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AlphaFold2 as a replacement for solution NMR structure determination of small proteins: Not so fast!

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ABSTRACT

The determination of a protein's structure is often a first step towards the development of a mechanistic understanding of its function. Considerable advances in computational protein structure prediction have been made in recent years, with AlphaFold2 (AF2) emerging as the primary tool used by researchers for this purpose. While AF2 generally predicts accurate structures of folded proteins, we present here a case where AF2 incorrectly predicts the structure of a small, folded and compact protein with high confidence. This protein, pro-interleukin-18 (pro-IL-18), is the precursor of the cytokine IL-18. Interestingly, the structure of pro-IL-18 predicted by AF2 matches that of the mature cytokine, and not the corresponding experimentally determined structure of the pro-form of the protein. Thus, while computational structure prediction holds immense promise for addressing problems in protein biophysics, there is still a need for experimental structure determination, even in the context of small well-folded, globular proteins.

1. Introduction

Proteins perform many functions that are essential to life, and in the many cases where these biomolecules fold into a well-defined conformation, function is intimately connected to three-dimensional structure, or fluctuations therein. Thus, elucidation of a folded protein's structure is often a necessary first step in establishing how it functions at the atomic level. In recent years, significant advances have been made in computational protein structure prediction, highlighted by the remarkable performance of AlphaFold2 (AF2) in the 14th Critical Assessment of Protein Structure Prediction (CASP14) competition [12]. In this blind test of computational structure prediction methods, AF2 structures had a median backbone accuracy of 0.96 Å $RMSD_{95}$ (Cα RMSD at 95 % residue coverage) [12], indicating that AF2 is capable of producing structures with an accuracy similar to that of experimental structures in many cases. This was further demonstrated by Tejero *et al.*, where it was shown that for six small proteins, AF2-predicted structures yielded comparable agreement with experimental NMR data as the corresponding NMR or X-ray crystal structures [21]. A similar study by Li *et al.* corroborated this result on a set of "blind" targets; that is, proteins having solved solution NMR structures which were (i) not used in AF2's training set and (ii) did not have homologous structures in the Protein Data Bank at the time of AF2's training [14]. In addition, Robertson *et al.* have shown close agreement between residual dipolar couplings measured on the SARS-CoV-2 main protease and those calculated from AF2's predicted structure of the protease, exceeding calculations based on most high-resolution X-ray structures in regions of well-defined secondary structure [18]. Since its public availability following CASP14, AF2 predictions have been beneficial to many researchers. For example, in several cases, AF2 predictions have been used to aid in structure determination via cryoelectron microscopy where maps had poor density for certain protein regions [11,16,9]. Further, the predictive power of AF2 beyond modeling single-chain protein structures has been tested, showing promise for the prediction of protein–protein interactions [5,24]. These examples clearly demonstrate the ability of AF2 to accurately predict protein structures in many cases and highlight the value of machine learning-based approaches for solving problems in protein biophysics.

The remarkable success of AF2 structure prediction is not ubiquitous, however. Although many regions in proteins that are predicted with

