The extent to which proteins are protected from hydrogen deuterium exchange (HDX) provides valuable insight into their folding, dynamics and interactions. Characterised by mass spectrometry (MS), HDX benefits from negligible mass restrictions and exceptional throughput and sensitivity but at the expense of resolution. Exchange mechanisms which naturally transpire for individual residues cannot be accurately located or understood because amino acids are characterised in differently sized groups depending on the extent of proteolytic digestion. Here we report HDXmodeller, the world’s first online webserver for high-resolution HDX-MS. HDXmodeller accepts low-resolution HDX-MS input data and returns high-resolution exchange rates quantified for each residue. Crucially, HDXmodeller also returns a set of unique statistics that can correctly validate exchange rate models to an accuracy of 99%. Remarkably, these statistics are derived without any prior knowledge of the individual exchange rates and facilitate unparallel user confidence and the capacity to evaluate different data optimisation strategies.
Hydrogen deuterium exchange mass spectrometry (HDX-MS) is a biophysical technique that probes time-dependent mass changes in proteins arising from the spontaneous exchange of labile protons for deuterium in D₂O solvent. Information on the kinetics of isotope exchange can reveal important information on protein dynamics, structure, and interactions. Recent commercialisation has facilitated greater accessibility of HDX-MS to non-specialists contributing to an upsurge in popularity of the technique. HDX-MS benefits from high sensitivity and throughput and this, coupled to an exceptional mass range and a tolerance for background contaminants such as lipids, has helped HDX-MS become established as an enabling method to investigate challenging protein systems of high biological importance. A significant limitation of HDX-MS is its poor resolution and while HDX occurs for every amino acid except proline, exchange rates are evaluated by MS as time-dependent mass shifts in proteolytically cleaved peptides. Isotope uptake cannot be pinpointed to individual residues and important metrics of protein stability and folding, such as HDX protection factors (PFs), cannot be determined. HDX-MS is limited to asking qualitative questions about changes in protein behaviour such as those arising from point mutations or binding. While this information can provide significant insight into protein function, the utility of HDX-MS would be extended significantly if exchange rates could be characterised for each residue.

Extracting residue resolved exchange rates from experimental HDX-MS data requires some form of exchange rate modelling. Unfortunately, modelling HDX-MS data has proven challenging and in the few cases where systematic validation has been provided, the accuracy of modelled outputs have not been encouraging. Underdetermination is the main limitation as the variables typically greatly outnumber the constraints such that many different microscopic exchange rate models are equally consistent with an experimental profile. A potential remedy for this limitation is to use additional restraints encoded by the peptide ion envelopes which can reveal clues regarding the distribution of isotope along a peptide. However, the interpretation of ion spectra can be challenging and any changes in peak shapes arising from extraneous isotope exchange may be impossible to properly account for. A related and often overlooked problem with modelling HDX-MS data relates to insufficient understanding of what constitutes appropriate input data for modelling, beyond an acceptance that a high peptide redundancy is preferable. Differences in the success of exchange rate modelling should be anticipated for different datasets, but it is currently impossible to deduce the utility of any given input file or evaluate the accuracy of a model output without prior knowledge of the residue resolved rates. An acceptance of the challenges associated with modelling HDX-MS data, combined with an inability to validate exchange rate models, has resulted in scepticism towards these approaches. High-resolution HDX-MS would represent a significant breakthrough in the field but at present this challenge remains unresolved.

Here we report HDXmodeller the world’s first online webservice for high-resolution HDX-MS. HDXmodeller is a fully automated advanced programming tool capable of calculating residue resolved HDX protection factors (PFs) from peptide level HDX-MS input data. Through an extensive search of different algorithms and procedures, HDXmodeller is able to provide the most accurate high-resolution exchange rate models currently reported, depending on the input. The standout feature of HDXmodeller however, is an auto-validation function that takes into consideration the quality of the entire optimisation process through the use of a novel method based on a covariance matrix over different replicates. Crucially, the auto-validation feature can quantify the fidelity of the model output to an accuracy of 99% without prior knowledge of the underlying residue exchange rates. HDXmodeller will provide the growing number of HDX-MS practitioners easy access to high-resolution HDX-MS along with essential insight into the reliability of their data.

