Molecular modeling of the oxidized form of nuclear factor-κB suggests a mechanism for redox regulation of DNA binding and transcriptional activation

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Abstract:

NF-κB is an important transcriptional regulator of numerous cellular genes, as well as viruses such as HIV-1. Oxidative stimuli in the cytosol are associated with nuclear translocation of NF-κB, whereas in the nucleus, reductive activation by thioredoxin is required for NF-κB to bind to DNA and activate target genes. Experimental structures of the reduced form of NF-κB bound to its DNA targets are available, from which we have modeled the oxidized form of NF-κB homodimer by removal of bound DNA, and modification via a hinge movement of a linker between the dimerization and DNA-binding domains of each subunit. These torsional motions enabled the formation of an inter-subunit disulfide bond between the Cys62 residues of each monomer; the resulting structure was refined using molecular dynamics simulation. The final model of oxidized, disulfide-bridged NF-κB is more compact than the open, reduced form. This may facilitate its nuclear translocation through small pores in the nuclear envelope, in response to oxidative stimuli in the cytosol. Furthermore, the inter-subunit disulfide blocks DNA from entering the active site of the oxidized dimer, explaining why subsequent reduction to the thiol form in the nucleus is essential for DNA binding and transcriptional activation to occur.

Keywords: DNA-binding protein | Gene expression | Nuclear factor-κB (NF-κB) | Redox regulation | Thioredoxin | Transcription factor

Article:

1. Introduction

Nuclear factor-κB (NF-κB) is a latent, primary cellular transcription factor that regulates the expression of a wide variety of cellular genes, particularly those involved in host defense [1], [2], [3]. It plays a role not only as an evolutionarily conserved coordinating element in an organism's response to situations of infection, stress and injury, but also as an activator of a number of viral genes including those of HIV-1 [4]. While NF-κB acts as the central mediator of immune response in many cells, multiple families of viruses promote their replication, prevent

virus-induced apoptosis and mediate the immune response to the invading pathogen by activating NF-κB [5], [6]. Thus the target genes under the regulation of NF-κB include a variety of cellular as well as viral genes.

NF-κB is composed of homo- or heterodimers of different subunits, which are members of a family of structurally related proteins (Rel/NF-κB proteins). Five different Rel proteins have been identified so far: p50, p52, p65, RelB, and c-Rel. Members of the Rel/NF-κB protein family are characterized by the presence of a Rel Homology Domain (RHD), which contains a nuclear localization sequence and is involved in sequence-specific DNA binding, dimerization and interaction with inhibitory I-κB proteins [7], [8]. NF-κB predominantly exists as a heterodimer of p50/p65 subunits in the cytoplasm, although p50 homodimers are also commonly observed. Each subunit of the dimer consists of two major domains. The DNA-binding domain, consisting of 180 amino acids at the amino-terminal end of the molecule, is responsible for base-specific DNA contacts, whereas the carboxyl-terminal domain of approximately 100 residues mediates dimerization, I-κB binding, and nuclear targeting via a nuclear localization sequence (NLS). The NLS is a cluster of basic amino acid residues, responsible for targeting the molecule into the nucleus [9], [10]. A flexible short linker of about 10 amino acids connects the DNA-binding and dimerization domains.

In unstimulated cells, NF-κB dimers are retained in the cytosol in an inactive form as a consequence of their association with members of another family of proteins called I-κB (inhibitors of κB) [11], [12], [13]. In response to a variety of stimuli including physical and chemical stresses, cytokines, reactive oxygen intermediates (ROI) [14] and ultraviolet light, the latent cytoplasmic NF-κB/I-κB complex is activated by the multisubunit I-κB kinase (IKK) complex [15], [16]. The IKK complex causes proteolytic degradation [17] of I-κB through sequence-specific phosphorylation and ubiquitination of the I-κB molecule [18], [19], [20].