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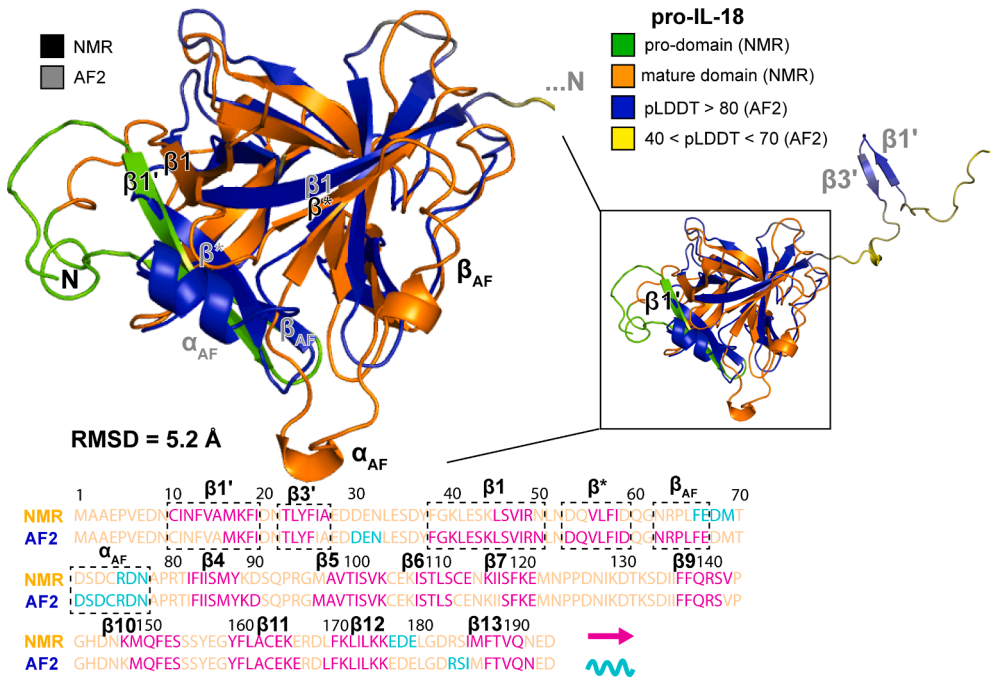

Fig. 1. Solution NMR structure of pro-IL-18 differs significantly from a high confidence predicted AF2 model. Overlay of the solution NMR structure of pro-IL-18 (pro-domain of pro-IL-18 in green, mature domain of pro-IL-18 in orange) and the AF2 prediction of this structure (yellow-blue gradient encoding predicted local-distance difference test (pLDDT) confidence values of 43–100). An RMSD value of 5.2 Å is calculated using backbone heavy atoms over the following residue ranges: 45–49 (β1), 55–58 (β*), 82–88 (β4), 97–103 (β5), 107–113 (β6), 115–121 (β7), 137–142 (β9), 148–153 (β10), 159–165 (β11), 170–176 (β12), and 185–190 (β13), corresponding to the NMR-determined β strands from the mature domain of pro-IL-18 only [8]. These same atoms were used to align the two structures. Elements which are distinct between the NMR structure and AF2's prediction are labeled in black (NMR) and grey (AF2). β3′ in the NMR structure is not marked, as it is not visible in this view. However, it does hydrogen bond with β1′ as in the AF2 prediction. In addition, the locations of α_{AF} and β_{AF}, as predicted by AF2, are displayed on the solution NMR structure (as well as on the AF2 model), and the position of β1* (established experimentally) is indicated on the AF2 model. Beta strands (magenta) and alpha helices (cyan) are shown on the amino acid sequence, with significant differences between the NMR and predicted structures highlighted by the dashed boxes.

very low confidence by AF2 are, in fact, intrinsically disordered [15], "false positives" emerge as well, with AF2 confidently assigning structures to about 15 % of the intrinsically disordered regions (IDRs) found in human proteins. In these cases, the predicted structures are often those assumed when the IDR binds to a target (*i.e.*, IDRs that "conditionally fold") [2]. It has also been shown that AF2 performs poorly in predicting structures of long loop regions of structured proteins [20]. In cases where a protein adopts multiple conformers, AF2 typically predicts only one of the structures, failing to capture the conformational diversity seen in the protein [19]. In addition, AF2 has been shown to lack the ability to predict structural changes induced by point mutations [6], struggles in some cases to predict structures of membrane proteins [3], and does not include post-translational modifications in its predictions [17]. Finally, AF2 predicts only the ground state structure of a protein, and not transiently accessed configurations, the latter of which can be critically important for function [4,10,13,23]. Despite these shortcomings, the utility of AF2 as an invaluable aid to structural biology is well-established.