Results

The optimisation method. The time-dependent mass shifts of proteolytically cleaved peptides, commonly reported as the ratio of the observed mass change to the total possible mass change or relative fractional uptake (RFU), provide a potential framework for exchange rate modelling because multiple peptides typically sample the same amino acids. Global optimisation of HDX-MS data should, therefore, be feasible but will depend on poorly understood parameters relating to the quality of the input data. A bottom-up optimisation strategy was utilised to develop HDXmodeller wherein HDX-MS data were built using experimental protein-peptide maps onto which RFU were projected using predefined exchange rates for each residue. This allowed the preparation of reference HDX-MS data for which the underlying were known, such that the accuracy of HDXmodeller could be evaluated fully for every residue within a dataset across a wide range of exchange rates. Simulated datasets were used to be certain that all reference data are error-free thereby providing an unambiguous benchmark based on currently accepted HDX theory. To ensure that simulated datasets best maintained the overall character of HDX all lnP values were simulated from protein structures using well-known expressions of protein HDX behaviour. (Methods, Supplementary Fig. 1).

HDXmodeller is based on constrained nonlinear optimisation and utilises sequential quadratic programming (SQP) to solve an objective function defined by the error between the model and input RFU across all timepoints. Minimisation is initiated with random initial guess data for which are then optimised sequentially with respect to the objective function. Multiple replicate runs are made each with different initial guess values for after which the data from all replicates are combined. In all cases is expressed as the natural log of the PF (lnP) which considers the chemical exchange rate of each residue, Eq. (1) (Methods).

\[
PF = \frac{k_{\text{ch}}}{k_{\text{obs}}}
\]

Critical to the success of HDXmodeller is an advanced algorithm which manages the optimisation process, and which allows automatic handling of the constraints over the objective function in every iteration. During the development of HDXmodeller, many different reference datasets were tested across different peptide maps and varying RFU timepoints. Following optimisation, the performance of HDXmodeller was remarkable with R² between model and reference lnP >0.9 in some instances and with >80% of the projected values within ±1 lnP of reference data. The ability of HDXmodeller to accurately calculate lnP values varied throughout different HDX-MS datasets (Fig. 1a, b). Changes in the fidelity of modelled lnP was anticipated, however, and presumably reflects variations in redundancy, which reports the number of different peptides that are occupied by each residue. High redundancy is considered important for good optimisation outcomes as it should constrain the range of available increasing precision across multiple optimisation replicates. There is also potential for the variation introduced from poorly constrained regions to propagate into the amino acids of neighbouring peptides, such that the elimination of
peptides that comprise these regions may be preferable. To account for this, a quality control measure was introduced to score peptides based on their occupancy, which is the sum of residue redundancies for any peptide, or density, divided by the number of residues excluding proline and the amino-terminus (Eq. (2)), where $D$ and $n$ represent the density and total number of amino acids numbers, respectively.

$$\text{Occupancy} = \frac{1}{n} \sum_{k=1}^{n} D_k \quad (2)$$

An occupancy threshold of 2.5 was deemed optimal for the identification of weak peptides that should be excluded prior to analysis (Fig. 1h–i, Supplementary Fig. 2). Where possible, HDX-MS data were also split into different subsections and each subsection optimised independently.

Because the accuracy of HDXmodeller naturally varies in response to changes in the quality of input data, subdivided inputs provide more scope for guiding potential users on anticipated modelling outcomes at a local level. However, the ability to provide this information requires a greater understanding of the relationship between input data and the accuracy of the model outputs. No significant deterioration in the performance of HDXmodeller was observed when protein data was optimised by subsection and in many cases, this improved the accuracy of the model lnP (Fig. 1a–d).