A key regulator of NF-κB is the cellular reducing catalyst, thioredoxin (Trx). Trx is a small endogenous molecule having a characteristic CXXC active site amino acid sequence, Cys-Gly-Pro-Cys, in which the two cysteine residues form a redox center with the ability to reduce disulfide bonds [21]. Trx plays dual and opposing roles in the regulation of NF-κB. Via its antioxidant effects, Trx can suppress NF-κB activation in the cytoplasm, by interfering with the signal to I-κB kinases and thereby blocking the degradation of I-κB. In contrast, in the nucleus, reduction by Trx enhances NF-κB-dependent transcription, by enabling its ability to bind to DNA [22], [23]. Thus, transcriptional activation via NF-κB can be considered as two distinct steps with respect to the cellular compartment, with Trx playing distinct and opposed roles in the cytoplasm and in the cell nucleus. Activation and regulation of the NF-κB complex is thus a redox-sensitive process dependent on the activity of reducing proteins like Trx [24], [25].

In the cytoplasm, ROI mediate the degradation and release of I-κB from NF-κB, and in those oxidizing conditions, the reactive SH groups in NF-κB would be oxidized. The p50 subunit of NF-κB has seven cysteine residues at positions 62, 88, 119, 124, 162, 262 and 273. Among these, the DNA-binding loop of NF-κB contains a characteristic sequence motif with a cysteine (Cys62) and three arginine residues. These cysteine residues in each of the two subunits are susceptible to oxidation. In order for NF-κB to bind to DNA, it is necessary for these cysteine residues to be in the reduced state [26]. Studies have shown that the DNA-binding activity of p50

correlates well with the redox states of Cys62, and that Trx regulates the DNA-binding activity of NF-κB by reduction of a disulfide bond involving Cys 62 [27]. The role of Cys62 of NF-κB as a specific target of Trx is also supported by an NMR structure of a small NF-κB peptide spanning this cysteine, covalently bound to the active site of Trx via a disulfide bond [28]. Thus, the oxidized form of Cys62 in NF-κB that is relevant for the mechanism of redox regulation of DNA binding is a disulfide, as opposed to other possible oxidation products of cysteine—and *the most viable candidate for Cys62 to pair with is the corresponding cysteine residue on the other subunit of NF-κB*.

Despite extensive information available regarding the structure of NF-κB and the regulation of its signaling, there is still limited understanding of the in vivo dynamics of this pathway. Although it is has been established that NF-κB must be reduced by Trx in the nucleus in order to bind to DNA [28], the precise structure of the oxidized form of NF-κB when it enters the nucleus is still not known. Thus, in order to understand how Trx recognizes and binds to NF-κB in the nucleus, reduces the disulfide bond and activates it for DNA binding, a structural model of the oxidized form of NF-κB would be highly desirable. The crystal structures available so far are of the reduced form of homodimeric and heterodimeric NF-κB complexes bound to their DNA targets [29], [30]. These crystal structures present a static picture of the transcription factor bound to DNA, whereas the structure of the oxidized form would provide an intermediate structure in this pathway and provide insights into the molecular mechanisms involved prior to transcriptional activation. Similarly, studies have suggested the importance of ROI in stimulating degradation of the NF-κB/I-κB complex [14], [31], but the ways in which ROI and redox-active cysteine residues may contribute to the nuclear translocation of NF-κB are not fully understood. Since NF-κB dimers assume different conformations when bound to I-κB versus the free or DNA-bound structures, knowledge of the various intermediate structures is essential for understanding the dynamic nature of these molecules and the mechanistic details of the NF-κB pathway. To that end, in this study, computational modeling and molecular dynamics simulation techniques were applied to the existing experimental structural information to develop a preliminary structural model of the oxidized form of NF-κB.

2. Methods

2.1. Model building

The crystal structure of the transcription factor NF-κB p50 homodimer bound to a kappa B sequence of DNA (PDB entry: 1NFK) [30] was used as the starting structure for building the oxidized model. After deleting the bound DNA helix from the dimer, the structure was examined to identify the peptide region that confers flexibility to the dimer and causes the movement of the two subunits. Analysis of the protein structure showed that the peptide linker connecting the amino and carboxyl-terminal domains of NF-κB could be involved in a hinge movement that produces a change in the orientation of the DNA-binding domains of the two subunits, bringing the two Cys62 residues within disulfide bonding distance (Fig. 1).

