A sequence-based foldability score combined with AlphaFold2 predictions to disentangle the protein order/disorder continuum

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Abstract  
Order and disorder govern protein functions, but there is a great diversity in disorder, from regions that are – and stay – fully disordered to conditional order. This diversity is still difficult to decipher even though it is encoded in the amino acid sequences. Here, we developed an analytic Python package, named *pyHCA*, to estimate the foldability of a protein segment from the only information of its amino acid sequence and based on a measure of its density in regular secondary structures associated with hydrophobic clusters, as defined by the Hydrophobic Cluster Analysis (HCA) approach. The tool was designed by optimizing the separation between foldable segments from databases of disorder (DisProt) and order (SCOPe (soluble domains) and OPM (transmembrane domains)). It allows to specify the ratio between order, embodied by regular secondary structures (either participating in the hydrophobic core of well-folded 3D structures or conditionally formed in intrinsically disordered regions) and disorder. We illustrated the relevance of *pyHCA* with several examples and appliedit to the sequences of the proteomes of 21 species ranging from prokaryotes and archaea to unicellular and multicellular eukaryotes, for which structure models are provided in the AlphaFold2 databases. Cases of low-confidence scores related to disorder were distinguished from those of sequences that we identified as foldable but are still excluded from accurate modeling by AlphaFold2 due to a lack of sequence homologs or to compositional biases. Overall, our approach is complementary to AlphaFold2, providing guides to map structural innovations through evolutionary processes, at proteome and gene scales.

Keywords  
Protein foldable segments, Hydrophobic Cluster Analysis, soluble and transmembrane domains, IDPs/IDRs, AlphaFold protein structure database

Abbreviations

aa : amino acids, AF2 : AlphaFold2, AFDB : AlphaFold Protein Structure DataBase, CAID : Critical Assessment of protein Intrinsic Disorder, DisProt : dabase of intrinsically Disordered Proteins, HCA : Hydrophobic Cluster Analysis, IDPs/IDRs : Intrinsically Disordered Proteins/Regions, OPM : Orientations of Proteins in Membrane, PDB : Protein Data Bank, pLDDT : predicted Local Distance Difference Test, RSSs : Regular Secondary Structures, SCOPe : Structural Classification of Proteins – extended, TM : Transmembrane

Introduction

Protein order has been largely explored by experimental approaches so that the protein fold universe has been widely mapped 1-3. This has led to a comprehensive inventory of the combinations according to which regular secondary structures (RSSs) are assembled to form compact, well-organized 3D structures, associated with specific functions 4. Over the years, the structure-function paradigm has however evolved to integrate intrinsically disordered proteins/regions (IDPs/IDRs), which lack well-defined 3D structures under physiological conditions but fulfill a variety of functions, in particular in signaling and regulatory pathways 5-8. IDPs/IDRs are characterized by a heterogeneous spatiotemporal structural organization 9. They correspond to very diverse entities, including short linear motifs and longer regions promoting molecular recognition and protein-protein interactions, which have led to elaborate specific classification schemes related in particular to their amino acid sequence characteristics 6,10. IDPs/IDRs were also shown to play a key role in the formation of higher-order assemblies and in the control of many cellular processes via their participation in biomolecular condensates, through multivalent interactions leading to liquid-liquid phase separation 11,12. IDPs/IDRs are generally defined by a heterogenous ensemble of conformations, undergoing rapid interconversion 13,14; some can fold into a unique conformation upon binding with a partner or within oligomeric complexes 15, while some cases of disorder maintained in the bound state were also described 16.

Characterization of IDPs/IDRs is challenging as they are generally “unseen” by traditional structural biology methods and are therefore considered as the dark side of protein universe 17. Their identification at large scale relies on computational methods that predict them directly from the information of the amino acid sequence 18. Some predictors are trained on experimental annotations of protein disorder, as stored in the DisProt database 19, while others are not, relying on physicochemical properties and predicting disorder as lack of, or deviation from order 20,21. Using such predictive tools, IDPs/IDRs were found abundant at the proteome level, making up approximately 30 % of residues in the human proteome and up to 50 % in some unicellular eukaryotes such as parasitic protozoa, enriched in long IDRs (with at least 30 consecutive disordered residues) 22-25. In contrast, the fractions of disordered residues represented less than 28% in most archaeal and bacterial proteomes. The quality of disorder predictors has improved over time, in particular by considering deep learning techniques and evolutionary information, as illustrated in the recent Critical Assessment of protein Intrinsic Disorder (CAID) 26. The predicted Local Distance Difference Test (pLDDT) introduced in the recent AlphaFold2 (AF2) predictor, a deep learning program which predicts 3D structures with an unprecedented accuracy 27 and which was applied at proteome scale 28, was also shown to provide a good metric for identifying order and disorder 29-31.

Only a few computational approaches have addressed the issue of the predictions of different, multiple states (or flavors) of IDPs/IDRs, while not considering evolutionary information (*e.g*. 32,33). Here, we propose an approach for appreciating the disorder/order degree in a protein segment from the only information of its amino acid sequence, which is based on a measure of the overall density in regular secondary structures, as predicted through Hydrophobic Cluster Analysis (HCA). HCA-based hydrophobic clusters match the positions of regular secondary structures constituting the building blocks of folded domains 34-38. The hydrophobic alphabet and rules used for the definition of hydrophobic clusters have been supported by comparison with experimental data, and the method has been successfully applied, for instance, to the identification of remote relationships based on the conservation of 2D signatures associated with hydrophobic clusters (see **Supplementary Dataset S1** for a complete description of the method). The use of a simple hydrophobic/non-hydrophobic dichotomy (rather than an hydrophobicity scale), associated with the use of a two-dimensional net for defining the amino acid neighborhood, offers an efficient way to reveal these signatures in remotely related sequences 39. A tool, called *SEG-HCA*, was previously developed for the delineation of regions with a high density in hydrophobic clusters, which have been shown to correspond to domains which have the ability to fold, either in an autonomous way or upon contact with partners 40,41. Contrasting with otherwise performant methods based on evolutionary couplings (e.g. 42) or even earlier tools based on propensities to be in ordered or disorder states (e.g. 43), the advantage of *SEG-HCA* is to allow the prediction of foldable domains from the only information of a single amino acid sequence, without the prior knowledge of homologous sequences or consideration of pre-calculated propensities. At proteome scale, *SEG-HCA* “order” predictions totalize more amino acids than the “undisordered” predictions performed by the popular IUPRED tool 44,45, which captures the inter-residue interaction capacity by energy estimation 41. The overlap between order and disorder detected by this comparison concentrates small sequences that are able to undergo disorder-to-order transitions 41.