We next investigated different HDX-MS datasets to understand aspects that had the greatest impact on the optimisation. Given the dependence between model accuracy and input data, guidance regarding anticipated optimisation outcomes is essential for user confidence. Contrary to our expectations, redundancy is a poor guide of HDX-MS data quality. Although it is apparent that the overall redundancy should exceed a certain threshold it has little additional bearing on the quality of model outputs. HDX-MS datasets with the same overall redundancy scores can yield markedly different errors in lnP estimations and data with exceptionally high redundancy can perform significantly less well than data for which the redundancy score is low (Fig. 1c–g). We developed our own metrics to score input data that were based on redundancy, but which also considered the overall peptide distribution. Many different methods were developed and tested but predicting the quality of modelling outcomes from the HDX-MS peptide maps was not possible. To understand this further three different RFU datasets were prepared and projected onto an identical peptide map prior to optimisation by HDXmodeller. Despite these data being built from the same peptides there were large differences in the capacity of HDXmodeller to accurately model the lnP (Supplementary Fig. 3). This indicates that modelling outcomes cannot be predicted at the peptide level alone and have a strong additional dependence on the RFU. This is a problem because RFU values cannot be easily separated from the underlying $k_{obs}$ of each residue on which they depend. The capacity to successfully gauge modelling outcomes deductively from HDX-MS data may not, therefore, be possible as it could rely on knowledge of the microscopic exchange rates for which HDX-MS cannot provide direct access.

The auto-validation method. Although it was not possible to predict the quality of modelling outcomes directly from input data, gauging the accuracy of modelled lnP post-optimisation may be feasible. To achieve this, we developed a novel auto-validation matrix that calculates the pair-wise correlation

![Fig. 1 Example optimisation outputs for HDXmodeller.](https://doi.org/10.1038/s42003-021-01709-x)
coefficients for every replicate over the course of a whole optimisation run. A library of reference HDX-MS data was prepared comprising of 30 different input files encompassing a total of over 500 peptides with a broad range of different sizes, redundancies, peptide distributions, RFU and lnP (Fig. 2a, b). Each dataset was submitted for optimisation by HDXmodeller and an auto-validation matrix prepared for each output from which the mean correlation coefficient (R-matrix) was obtained (Fig. 2d, e). The mean correlation coefficients were then recalculated for each replicate run but with the values obtained using the reference lnP values (R-reference) rather than pairwise between replicates (Methods). The R-matrix and R-reference scores were then compared across all datasets to see if the accuracy of the optimisation could be predicted from the matrices. On comparison, the ability of the auto-validation matrices to predict the accuracy of the optimisation was outstanding with a R² value of 0.99 (Fig. 2c). We also compared the accuracy of different central tendencies and clustering approaches including the mean, median, and k-means clustering. For data bins with R-matrix values of 1.0–0.7, 0.5–0.69, and 0–0.49, the median was the best performing with regard to overall accuracy and ability to distinguish between the different R-matrix bin classifications (Fig. 2f). Without prior knowledge of the underlying exchange rates the R-matrix score can provide highly accurate information on the fidelity of modelled outputs allowing HDXmodeller to assign confidence to the models. Furthermore, since the matrices operate post-optimisation they have the additional advantage of providing potential users with the flexibility to test and score different optimisation strategies for their data.

The workflow for HDXmodeller entails data optimisation following calculation of R-matrix values to ascertain the accuracy of the modelled lnP. Data should also be submitted in subsections to provide more local guidance on the fidelity of the outputs. However, even poorly restrained input data, that yield low R-matrix values, can contain many highly accurate model lnP accounting for 50% of the residues with values that are within ±1.0 lnP of the true value. Conversely, many datasets with strong optimisation outcomes contain a small fraction of outlying data with lnP values >2.0. It would be useful therefore, if the capabilities of HDXmodeller could be extended so that it was capable of capturing individual residues with highly accurate lnP or was able to highlight outliers. We prepared optimisation histograms for each residue which report the density of lnP values over all replicates. These histograms serve as the primary guide for the accuracy of each residue and in-house benchmarking has shown that a high degree of confidence should be ascribed to residues that produce unimodal histograms with narrow distributions.