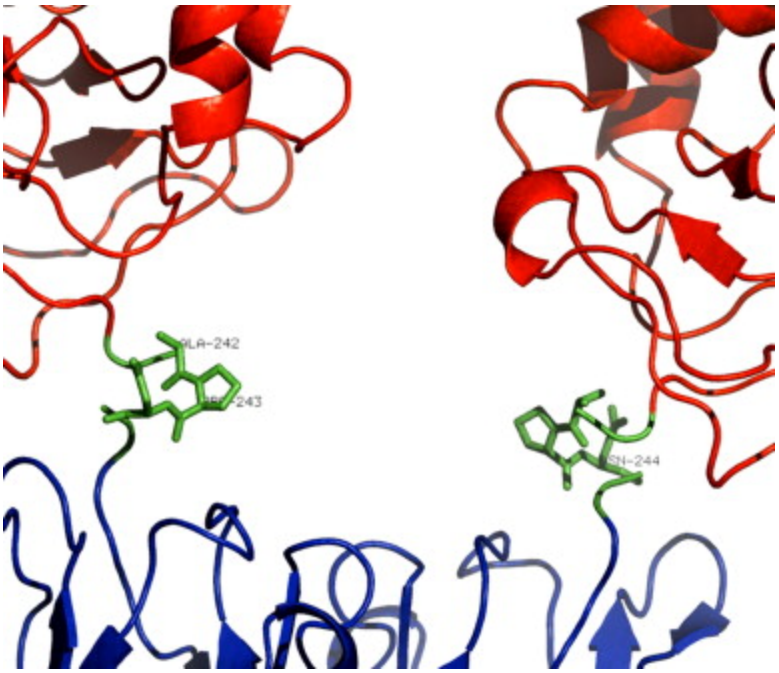

Fig. 1. Three-dimensional structure of NF-κB p50 homodimer (PDB code: 1NFK) with the DNA removed. The DNA-binding domain and the dimerization domain are shown in red and blue, respectively; the residues (Ala242-Asn244) in the linker region that contribute to the hinge movement are shown in green.

Subsequently, in an effort to model the oxidized, disulfide-bridged structure of NF-κB, the backbone torsion angles of the linker region were modified to achieve the desired conformation. Any reasonable mechanism of conformational change would involve low energy expenditure, and would therefore occur through minimum torsional angle modifications to the backbone. Keeping this in mind, systematic search of torsional angle space in the linker region pointed to residues Ala242-Asn244 (1NFK numbering) that produced significant conformational change to the protein structure upon small torsional modifications. Since the linker region was relatively unconstrained by packing and was not part of an α-helix or a β-sheet, the hinge movement did not impose any restrictions on hydrogen bonding. The only structural constraint was that the torsion angles remain in the allowed regions of the Ramachandran plot. Two torsions, the ψ and ω angles around Pro243, were modified by 30° and 12°, respectively to produce a conformation with minimum expenditure of energy and having the least steric hindrance. This localized torsion angle modification induced a large movement about the hinge thereby bringing the Cys59 residues (1NFK numbering) of the two subunits closer to each other, for disulfide bonding. The rest of the protein however rotated as a rigid body, with the axis of rotation passing through the hinge region (Fig. 2, Fig. 3).

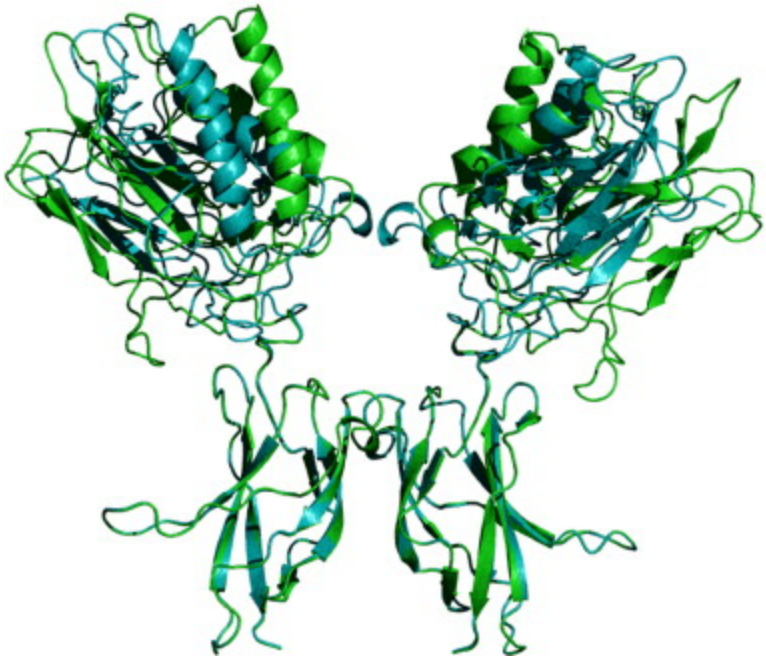

Fig. 2. Superposition of the NF-κB dimers before (green) and after (cyan) hinge movement. Hinge movement through torsion angle modification brings the DNA-binding domains closer, while the dimerization domain is unaffected.