We optimized the residue weights of the here-proposed density metric for an optimal separation of foldable domains found in reference databases of order (soluble and transmembrane domains extracted from SCOPe and OPM, respectively) and disorder (DisProt,), thereby deconvolving the spectrum of disorder according to the order/disorder ratio and specifying different types of disorder. Using this scoring scheme, we analyzed the per-residue confidence (pLDDT) scores of AF2 structural predictions in 21 reference proteomes, for species ranging from prokaryotes and archaea to unicellular and multicellular eukaryotes with different lifestyles 28,46. Our analysis provides new elements to distinguish cases where low-confidence structural predictions are indeed related to disorder, as now commonly reported 20,21, from those of domains which are foldable but whose structures cannot be accurately predicted due to AF2 intrinsic limitations. Overall, the complementarity of *pyHCA* and AF2 provides guides to map structural innovations through evolutionary processes, at proteome and gene scales.

Materials and Methods

Delimitation of foldable segments within protein sequences

The HCA methodology is described in details in **Supplementary Dataset S1**. SEG-HCA 41 was previously developed to automatically delineate regions with high density in hydrophobic clusters, constituting potential “foldable” domains within protein sequences. The new version of SEG-HCA was rewritten for speed and includes new functionalities, including the calculation of HCA score (see below). In addition, we rewrote and packaged in a python module the tools SEG-HCA (in a function named segment), TREMOLO-HCA (Traveling through REMOte homoLOgy) 47 and the HCA drawing, as well as provided some utility scripts to analyze their results, adding information on amino acids conservation and taxonomy. These tools can significantly help detection of hidden relationships between sequences, as repeatedly performed using the HCA approach in an expert-based way (see references in 40 and Supplementary Dataset S1). The package can be used as a Python library, named pyHCA, or as a standalone tool, named HCAtk, and is provided at <https://github.com/DarkVador-HCA/pyHCA> under the CeCILL-C license agreement.

HCA score implementation

The HCA score was introduced in order to describe the general composition in hydrophobic clusters and hydrophobic amino acids in foldable segments. Each residue of a protein sequence is associated with a class regarding the residue type and hydrophobicity. Such a residue is either (i) in a hydrophobic cluster and hydrophobic according to the common HCA alphabet, which considers as strong hydrophobic the seven amino acids V, I, L, M, F, Y, and W, (ii) in a hydrophobic cluster and non-hydrophobic, or (iii) outside a hydrophobic cluster. A value is attributed to each class and the HCA score (𝑆𝐻𝐶𝐴) is computed as follow:

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with 𝑤𝐻𝐶𝐴(𝑠𝑒𝑞𝑖, 𝑐𝑙𝑎𝑠𝑠) the weight associated with the i amino acid of a sequence from a given class, and N the sequence length.

Therefore, the HCA score scales with the density in hydrophobic clusters and in strong hydrophobic residues inside the clusters. As the HCA score calculation motivation is to provide an estimation of the globular character, i.e. the foldability of a domain, the weight of each residue was optimized inside each of the three classes to minimize the overlap between the distributions of the HCA scores computed on non-redundant disordered foldable segments from DisProt v8.0.2 sequences and non-redundant foldable segments of globular proteins from the SCOPe (soluble domains) and OPM databases (transmembrane domains), respectively (see below for details on the datasets). During the optimization step, the allowed possible values of the weights were discrete inside the [-10; 10] interval ([- 1; 1] scaled to one order of magnitude for better readability). For a given combination of weights, the distribution overlap (*i.e.* the criteria to be minimized) was estimated by histogram intersection as follows:

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with B the number of histogram bins (set to 60), and H1(i) and H2(i) the values in normalized histograms of HCA score for non-redundant dataset Disprot v8.0.2 and non-redundant dataset SCOPe and OPM for bin i, respectively. The optimization was achieved using 10-fold cross-validation, repeated 10 times. We selected the set of parameters that was the most frequently the optimal one out of the 10 iterations, and that allowed to achieve the lowest overlap between disorder and order.

This implementation is an updated version (v2) of a previous one, described in 48 and applied in investigations such that reported in 49, where the disorder dataset corresponded to sequence segments as predicted by Mobi-DB 50 on the DisProt v7.0 sequences. The *pyHCA* GitHub repository has been updated accordingly (<https://github.com/DarkVador-HCA/pyHCA>).

Sequence datasets

(a) Sequence redundancy filters

For each dataset described below (b-e), sequence redundancy was addressed using mmseqs2 51 clustering (mode 1, sensitivity 8) with a sequence identity threshold of 30% and a coverage threshold of 90%. The non-redundant sets of sequences (Supplementary Dataset S2) comprised the representative sequences of each cluster.

(b) Disordered segments

Disordered sequences, as assessed from experiments and manually curated, were extracted from the reference database DisProt v8.0.2 (8 254 sequences, 19; <https://disprot.org/>). The corresponding non-redundant set DisProt comprises 3 166 sequences.

(d) Soluble domains

27 543 sequences of soluble domains with known 3D structures were collected from the Structural Classification of Proteins — extended (SCOPe) v2.0.7 database (52; <https://scop.berkeley.edu/>) as provided by Astral repository with 95% identity filter. The SCOPe classification of these entries according to their content in secondary structures was as follows: 4 974 all-alpha domains (class a), 7 622 all-beta domains (class b), 8 250 alpha/beta domains (class c), 6 697 alpha+beta domains (class d). Our non-redundant dataset SCOPe comprises 10 885 domain sequences with the SCOPe a-d classes represented by 2 507, 2 511, 2 841 and 3 026 entries, respectively.

(e) Transmembrane domains

The Orientations of Proteins in Membranes (OPM) database (53; <https://opm.phar.umich.edu/>) was evidenced to include the largest number of membrane proteins with known 3D structures 54. The OPM entries annotated as transmembrane domains were downloaded on August 30, 2021 and include 3 classes: alpha-helical polytopic or multi-pass (140 superfamilies, 5 381 entries), bitopic or single-pass (69 superfamilies, 1 151 entries) and beta-barrels transmembrane (35 superfamilies, 601 entries) domains. 35 sequences only formed by unknown residues (replaced in OPM by alanines) were suppressed. We performed a first redundancy treatment to suppress repeated sequences (using *mmseqs2* clustering with a 0.95 identity threshold and keeping the longest sequence of the cluster), leaving only 3 051 unique TM domains in total. For each domain, OPM provides the information of the calculated transmembrane (TM) segment boundaries. In order to delineate the whole membrane-spanning domains, we included loops with length smaller than or equal to 30 residues in our sequence dataset. According to the distribution of loop lengths in TM domains and to the minimum size of known globular domains, TM segments separated by more than 30 residues are probable to encompass nested soluble domains 55. In order not to include these cases of large loops in our sequence dataset, we only kept the first 15 residues after the first TM segment and the last 15 before the second TM segment. If an extended segment boundary falls inside a hydrophobic cluster, we moved it to include the whole hydrophobic cluster and the 4 following residues (*i.e.* the minimal distance considered to separate contiguous hydrophobic clusters, see **Supplementary Dataset S1**). This trimming needed to be applied to 1 882 sequences (see Supplementary Dataset S2 for details). The non-redundant dataset OPM comprises 1 698 sequences: 1330, 165, and 203 annotated as alpha-helical polytopic, bitopic and beta-barrels transmembrane domains, respectively.

