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Rosetta Protein Structure Prediction from Hydroxyl Radical Protein Footprinting Mass Spectrometry Data

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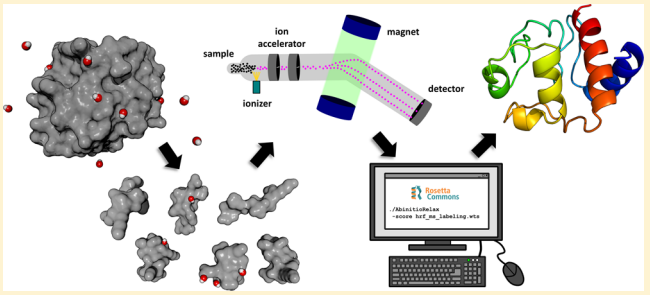
Supporting Information

ABSTRACT: In recent years mass spectrometry-based covalent labeling techniques such as hydroxyl radical footprinting (HRF) have emerged as valuable structural biology techniques, yielding information on protein tertiary structure. These data, however, are not sufficient to predict protein structure unambiguously, as they provide information only on the relative solvent exposure of certain residues. Despite some recent advances, no software currently exists that can utilize covalent labeling mass spectrometry data to predict protein tertiary structure. We have developed the first such tool, which incorporates mass spectrometry derived protection factors from HRF labeling as a new centroid score term for the Rosetta scoring function to improve the prediction of protein tertiary structures. We tested our method on a set of four soluble benchmark proteins with known crystal structures and either published HRF experimental results or internally acquired data. Using the HRF labeling data, we rescored large decoy sets of structures predicted with Rosetta for each of the four benchmark proteins. As a result, the model quality improved for all benchmark proteins as compared to when scored with Rosetta alone. For two of the four proteins we were even able to identify atomic resolution models with the addition of HRF data.



Historically, mass spectrometry has been used as a tool to quantify the mass and oligomeric distribution of proteins and protein assemblies.[1,2] More recently, advances have been made that allow mass spectrometry experiments to yield three-dimensional structural information on proteins and their complexes. By itself, there is no one mass spectrometry technique that can unambiguously elucidate the atomic-resolution tertiary structure of a protein or protein complex. Hence, a combination of multiple different techniques is generally required.[3−5] Several techniques have been particularly successful in probing the tertiary structure of proteins and their complexes. Hydrogen−deuterium exchange (HD/X) is based upon measuring the extent of isotopic exchange of backbone amide hydrogens.[6,7] Chemical cross-linking involves studying the structurally defined distances by covalently pairing functional groups within a protein.[8,9] Noncovalent interactions between lysine residues and 18-crown-6 ether (a cyclic organic compound) can provide lysine solvent accessibility within proteins.[10] Finally, covalent labeling (sometimes referred to as "protein footprinting") involves exposing a protein in solution to a small labeling reagent that will covalently bond to select amino acid side chains that are exposed to solvent, whereas side chains buried within the core of the protein or occluded by interacting protein subunits will not get labeled.[11−13] This provides information about the relative location of certain amino acids with respect to the solvent (either on the surface and solvent exposed or buried within the protein or protein complex structure). A variety of different labeling reagents exist; some are highly specific as to which amino acid(s) can react with the reagent, and others have a much broader range of potential target residues. These techniques have been successfully employed with mass spectrometry to analyze protein structures.[14−22]

One covalent labeling method which has been increasingly widely used recently is hydroxyl radical footprinting (HRF).[23,24] This method involves exposing a solvated protein of interest to hydroxyl radicals generated from one of a variety of sources. Initially, oxidative labeling was performed using a synchrotron that ionized water to form the hydroxyl radicals.[25] With recent advancements, a new method of hydroxyl radical labeling, fast photochemical oxidation of proteins (FPOP), has been developed.[26,27] With FPOP, a pulsed laser is used to photolyze hydrogen peroxide on a microsecond time scale, which is faster than the unfolding of a protein. This ensures that the labeling process does not disrupt the native state of the protein. In conjunction with mass spectrometry, FPOP provides important insight into the structure of proteins. This labeling method is quite broad in that it can label 19 of the 20 different amino acids, yielding extensive structural information. Despite the wealth of information provided by FPOP, the data

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itself is sparse, meaning that the solvent exposure information on a set of protein residues cannot provide an unambiguous determination of the protein structure. There remains a critical need for computational methods that can facilitate and compliment the structural interpretation of mass spectrometry FPOP labeling data.

Over the years, numerous experimental techniques have been successfully combined with computational methods to predict protein structures. Some examples of this are sparse experimental data from site-directed spin labeling electron paramagnetic resonance (SDSL-EPR) in conjunction with Rosetta to improve protein structure predictions,[28,29] nuclear magnetic resonance spectroscopy (NMR),[30,31] small-angle X-ray scattering (SAXS),[32−35] and cyro-electron microscopy (cryo-EM).[36−43] Mass spectrometry techniques have also been utilized in conjunction with computational methods. Malmström and co-workers have made significant contributions by incorporating data from MS chemical cross-linking experiments as inputs into computational methods for protein structure prediction.[15,44−47] The work of Sali and co-workers has contributed greatly to the field with the development of the Integrative Modeling Platform (IMP), an open source platform that integrates experimental data into computational methods.[19,35,48−52] IMP is designed as a set of self-contained modules that can be mixed and matched based on a user's preference. Models are generated and scored based on spatial restraints that are derived from multiple sources of experimental data. Currently IMP supports the use of experimental data gathered from sources such as SAXS profiles, EM images and density maps, NMR, chemical cross-linking, HD/X, and chromosome conformation capture. With IMP, both monomeric and multiunit protein structures can be studied. Finally, Yang and co-workers have developed an integrative method, iSPOT, to determine protein−protein complexes that combines SAXS, hydroxyl radical footprinting, and computational docking of either rigid-body or molecular dynamics models.[32]