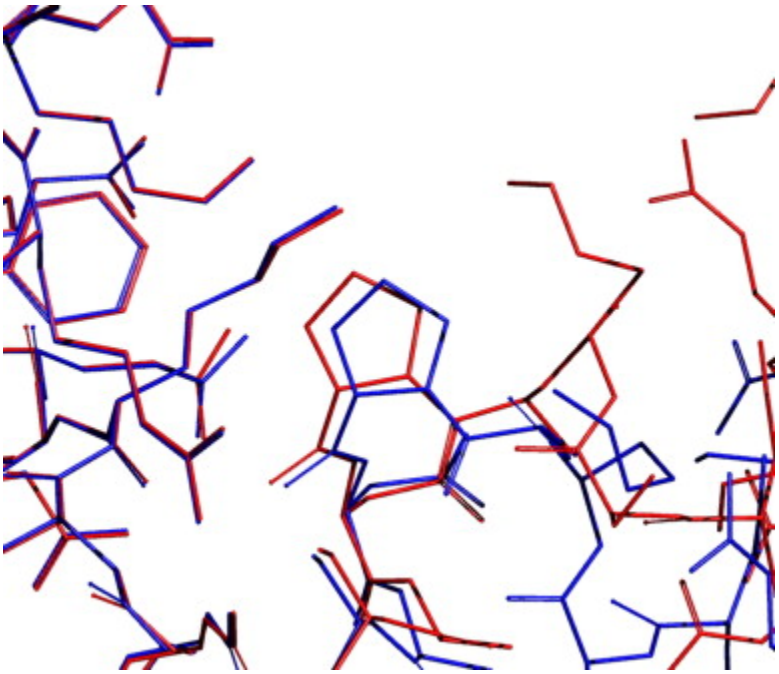

Fig. 3. Close-up view of the hinge region residues before (blue) and after (red) torsional modification. The ψ and ω angles around Pro243 were modified by 30° and 12°, respectively.

2.2. Model refinement

Molecular dynamics (MD) simulation was carried out after hinge movement, in order to remove unfavorable steric interactions and refine the positions of side chains in the modeled oxidized

structure. To pre-condition the modeled structure, energy minimization using the Kollman all-atom force field [32] as implemented in SYBYL 7.0 (Tripos Inc., Louis, MO) was performed for 2000 steps of steepest descent and 3000 steps of conjugate gradient minimization. A distance-dependent dielectric function was used with the dielectric constant set to *r* (the distance in angstroms between atom pairs) and the non-bonded cutoff was set to 8 Å. The resulting structure was used as the starting structure for the MD simulation, performed using AMBER 7.0 molecular simulation package [33]. A complete MD study of the NF-κB dimer requires simulation performed when the protein is adequately solvated. However, due to the large size of the system after adding water molecules, it was decided to simplify the model of the protein so that computing resources could be conserved. Also, the sampling of configurational space for the whole protein is too slow to allow sufficient sampling in the available time. But when structural restraints are used to restrict atomic freedom, the required sampling is less extensive. Therefore the simulation was carried out in vacuo with a simple distant-dependent dielectric solvation model, using the BELLY option, in which only the atoms of residues within 30 Å of the disulfide bridge were allowed freedom of motion during the course of the MD trajectory, while the residues outside that radius were restrained. At physiological pH, the starting structure was positively charged; thus, in order to make the simulation system electrically neutral, counter ions (Cl^-) were added using the LEAP module. As a preparatory step for the simulation a 1000-step minimization was run using a combination of steepest descent and conjugate gradient method to relax the chloride ions and to remove the strong initial forces due to collisions generated by the ions insertion process. A 3 ns simulation was performed under constant pressure conditions using the parm99 force field to describe the interactions between the protein atoms [34]. As in the minimization, the non-bonded cutoff for electrostatic and van der Waals was set to 8 Å and a distance-dependent dielectric function with the dielectric constant set to *r* was used. The simulation was carried out using a 2 fs time step for a total simulation time of 3 ns. The energy profile was then analyzed to compare the structures of the reduced and oxidized models.

