation (2) obtained from EMP8 bound EPOR. H. $\psi$ and $\phi$ angles (1) and average relative orientations according to the model in Figure 4 from the last 5 ns of simulation (2) obtained from EMP28 bound EPOR.
6 References