However, most residues with accurate lnP yield more varied histograms from which the accuracy of the output is more difficult to assign. This is due to the requirement to report a central tendency which can occupy a range of distances from the true value depending on the details of each optimisation (Fig. 3a, b). We tested a range of metrics to report the accuracy of the model.
InP for each residue, but none were successful. Ultimately, we returned to the auto-validation matrices and used them to report the change in the $\hat{R}$-matrix value ($\Delta \hat{R}$-matrix) for a dataset following the sequential removal of each residue. A positive $\Delta \hat{R}$ value should result following the deletion of residues with low error and the opposite should be true for less accurate amino acids. Equivalent calculations were then made but with $\Delta \hat{R}$ calculated against the reference InP (Methods). In most datasets the trend in $\Delta \hat{R}$-reference was mirrored by $\Delta \hat{R}$-matrix showing that information regarding the accuracy of each residue could be predicted from the $\Delta \hat{R}$-matrix score (Fig. 3c). We then calculated the direction of $\Delta \hat{R}$-matrix for all residues in the HDX-MS reference library and investigated the capacity of this metric to rank individual residues by their error (Methods). The results indicated that the error in the model InP of each residue could assign positive and negative $\Delta \hat{R}$-matrix values with an accuracy of 72%. Over the whole HDX-MS reference library residues with positive $\Delta \hat{R}$-matrix values contained 60% more highly accurate InP with RMSE <1.0 InP. Conversely, outlier InP values with errors >2.0 InP were approximately three times more likely to occur in residues with a negative $\Delta \hat{R}$-matrix (Fig. 3d). Gauging the accuracy of modelled data at residue resolution is extremely challenging and no readymade method can be successfully applied to this problem. Nevertheless, the $\Delta \hat{R}$-matrix value of a residue can provide important clues regarding model accuracy and confidence to the lnP of individual amino acids.

Discussion
HDXmodeller is available at https://hdxsite.nms.kcl.ac.uk/. The website provides guidance on data submission and processing along with all of the reference datasets used for code development including all the simulated lnP values and their associated peptide/RFU data. Users should upload their HDX-MS data as text files reporting the RFU of each isotope labelling time. It is critical that users correct their HDX-MS data for extraneous exchange prior to optimisation. Results obtained from data where the RFU have not been corrected for back and forward exchange will produce unreliable results and we recommend the method of Zhang for data correction. After users have uploaded their input files they are sent to a production area for optimisation and the outputs returned to users by email once the calculations are complete.

HDXmodeller was specifically developed with a recognition of the innate variations in the quality of input data, with regard to differences in the restraints and to our knowledge is the first HDX-MS optimisation method capable of reporting on model fidelity. The exceptional accuracy with which HDXmodeller is able to validate HDX-MS optimisation runs allows users to adopt a sandbox approach to modelling their data, and we encourage potential users to test different input and optimisation strategies. However, after testing many different methods and input files in-house, our recommended workflow for the use of HDXmodeller is as follows. Prior to a full production run, we recommend that protein data is first split into different subsections which should then be submitted as separate jobs. Subsections may be present due to natural breaks in the HDX-MS data or created after the elimination of peptides with low occupancy or following the deletion of bridging peptides that connect two otherwise independent subsections. https://hdxsite.nms.kcl.ac.uk/Utility: A utility workspace in HDXsite contains many useful ancillary tools including a $k_{ch}$ calculator ($k$-intrinsic), an R-matrix evaluator for customised domains (R-evaluator) and a tool for determining peptide occupancy (Occupier), which can be instructive in the preparation of data subsections. It is difficult to provide a general guide for the preparation of data subsections. However, since each subsection yields a unique validation score we recommend that users attempt to maximise data subdivision where possible as this will best enable localised validation feedback throughout input data. Nevertheless, users should also refrain from needless peptide deletion in the preparation of data subsections as this may negatively impact the level of constraint and the accuracy of model outputs (Fig. 4a). Our recommendation is to test different input strategies utilising the auto-validation outputs as a guide. After the preparation of input files, we recommend that data are first subjected to a short evaluation step involving optimisation for 10 replicates with default settings. Users will also need to upload a separate $k_{ch}$ file for these calculations which can be prepared from sequence data within our HDXutilities workspace. Following this evaluation step the R-matrix score of each dataset should be inspected for guidance on anticipated modelling outcomes at the production phase. In-house benchmarking has
Occupancy scores can also assist in the preparation of data subsections. Peptide filtering should also be performed using the peptide occupancy tool to identify poorly constrained peptides which we recommend eliminating from input production runs with evaluation and production R
datasets to be split into different subsections and each subsection submitted as a separate job. Peptide filtering should be performed using the peptide occupancy tool to identify poorly constrained peptides which we recommend eliminating from input files. The deletion of weak peptides with poor occupancy scores can also assist in the preparation of data subsections. b A quality evaluation step is recommended involving 10 replicate calculations using default settings. Following the evaluation stage, the Δ̂-matrix value of each output should be inspected for guidance relating to anticipated accuracy at the production phase. Production runs for data that obtain Δ̂-matrix values <0.5 at the evaluation stage is not recommended (refer to main text). c Production runs entail data optimisation using 50 replicates and users should inspect all outputs once the calculations are complete. HDXmodeller prepares several data outputs including various text files as well as plots of the lnP of each residue along with the interquartile range. A range of plots for data validation and scoring are also prepared, including the correlation matrix for the whole subsection as well as optimisation histograms and the Δ̂-matrix plot for all residues.