3. Results and discussion

3.1. Effect of torsion angle modifications

The large conformational change occurring in NF-κB upon torsional modification can be described as a rigid body movement of one domain in relation to the other with very little change to the internal conformation of either domain. After superposition of the backbone atoms of dimerization domains of the reduced and oxidized structures, the RMS deviation was only 0.16 Å in the dimerization domain (reflecting some minor changes during energy minimization), whereas it was 5.86 Å for the DNA-binding domain, resulting from a change in the relative orientation of this domain relative to the (fixed) dimerization domain. Thus, this number (5.86 Å) represents the mean atomic displacement for atoms in the DNA-binding domain after the hinge motion. The large relative movement of the two subunits is a consequence of small rotations of two backbone torsion angles in the connecting strand. These relatively small changes to the backbone torsions yielded a significant change in the relative orientation of each of the subunits, causing an en bloc movement of entire domains. At the same time, these torsional changes kept the secondary structural features intact. The distance between sulfur atoms of the Cys62 residues in the two subunits was reduced from 19.9 Å (SG–SG) in the starting crystal structure to 3.8 Å after the torsional modifications alone, prior to minimization and dynamics,

after which the S–S distance was reduced to 2.3 Å, a typical S–S bonding distance. The transcription factor undergoes a dynamic change between the open and closed conformation due to the flexible linker region between the DNA-binding and the dimerization domains. This kind of behavior does not appear to be specific for NF-κB alone: a similar opening–closing movement of domains has been observed in many binding proteins and the peptide region connecting the domains is primarily responsible for the conformational change [35]. It could be argued that the domain movements would involve a more complex mechanism than simple backbone torsional modification to produce such a large change in conformation. However, this hinge movement is consistent with domain movements observed in several other proteins, indicating that the flexibility of protein structures is important for their catalytic activity [36], [37].

In fact earlier crystallographic studies have shown that in NF-κB, the p65 subunit is remarkably flexible and adopts a different conformation upon binding to I-κBα. Compared to its DNA-bound open conformation, the I-κBα-bound p65 amino-terminal domain rotates almost 180° and translates 38 Å towards its carboxyl-terminal domain [12]. This movement is also entirely due to the flexible peptide linker connecting the DNA-binding and dimerization domains. These large relative domain movements, as observed in many protein structures, provide examples of protein flexibility.

It is also notable that a new dimer interface is formed in the region of contact around the new disulfide bridge. This is actually a fairly limited area of contact, local to the two cysteine residues (as seen in Fig. 2, Fig. 6b). The NF-κB dimer is more than adequately held together by its dimerization domains, and this new connection between the DNA-binding domains in the oxidized form probably serves primarily to block DNA entry, serving as the “off” switch for NF-κB, and therefore must be easily reversible on reduction by Trx. Thus, an excessively strong contact here could be counterproductive. The extent of the new contact surface was calculated (from the decrease in total solvent-exposed surface) as being at most 100 $Å^2$ per monomer, which is not a large contact area. The details and energy contributions to this contact are discussed further in the following section.

3.2. Refinement using molecular dynamics simulation

A number of bad steric contacts are typically introduced during torsion angle modification, so to eliminate these bad contacts and establish an accurate structural model, a MD simulation was carried out. In our simulation, the primary focus was on the movement of the DNA-binding domain; since NF-κB is a large protein with 624 amino acids, the objective was to simulate a truncated portion of the molecule, which would allow for more efficient use of computing time. Therefore, we used a model in which only the atoms within 30 Å distance from the disulfide bridge were included in the simulation. Bad steric contacts were removed and side chain rotamer distributions were improved after a 3 ns simulation. The Ramachandran plot of the backbone angles for the oxidized model was computed with PROCHECK to locate any abnormal peptide linkages or other unlikely bond angles [38]. Examination of the plot (Fig. 4) shows that the residues fall in the allowed regions of phi–psi space and the model is structurally refined after the MD simulation.