(f) Proteomes from **AlphaFold Protein Structure database** v1

The amino acid sequences, the 3D structure predictions and the corresponding per-residue model confidence values (pLDDT) were downloaded from the AlphaFold Protein Structure database (AFDB) v1 (46, [https://alphafold.ebi.ac.uk](https://alphafold.ebi.ac.uk/), downloaded on July 21, 2021) for the reference proteomes of 21 model organisms. pLDDT values estimate on a per residue basis how well the predicted structure would agree with the experimental 3D structure and is scaled between 0 and 100 as follows: very low (pLDDT ≤ 50), low (50 < pLDDT ≤ 70), confident (70 < pLDDT ≤ 90), very high (pLDDT > 90).

Figure creation and statistical analyses

3D structures were visualized with the UCSF Chimera package 56. Statistical analyses were performed using the R software, version 4.1.2 (57) and the Python Language ([http://www.python.org](http://www.python.org/)), versions 3.7.6 (for the HCA score implementation and AFDB v1 analyses) and 3.6.3 (for the classification of order and disorder from DisProt, SCOPe and OPM databases). Standardized principal component analysis (PCA) and hierarchical clustering on principal components were performed using the R package Factoshiny, version 2.4 (<https://cran.r-project.org/web/packages/Factoshiny>). Mean comparisons by non-parametric Mann-Whitney U test were performed using the Python scipy library, version 1.4.1 ([https://scipy.org](https://scipy.org/)). Graphics were generated using the Python libraries matplotlib, version 3.0.3 ([https://matplotlib.org](https://matplotlib.org/)) and seaborn, version 0.11.2 ([https://seaborn.pydata.org](https://seaborn.pydata.org/)).

Results

Exploring the degree of order and disorder in reference databases using HCA score

HCA score was introduced in order to provide a global estimation of the density of a given amino acid sequence in hydrophobic clusters and hydrophobic amino acids within hydrophobic clusters, as a proxy for order/disorder ratio in protein segments. Hence, we focused on foldable segments (as defined by the segment function of the pyHCA package) of the reference datasets and optimized the weights of this metrics to best separate the different categories according to structural features.

We optimized the weights of this metrics for separating at best order and disorder relative to two datasets: (i) a set of 16489 non-redundant foldable segment sequences from globular proteins from the reference databases SCOPe for soluble domains (14624 segments) and OPM for transmembrane domains (1865 segments) and (ii) a set of 3276 non-redundant foldable segment sequences from disordered proteins from the reference DisProt database (v8.0.2) (**Supplementary Dataset S3**). The optimal HCA score was reached for a minimum overlap of 49% between the score distributions of the globular and disordered sequences, respectively, and with the weights of 9 for strong hydrophobic residues in hydrophobic clusters, 7 for non-strong hydrophobic residues in hydrophobic clusters and -10 for residues outside of hydrophobic clusters (see equation (1) in the Material and Methods section).

A vast majority of amino acids of the SCOPe and OPM non-redundant datasets are included in foldable segments (90 % and 96 %, respectively), while non foldable segments in these datasets mostly correspond to large, hydrophilic loops (see for instance the example shown in **Supplementary Figure S1a**). In contrast, only 50% of amino acids in the DisProt v8.0.2 dataset are included in foldable segments, the remaining ones (*i.e.* in non-foldable segments) can be thus considered as fully disordered (**Supplementary Figure S1b**). DisProt segments can thus be analyzed after being separated into two distinct categories (*i.e.* foldable and non-foldable segments). A few instances of poor coverage of folded domains of the SCOPe database by foldable segments are observed. These correspond to sequences rich in alanine (class a) and threonine/serine (class b), which are not included in the hydrophobic alphabet used for hydrophobic cluster definition, but participate in the hydrophobic core (**Supplementary Figure S1c and S1d**). Some other cases of low coverage actually correspond to domains stabilized by a ligand or for which the folding is cooperative or dependent of an oligomeric organization (obligate oligomers) (**Supplementary Figure S1e**).

The HCA score values range between -7 and 9 (Figure 1, Supplementary Dataset S3). Soluble domains have HCA scores included mostly in the [-1,3.5] interval (labeled b). As illustrated for the SCOPe b class, the lowest and highest values are mainly associated with the presence and absence of large loops (thus large inter-cluster linkers), respectively (Figure 2a). HCA scores for the beta-barrel transmembrane class are close to those of this SCOPe distribution, while those for the alpha-helical polytopic class are higher ([3.5,6.7] interval, (labeled c)) (Figure 1). This is consistent with the general properties of these two classes of proteins; the beta-barrel strands, albeit longer, are indeed characterized by a periodicity of 2 in hydrophobic amino acids, as for beta-strands from globular domains, while transmembrane alpha-helices from alpha-helical polytopic segments are associated with larger numbers of strong hydrophobic amino acids dotted with charged/polar amino acids, as well as tiny ones (Gly, Ala), ensuring tight packing (Figure 2b). Beta-barrel transmembrane sequences can be distinguished from soluble globular domains by their amino acid composition and hydrophobic cluster characteristics (**Figure 2b,** also see Discussion). Finally, a separate category exists for all reference databases, with HCA scores above a value of 7.6 (labeled d), including mostly short sequences covered by a single hydrophobic cluster. These correspond entirely to the foldable segments of OPM bitopic sequences (made of only one transmembrane segment, rich in strong hydrophobic amino acids, **Figure 2b** light blue) or are part of OPM polytopic or SCOPe soluble sequences, which have been fragmented into several parts due to the presence of large loops (see the example in **Supplementary Figure S1a)**. Overall, the HCA score represents a metric that allows to globally assess the order/disorder ratio in a single protein sequence and to unravel its main structural features.

The HCA score distribution of foldable segments from the DisProt database is wide, ranging from -7 to 9, due to the structural heterogeneity of the corresponding sequences, reflecting diverse order/disorder ratio. Some foldable segments have low HCA score values, having hydrophobic cluster densities below those observed for folded domains, which reflect their propensity to fluctuate between disorder and order and/or to interact with partners (labeled a and a’ in **Figure 1**). This is for instance the case of a foldable segment from the yeast nuclear pore complex NUP2 protein, which contains FxFG repeats (shown with a red circle on **Figure 3a**, HCA score of -5.36 (*a* region in **Figure 1**)). FxFG repeats are known to bind transport factors of the importin-beta/karyopherin-beta family, which function as carriers for many nuclear trafficking processes 58. They bind in hydrophobic pockets displayed at the surface of HEAT repeats, in which the phenylalalanine side chains are buried (modeled on **Figure 3a**, on the basis of a complex of FxFG peptides with importin-beta). A second example is related to foldable segments of the mouse nucleoporin ELYS, interacting with chromatin 59(**Figure 3b,** no 3D structure available, HCA score of -2.84 (*a’* region in **Figure 1**)). A third example is that of a cadherin 1 segment, whose 3D structure has been partially solved in complex with catenin beta 1 60 (**Figure 3c,** HCA score of -1.62 (*a’* region in **Figure 1**)). Some other DisProt foldable segments are falling into the “folded, soluble domain” category (*b* region in **Figure 1**), being characterized by hydrophobic clusters ratio typical of this kind of stable 3D structures. This is for instance the case of a foldable segment of the 7SK Sn RNA methylphosphate capping enzyme, which is partially disordered and undergoes a disorder-to-order conformational change upon RNA binding 61 (**Figure 3d,** HCA score of 0.40).Another example of a DisProt sequence, totally covered by a foldable segment, is shown in Supplementary Figure S2a, with a cluster composition and density similar to that observed in globular domains, albeit with a slightly low content (30%) in hydrophobic amino acids. This example corresponds to the yeast proteasome maturation factor Ump1, which is disordered when free in solution, as observed using various experimental techniques 62,63. It however forms well-ordered secondary structures in complex with the 20S core particle, playing a key role in the dynamical assembly of proteasome 64. An additional example of stabilization of 3D structures in complexes is provided in **Supplementary Figure S2b**.