Computational modeling using FPOP data is still in its early stages. Recently, an integrated workflow was developed by Xie and co-workers that successfully demonstrated correlation between experimental high-resolution hydroxyl radical footprinting data and residue solvent exposure (as measured by absolute average solvent accessible surface area) as well as differentiated between low and high RMSD models for the soluble proteins myoglobin and lysozyme.[53] This elegant work demonstrated that there is a strong potential for successfully incorporating HRF or FPOP experimental data into computational methods in order to improve the prediction of a protein structure. Despite the many advances and successes with using sparse data from various experimental methods for structure prediction, the use of covalent labeling mass spectrometry as the data source had yet to be accomplished.

In this work, we incorporated mass spectrometry derived protection factors from FPOP and synchrotron-based HRF labeling as a new score term for the Rosetta scoring function to improve the prediction of protein tertiary structure. Rosetta is one of the primary computational tools used for protein structure prediction.[54] To accomplish our goal, we compiled a set of four soluble benchmark proteins with known crystal structures and either published HRF/FPOP experimental results or internally acquired data. We developed an efficient metric to quantify residue-specific burial that correlated linearly to the natural logarithm of experimental protection factors derived from the labeling rates. A new Rosetta centroid score term, which utilizes residue-resolved protection factors as inputs, was developed. This score term was used in conjunction with the standard Rosetta scoring function to rescore large decoy sets of predicted structures for each of the four benchmark proteins. In this process of rescoring the quality of all models improved such that after rescoring the structures with the best score correlated more closely to the native structures. For two of the four proteins, we were even able to identify atomic resolution models using the HRF/FPOP data.

MATERIALS AND METHODS

Benchmark Data Set and Experimental Protection Factors. For this work, we used the protection factor (PF) which was first described by Chance and co-workers and is derived from a labeling rate constant as a metric for residue labeling.[55] PF is defined as the relative intrinsic reactivity of a given residue to hydroxyl radicals divided by the rate constant. The intrinsic reactivities of each amino acid type are well-defined in the literature.[24] The PF, as expressed on a natural logarithmic scale, has been shown to correlate with the solvent exposure of a given residue.[16,55,56] Within the literature, the PF has been defined multiple ways, but for our purposes we have defined the protection factor for residue i, where R_i is the intrinsic reactivity for residue i and k_i is the experimentally determined labeling rate constant, as defined by eq 1:

$$PF_i = \frac{R_i}{k_i} \tag{1}$$

As a benchmark set, four different proteins with available FPOP or HRF labeling data were utilized. These proteins were calmodulin (PDB: 1PWR), myoglobin (PDB: 1DWR), lysozyme (PDB: 1DPX), and cytochrome c (PDB: 2B4Z). The experimentally determined PFs for calmodulin were extracted from the published work of Kaur and co-workers, who generated radicals via a millisecond time scale synchrotron radiation method.[16] For myoglobin, the PFs were calculated from the reported labeling rate constants by Xie and co-workers[53] using the reactivities reported in the literature.[24] For this study, radicals were generated using submicrosecond FPOP with a dosimeter to provide varying doses of radicals. Finally, the experimental PFs for both lysozyme and cytochrome c were oxidatively modified by FPOP at a single radical dose as described in the Supporting Information.

For incorporation of the data into the newly developed score term, input files were created for each protein consisting of a heading line followed by two columns comprising the residue number and the natural logarithm of the protection factor, with each labeled residue on a new line. FPOP/HRF can label 19 of the 20 amino acids; however, data from the following residue types were omitted due to having either too low/high reactivity or unclear products: M, C, D, N, Q, T, S, A, G, R, K, and V. Of this list of omitted residues, it has been previously suggested by Xie and co-workers that the sequence context plays a role in whether or not these amino acid types are labeled. This is a complex issue and has not been examined in this current work. As a result, only eight of the 20 amino acids were considered in the analysis: I, L, P, F, W, Y, E, and H. These residues have intermediate reactivities and correspond with the residue types utilized in similar studies.[16,53]

Rosetta ab Initio Folding. In the absence of any experimental labeling data, decoy sets of 20000 structures

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were generated for each of the four benchmark proteins using the *AbinitioRelax* application within Rosetta.[57−59] The *AbinitioRelax* protocol consists of two main steps: (1) a coarse-grained fragment-based search of conformational space that uses a low-resolution "centroid"-based (treating each residue with backbone atoms defined explicitly and the side-chain represented as a single sphere) scoring function and (2) a high-resolution refinement using the full-atom Rosetta score function.