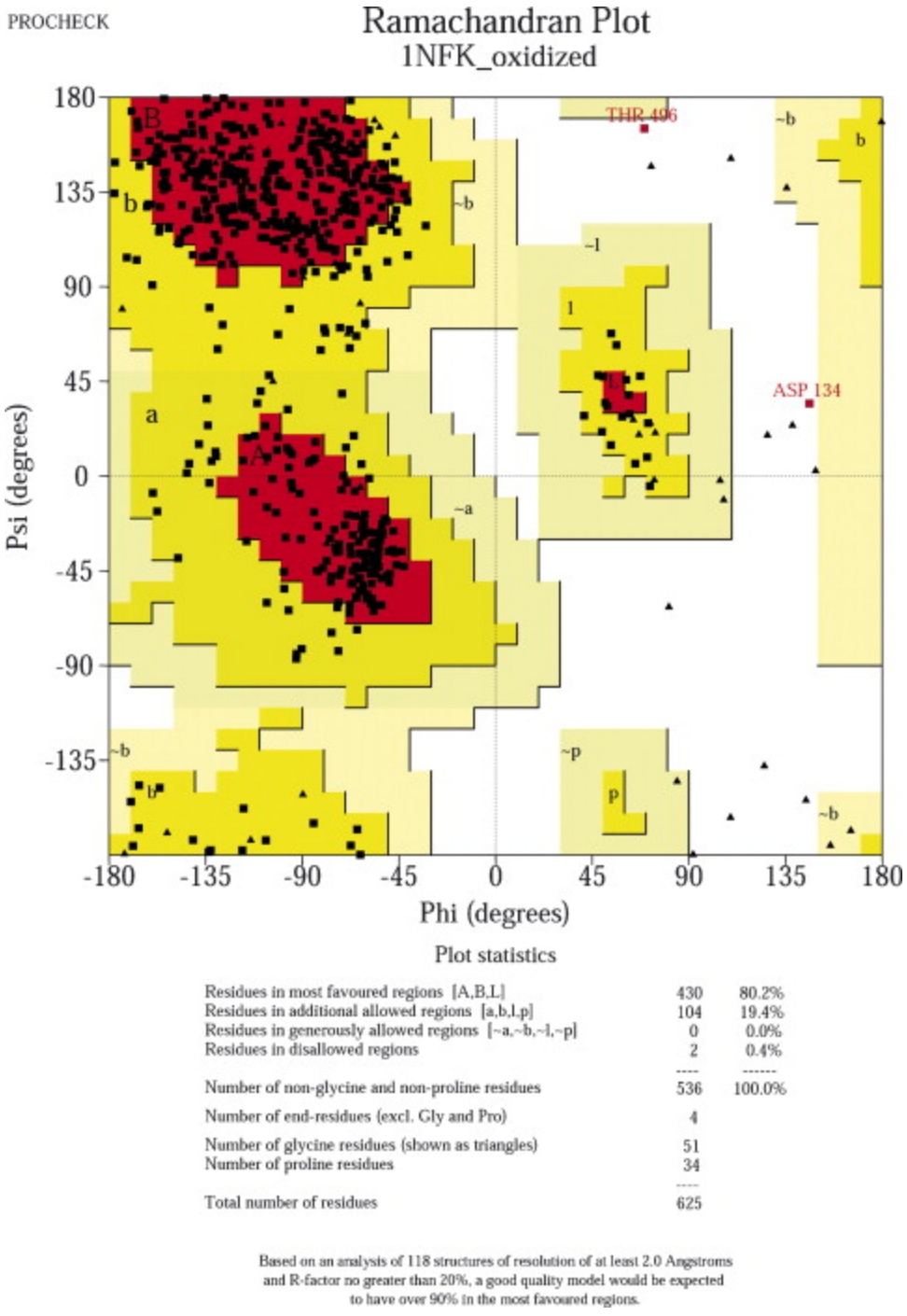

Fig. 4. Ramachandran plot for the oxidized form of NF-κB.

After the structural refinements, energies were calculated to see if there is a substantial energy penalty for forming the disulfide-bonded oxidized state of NF-κB. On the contrary, it was observed that the resulting oxidized structure was actually more energetically favorable than the reduced structure, by around 200 kcal/mol (although this is only a qualitative result considering the lack of explicit solvation in the calculations). This result nonetheless supports the hypothesis that the oxidized form is at least as energetically favorable as the reduced form, suggesting that oxidation does not impose any energy penalty as a prerequisite for nuclear translocation of NF-

κB. We analyzed the energy contributions to the relative stabilization of the oxidized form, and 87% of the difference is contributed by electrostatic terms. However, the new contact area is small (~100 $Å^2$ per monomer) and there are no explicit salt bridges formed, so, in addition to the formation of the covalent S–S bond, we conclude that this interaction is driven by overall electrostatic complementarity operating at medium distances.

