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6 **MAHOMES II: A webserver for predicting if a metal binding site is enzymatic**

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17

18 *Abstract:*

19 Recent advances have enabled high-quality computationally generated structures for
20 proteins with no solved crystal structures. However, protein function data remains
21 largely limited to experimental methods and homology mapping. Since structure
22 determines function, it is natural that methods capable of using computationally
23 generated structures for functional annotations need to be advanced. Our laboratory
24 recently developed a method to distinguish between metalloenzyme and non-enzyme
25 sites. Here we report improvements to this method by upgrading our physicochemical
26 features to alleviate the need for structures with sub-angstrom precision and using
27 machine learning to reduce training data labeling error. Our improved classifier identifies
28 protein bound metal sites as enzymatic or non-enzymatic with 94% precision and 92%
29 recall. We demonstrate that both adjustments increased predictive performance and
30 reliability on sites with sub-angstrom variations. We constructed a set of predicted
31 metalloprotein structures with no solved crystal structures and no detectable homology
32 to our training data. Our model had an accuracy of 90 - 97.5% depending on the quality
33 of the predicted structures included in our test. Finally, we found the physicochemical
34 trends that drove this model's successful performance were local protein density,
35 second shell ionizable residue burial, and the pocket's accessibility to the site. We
36 anticipate that our model's ability to correctly identify catalytic metal sites could enable
37 identification of new enzymatic mechanisms and improve *de novo* metalloenzyme
38 design success rates.

39

40 *Keywords:* Enzymes, Metalloenzymes, Metalloproteins, Machine Learning

41
42 *Significance statement:* Identification of enzyme active sites on proteins with unsolved
43 crystallographic structures can accelerate discovery of novel biochemical reactions,
44 which can impact healthcare, industrial processes, and environmental remediation. Our
45 lab has developed an ML tool for predicting sites on computationally generated protein
46 structures as enzymatic and non-enzymatic. We have made our tool available on a
47 webserver, allowing the scientific community to rapidly search previously unknown
48 protein function space.

49

50 *Abbreviations footnote:*

51 ML = machine learning

52 RBF = Radial Basis Function

53 CV = cross validation

54 MAHOMES = metal activity heuristic of metalloprotein and enzyme sites

55 DROPP = distribution overlap of a physicochemical property

56 PDB = Protein Data Bank

57 PDE = probability density estimate

58 pLDDT = predicted Local Distance Difference Test

59 MCC = Mathews correlation coefficient

60 TNR = true negative rate

61 TN = true negative

62 TP = true positive

63 FN = false negative

64 FP = false positive

65 1. Introduction

66 Enzymes are biological catalysts that are known to lower activation energy for over
67 8,000 reactions (McDonald, Boyce, and Tipton 2009). Furthermore, enzymes can
68 increase reaction rates by factors of up to 10^{17} -fold (Wolfenden, Ridgway, and Young
69 1998). Enzymes are also becoming increasingly prevalent in industrial processes due to
70 their greener chemistry (Sheldon and Woodley 2018). Despite the importance and
71 extent of enzymatic research, a reproducible physicochemical basis of catalysis remains
72 elusive. This unknown limits *de novo* enzyme design or even reliable identification of
73 enzyme active sites from structure.

74 We have recently used protein structure-based machine learning (ML) to distinguish
75 between very similar sites, metalloenzyme active sites and protein sites that bind metals
76 without any enzyme activity (Feehan, Franklin, and Slusky 2021). Our model, metal
77 activity heuristic of metalloproteins and enzyme sites (MAHOMES), uses an extra-trees
78 algorithm to achieve better performance metrics than available enzyme function
79 predictors. We attribute the classifier's success to training on structural physicochemical
80 properties of similar sites. By training on negative sites that were also in pockets and
81 also coordinated metals, rather than on all other sites on the protein, our classifier was
82 able to assign feature importance based on characteristics that were particular to
83 enzyme activity.

84 Using protein structure-based features enabled MAHOMES to focus on learning
85 physicochemical properties related to catalysis but it relied on structurally determined
86 proteins for its input. The PDB only has ~200,000 solved protein structures (Burley et al.