Additional options for data that is predicted to optimise poorly at the production phase is to merge different subsections into a single file and submit the combined data as one input. Some variations in modelling outcomes can be expected depending on whether subsections are submitted separately or merged and submitted as a single file. Following the data evaluation or production run, information regarding the quality of the optimisation for each subsection in the combined input can be gained using our stand-alone R-evaluator tool, located in the HDXutilities workspace. This tool can perform all matrix calculations on users-defined regions of a single file with no loss in accuracy provided that the evaluated regions represent discrete subsections (Supplementary Fig. 5). The matrix evaluator tool has additional applications, and users should be alerted to residues in either the evaluation or production stages with high lnP values that also display exceptionally broad inter-quartile range spanning 20–30 lnP. This may be indicative of data collapse for these residues and can occur in particular if the dataset contains insufficient RFU sampling at long timepoints and is a consequence of having a weak constraint on the lower optimisation bound (Methods). Ideally the longest isotope labelling timepoint should achieve an RFU as close to 1.0 as possible. This effect can generally be remedied if data subsections are combined and submitted as a single file with Δ̂-matrix values <0.5 at the evaluation stage is not recommended (refer to main text). c Production runs entail data optimisation using 50 replicates and users should inspect all outputs once the calculations are complete. HDXmodeller prepares several data outputs including various text files as well as plots of the lnP of each residue along with the interquartile range. A range of plots for data validation and scoring are also prepared, including the correlation matrix for the whole subsection as well as optimisation histograms and the Δ̂-matrix plot for all residues.

A final resort for data which is predicted to have a poor optimisation outcome is for users to continue to the production phase but only interpret lnP with positive Δ̂-matrix values. However, for data with poor scores our main recommendation is for the acquisition of additional experimental data.

For HDXmodeller production runs we recommend 50 replicates with default settings. Optimisations performed with >50 replicates or >1000 maximum iterations are unnecessary, and all in-house tests performed have reported no gains in accuracy for optimisations that exceed these values. Following optimisation, users will receive a compressed file containing their outputs comprising of various raw data files and interactive graphs summarising their results. Users will receive plots of the residue lnP with projected inter-quartile range along with different validation graphs including separate optimisation histograms for all residues, the auto-validation matrix and R̂-matrix score and a plot of the Δ̂-matrix values for each amino acid. The Δ̂-matrix score should first be inspected as a guide to the overall accuracy of the lnP across a data subsection, followed by examination of the optimisation histograms and Δ̂-matrix values for information regarding accuracy on the residue level. For datasets that achieve high Δ̂-matrix scores (>0.7) in-house benchmarking has reported a high abundance (60–85%) of highly accurate lnP that are within ±1.0 lnP of the true values. While a reduction in accuracy is expected for data with Δ̂-matrix scores <0.5 many of these outputs have been shown to contain >50% highly accurate lnP following in-house testing. Insight into the accuracy of model outputs at the residue level should first be understood by inspection of the optimisation histograms of all residues. Subsidiary information regarding residue resolved accuracy can also be obtained from examination of the Δ̂-matrix values for each residue (Fig. 4c).

Our motivation for the development of HDXmodeller was to provide a user-friendly fully automated online platform allowing high-resolution characterisation of proteins by HDX-MS. It is our aim to restore the fine details of HDX that are unfortunately lost.