3.3. Size of the molecule and ability to bind to DNA

NF-κB molecules act as substrates for the nuclear transport receptors, importin α3 and α4, and are delivered to the nucleus, where they stimulate transcription of genes. Following I-κB degradation, the peptide region containing the NLS is exposed in NF-κB and the nuclear import mechanism kicks in. NF-κB is translocated into the nucleus through the nuclear pores by association with the importin complex [39]. Studies on the influence of cargo size for molecules that are transported into the nucleus through this mechanism have shown that the rate of transportation is inversely proportional to the molecular size of the cargo. Small cargo sizes result in easier transportation, with increased rates as compared to larger cargo sizes. Analysis of the size of the modeled oxidized NF-κB structure shows that it is significantly more compact than the reduced form, which could facilitate its entry through nuclear pores (Fig. 5). Using the program Deepview [40], the size of the smallest box that can enclose the entire molecule or the molecular volume was calculated for both the structures and it was found that the smallest box capable of containing the oxidized structure had a volume that was 5032 $Å^3$ smaller than that required to contain the reduced form. This appreciable reduction in volume is expected to improve its ability to pass through the nuclear pore. We hypothesize that oxidation in the cytosol facilitates nuclear translocation of NF-κB, due to the more compact shape of the oxidized form. However, it is difficult from this study to determine unequivocally the extent to which the size reduction may help in the nuclear import mechanism.

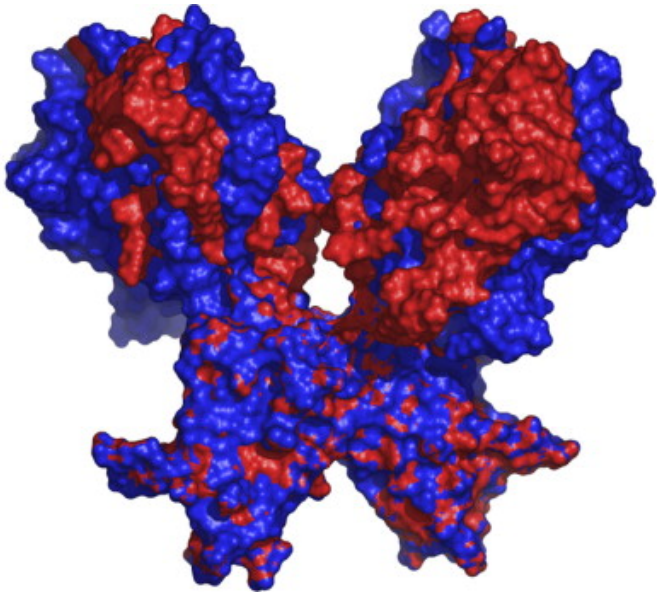

Fig. 5. Space-filling models of the oxidized (red) and reduced (blue) forms of NF-κB. The oxidized form is significantly more compact than the reduced form, which may facilitate its nuclear entry.

The observed decrease in volume from the surface and core of the protein shows that the volume of the DNA-binding cleft is also reduced in the oxidized form, making it impossible for DNA to enter and bind to NF-κB, as a result of steric hindrance. It is exactly what is expected from the convergence of two subunits, resulting in a decrease in the volume of the binding pocket, making it difficult for DNA to interact with the residues in the pocket and in fact making it impossible for DNA to enter the binding cleft when the inter-subunit disulfide bridge is present. This demonstrates the need for Trx to reduce the disulfide bond in order to enable DNA binding and transcriptional activation. The oxidized structure thus highlights the intricate and multi-faceted relationship between redox status and NF-κB activation, highlighting the importance of observations showing that NF-κB responds to different stimuli in the cytoplasm and in the cell nucleus.