Next, higher HCA scores values, typical of polytopic membrane domains (*c* region in **Figure 1**), are also encountered in DisProt sequences. In a general way, these segments have long hydrophobic clusters (which are common in membrane domains), but form long helical structures involved in oligomeric coiled-coils. This is the case for instance of the HR2 domain of SARS-CoV-2 spike glycoprotein, which form an elongated six-helix bundle together with the HR1 domain 65 (**Figure 3e**, HCA score of 5.66). Another example is the flexible C-terminal part of the *E. coli* antitoxin ParD, which is involved in the binding and neutralization of the ParE toxin66 and forms upon binding long helices docking into the groove of the partner 67 (DP0033r012, HCA score:5.15, 27 amino acids).

Finally, HCA score values above 7.6 (*d* region in **Figure 1**) include foldable segments with a single hydrophobic cluster, as for similar segments of the SCOPe and OPM databases. Such segments frequently correspond to preformed secondary structures (MORFs) or short linear motifs (SLIMs), which fold upon binding, as illustrated here with the short linear motif of the *C. elegans* EGL-1, whose binding to CED-9 initiate cell programmed death 68 (**Figure 3F,** HCA score of 8.23).

Overall, applied to disordered sequences extracted from the DisProt database, the HCA score also allows to globally assess the order/disorder ratio and unravel the wide diversity (or different flavors) of disorder.

Leveraging AlphaFold2 predictions with *pyHCA* package

The recent development of the AlphaFold2 (AF2) deep-learning approach was a huge step forward in the high-throughput prediction of 3D structures for folded regions, and was even announced as a disorder prediction tool, as region with very low confidence largely overlap with IDRs 29-31,69. We wanted here to compare the AF2 predictions to those of foldability and structural states, which can be made using *pyHCA*. Each proteome processed by AF2, provided by AlphaFold Protein Structure Database (AFDB 46, [https://alphafold.ebi.ac.uk](https://alphafold.ebi.ac.uk/)), can be described in terms of foldability and order/disorder content by the pyHCA package: the segment function allows first to quantify the proteome coverage in foldable segments, then to compute the HCA score on foldable segments, leading to their assignment to a structural class related to their position within the order-disorder continuum, as inferred in **Figure 1**. The link between these two metrics (one, binary, and the other, discretized from a continuum) and the per-residue metrics of uncertainty (pLDDT) defined by AF2 was here explored. Four main classes of pLDDT values, reflecting the confidence in the AF2 structural predictions, are generally considered: very high (pLDDT > 90), high (90 ≥ pLDDT > 70), low (70 ≥ pLDDT > 50) and very low (pLDDT ≤ 50).

We first analyzed the confidence in the AF2 structural predictions for residues in versus outside of the foldable segments, for the 362 094 sequences of the 21 reference proteomes from AFDB v1. Among this whole dataset, 81.9% of the residues are part of foldable segments (ranging from 73.1% to 96.3% for the proteomes of the parasitic protozoa Leishmania infantum and the autotrophic hyperthermophilic archaeon Methanocaldococcus jannaschii, respectively), corresponding mostly to confident predictions: 45.7% and 27.1% residues with very high and high pLDDT, respectively (Figure 4a). Instead, the residues located outside of foldable segments (hereafter described as non-foldable segments, representing 18.1% residues of the whole dataset) correspond mostly to low confident predictions (61.3% and 17.1% residues with very low and low pLDDT, respectively, Figure 4a). These trends are also observed for each organism separately, at the exception of Plasmodium falciparum whose proteome is dominated by low confident predictions, even in the foldable segments (39.7% and 13.1% residues with very low and low pLDDT, respectively) (Supplementary Figure S3, Supplementary Table S1). The four prokaryotic proteomes are instead largely dominated by high confident predictions, in foldable (>72% residues with very high pLDDT) but also in non-foldable segments (<40% residues with very low or low pLDDT). Overall, low and high pLDDT scores are thus mostly observed for amino acids in non-foldable (full disorder) and foldable segments, respectively (Figure 4b, Supplementary Table S1), thereby independently supporting the observation that AF2 low confidence predictions are significantly enriched in intrinsically disordered regions 29,30. This is consistent with the recently reported distributions of pLDDT scores for the per-residue predictions of order and disorder 69,70.

In order to further explore the confidence of AF2 predictions for foldable and non-foldable segments separately, we considered regions of contiguous residues (2 aa as minimum length) within these segments where the residues are all affiliated to the same class of pLDDT values (4 classes, see above). In the non-foldable segments, 96.1% residues with a very high pLDDT are located in such regions of homogeneous prediction confidence (3.9% residues with a very high pLDDT in non-foldable segments are thus isolated within regions with a lower prediction confidence).

The sequence length distribution of these regions of very high prediction confidence in non-foldable segments is illustrated in Supplementary Figure S4 (only 13 sequences with length greater than 100 amino acids are observed, corresponding to 6 different proteomes). These regions fall into two distinct categories, corresponding to: *(i)* large loops within folded domains (Supplementary Figure S5a) or linkers between folded domains (Supplementary Figure S**5**b), which are generally disordered or flexible in isolated proteins but conditionally folded in presence of specific interactions, *(ii)* regions which are well folded but independent of the presence of strong hydrophobic amino acids (Supplementary Figure S5c-f). These sequences correspond to either *(a)* long coiled-coils, which form extended rod-like structures and are rich in alanine (Supplementary Figure S5d) or made of acidic and basic-rich heptad repeats (Supplementary Figure S5c), and to various structural repeats (left-handed parallel beta helix repeats (Supplementary Figure S5e), tetratricopeptide repeats, armadillo repeats, ...) or *(b)* domains with ion-dependent folding (calcium (Supplementary Figure S5f), zinc, ...). Non-foldable sequences which form well-stable structures thus correspond to particular cases in which the fold is not conditioned by the presence of a core of strong hydrophobic amino acids, but which possess clear, distinctive amino acid composition. Conversely, as discussed in 70, foldable segmentsdo include sequences with disorder potential, for which AF2 prediction however capture some structural features formed upon interaction (already depicted in experimental structures).