Fig. 4 Recommended workflow for HDXmodeller. a Input files should be in the form of RFU for each isotopic labelling timepoint and peptide that have been corrected for back and forward exchange. To increase the resolution to which optimisation outputs can be validated it is recommended for whole datasets to be split into different subsections and each subsection submitted as a separate job. Peptide filtering should be performed using the peptide occupancy tool to identify poorly constrained peptides which we recommend eliminating from input files. The deletion of weak peptides with poor occupancy scores can also assist in the preparation of data subsections. b A quality evaluation step is recommended involving 10 replicate calculations using default settings. Following the evaluation stage, the Δ̂-matrix value of each output should be inspected for guidance relating to anticipated accuracy at the production phase. Production runs for data that obtain Δ̂-matrix values <0.5 at the evaluation stage is not recommended (refer to main text). c Production runs entail data optimisation using 50 replicates and users should inspect all outputs once the calculations are complete. HDXmodeller prepares several data outputs including various text files as well as plots of the lnP of each residue along with the interquartile range. A range of plots for data validation and scoring are also prepared, including the correlation matrix for the whole subsection as well as optimisation histograms and the Δ̂-matrix plot for all residues.
as a consequence of characterisation by mass spectrometry. The most prominent feature of HDXmodeller is its capacity for post-optimisation validation which provides users with important feedback on the anticipated accuracy of modelled outputs. The auto-validation feature uses a covariance matrix to compare pairwise optimisation replicates and from this HDXmodeller can quantify the degree to which input data is constrained considering the peptide map, RFU distribution, and the underlying exchange rates. We propose that the auto-validation matrix is an image of the error surface confronted during optimisation and that high R-matrix scores are indicative of optimisations where the global minimum can be found more readily. We expect continued development of HDXmodeller to increase overall performance and model accuracy as well as enhance the capacity to assign confidence to the exchange rates of individual residues. The overarching objective is for HDXmodeller to become a routine tool for all HDX-MS post-processing workflows allowing the maximum possible understanding of protein function by HDX-MS.

Methods

Preparation of reference HDX-MS data. Reference HDX-MS data were prepared using previously obtained HDX-MS peptides maps of alpha lactalbumin, barnase, enolase, and serum amyloid P component with lnP simulated according to pre-known expression of protein HDX behaviour3,23. The simulated lnP were then used to calculate kobs for each residue allowing the RFU of each peptide to be determined according to the following polynomial function where, n and t represent number of amino acids and experimental time point, respectively (Eq. (3)).

\[ \text{RFU} = \frac{1}{n} \sum_{i=1}^{n} 1 - \exp(-k_{\text{obs}} t) \]  

(3)

RFU values were projected at 0.25, 1, 5, 20, 60, 240, and 480 min with proline reference RFU values being assigned from protein structures according to an in-house version of a well-known expression of protein HDX behaviour24. The simulated lnP were then used to calculate kobs for each residue allowing the RFU of each peptide to be determined according to the following polynomial function where, n and t represent number of amino acids and experimental time point, respectively (Eq. (3)).

The maximum possible understanding of protein function by HDX-MS.

Auto-validation matrix. The validation score (R-matrix) is calculated in the terms of pairwise correlation coefficients (R) between the lnP values of replicate runs and is based on a covariance matrix (C) as defined in Eq. (6) where, R and C represent the respective correlation coefficient and covariance matrix between replicates i and j, and Eq. (7) where PE and the means including model lnP values (x) for all replicate runs and pair-wise correlation coefficient (R) values, respectively.

\[ R_{ij} = \frac{\sum_{k=1}^{n} (x_{ik} - \mu_i)(x_{jk} - \mu_j)}{\sqrt{\sum_{k=1}^{n} (x_{ik} - \mu_i)^2 \sum_{k=1}^{n} (x_{jk} - \mu_j)^2}} \]  

(6)

Following pairwise analysis R-matrix is reported as the arithmetic mean of all R values in the matrix (Eq. (8)), where R is the correlation coefficient between different replicate runs in the matrix and j is the total number of replicate runs.