The generated decoy sets act as benchmarks to compare the structure prediction capabilities of Rosetta in the absence of FPOP/HRF labeling data. Specifics of the protocol have been detailed extensively in the literature.[60] The fragment libraries for this work were generated using the Robetta online server.[61] The required FASTA formatted sequences and native protein structures were extracted from each protein's respective PDB file. The fragment libraries, FASTA sequences, and native PDB structures (used solely for determining the deviation of the generated models from the native) were used as inputs for Rosetta's *AbinitioRelax* application. For lysozyme, disulfide bonds were present between the following residues: 6 and 127, 30 and 115, 64 and 80, and 76 and 94. An additional input file was provided to specify the residues that are a part of the disulfide bonds. The generated structures were scored using the Rosetta energy function (Ref15), where the score is an approximation of the energy of the protein or complex.[62] The scores and respective root-mean-square deviation (RMSD) to the native crystal structure were extracted from the output score file. Structures were ranked based on their scores, with lower scores anticipated to correspond to models closer in structure to the native structure. Rosetta scores versus RMSD to the native protein were generated to demonstrate this correlation.

For each of the benchmark proteins, two small sets of representative structures were generated. The first set represented ten native-like conformations of each protein which were obtained by relaxing each crystal PDB in the Rosetta force field using the *relax* application.[63,64] We will refer to these structures as the ten native-like models or the native-like model set. The second set contained models that scored well with the Rosetta energy function but had high RMSDs compared to the crystal native structures. These were obtained by extracting the top ten scoring models with RMSD > 10 Å for each protein from the initial ab initio calculations. We will refer to these structures as the good scoring/high RMSD model set. Together, these sets represented the two extremes of potential models that we desired to efficiently differentiate between using our new score term.

Residue Exposure Metric. To compare the protection factors extracted from the FPOP/HRF labeling data to the residue exposure in the protein models, a corresponding residue exposure measure was developed which enabled calculation of the level of exposure of every labeled residue in a protein model. The PF has been shown to correlate to a residue-level solvent accessible surface area (SASA).[16,53,56] Because residue-level SASAs are expensive to calculate,[65,66] we explored other metrics, aside from SASA, that were less computationally expensive and provided even stronger correlation to the natural logarithm of the experimental FPOP/HRF PFs. Assuming solvent exposed residues are preferentially labeled, we sought to find a residue burial/exposure metric that showed correlation to the natural logarithm of the PFs. Several methods, such as weighted neighbor count and SASA,[65,67] were investigated. For reference, the correlation between SASA and the natural logarithm of the PFs can be found in Figure S-1. However, the burial measure found to give the strongest correlation to the experimental data was a neighbor count determined for each labeled residue. A residue with a high neighbor count can be thought of as buried, whereas a residue with a low neighbor count can be considered solvent exposed. For this analysis, a low-resolution model of the protein was used where all of the backbone atoms were represented explicitly and the side-chain was represented as a single sphere called a centroid. To calculate a residue's neighbor count, the distances between the labeled residue's centroid (residue i) and all of the other residues' centroids (residues $j \neq i$) were measured. The distance, r_{ij}, was then used in a sigmoid function that defined a value between 0 and 0.7, as shown in Figure S-2, representing the amount of contribution of a neighboring residue j to the total neighbor count of the target residue i. The closer a residue j's centroid is to labeled residue i's centroid, the more it contributed to the overall neighbor count; conversely, the further away it is, the less it contributed. The total neighbor count for each labeled residue i was then defined as the sum of every residue's contribution to the neighbor count:

$$\text{neighbor count}_i = \sum_{j \neq i}^{\text{total \# of residues}} \frac{1.0}{1.0 + \exp(0.1(r_j - 9.0))} \tag{2}$$

We developed a new Rosetta application, *burial_measure_centroid*, which calculated the neighbor counts (as defined in eq 2) for arbitrary protein structures. For each of the 80 models comprising the native-like and good score/high RMSD model sets, the neighbor counts were calculated using the *burial_measure_centroid* Rosetta application. The neighbor counts for the ten native-like structures of calmodulin (1PRW) were used to perform a linear regression with the corresponding experimental ln PF values. The linear fit obtained was then used as a prediction function to predict the neighbor count for all 80 representative models with their respective experimental ln PF values as inputs.

hrf_ms_labeling Score Term. A new score term, *hrf_ms_labeling*, was developed to be incorporated into Rosetta to assess the agreement of Rosetta models with experimental FPOP/HRF labeling data. This score term is defined as a centroid score term that rewards protein conformations that show agreement with the experimental labeling data. By treating the score term in a Bayesian fashion, the total Rosetta score was derived (as shown explicitly in the Supporting Information) to be the sum of the weighed score term and the current Rosetta score:

$$\text{total score} = (w_{\text{hrf}})(hrf\_ms\_labeling) + \text{Rosetta score} \tag{3}$$

The score term *hrf_ms_labeling* was implemented using the linear prediction function obtained by correlating the observed neighbor counts and experimental ln PF for the benchmark protein calmodulin (see the previous section, Residue Exposure Metric). A value for *hrf_ms_labeling* was calculated by summing the per-residue neighbor scores over the set of labeled residues and was defined as

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$$hrf\_ms\_labeling = \sum_{i}^{\#\ \text{of labeled residues}} \frac{-1.0}{1.0 + \exp(2.0(|\text{diff}|_i - 7.5))} \quad (4)$$

where $|\text{diff}|_i$ is the absolute value of the difference between the observed neighbor count (calculated using eq 2 for the modeled protein) and the predicted neighbor count (calculated using the linear prediction function) for labeled residue *i*. Using the definition in eq 4, each labeled residue contributed a per-residue score ranging from −1 to 0, with a value of −1 in the case of strong agreement with the experiment and a value of 0 in the case of complete disagreement. If the value of $|\text{diff}|_i$ fell between 5 and 10 (which corresponded to the same cutoffs as the delta lines used in analyzing the prediction function), the residue received a logistically increasing value ranging from −1 to 0. The per-residue score (function found within the summation in eq 4) is depicted in Figure 1 with all relevant points highlighted.