The methodology developed here allows us to put the AF2 prediction made at the residue level into the context of the foldable segment to which it belongs and of its position in the order/disorder continuum. We thus calculated the distribution of residues in each pLDDT category as a function of the HCA scores of the foldable segments in which they are included (**Supplementary Dataset S4**). Overall, we observed differences in the relative proportions of each pLDDT category as regards to the structural states (labeled a to d), previously defined based on the HCA score (Figure 5a). The highest proportion of residues with very high pLDDT (51.8%) is observed for the foldable segments with an HCA score within ]1;2] interval, corresponding to the typical score of soluble domains (*b* region, as defined in Figure 1). Moreover, the residues with very high pLDDT represent 48.3% residues included in foldable segments with a HCA score in ]-1;6], corresponding to the soluble/beta-barrel and alpha-transmembrane domains (*b and c regions*). Instead, residues with very low pLDDT dominate the likely disordered foldable segments with a HCA score in ]-7;-4] (74.8%), but also those with a HCA score in ]+7;+9] (63.7%) corresponding to local maxima in the HCA score distribution for DisProt disordered sequences (see **Figure 1**). We should notice the uniformity of the length of the foldable segments with HCA score lower than +7, with a median length of 84 aa, as evidenced by the tight correlation between the number of residues and the number of foldable segments within the corresponding intervals of HCA score (see top graph in **Figure 5a**, with black bars and grey line, respectively). Instead, the foldable segments with HCA score higher than +7 are much shorter, with a median length of 6 aa.

Although these global trends of relationship between pLDDT and HCA score of foldable segments are conserved across the 21 individual proteomes, several differences should be noticed, as illustrated by five representative patterns in Figure 5b-f. The 21 proteomes have been clustered according to the principal component analysis (PCA) built of their relative proportion of residues of each pLDDT category for foldable segments of each interval of HCA score values (Figure 6). The first principal component explained 56.4% of the total variance and showed that the foldable segments of prokaryotic and eukaryotic proteomes differ for almost all intervals of HCA scores, with higher proportions of very high and very low pLDDT residues, respectively (Supplementary Figure S6a-d). At second order and according to the second principal component (explaining only 11.7% of the total variance), the proteome of the archaeon Methanocaldococcus jannaschii slightly differs from the 3 bacterial proteomes, in particular due to the absence of sequences with HCA scores lower than -4 (corresponding to full disorder) in M. jannaschii (**Figure 5b-c**).

The proteomes of eukaryotes are split into 3 groups, cluster 4 includes the four plant proteomes (*Arabidopsis thaliana*, *Oryza sativa*, *Glycine max*, *Zea mays*), the three *Ascomycota* are separated according to their taxonomy: the two *Saccharomycetaceae* (*Candida albicans* and *Saccharomyces cerevisiae*) belongs to cluster 3 whereas *Schizosaccharomyces pombe* is in cluster 4. Cluster 4 is characterized by a higher proportion of low and very low confidence regions in segments with HCA score in ]-1;6] (corresponding to globular sequences), and by a lower proportion of very low confidence predictions in segments corresponding to disorder (HCA score lower than -5) (**Figure 5d-e**). Finally, the proteome of Plasmodium falciparum forms another last group, being characterized by predictions of globally lower confidence (**Figure 5f**). This is consistent with the high level of dark sequences in the Apicomplexa proteomes, for which remote homologs cannot easily been detected for efficient covariation analyses.

In the foldable sequences, we observed two major types of apparent discrepancies between the confidence score of AF2 structural prediction and our classification of structural states based on HCA score. These types indeed deviate from the current assumption that the structure of folded regions would be predicted with high confidence, while disordered regions would not. First, we reported cases of regions of contiguous amino acids with high pLDDT values with low HCA scores, that we suggested to correspond to disorder (785 sequences with length greater than 100 amino acids, corresponding to 20 different proteomes, *i.e.* at least one sequence in all AFDB v1 proteomes except *Methanocaldococcus jannaschii*; **Supplementary Figure S7a**). These regions include the two categories depicted before for non-foldable segments with high pLDDT values, i.e. (a) disordered sequences included in large loops and/or undergoing conditional folding (AF2 then capturing the structure of the complexed state), (b) long, repetitive structure with a particular abundance of (alpha-forming) alanine or (beta-forming) serine/threonine and sequences with an ion-dependent folding (Supplementary Figure S8). Low HCA scores are also observed in some enzymes, which depend on ions for their catalytic activity (e.g. one of the longest regions (224 amino acids) in the human carbonic anhydrase (P00915, HCA score of -0.62)). Apolar but non-strong hydrophobic amino acids are also particularly abundant in these low HCA score regions within standard globular domains, completing the hydrophobic clusters participating in the hydrophobic core.

Second, we reported cases of regions of contiguous amino acids with very low pLDDT values but with HCA scores typical of folded domains (9722 sequences with length greater than 100 amino acids, corresponding to 21 different proteomes; Supplementary Figure S7b-c). Only 54 regions of more than 30 amino acids are found in the E. coli proteome (Figure 7), among which a whole protein reported as a recent and rare case of emerging gene by overprinting in this bacterial species (MbiA 71; Figure 7a), small domains in multidomain proteins (RhsC - Figure 7b, YdfE -Figure 7c) or a protein segment (YjfZ - Figure 7d). RSSs are more or less well predicted by AF2 although their assembly cannot be validated. Of note however is the case of MbiA for which only a part of the RSS is predicted, while the HCA plot clearly indicates several others, associated with hydrophobic clusters typical of beta-strands. This failure of RSS prediction appears recurring in several AF2 predictions of such regions in the Plasmodium falciparum proteome, as illustrated in Figure 7e. These results indicate that regions with AF2 very low confidence do not always correspond to disordered regions, in line with studies that have compared AF2 to order/disorder predictors 69,70.

Discussion  
The remarkable progress that has recently been made in the field of structure prediction 72 owes its success to the use of machine learning approaches and the consideration of pre-existing knowledge at the protein sequence and structure levels. In particular, the wealth of evolutionary information was leveraged to an optimal level to extract key features of inter-residue contacts and distances, which have paved the way to unprecedented levels of accuracy in the prediction 27,73. Considering evolutionary information was also instrumental in the recent advances made in the field of disorder prediction 26.

Among the key questions that evolutionary-based methods applied to structure prediction cannot easily address, at least with the expected accuracy, is that of regions lacking known homologous sequences and falling outside family annotations, which constitute the dark proteome 74-76. Contrary to what it could have been expected for the globally lower conservation of IDPs/IDRs sequences (except for the conditionally folded IDPs/IDRs), the dark proteome contains an important part of non-disordered sequences 74,76. A recent survey of the human dark proteome before and after AF2 development has indicated that only a part of these non-disordered sequences was predicted with good accuracy 77, thereby supporting the ever-present need to develop tools for better characterizing the foldability potential and structural features from the only information of single amino acid sequences and applicable to any proteome, even the darkest ones.