\[ R_{\text{matrix}} = \frac{1}{j} \sum_{j=1}^{j} R_{ij} \]  

(8)

Auto-validation matrix. The validation score (R-matrix) is calculated in the terms of pairwise correlation coefficients (R) between the lnP values of replicate runs and is based on a covariance matrix (C) as defined in Eq. (6) where, R and C represent the respective correlation coefficient and covariance matrix between replicates i and j, and Eq. (7) where PE and the means including model lnP values (x) for all replicate runs and pair-wise correlation coefficient (R) values, respectively.

\[ R_{ij} = \frac{\sum_{k=1}^{n} (x_{ik} - \mu_i)(x_{jk} - \mu_j)}{\sqrt{\sum_{k=1}^{n} (x_{ik} - \mu_i)^2 \sum_{k=1}^{n} (x_{jk} - \mu_j)^2}} \]  

(6)

Following pairwise analysis R-matrix is reported as the arithmetic mean of all R values in the matrix (Eq. (8)), where R is the correlation coefficient between different replicate runs in the matrix and j is the total number of replicate runs.

\[ R_{\text{matrix}} = \frac{1}{j} \sum_{j=1}^{j} R_{ij} \]  

(8)
the ΔR-matrix scores. The ΔR-matrix direction was calculated for all residues in the reference library accounting for almost 800 amino acids. For each residue the lnP RMSE was also calculated using the reference lnP values from which each dataset was constructed. A receiver operating classification (ROC) curve was then prepared which yielded an AUC value of 0.72 indicating that the error in the lnP of each residue could correctly classify the direction of ΔR-matrix to an accuracy of 72%. Data were also pooled for amino acids depending on the direction of their ΔR-matrix scores and histograms prepared for the RMSE error with bin sizes of 1 lnP. The histograms demonstrated that highly accurate model lnP with RMSE < ±1.0 were 60% more frequent for residues with positive ΔR-matrix whereas outlier amino acids with lnP > ±2.0 were 300% more likely in residues for which ΔR-matrix was negative.

Statistics and reproducibility. ROC plot statistics were calculated using embedded macros in SigmaPlot v14 (Systat Software Inc. USA) and default settings. Data were ranked according to their RMSE and classified as either positive or negative depending on the direction of the ΔR-matrix.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
All data used in this study is available to download https://hdxsite.nms.kcl.ac.uk/.

Code availability
All codes may be accessed for use as is from our webserver https://hdxsite.nms.kcl.ac.uk/.

Received: 20 February 2020; Accepted: 29 December 2020; Published online: 15 February 2021