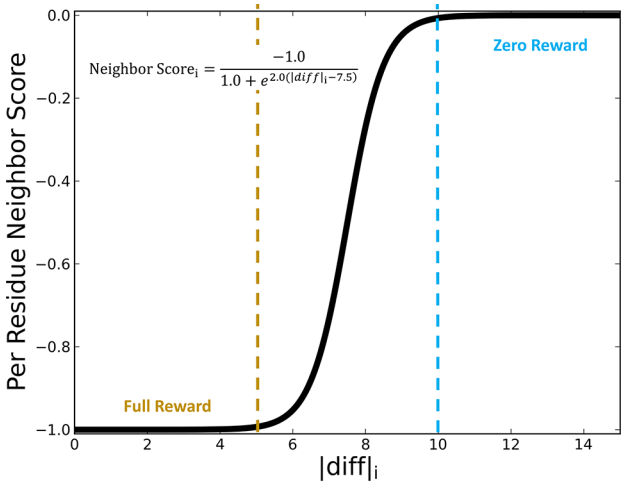

Figure 1. Plot of the per-residue neighbor score for labeled residue *i* as a function of the absolute difference between its observed and predicted neighbor counts ($|\text{diff}|_i$). The score function fully rewarded (with a score of −1) residues that have an $|\text{diff}|_i < 5$ and gave no reward (a score of 0) to residues that have an $|\text{diff}|_i > 10$.

Rescoring of Rosetta Structures. To test the capability of our new score term to improve Rosetta model quality, the 20000 Rosetta models initially generated as part of the ab initio folding for each benchmark protein were rescored with the *hrf_ms_labeling* score term. The calculated *hrf_ms_labeling* score was weighted by a value of 6.0 and added to the Rosetta score calculated using Rosetta's Ref15 energy function:

$$\text{total Rosetta score} = \text{Ref15 Rosetta score} + 6.0(hrf\_ms\_labeling) \quad (5)$$

A weight of 6.0 was the lowest possible value that showed the greatest improvement. We iterated through all integer values from 1 to 36 and determined the top scoring models' RMSDs at each weight. The results of this analysis are shown in Figure S-3. To calculate the *hrf_ms_labeling* contribution for each model, the *score* Rosetta application was run on each of the 80000 models using the output structures from the initial ab initio model generation as the input. For each of the 80000 rescored models, the total Rosetta scores, the RMSD to the native structure, and the *hrf_ms_labeling* scores were extracted.

Model Evaluation. Several different metrics were used to evaluate the performance of both Rosetta and the score term. Those metrics were based upon the concept of an energy funnel, i.e., that within the overall energy landscape, low RMSD models can be distinguished from high RMSD models due to their lower energy (Rosetta score).[68] The first metric used was a simple determination of the top scoring model's RMSD to the native structure. In practice, the Rosetta model with the lowest (most favorable) Rosetta score is assumed to be closest in structure to the native. Because all of the benchmark proteins chosen for this study had crystal PDB structures available, an RMSD for that model can be calculated.

The second metric used was the goodness-of-energy-funnel metric P_{near}, as defined by Bhardwaj and co-workers.[69] A value of P_{near} was calculated for each Rosetta score versus RMSD distribution using the following equation:

$$P_{near} = \frac{\sum_{m=1}^{N} \exp\left(-\frac{rmsd_m^{\,2}}{\lambda^2}\right)\exp\left(-\frac{E_m}{k_B T}\right)}{\sum_{m=1}^{N} \exp\left(-\frac{E_m}{k_B T}\right)} \quad (6)$$

where N is the total number of models and E_m and $rmsd_m$ are the Rosetta score and RMSD of model m, respectively. The parameter λ was given a value of 2.0 and controlled how similar a model needed to be to the native to be considered native-like. The final parameter, $k_B T$, was set to 1.0 and governed how the shallowness or depth of the funnel affects P_{near}. Values of P_{near} can range from 0 (very non-funnel-like) to 1 (funnel-like).

The final metric used was a comparison of the number of top 100 scoring models with RMSD's below 10.0 Å. By comparing this metric between different Rosetta scores versus RMSD distributions, we were able to investigate how well (or poorly) the addition of *hrf_ms_labeling* was at improving model quality.

RESULTS AND DISCUSSION

Generation of Control ab Initio Model Set for Benchmark Proteins using Rosetta. To establish the baseline performance of Rosetta's Ref15 scoring function at predicting protein structures without any additional experimental knowledge, decoy sets consisting of 20000 models were generated for each of the four benchmark proteins. The four proteins selected for the benchmark were calmodulin (PDB: 1PWR), myoglobin (PDB: 1DWR), lysozyme (PDB: 1DPX), and cytochrome c (PDB: 2B4Z). Table 1 summarizes the benchmark proteins. These proteins ranged in size from 104 to 153 amino acids in length. Contact orders (CO) were calculated for each of the proteins.[70] The contact orders for all four proteins were low, ranging from 10.7 to 13.7. The secondary structure content for the four proteins were relatively high, ranging from 41% to 74%. Because these proteins were all relatively small (approximately fewer than 150 amino acids) and had high secondary structure content and low contact