The (non)foldability of proteins is encoded in their amino acid sequences, with two global physicochemical patterns, the absolute mean charge and the mean hydropathy, primarily accounting for the differences between two classes 78. This issue of the foldability potential was addressed here in two steps, based on the consideration of this basic hydrophobic/non-hydrophobic dichotomy, enriched by the information on local structure through the use of a two-dimensional representation of amino acid sequences. Capitalizing on the proven ability of HCA to highlight structural invariants in a context of high evolutionary divergence 78, we do not explicitly use an hydrophobicity scale especially in order to take into account at the best this dichotomy and the driving role of strong hydrophobic amino acids in the formation of regular secondary structures, regardless of their specific physico-chemical features. The first step of our procedure relies on a binary definition of foldability, with the delineation of homogeneous regions in terms of general properties related to order (foldable regions) or disorder (non-foldable regions). The second step, focusing on the foldable regions, estimates their degree of foldability in a continuum. This combined approach goes thus beyond a binary and per-residue order/disorder dichotomy and is independent of the consideration of a set of homologous sequences. *pyHCA* is in line with the spirit of polymer scaling behavior, which combines hydropathy and charge patterning and is used for characterizing the structural properties of IDPs/IDRs 79. It is also to be compared to ODiNPred, a sequence order/disorder predictor which uses a deep neural network trained on a database of an experimental, continuous-valued quantification of local disorder based on NMR chemical shifts and considering a large number of sequence features 32, among which foldable domains as predicted by *SEG-HCA*. The foldable segments defined by SEG-HCA are expected to fold spontaneously or conditionally into stable 3D structures through the participation in an hydrophobic core, while non-foldable segments correspond to full disorder, with the exception of regions whose stably fold without the need of a consistent hydrophobic core, but *e.g*. depending on ion binding 48. Conditional foldable regions, having transient residual structures or fold dependent on interactions or environment 8,10, can generally be distinguished from the autonomous folding units as these segments are often predicted as disordered by current disorder predictors 41. This category of transient disorder includes short linear motifs (SLIMs) 80 and Molecular Recognition Features (MoRFs) 81, which are generally embedded in large disordered regions. Their intermediate behavior can be highlighted using tools such as ANCHOR 82 and visualized with FELLS, an estimator of latent structures integrating *SEG-HCA* and *IUPred2* predictions 83. It is interesting to note that these sequences, which are predicted as disordered but foldable, are globally well predicted by AF2, capturing the folded state, however without detecting their structural plasticity 70.

In a global way, the HCA scoring scheme introduced here allows to appreciate the degree of foldability of protein segments, reflecting the relative abundance of loop/coil regions (disorder) and regular secondary structures (order). Thereby, we are able to disentangle the great diversity present within the IDPs/IDRs, which is reflected by a wide range of HCA score values, whereas folded domains are characterized by narrower ranges of values (Figure 1). The distinct behaviors between these two groups have also been evidenced in a recent study using a Gini index, which allows to estimate distribution uniformity 84.

Based on the HCA score, some foldable segments from DisProt clearly deviate from folded-like regions (regions *a - a’* in **Figure 1**) while having the capacity to conditionally fold. This is the case, for instance, of the segments shown in **Figure 3a to c**, with low HCA scores. These segments can thus be easily distinguished from those that are closer to a soluble, globular domain behavior (region *b* in **Figure 1**). These last ones are however generally shorter than typical well-folded globular domains extracted from the SCOPe database, which may explain that they are unable to fold stably in absence of partners. Indeed, only 48.4% of sequences from this category in DisProt have length greater than 30 amino acids (mean length 81.6 aa), against 85.1 % in SCOPe (mean length 133.3 aa). In cases of longer IDPs/IDRs from this category, amino acid composition may help to distinguish them from well-folded globular domains. Indeed, we observed that segments from DisProt of this category (region *b* in **Figure 1**) are enriched in polar amino acids that have been previously described as disorder-promoting (Gln, Lys, Ser, Glu) 85 (**Supplementary Figure S9a**). Remarkably, they have composition in strong hydrophobic amino acids comparable to that found in soluble domains. This composition, consistent with high HCA scores, thus defines a specific class of long disordered sequences, reflecting their propensity to fold upon constraint (**Supplementary Figure S10**). The example of AF4-AF9 complex, discussed in 48 also suggested that some sequences of IDPs/IDRs might be stabilized in absence of interacting partners by intra-molecular interactions mediated by sequences located at long-range distance in the protein. In this region *b*, one can also observe that the composition of well-folded soluble domains is different from that of also well-folded transmembrane beta-barrels, characterized by similar HCA scores values (**Figure 1**). These have indeed distinctive features such as dyad-repeat patterns and a high abundance in aromatic amino acids at the bilayer interface 86, allowing their accurate predictions by dedicated tools (e.g. 87,88).  Here, we also evidenced an enrichment of beta-barrel foldable segments relative to soluble domain ones in Tyr and Trp, as well as in small and polar amino acids (Gly, Asn, Ser, Thr~~,~~) (**Supplementary Figure S9a)**, consistent with previous observations 86. In the *c* region, the DisProt foldable domains can also be distinguished of well-folded alpha-helical membrane domains by their amino acid composition, as the former ones are also enriched in polar/charged amino acids (Asp, Glu, Asn, Gln, Arg, Lys, His, Ser, Thr) (**Supplementary Figure S9b)**. Examples extracted from this category of DisProt segments highlighted sequences with long hydrophobic clusters (length similar to transmembrane helices), but forming elongated, soluble coiled-coils. First attempts to develop tools for sorting sequences in the *b-c* regions, based on these amino acid composition differences, are encouraging but further investigations are needed to analyze the content of these sequences in hydrophobic clusters and understand the molecular basis of their particular structural behavior, especially in terms of both fuzziness (typified by the co-existence of several minima of free-energy content) 89,90 and frustration 91,92.

The combination of HCA score and AF2 pLDDT applied to proteome-wide analysis shed light on the relative part of each given proteome in which order is still hidden, corresponding to very low AF2 pLDDT values but with HCA scores typical of globular-like regions (regions *b - c* in **Figure 1**). Examination of particular cases of hidden order indicates that AF2 is able in some situations to predict RSSs, which correlate with hydrophobic clusters (as observed in the E. coli sequences presented in Figure 7), nonetheless without confidence in the way they are associated. The overestimation of disorder by AF2 is minimum in the case of prokaryotic proteomes (**Figure 8a**), where large amount of known 3D structures and sequences are available. In contrast, the accuracy of disorder prediction by AF2 is much lower in case of *Dictyostelium discoideum* and parasitic organisms such as *Trypanosoma cruzi*, *Leishmania infantum* and *Plasmodium falciparum*. For this latter proteome, a percentage of amino acids as high as 40.8% identified in the globular-like category by *pyHCA* correspond to AF2 very low pLDDT scores. Some hypothetical proteins from Plasmodium falciparum escape RSS prediction by AF2 and are represented as fully disordered, although they possess hydrophobic clusters with HCA scores typical of well-folded domains. In fact, the higher proportion of amino acids with very low pLDDT values in the Plasmodium falciparum proteome (46.0%) cannot be explained in a straightforward way by a higher proportion of disorder, but instead by the compositional bias and low complexity regions leading to mask the order characteristics and to leave a large number of sequences in the dark 93,94. *pyHCA* is not affected by these biases and estimates a similar foldability trend for *Plasmodium falciparum* as for other eukaryotes such as human (80-85% aa within foldable segments, **Table S1**). It allows therefore to unravel the characteristics of the hidden order, as already applied to the identification of hidden actors of the transcription machinery 95.