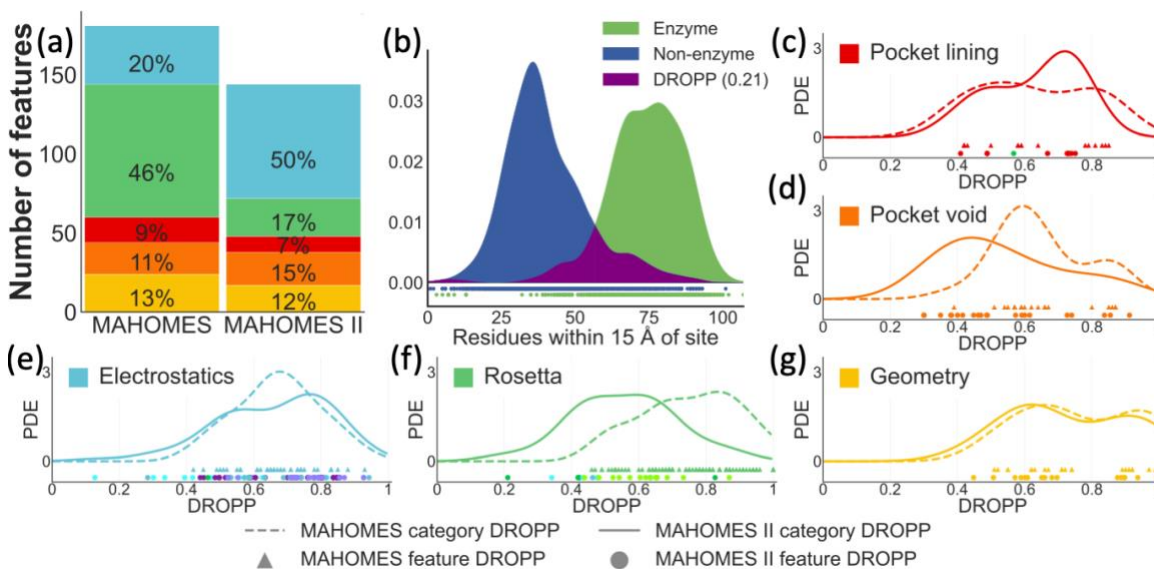
87 2019) thereby limiting MAHOMES utility. Recently, the ML tool AlphaFold2 generated
88 quality protein structure predictions (Jumper et al. 2021) and now two hundred million
89 predicted structures are available for download from AlphaFoldDB (Tunyasuvunakool et
90 al. 2021). However, it remained unclear if these structures could be used for identifying
91 catalytic sites. This concern was compounded by the finding that a relatively low
92 percentage of AlphaFold models have a high enough confidence to be recommended
93 for characterizing binding sites (Thornton, Laskowski, and Borkakoti 2021).

94 To test usage of the computationally generated structures, we updated the calculation
95 methods used for several of MAHOMES features to reduce the need for sub-angstrom
96 accuracy. Then, we use the new features when cross validating ML models to reduce
97 labeling error in our training data labels. The improved features and training data were
98 used with a variety of ML classifiers and techniques. We found that both the feature
99 improvement and reduced labeling error led to increased performance for ML models.
100 Our best ML model, MAHOMES II, outperformed its predecessor on our holdout test-set
101 with 94% precision and 92% recall. Furthermore, MAHOMES II's predictions were more
102 reliable for different input structures of the same site with sub-angstrom differences. We
103 evaluated MAHOMES II on a new set of predicted metalloprotein structures, where it
104 scored 97.5% accuracy on high confidence structures. Finally, we examined the
105 features that MAHOMES II found to be the most important for making successful
106 enzyme or non-enzyme predictions and found a preference for features describing
107 enzyme sites to be densely packed, have buried second shell residues, and pockets
108 that were highly accessible to the metal. MAHOMES II can be accessed online

109 (<https://mahomes.ku.edu>), allowing easy use for the scientific community, regardless of
 110 computational expertise.