Table 1. Summary of the Four Benchmark Proteins

protein	PDB entry	no. of amino acids	no. of labeled residues	contact order	secondary structure content (%)
calmodulin	1PRW	148	25	10.7	61
cytochrome c	2B4Z	104	9	11.6	41
myoglobin	1DWR	153	25	12.4	74
lysozyme	1DPX	129	6	13.7	51

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Table 2. Rosetta ab Initio Prediction and Rescoring Results Summary with and without the Addition of *hrf_ms_labeling*

	Rosetta ab initio results		Rosetta + *hrf_ms_labeling* rescore results		
protein	top scoring model RMSD to native (Å)	P_{near}	top scoring model RMSD to native (Å)	P_{near}	confidence measure (P_{near} to top scoring RMSD)
calmodulin (1PRW)	11.8	2.10×10^{-8}	10.2	1.17×10^{-6}	4.18×10^{-5}
cytochrome c (2B4Z)	5.5	0.0805	2.2	0.238	0.038
myoglobin (1DWR)	5.0	0.00208	1.8	0.378	0.0089
lysozyme (1DPX)	15.2	3.04×10^{-7}	7.2	1.89×10^{-6}	3.079×10^{-9}

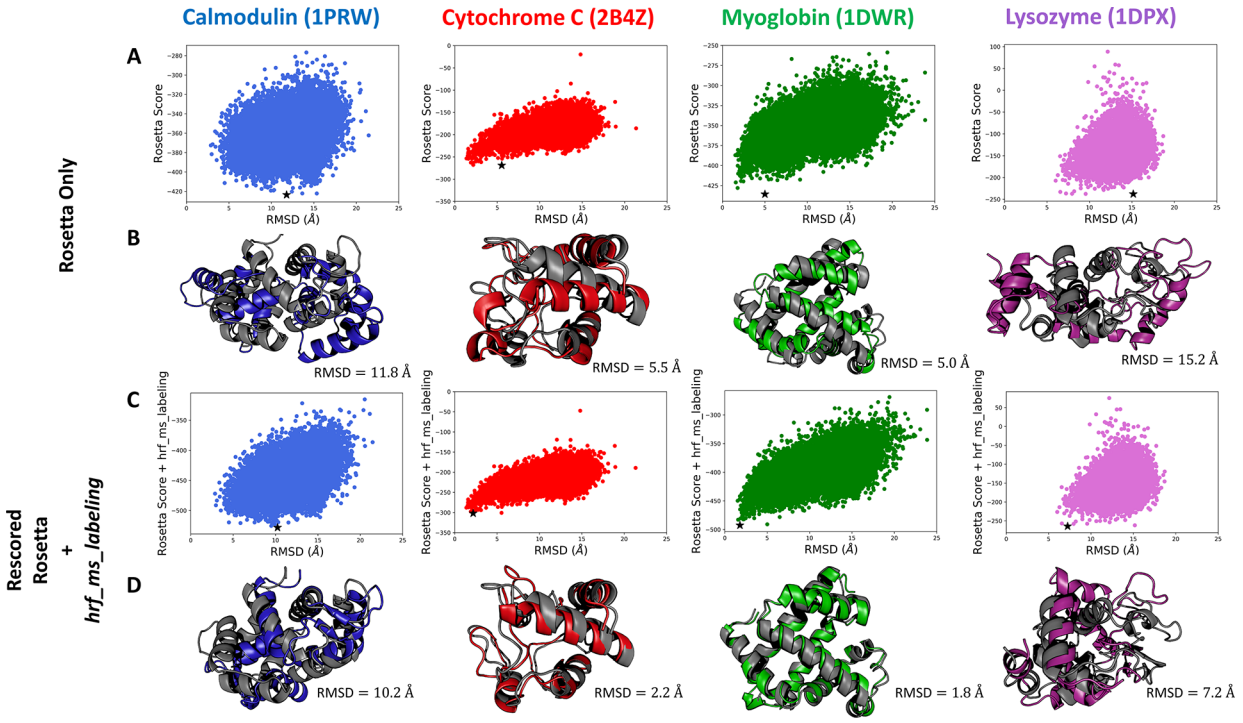

Figure 2. (A) Rosetta score versus RMSD to the native structure plots for 20000 models generated using Rosetta ab initio for each of the four benchmark proteins. The top scoring model is represented as a star on each plot. (B) The top scoring models from the Rosetta score versus RMSD distributions in A (color) superimposed on the respective native model (gray). (C) Rosetta score + *hrf_ms_labeling* versus RMSD to the native structure plots for each of the four benchmark proteins after rescoring with the new score term. The top scoring model is represented as a star on each plot. (D) The top scoring models from the Rosetta score + *hrf_ms_labeling* rescoring distributions in C (color) superimposed on the respective native model (gray).

orders, we concluded that they were amendable to Rosetta ab initio protein structure predictions.