In this Communication, we share a case where the incorrect structure predicted by AF2 does not fall under any of these known limitations. Pro-interleukin-18 (pro-IL-18), the precursor form of the pro-inflammatory cytokine IL-18, has been shown to adopt a compact, β-trefoil structure [8], while AF2 predicts a lengthy N-terminal disordered region protruding from the trefoil. Further, the structured regions are predicted with high confidence by AF2, despite not matching the experimentally determined structure. This example emphasizes that while computational methods for protein structure prediction have made tremendous strides in recent years and hold immense promise, the problem of structure prediction, even for small and compact proteins, is not quite "solved" yet.

2. Results and discussion

We recently reported the solution NMR structure of pro-IL-18 [8], and we and others have described the cryo-EM structure of pro-IL-18 bound to the proteases caspase-1 and caspase-4 [8,7]. Pro-IL-18 is compact and well-folded in the experimental structures with β-trefoil folds in both apo and bound states. Notably, the pro-domain (residues 1–36, which are cleaved to form the mature, active cytokine by caspases-1/-4) contains beta strands which are incorporated into the trefoil. The AF2 prediction for pro-IL-18 is very different, with a backbone heavy atom RMSD of 5.2 Å over ordered residues (see legend of Fig. 1) relative to the NMR-derived fold. Further, rather than being incorporated into the trefoil, the pro-domain is predicted to protrude from the rest of the molecule (Fig. 1). Consequently, the N-termini of the predicted and experimentally derived structures reside on opposite ends of the aligned structures (Fig. 1). Low confidence scores for the AF2-calculated structure in this region (40 < pLDDT < 70) suggest that the pro-domain is largely disordered, with the exception of two beta strands. These beta strands are mostly comprised of the same residues which form the beta strands in the NMR structure, though β1′ in our solution NMR structure is much longer. There are significant differences outside of the pro-domain as well, despite the fact that the rest of the structure is predicted with high confidence by AF2 (pLDDT > 90 for beta strands); β1 in the AF2 prediction is much longer than in the experimental structure and is positioned differently within the trefoil (Fig. 1). Further, there are additional stretches of secondary structure, β_{AF} and α_{AF}, which are within what is a mostly disordered region in the solution NMR structure, yet appear in core, structured regions in the predicted model. In addition, a short β strand (β*, V55-I58) that is hydrogen bonded to β4 in the experimental structure, is replaced by a longer strand extending from

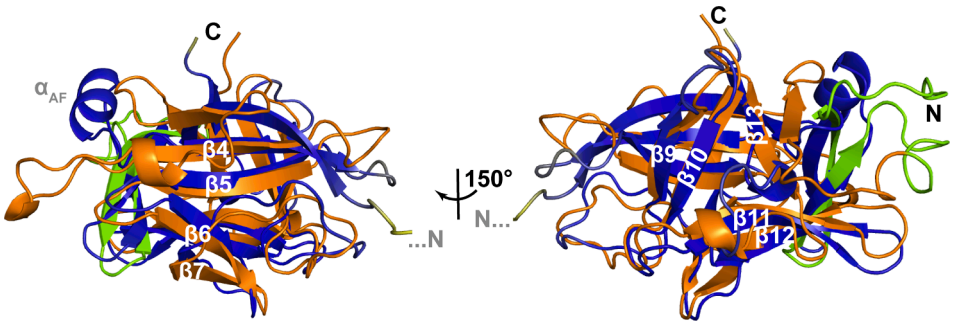

Fig. 2. C-terminal halves of solution NMR and AF2 predicted structures show significantly better agreement than N-termini. An overlay of the solution NMR structure of pro-IL-18 and the AF2 predicted model is displayed using the same color scheme as Fig. 1. Beta strands which are well-superimposed for the two structures are labeled in white. Beta strands β4-β13 from the C-terminal half of the protein are labeled, and can be seen to be in reasonable agreement, in contrast to the structural elements in the N-terminal half highlighted in Fig. 1. While the backbone heavy atom RMSD of β1 and β* is 13.3 Å, the remaining β strands (β4-β13) have a backbone heavy atom RMSD of only 2.0 Å. If the structures are aligned using only β4-β13 (see legend of Fig. 1 for residue ranges), this RMSD is reduced to 1.7 Å.