111 2. Results

Figure 1: Feature category input space and DROPP (a) The number of features used by MAHOMES and MAHOMES II for each feature category: blue for electrostatics, green for Rosetta energy terms, red for pocket lining, orange for pocket void, and yellow for coordination geometry. (b) Example of DROPP calculation for the number of residues within 15 Å of the site feature. Kernel density estimators for the feature's dataset values of enzymes (green) and non-enzymes (blue) are made and the overlapping region (purple) is calculated to give the features DROPP. (c-g) Comparison of MAHOMES and MAHOMES II DROPP probability density estimate (PDE) for each feature category: (c) pocket lining category, (d) pocket void category, (e) electrostatics category, (f) Rosetta energy terms category, and (g) geometry category. Dotted lines and triangles represent MAHOMES features. Solid lines and circles represent features used by MAHOMES II. Circles are colored by feature groups shown in figure 4b.



112 2.1 New and improved feature calculations

113 To transform metal sites into input for ML algorithms, we identified features belonging to
 114 five categories which have previously been linked to enzymatic activity— coordination
 115 geometry, electrostatics, pocket lining, pocket void, and Rosetta energy terms (Figure 1
 116 a and b).

117 We use a metric to quantify how much a feature's values are similar between enzymatic
118 and non-enzymatic sites. This metric, DROPP (distribution overlap of a physicochemical
119 property) identifies how similar the distribution is between enzymatic and non-enzymatic
120 sites(Figure 1b)(see methods). DROPP was previously found to be lower for features
121 that are more important for predicting enzyme sites.(Feehan, Franklin, and Slusky
122 2021). Therefore, when trying to improve our features, we used DROPP as indicator of
123 feature improvement (Figure 1 c-g). We made efforts to improve all feature classes
124 except coordination geometry, though some improvements were more successful than
125 others.

126 *Electrostatics features expansion:* The most important feature used by the original
127 MAHOMES model was an electrostatic feature (Feehan, Franklin, and Slusky 2021) ,
128 which was the mean second moment of the of the theoretical titration curve's first
129 derivative for ionizable residues in the second shell (3.5-9Å). We modeled this feature
130 after the THEMATIC calculations, which have been used to identify catalytic residues
131 due to their deviations from Henderson Hasselback titration behavior (Somarowthu,
132 Yang, et al. 2011; Tong et al. 2009; Ko et al. 2005).

133 To improve our enzyme activity predictions and further our understanding of
134 electrostatic properties responsible for catalytic activity, we expanded our electrostatic
135 features category from 37 features to 152 features (Sup. Figure S1). To further
136 investigate the success of electrostatics in MAHOMES, we added the Z-score
137 calculations which are used by THEMATICs to measure the relative deviation of the
138 theoretical titration curve's first derivative's second, third, and fourth moments (Ko et al.
139 2005). We also added variables output by the generalized Born program we use for

140 generating theoretical titration curves, BLUUES (Fogolari et al. 2012b). Moreover, since
141 catalytic residues often show interesting shifts in pKa (Pérez-Cañadillas et al. 1998;
142 Bate and Warwicker 2004), we added features for the pKa shift from ideal amino acid
143 values, the pKa shift due to desolvation, the pKa shift due to the interaction with other
144 charges in the molecule with all titratable sites in their neutral state, and the pKa shift
145 due to the interaction between titratable sites. Additional added features in the
146 electrostatic category are the generalized Born atomic radii, a solvation exposure
147 parameter, and solvation energies. After removing redundant features (see methods),
148 72 electrostatic features are used by MAHOMES II (Figure 1a). Six of these new
149 features had lower DROPP than any of the 37 previously used electrostatic features
150 (Figure 1e).

151 *Rosetta features reduction:* In contrast to the expansion of the electrostatic feature
152 space, we reduced the Rosetta feature space while also improving the features and
153 improving our model reproducibility. We calculated Rosetta features in MAHOMES
154 based on spheres with defined radii from the center of the site. In benchmarking
155 MAHOMES, we found that sub-angstrom differences between relaxed structures of the
156 same site caused large shifts in Rosetta feature values. To prevent sub-angstrom
157 differences from significantly changing calculated feature values for the same site, we
158 switched to a radial basis function (RBF) calculation for the Rosetta energy term
159 features. The RBF calculation uses distance to weight each residue's influence on the
160 calculated feature, which prevents subtle changes in the structure from having a
161 significant impact on the calculated value. The RBF Rosetta energy terms category

162 decreased DROPP (Figure 1f) and the number of features used by the category (Figure
163 1a).