Using Rosetta to generate 20000 models for each of the four proteins resulted in the selection of best-scoring structures with RMSDs ranging from 5.0 to 15.2 Å, as summarized in Table 2 and indicated on the Rosetta score versus RMSD to native structure plots in panel A of Figure 2 by stars. The two proteins with top scoring structures that were closest to their respective native structures were myoglobin (RMSD = 5.0 Å) and cytochrome c (RMSD = 5.5 Å). The predictions for the remaining two proteins, calmodulin and lysozyme, were poor, yielding top scoring models with RMSDs of 11.8 and 15.2 Å, respectively. Considering the size of the benchmark proteins, none of these best-scoring models were high-quality, near-atomic resolution models. For two of the proteins, even an incorrect topology was identified. However, as can be seen in Figure 2A, models with significantly lower RMSDs to the native structure were built for all four proteins. For calmodulin, the RMSDs for the generated models ranged from 2.9 to 21.5 Å. Similar ranges were sampled for cytochrome c and myoglobin, with RMSDs ranging from 1.4 to 21.3 Å and 1.5 to 27.3 Å, respectively. Lysozyme had the poorest sampling, where model RMSDs ranged from 6.0 to 18.7 Å. This indicated that better, and in some cases even near-atomic, resolution models were in fact generated for all proteins, but they were generally not identified by the lowest score.

The goodness-of-energy-funnel metric, P_{near}, was used to evaluate the funnel quality of each of the distributions. As can be seen in Table 2, none of the distributions had P_{near} values greater than 0.1, strongly suggesting that none of the ensembles of the models exhibited funnel-like score distributions. This lack of a funnel in the Rosetta score versus RMSD to native structure plots made structure prediction and, particularly, native structure identification challenging. On the basis of these ab initio structure prediction results, we concluded that incorporation of experimental data, such as HRF/FPOP labeling data, had the potential to improve identification of low RMSD models by score.

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Rescoring Model Sets using *hrf_ms_labeling*. The overall goal of this work was to utilize experimental HRF/FPOP labeling data in order to improve models predicted by Rosetta. To accomplish this, a new Rosetta score term, *hrf_ms_labeling*, was developed that incorporated experimental HRF/FPOP protection factors (PFs). After developing *hrf_ms_labeling*, we confirmed that incorporation of HRF/FPOP labeling data did enable discrimination of near-native and high RMSD models and that combination of this score with the total Rosetta Ref15 score did improve the quality of the models selected from the structure ensembles.

The first step in this process was to demonstrate that a correlation existed between the experimental labeling data (the PFs) and a residue solvent exposure metric derived within Rosetta. The metric that demonstrated the best correlation was the per-residue neighbor count, as defined in the Materials and Methods. The calculated neighbor count for every labeled residue within calmodulin (1PRW), one of our benchmark proteins, was plotted against the natural logarithm of the respective PF values. The positive correlation, as seen in Figure 3, had an R^2 of 0.48 and p value of 1.36×10^{-36}. The observed

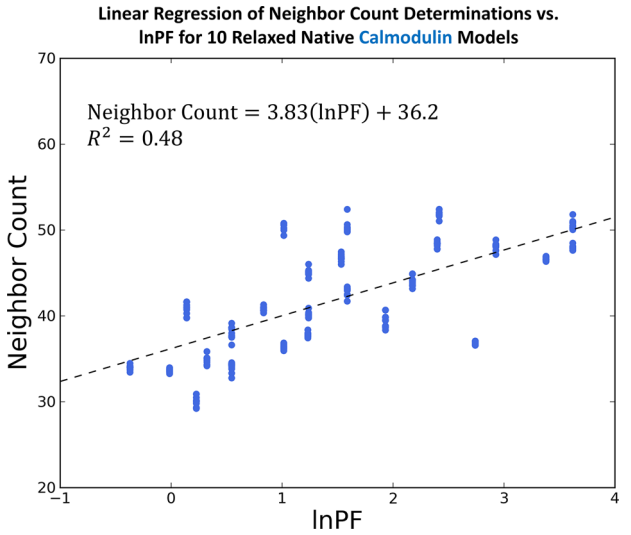

Figure 3. Linear regression between the neighbor count and the natural logarithm of the experimental protection factor (ln PF) for ten relaxed native models of calmodulin. The linear fit along with its coefficient of determination are indicated on the plot.

trend matched our expectation where residues with a low ln PF also showed a low neighbor count (suggesting a higher solvent exposure) and residues with a high ln PF showed a high neighbor count (suggesting a lower solvent exposure). The derived relationship between PFs and neighbor count was used to predict neighbor counts for all four benchmark proteins based on the experimental HRF/FPOP protection factors. For comparison, observed neighbor counts for two small sets of representative structures (the native-like model sets and the good scoring/high RMSD model sets) were calculated from each PDB structure using *burial_measure_centroid*. The predicted neighbor counts have been plotted against the observed neighbor counts (calculated directly from representative structures of the four benchmark proteins) in Figure 4. In order to quantify the accuracy of the prediction, two delta lines were defined (d_1 = 5.0 and d_2 = 10.0). These delta lines represent how close the predicted neighbor counts were to the actual observed values. Using the native-like model sets for all four proteins, an average of 81% and 59% of the labeled residues fell within d_2 and d_1, respectively, whereas only 67% and 38% of those belonging to the good scoring/high RMSD model sets did. This demonstrated that we predicted the majority of the labeled residues in native-like models within the delta lines and simultaneously excluded the majority of residues in the high RMSD models from within the delta lines. This suggested that agreement between a model's residue exposure and the neighbor count metric derived from experimental FPOP/HRF mass spectrometry data can indeed distinguish between low and high RMSD models and can thus be used to rescore protein models built in the absence of experimental FPOP/HRF labeling data. To be able to rescore protein models, a *hrf_ms_labeling* score term was developed for incorporation into Rosetta.