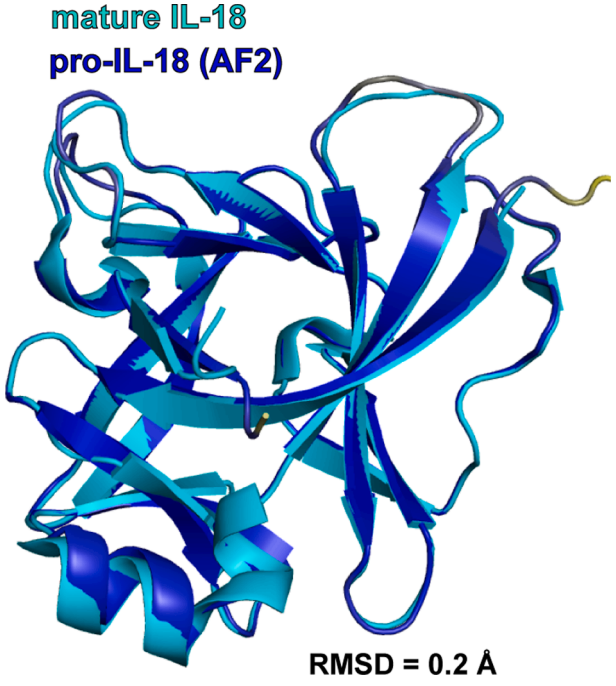

Fig. 3. AF2 prediction of pro-IL-18 is virtually identical to the structure of mature IL-18. Overlay of the structure of mature IL-18 (cyan, PDB ID 3WO2) and the AF2 prediction of pro-IL-18, omitting the pro-domain of pro-IL-18 (first 36 residues). An RMSD value of 0.2 Å is calculated using the same backbone heavy atoms listed in the legend of Fig. 1.

D53-D59 in the AF2 model, that is, in turn, localized to a different region of the structure. The differences between the NMR and AF2 derived structures are largely confined to the N-terminus (for example, a backbone heavy atom RMSD of 13.3 Å is calculated for β1 and β*), with much better agreement observed in the C-terminal half of the molecule (backbone heavy atom RMSD of 2.0 Å for β4-β13, see Figs. 1 & 2). These topological differences are virtually identical to those seen between our NMR structure of pro-IL-18 and that of either the crystallographic or AF2 derived structures of the mature cytokine. Indeed, the structure of mature IL-18 (PDB ID 3WO2) [22] and the AF2 prediction of pro-IL-18 (excluding the pro-domain) are in excellent agreement, with a backbone heavy atom RMSD of 0.2 Å over ordered residues (Fig. 3).

It is of interest to speculate as to why nature might have designed the pro-IL-18 structure in the way that she did, rather than simply placing the pro-domain on the periphery of the mature region, as the AlphaFold2 model predicts. An answer is obtained by examination of the cryo-EM structures of the pro-IL-18:caspase-1 or caspase-4 complexes which show the importance of interactions between substrate (pro-IL-18) and enzyme (caspase) that are only accommodated with the correct global fold of the pro-form of IL-18. As described previously, there are two surfaces of interaction between pro-IL-18 and caspases-1 and -4: one at the active site of the enzyme where the tetrapeptide sequence of pro-IL-18 (33-LESD-36) is recognized, and another termed the "exosite" which involves an interaction between the sidechain of I48 of pro-IL-18 and a tryptophan residue of the caspase [8,7]. In order for both of these surfaces to interact simultaneously, the tetrapeptide and I48 of pro-IL-18 must be positioned appropriately relative to each other. In the pro-IL-18:caspase-1 complex, the distance between the Cα atoms of L33 and I48 is found to be 19.1 Å (19.6 Å in pro-IL-18:caspase-4). In our NMR structure of pro-IL-18, these residues are similarly positioned with a Cα-Cα distance of 15.8 Å. However, in the AF2 predicted structure, this distance, 36.7 Å, is much larger (Fig. 4). Consequently, if pro-IL-18 were structured as in the AF2 prediction, the active site and exosite interactions with caspases-1/-4 would not be able to form simultaneously. Further, in the NMR structure the tetrapeptide sequence is contained within a flexible loop, and it is possible that this flexibility is necessary for the tetrapeptide to be accessible to the active sites of caspases-1/-4. In the AF2 prediction, on the other hand, the tetrapeptide sequence is immediately adjacent to β1, which may be detrimental to forming the proper interactions at the active site. Together, these observations suggest that if pro-IL-18 were structured as in the AF2 prediction, it is unlikely that it could be processed by caspase-1/-4 to produce the active from of the cytokine. Finally, an unstructured pro-domain might be readily cleaved by other cellular proteases, circumventing the role of the caspases, and leading to the aberrant regulation of the maturation pathway of IL-18.