References
1. Zhang, Z. & Smith, D. L. Determination of amide hydrogen exchange by mass spectrometry: a new tool for protein structure elucidation. Protein Sci. 2, 522–531 (1993).
2. Zheng, J., Strutzenberg, T., Pascal, B. D. & Griffin, P. R. Protein dynamics and conformational changes explored by hydrogen/deuterium exchange mass spectrometry. Curr. Opin. Struct. Biol. 58, 305–313 (2019).
3. Borysiak, A. J. Simulated isotope exchange patterns enable protein structure determination. Angew. Chem. Int. Ed. Engl. 56, 9396–9399 (2017).
4. Li, K. S. et al. Hydrogen-deuterium exchange and hydroxyl radical footprinting for mapping hydrophobic interactions of human bromodomain with a small molecule inhibitor. J. Am. Soc. Mass Spectrom. 30, 2795–2804 (2019).
5. Nielsen, A. K. et al. Substrate-induced conformational dynamics of the dopamine transporter. Nat. Commun. 10, 2714 (2019).
6. Zhu, S. et al. Hydrogen-deuterium exchange epitope mapping reveals distinct neutralizing mechanisms for two monoclonal antibodies against diphtheria toxin. Biochemistry 58, 646–656 (2019).
7. Trabjerg, E. et al. Conformational analysis of large and highly disulfide-stabilized proteins by integrating online electrochemical reduction into an optimized H/D exchange mass spectrometry workflow. Anal. Chem. 87, 8880–8888 (2015).
8. Redhair, M., Clouser, A. F. & Atkins, W. M. Hydrogen-deuterium exchange mass spectrometry of membrane proteins in lipid nanodiscs. Chem. Phys. Lipids 230, 14–22 (2019).
9. Masson, G. R. et al. Recommendations for performing, interpreting and reporting hydrogen deuterium exchange mass spectrometry (HDX-MS) experiments. Nat. Methods 16, 595–602 (2019).
10. Althaus, E. et al. Computing H/D-exchange rates of single residues from data of proteolytic fragments. BMC Bioinf. 11, 424 (2010).
11. Zhang, Z., Zhang, A. & Xiao, G. Improved protein hydrogen/deuterium exchange mass spectrometry platform with fully automated data processing. Anal. Chem. 84, 4942–4949 (2012).
12. Saltzberg, D. J. et al. A residue-resolved Bayesian approach to quantitative interpretation of hydrogen-deuterium exchange from mass spectrometry: application to characterizing protein-ligand interactions. J. Phys. Chem. B 121, 3493–3501 (2017).
13. Gessner, C. et al. Computational method allowing hydrogen-deuterium exchange mass spectrometry at single amide resolution. Sci. Rep. 7, 3789 (2017).
14. Skinner, S. P., Radou, G., Tuma, R., Houwing-Duistermaat, J. J. & Paci, E. Estimating constraints for protection factors from HDX-MS data. Biophys. J. 116, 1194–1203 (2019).
15. Walters, B. T. Empirical method to accurately determine peptide-averaged protection factors from hydrogen MS data. Anal. Chem. 89, 1049–1053 (2017).
16. Kan, Z. Y., Walters, B. T., Mayne, L. & Englander, S. W. Protein hydrogen exchange at residue resolution by proteolytic fragmentation mass spectrometry analysis. Proc. Natl Acad. Sci. USA 110, 16438–16443 (2013).
17. Babic, D., Kazzazic, S. & Smith, D. M. Resolution of protein hydrogen/deuterium exchange by fitting amide exchange probabilities to the peptide isotopic envelopes. Rapid Commun. Mass Spectrom. 33, 1248–1257 (2019).
18. Zhang, Z. Complete extraction of protein dynamics information in hydrogen/deuterium exchange mass spectrometry data. Anal. Chem. 92, 6486–6494 (2020).
19. Houde, D., Peng, Y., Berkowitz, S. A. & Engen, J. R. Post-translational modifications differentially affect IgG1 conformation and receptor binding. Mol. Cell Proteom. 9, 1716–1728 (2010).
20. Englander, S. W. & Kallenbach, N. R. Hydrogen exchange and structural dynamics of proteins and nucleic acids. Q Rev. Biophys. 16, 521–655 (1983).
21. Bai, Y., Milne, J. S., Mayne, L. & Englander, S. W. Primary structure effects on peptide group hydrogen exchange. Proteins 17, 75–86 (1993).
22. Harris, M. J., Raghavan, D. & Borysiak, A. J. Quantitative evaluation of native protein folds and assemblies by hydrogen deuterium exchange mass spectrometry (HDX-MS). J. Am. Soc. Mass Spectrom. 30, 58–66 (2019).
23. Vendruscolo, M., Paci, E., Dobson, C. M. & Karplus, M. Rare fluctuations of native proteins sampled by equilibrium hydrogen exchange. J. Am. Chem. Soc. 125, 15686–15687 (2003).
24. Nguyen, D., Mayne, L., Phillips, M. C. & Walter Englander, S. Reference parameters for protein hydrogen exchange rates. J. Am. Soc. Mass Spectrom. 29, 1936–1939 (2018).
25. Nocedal, J. & Wright, S. J. Numerical optimization. 2nd edn, (Springer, 2006).

Acknowledgements
This work was funded by the Biotechnology and Biological Sciences Research Council (BBRSC). We also gratefully acknowledge the use of the research computing facility at King’s College London, Rosalind (https://www.rosalind.kcl.ac.uk). We also thank Andy McAllister for design of the website logos.

Author contributions
R.E.S. and A.J.B. designed the experiments, protocols, analysed data, and wrote the manuscript. R.E.S. developed and optimised the code and the website.

Competing interests
The authors have no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s42003-021-01709-x.

Correspondence and requests for materials should be addressed to A.J.B.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.