We next demonstrated that the new score term was effective in improving model prediction. The 20000 model decoy sets generated for each of the four benchmark proteins were rescored with the *hrf_ms_labeling* term added to the Ref15 Rosetta score. For each set of models, Rosetta score + *hrf_ms_labeling* versus RMSD plots were generated. On the basis of the rescored structures, new top scoring models were selected. As shown in Table 2, the RMSDs of the top scoring models improved for all four proteins, while for two of the proteins near-atomic resolution models were identified. The biggest increases in top scoring model quality were observed for lysozyme. Addition of HRF/FPOP labeling data improved the RMSD of the top scoring lysozyme model from 15.2 to 7.2 Å, a significant improvement in the model's quality. Although a model with an RMSD of 7.2 Å is not usually considered high quality, considering that the best lysozyme ab initio model had an RMSD of 6.0 Å, one of the best existing models was identified. Both myoglobin and cytochrome c showed decreases in their RMSDs to near-atomic resolution models (2.2 and 1.8 Å respectively), thus models were identified with RMSDs close to the best existing models within the 20000 structures. Calmodulin had the least improvement with a change in RMSD from only 11.8 to 10.2 Å. When we superimposed the top scoring models onto their respective native structures, as depicted in panels B and D of Figure 2, a significant increase in model quality could be observed as a result of the addition of *hrf_ms_labeling*. All top scoring models now identify the correct protein topology.

In addition to analyzing the RMSD of the top scoring models, the overall energy landscape of the structures was analyzed. Values of P_{near} were calculated for each score versus RMSD distribution, identical to what was done without the addition of *hrf_ms_labeling* (see Table 2). With the addition of the *hrf_ms_labeling* term to the scoring function, there was an increase in P_{near}, i.e., an increase in funnel quality of the score versus RMSD plots, for all four proteins. As can be seen in panel C of Figure 2, the distributions appear more funnel-like, with lower RMSD models receiving lower scores. Interestingly, the P_{near} values of the two proteins for which near-atomic resolution models were identified (myoglobin and cytochrome c) were several orders of magnitude higher than those of the other proteins. We thus speculated that P_{near} might be used as a confidence measure to identify cases for which near-atomic resolution models were identified. To explore this idea, we recalculated the score versus RMSD plots with respect to the lowest scoring structure (to obviate the necessity for knowledge of the native structure) and measured P_{near} values for these

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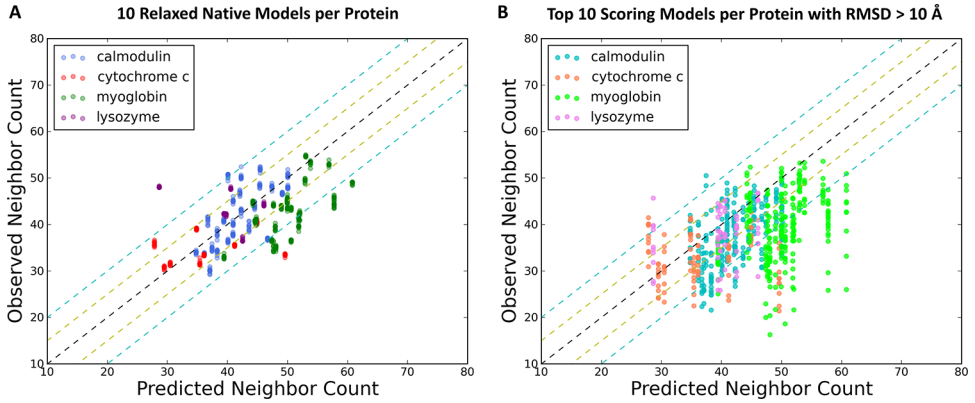

Figure 4. (A) Plot of predicted and observed neighbor counts for ten relaxed native models for each of the four benchmark proteins. (B) Plot of predicted and observed neighbor counts for ten models with good Rosetta scores and high RMSD values (>10 Å) as compared to their respective natives for each of the four benchmark proteins. For both plots, the dashed black line represents the theoretical perfect fit (the predicted matches the observed perfectly) and the yellow and cyan lines represent the inner (d_1 = 5) and outer (d_2 = 10) delta lines, respectively.

distributions as shown in the last column of Table 2. While the trend was not as pronounced as before, this P_{near} value still served as a confidence measure in that the P_{near} values of the two proteins for myoglobin and cytochrome c were more than 2 orders of magnitude higher than those of the other proteins. Upon rescoring with *hrf_ms_labeling*, the overall distribution of the structures did not shift to a lower RMSD because *hrf_ms_labeling* was simply used to rescore previously generated models. Plots of *hrf_ms_labeling* versus RMSD are shown in Figure S-4. For all four proteins, models with poor (i.e., high, closer to 0) *hrf_ms_labeling* scores also had a higher RMSD. Likewise, some of the models with a better *hrf_ms_labeling* score tended to have a lower RMSD. There were a fair number of models, however, that had good *hrf_ms_labeling* scores but a high RMSD. This trend is not concerning because the information obtained from the HRF/FPOP labeling experiments are not all encompassing of a protein's structure. Individual score terms within Rosetta generally do not exhibit the exact trend of low score/low RMSD and high score/high RMSD. Combination of this score term with the Rosetta scoring function, however, generated the desired trend.