*pyHCA* biases mainly rely on regions which stably fold without the need of a consistent hydrophobic core (**Supplementary Figure S5**). The analysis of AF2 pLDDT scores outside of foldable segments provides therefore a useful way to evaluate the overestimation of full disorder by *pyHCA* (**Figure 8b**). At the proteome scale, this bias is minimum for *Dictyostelium discoideum* and the three parasitic organisms (in case of *Plasmodium falciparum*, 3.3% aa outside of foldable segments correspond to a very high pLDDT in AF2 predictions) while ranging to a maximum for the four prokaryotic organisms (51% in *E. coli*). Reminding that these latter proteomes are mostly covered by regions identified as foldable by *pyHCA* (84-96% aa), this overestimation of disorder by *pyHCA* is however quite low when considering the number of long regions (length higher than 30 aa) capable to fold (*e.g.,* corresponding only to 8 different proteins for *Methanocaldococcus jannaschii*).

Overall, combining *pyHCA* with AF2 provides a revised estimation of the full disorder content in proteomes, corresponding to not only non-foldable segments which are not well-predicted by AF2, but also foldable segments with HCA score lower than -4.7. The conditionally folded regions, corresponding to foldable segments with higher HCA score values, are thus discarded from this estimation. Compared to previous studies (*e.g.* 96), we thus suggest a lower disorder content, as long IDRs (length higher than 30 aa) were found in 12 to 40.4% of long proteins (length higher than 60 aa) in the eukaryotic proteomes in AFDB, and in 0.6 t 4.7% for the prokaryotic ones. Our approach also allows to explore the order/disorder content of proteomes in relation to organism ecological traits, in a complementary way to previous works 23,97. In addition to a specific behavior of parasitic organisms within eukaryotes (see above and **Figure 6**) that remains to be explained, our study also detected a particularly low disorder content in the proteome of the hyperthermophilic archaeon *Methanocaldococcus jannaschii*, likely related to the high thermal stability constrained by the environmental conditions.

Finally, the scoring scheme offered by *pyHCA* can be used for understanding the protein evolutionary trajectories at the proteome level. It is particularly well-suited to study the structural properties of proteins encoded by *de novo* emerginggenes and how such properties have influenced their early emergence and long-term retention. In particular, it can bring new light to the debate of whether *de novo* proteins have much intrinsic disorder 98 or are aggregation-prone 99 and whether retention of *de novo* gene precursor is driven by such properties or remains a stochastic process 100,101. Several works have already used the concept of foldable segments to investigate such properties 102-104, and a recent work considering a preliminary version of HCA scoring systems has evidenced that most yeast intergenic ORFs contain the elementary building blocks of protein structures 49. The example of *E. coli* MbiA, an overlapping (protein-coding) orphan gene which has recently evolved by overprinting and was shown to share the structural properties of globular-like domains although not predicted by AF2 (**Figure 7a**), well illustrates the interest of our approach to decipher structural features in absence of homologs and uncover dark sides of protein evolution.

Data availability

All data and scripts used in the present work are available in a GitHub repository at https://github.com/DarkVador-HCA/Order-Disorder-continuum. The package *pyHCA* is provided at <https://github.com/DarkVador-HCA/pyHCA> under the CeCILL-C license agreement.

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**dataset class sequences aa sequences with FS sequences without FS aa in FS aa outside of FS**

SCOPe a 2507 347268 2507 (100.0) 0 (0.0) 309100 (89.0) 38168 (11.0)

SCOPe b 2511 366584 2511 (100.0) 0 (0.0) 322970 (88.1) 43614 (11.9)

SCOPe c 2841 688643 2841 (100.0) 0 (0.0) 633955 (92.1) 54688 (7.9)

SCOPe d 3026 455400 3026 (100.0) 0 (0.0) 410347 (90.1) 45053 (9.9)

DisProt v8.0.2 3166 209812 2418 (76.4) 748 (23.6) 105390 (50.2) 104422 (49.8)

OPM polytopic 1330 186220 1328 (99.8) 2 (0.2) 181396 (97.4) 4824 (2.6)

OPM bitopic 203 4390 202 (99.5) 1 (0.5) 4025 (91.7) 365 (8.3)

OPM beta 165 37320 164 (99.4) 1 (0.6) 33986 (91.1) 3334 (8.9)

**Table 1. Foldable segments (FS) in the non-redundant sequence datasets.**

Total number of sequences and amino acids (aa) of each dataset, as well as their number (and percentage) of sequences with and without foldable segment(s), and the number (and percentage) of residues found in or outside of foldable segments. The datasets are those of soluble domains with known 3D structures (SCOPe), transmembrane domains with known 3D structure (OPM) and disordered segments (DisProt v8.0.) (see *Materials and Methods* for details). OPM classes have been shortened to polytopic for alpha-helical polytopic domains, bitopic for alpha-helical bitopic domains and beta for beta-barrels.

Figure and table captions

**Figure 1: Distribution of the HCA scores calculated for the foldable segments from disordered protein regions (DisProt) and ordered protein domains (soluble domains from SCOPe and membrane domains from OPM).** The considered non-redundant datasets are: DisProt v8.0.2 (HCA scores ranging from -6.95 to 9 and peaking at 0.5 and 8.1), SCOPe (HCA scores ranging from -4.67 to 9 and peaking at 1.4 and 8.1), OPM alpha-helical polytopic and bitopic categories (HCA scores ranging from -3.32 and 0.97 to 9 and peaking at 4.9 and 8.3, respectively) and OPM beta-barrel category (HCA scores ranging from -3.86 to 9 and peaking at 1.2. And 7.9). Several thresholds were fixed from these distributions, allowing to define four main classes of foldable segments (a-d). The threshold of 3.5 better discriminates the HCA scores of SCOPe foldable segments from those of alpha-helical OPM foldable segments. -1 is the threshold above which are found 95% of the HCA scores computed for foldable segments from SCOPe protein domains.