Protein structure prediction has advanced rapidly in recent years, with AF2 emerging as the primary tool used by researchers for this purpose. While it has been demonstrated that AF2 generally predicts accurate structures of folded proteins, both in the CASP14 competition as well as in follow up studies by other research groups [12,21,14,18], we have presented here a small, folded protein whose structure is not accurately predicted by AF2. Further, AF2 predicts most of the structure of this protein, pro-IL-18, with high confidence, despite the considerable discrepancies with our experimentally determined NMR structure [8]. Pro-IL-18 is the precursor form of the cytokine IL-18, where the first 36 amino acids of pro-IL-18 are cleaved to produce the mature, active cytokine. Interestingly, the structure of pro-IL-18 predicted by AF2 is virtually identical to that of mature IL-18, with the first 36 residues of pro-IL-18 appended as a largely disordered (low confidence) tail, rather than incorporated into the trefoil as observed experimentally. Notably, numerous structures of mature IL-18, but no structures of pro-IL-18, were present in the Protein Data Bank at the time of AF2's training.

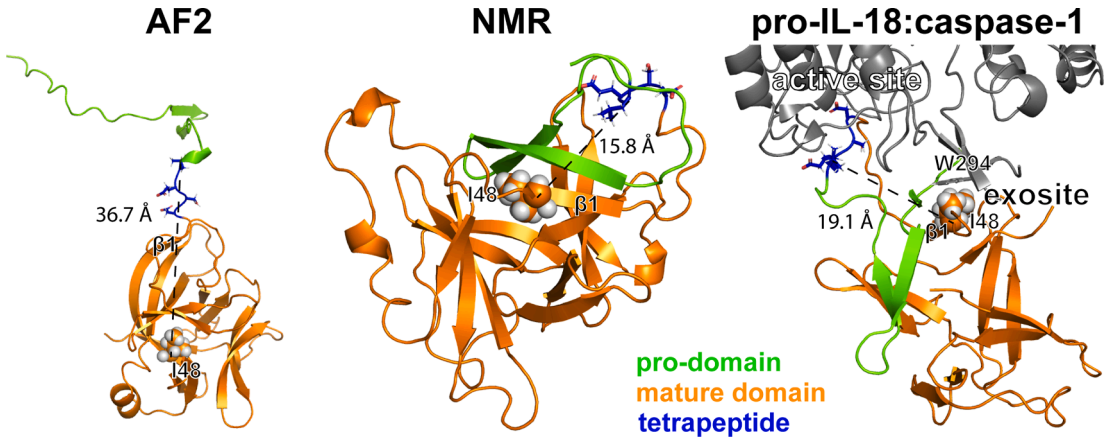

Fig. 4. AF2 predicted structure of pro-IL-18 is likely incompatible with caspase binding. Pro-IL-18 structures predicted by AF2 (left), determined by solution NMR (middle, PDB 8URV), and in complex with caspase-1 as established by cryo-EM (right, PDB 8SV1) are shown. The I48 sidechain is shown in spheres, while the tetrapeptide is shown in blue sticks. The sidechain of W294 from caspase-1 is shown in grey sticks. Dashed lines connect the Cα atoms of I48 (exosite) and L33 (tetrapeptide, active site), with the distance between these atoms noted. This distance is much larger in the AF2 predicted structure than in the complex with caspase-1, suggesting that the AF2 predicted structure would not be able to interact with both the active site and exosite of caspases-1/-4 simultaneously.