We finally investigated whether a larger set of top scoring models after the rescoring were of increased quality. Histograms were generated showing the RMSD frequency of the top 100 scoring models for the distributions pre- and post-addition of *hrf_ms_labeling*. On the basis of these histograms shown in Figure 5, there was a definite shift in the model quality for calmodulin and myoglobin, with more models scoring well with low RMSDs. The percentage of the top 100 scoring models that had a RMSD < 10 Å increased from 35% to 68% for calmodulin with the addition of *hrf_ms_labeling*. This illustrates that despite not identifying a near-atomic resolution model for calmodulin, addition of the labeling information significantly improved the model quality. Myoglobin demonstrated an increase in the percentage of models in the top scoring 100 with RMSD < 5 Å from 47% to 70%. A shift in model quality of the top 100 scoring models was also seen with for lysozyme and cytochrome c, albeit it was much less significant.

The *hrf_ms_labeling* score term has shown great success in rescoring structures based on experimental HRF/FPOP labeling data and has been designed efficiently. A centroid form of the score term was chosen for two reasons. First, this implementation showed the highest correlation between the

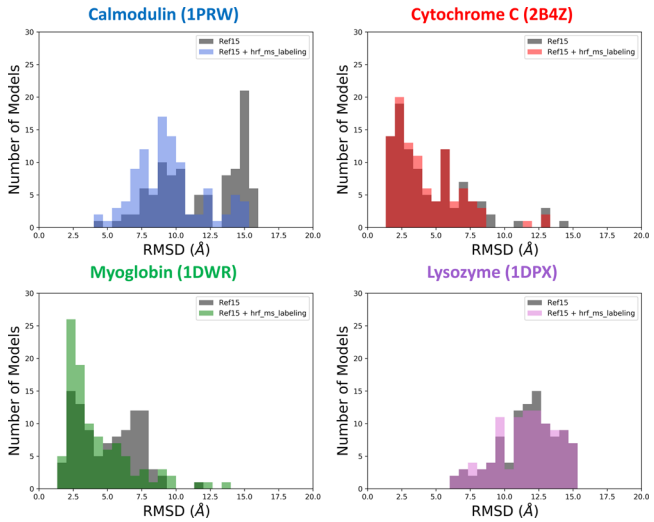

Figure 5. Histograms for each of the four benchmark proteins showing the RMSD frequency of the top 100 scoring models from both the ensembles generated using Rosetta and the ensembles obtained after rescoring with *hrf_ms_labeling*. The histograms are plotted in the range of 0−20 Å with bin widths of 0.67 Å.

centroid-based neighbor count and experimental ln PFs. Second, a centroid-based score function is crucial in predicting structures within Rosetta's *AbinitioRelax* protocol. Within this protocol, the main sampling of conformational space occurs during the centroid scoring phase. Thus, *hrf_ms_labeling* would have maximal impact on predicting structures ab initio if it was utilized during the centroid scoring phase. Future work will focus on developing these ab initio capabilities.

CONCLUSION

In this work, a new Rosetta score term, *hrf_ms_labeling*, was developed. This score term utilizes residue-resolved protection factors from hydroxyl radical labeling (HRF/FPOP) mass spectrometry data and assesses agreement of the protein model with the experimental data. Four proteins (calmodulin, cytochrome c, myoglobin, and lysozyme) that had both available experimental data and known crystal structures were used to benchmark the performance of the score term. Using the linear correlation between the natural logarithm of the experimental protection factors and calculated neighbor counts

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for one of the benchmark proteins, calmodulin, a prediction function was generated to predict the neighbor counts for the other proteins using their respective ln PFs. This prediction function was used as the basis of the new score term *hrf_ms_labeling*. The new score term was used to rescore sets of 20000 models for each protein generated using Rosetta's *AbinitioRelax* application. As a result, the top scoring model increased in quality for all four proteins. The method used for radical generation did not adversely affect the modeling. For two of the four proteins we were even able to identify atomic resolution models using the HRF/FPOP data. In addition, the overall distribution of models moved more toward a funnel-like energy landscape, indicating that good scoring models were closer in structure to their respective natives. Finally, we were able to identify a confidence measure that has the potential to identify successful models without having to know the native structure.

To our knowledge, we are reporting the first method to incorporate experimental HFR/FPOP labeling data in protein structure prediction. This marks an important first step in fully utilizing mass spectrometry-based covalent labeling techniques in quantitative structure predictions, rather than just qualitative explanations. By demonstrating the potential of covalent labeling in conjunction with the protein structure prediction capabilities of Rosetta, these techniques will be elevated to be comparable in utility to other structural biology techniques such as EPR or FRET. The scoring term and applications discussed in this paper are freely available and easily accessible through Rosetta. We have added a tutorial, including a summary of necessary files and command lines, to the Supporting Information.

Future work will focus on extending this methodology to other labeling techniques. While this particular scoring term is specific to HRF, we plan to implement the capability to use labeling data from other mass spectrometry-based covalent labeling experiments in the future. A second direction of our future efforts will be to develop covalent labeling-guided ab initio structure prediction, where the labeling data are used as part of the actual structure generation as opposed to rescoring structures generated in the absence of the experimental data.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b01624.

Experimental methods, Bayesian derivation of *hrf_ms_labeling*, and additional figures (PDF)

Tutorial for the use of the new score term in Rosetta (ZIP)

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Notes

The authors declare no competing financial interest.

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