4. Conclusions

Although it has been established that Trx regulates the DNA-binding activity of NF-κB through reduction of a disulfide bond involving Cys62 residues [26], no detailed structure of NF-κB in its oxidized form has been presented until now. Despite the relatively large number of crystal structures available for the bound conformations of NF-κB with the DNA or I-κB, there have been no structures available for the unbound conformation. In this study we have derived a model using computational modeling and simulation methods that describes the structure of NF-κB in its oxidized state, prior to reductive activation by Trx (Fig. 2, Fig. 5, Fig. 6). Our model is consistent with previous reports showing that these critical cysteine residues need to be reduced by Trx in order for NF-κB to bind to DNA.

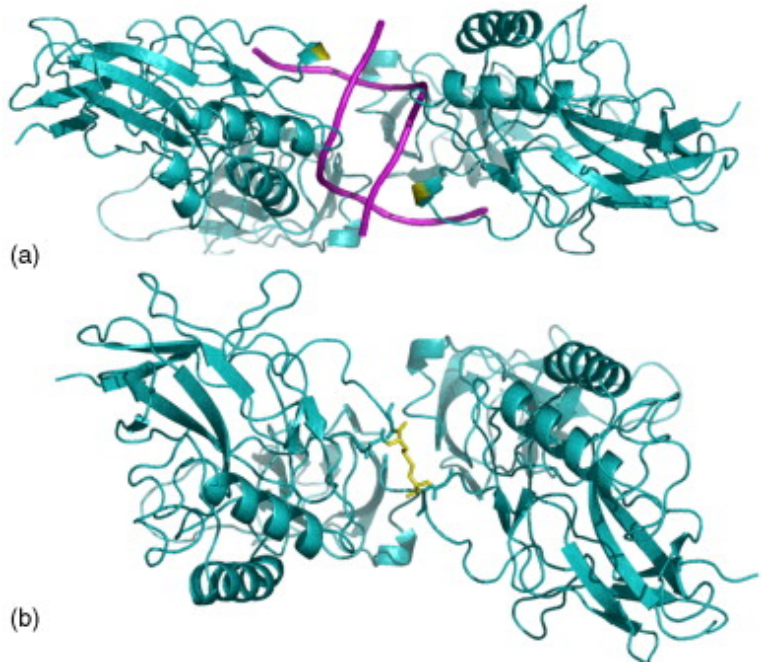

Fig. 6. Comparison of the DNA-binding cleft of the reduced and oxidized forms (a) reduced form of NF-κB (PDB code 1NFK) with the bound DNA. The Cys62 (Cys59 in PDB file) residues in the two subunits are shown in yellow. (b) Modeled structure of the oxidized form of NF-κB showing the inter-subunit disulfide bond (in yellow).

This model of the oxidized form of NF-κB indicates that the linker region that connects the dimerization and DNA-binding domains provides flexibility to the protein structure necessary for the relatively large movements upon oxidation and reduction. This connecting strand can readily act as a hinge between the two domains, providing conformational flexibility to the molecule.

Also, it is known that disulfide bridges often play functional roles in proteins and studies have shown that the enzyme Trx acts as a regulatory switch of target proteins by reducing their disulfide bonds. The structural data from this study suggests that the disulfide bond formation that results in the closed conformation of the dimer is the form that must be targeted and recognized by Trx in order to be reduced so that it can bind to its cognate DNA target. After the Cys62 residues that are involved in disulfide bonding are reduced, the DNA-binding regions of the two subunits which were in contact with each other must move farther apart, opening the binding cleft. The DNA can then enter the cleft formed upon reduction of NF-κB, where it can be recognized and initiate the process of transcription. An accurate picture of the binding mode of Trx on oxidized NF-κB, and the dynamic trajectory of the reduction itself, are important goals for the next phase of modeling studies that are being undertaken in our laboratory.

In conclusion, the model for the oxidized form of NF-κB proposed here provides structural explanations for questions that were not answered by previous experimental studies that showed NF-κB bound to either DNA or I-κB molecules. This snapshot of the oxidized form provides insight into an intermediate structure of the NF-κB activation pathway and also highlights the importance of redox signaling in controlling transcriptional activation. However, in different cell types under various conditions there may be not only different NF-κB dimers, but a number of potential interactions between I-κB, DNA, NF-κB species and Trx contributing to the complexity of the system. This complexity highlights the need for an integrated approach, in which modeling is used to extrapolate between the available experimental structures. Not only additional modeling studies, but also much further experimental work including NMR studies, will be required to solve and confirm more of the intermediate structures, in order to better understand the dynamic nature of the NF-κB pathway.

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