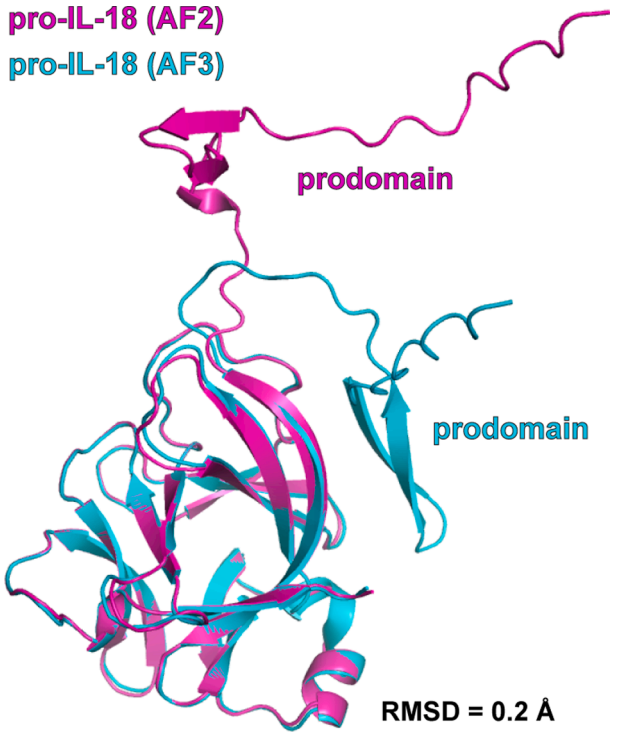

Fig. 5. AF3 prediction of pro-IL-18 is very similar to that of AF2. An overlay of the AF2 (magenta) and AF3 (cyan) structure predictions of pro-IL-18 is shown. Aside from the orientation of the pro-domain relative to the mature domain, the predictions are virtually indistinguishable. Indeed, an RMSD of 0.2 Å is calculated using the same backbone heavy atoms listed in the legend of Fig. 1. The AF3 prediction was made with 50 Na^+ and Cl^- ions included, however, the prediction outside the pro-domain is unaffected by the addition of these ions.

Our finding may raise some concerns regarding the accuracy of AF2 predictions of other, similarly post-translationally processed proteins. Naturally, cases where two proteins are comprised of nearly identical amino acid sequences (in the case here identical sequences past the pro-domain), yet have considerable structural differences, pose a unique challenge to algorithms designed to predict structure from a given amino acid sequence.

During preparation of this manuscript, a new and improved predictor of biomolecular structure – AlphaFold3 (AF3) – was released [1]. This new version shows significant improvements in structural predictions of biomolecular complexes, including those involving protein:protein and protein:nucleic acid interactions. We wondered whether a more powerful predictor of protein:protein contacts might be able to imbed the pro-domain properly into the structure of the mature domain, as observed experimentally. However, even for this updated version of AlphaFold we find that the erroneous structure prediction of pro-IL-18 persists (Fig. 5).

In summary, the example of the pro-IL-18 structure highlights that while AlphaFold has been shown to generally predict structures of folded proteins with high accuracy, there are likely to be certain cases, even ones involving small, globular proteins, where experimental structure determination still remains essential.

CRediT authorship contribution statement

Jeffrey P. Bonin: Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **James M. Aramini:** Methodology, Formal analysis. **Ying Dong:** Writing – review & editing. **Hao Wu:** Writing – review & editing, Conceptualization. **Lewis E. Kay:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [H.W. is a co-founder and chair of the scientific advisory board of Ventus Therapeutics. This relationship did not influence this study].

Data availability

Coordinate files are available on Zenodo repository (DOI given in acknowledgements)

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