**(a)** Below -4.7: only foldable segments from disordered regions, **(b)** from -1 to 3.5, globular soluble segments (SCOPe) or membrane beta-barrels (OPM**)**, **(c)** from 3.5 to 6.7, segments from alpha-helical transmembrane domains, **(d)** above 7.6, foldable segments composed of only one hydrophobic cluster. Two intermediate regions were also defined: **(a’)** between -4.7 and -1, in this range 62% of segments are from SCOPe and 36% from DisProt, they represent only 5% of SCOPe and 11% of DisProt; **(c’)** between 6.7 and 7.6, foldable segments with a dense composition in hydrophobic clusters and of short length (96% are shorter than 100 aa).

**Figure 2: Foldable segments extracted from the SCOPe and OPM databases: HCA scores, HCA 2D plots and experimental 3D structures.** The HCA plots of the sequences extracted from the SCOPe (all beta) **(a)** and OPM **(b)** databases are shown, with their foldable segments boxed (dashed lines). The corresponding HCA scores are reported below the boxes. Special symbols used in the HCA representation and the way to read the sequences and regular secondary structures (RSSs) are indicated in the inset. Positions of the RSSs, as experimentally observed from the corresponding 3D structures (ribbon representations), are reported in color below the HCA plots. The position of the 13 aa-long segment which has been removed for the OPM protein sequence in order to only keep membrane domains is highlighted in red (see *Materials and Methods* for details).

**Figure 3: Foldable segments extracted from the DisProt database: HCA scores, HCA 2D plots and experimental 3D structures.** The HCA plots of the sequences extracted from the DisProt database are shown (arrows indicate the N- or C-terminal limits of the DisProt segments). The foldable segments are boxed (dashed lines). The corresponding HCA scores are reported below the boxes. Special symbols used in the HCA representation and the way to read the sequences and regular secondary structures (RSSs) are indicated in the inset. 3D structures (in green, with their pdb identifiers) have been solved for only a few of the DisProt sequences (moreover often limited to only a part of the regions), stabilized by interaction with a partner (grey surfaces for two of them). One of the FXFG repeats of yeast NUP2 (red circles) has been modeled based on the experimental 3D structure of such a repeat in complex with importin beta-1 (*pdb 1O6O*).

**Figure 4. Distribution of AlphaFold2 per-residue prediction confidence scores (pLDDT) within and outside of foldable segments.**

**(a)** Distribution of residues from the AFDB 21 proteomes within foldable segments (top, 81.9 % of the total) and outside of foldable segments (bottom, 18.1% of the total) in the different categories of AF2 prediction confidence. **(b)** Distribution of pLDDT scores for residues within (dark grey) and outside of (light grey) foldable segments. The AF2 prediction confidence categories are highlighted following the same color code as in (a).

**Figure 5. Relationship between HCA score and AlphaFold2 per-residue prediction confidence score for foldable segments.**

**(a)** AFDB v1 21 proteomes, **(b-f)** 5 representative proteomes from AFDB v1 (see **Figure 6** and **Supplementary Figure S6** for details).

**Top:** Representation of the number of residues by the barplot (axis on the left, base 10 logarithmic scale) and number of foldable segments by the grey line and points (axis on the right, base 10 logarithmic scale) belonging to foldable segments with HCA scores in a given interval. **Bottom**: percentage of residues in each AF2 prediction confidence categories for each HCA score range. Below the barplot are represented the foldable segment types that are likely to be found according to their HCA score, as defined in **Figure 1**: (a) disorder only, (a’) mainly disorder, (b) globular domains/ membrane domains (beta-barrel), (c) membrane domains (alpha-helical), (d’) mainly foldable segments with one hydrophobic cluster, (d) foldable segments with one hydrophobic cluster.

**Figure 6. Principal component analysis (PCA) for the AFDB v1 21 proteomes according to HCA score and AF2 prediction confidence.**

The 64 variables correspond to the proportion of residues of foldable segments found in each pLDDT category (very high, confident, low, very low) for each of the HCA score intervals (from -7 to 9, in steps of 1) (as represented in **Figure 4**).

The plotrepresents the 21 AFDB proteomes in the factor map formed by the first and second principal components (explaining 59.24% of the dataset variability). The correlation circle of the variables is represented in **Supplementary Figure S6**. Five clusters of proteomes were defined using hierarchical clustering on the first four principal components (explaining 83% of the dataset variability).

ARATH: *Arabidopsis thaliana*, CAEEL: *Caenorhabditis elegans*, CANAL: *Candida albicans*, DANRE: *Danio rerio*, DICDI: *Dictyostelium discoidum*, DROME: *Drosophila melanogaster*, ECOLI: *Escherichia coli*, HUMAN: *Homo sapiens*, LEIIN: *Leishmania infantum*, MAIZE: Zea mays, METJA: *Methanocaldococcus jannaschii*, MOUSE: *Mus musculus*, MYCTU: *Mycobacterium tuberculosis*, ORYSJ: *Oryza sativa*, RAT: *Rattus norvegicus*, SCHPO: *Schizosaccharomyces pombe*, SOYBN: *Glycine max*, STAA8: *Staphylococcus aureus*, TRYCC: *Trypanosoma cruzi*, YEAST: *Saccharomyces cerevisiae.*

**Figure 7. Examples of very low AlphaFold2 confidence scores for globular-like domains.**

The examples shown are segments of contiguous amino acids with very low pLDDT values, fully included in foldable segments with HCA scores typical of well-folded domains (boxed in orange on the HCA plots, with the corresponding HCA scores and segment lengths (in aa) reported below). Other foldable segments of the sequences, taken from UniProt, are boxed in grey. Special symbols used in the HCA representation and the way to read the sequences and RSSs are indicated in the inset. The AF2 3D structure models of the corresponding segments are highlighted with orange circles, extracted from the models of the whole proteins (ribbon representations), colored according to the AF2 per-residue confidence metric. Positions of the RSSs, as predicted by AF2, are reported in color on the HCA plots.

**Figure 8. Classification of the AFDB v1 21 proteomes according to probable overestimation of disorder by AF2 and pyHCA in foldable and non-foldable segments, respectively.**

a) Proteomes are sorted in a top-down scale (ranging from 0 to 60%) according to the increasing proportion of residues predicted with a very low confidence by AF2 in segments with HCA scores typical of soluble globular HCA scores (category *b* defined in **Figure 1**). b) Proteomes are sorted in a top-down scale (ranging from 0 to 60%) according to the increasing proportion of residues found in non-foldable segments that are predicted with a very high confidence by AF2. For details on the proportion of residues in non-foldable segments, see **Supplementary Table S1** and **Supplementary Figure S3.**

The proteome of the archaeon *Methanocaldococcus jannaschii* harbors the lowest number of long regions (length > 30 aa) composed only by residues predicted with a very high confidence by AF2, included in non-foldable segments. The UniProt sequence accession numbers and the boundaries of these 8 regions are as follows: Q60356 [171-207], Q57673 [15-83], Q58130 [243-276], Q58560 [108-139], Q58991 [253-283], Q60317 [32-64], Q57676 [24-54] and Q58814 [28-61]. All have at least one homologous sequence with known 3D structure (data not shown).