164 *Pocket void and pocket lining improvements:* Our previous method, Rosetta pocket
165 measure (Johnson and Karanicolas 2013), did not detect surface pockets for 645
166 dataset sites, therefore 19% of the MAHOMES training data was missing values for
167 pocket void and pocket lining features. GHECOM (Kawabata 2019, 2010), a tool that
168 uses mathematical morphology for finding multi-scale pockets on protein surfaces,
169 generated pocket for 99.5% of the dataset sites. To improve the quality of training data,
170 we removed dataset sites that did not have pocket. Additionally, we added various
171 pocket descriptors, including output features from GHECOM which describe the
172 pocket's shallowness and size rank relative other pockets on the structure. Ultimately,
173 the pocket output by GHECOM lowered the DROPP for features in both the pocket
174 lining and pocket categories (Figure 1c and 1d).

175 2.2 Reduced training data labeling error

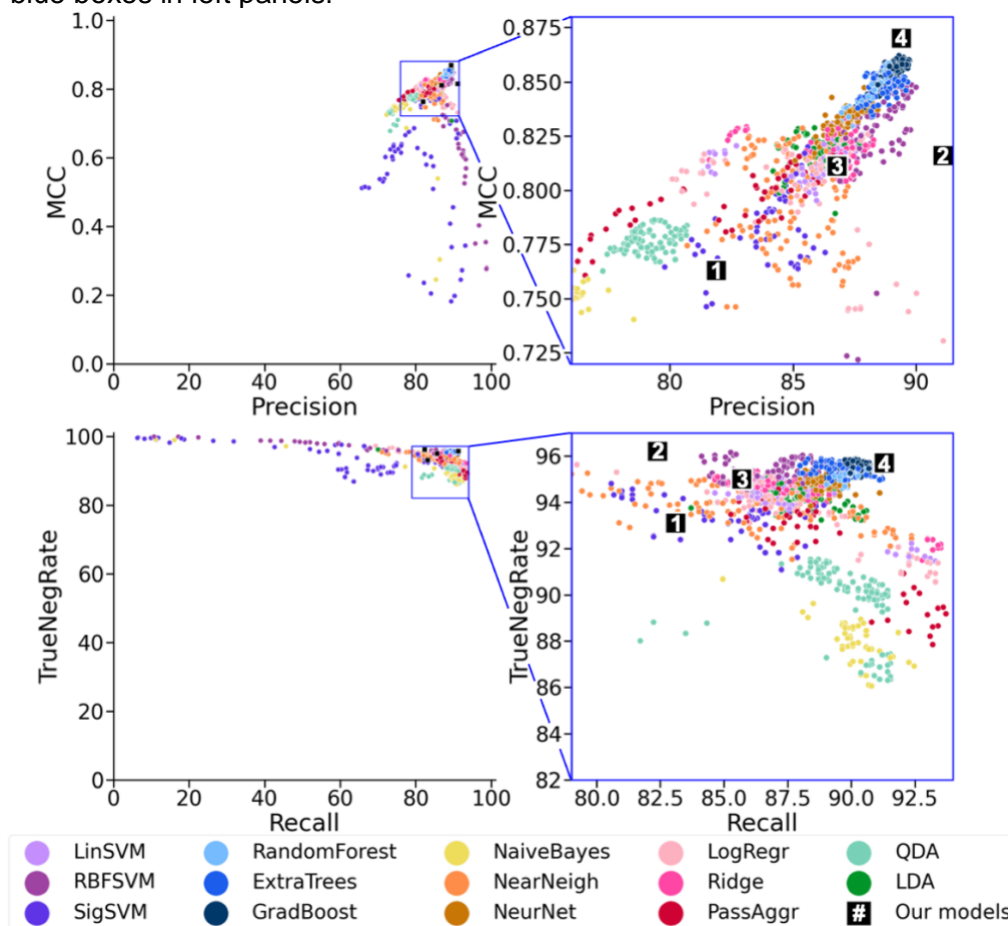
176 Using manual validation, we previously estimated that ~6% of our non-catalytic sites are
177 mislabeled and that ~0% of our catalytic sites were mislabeled (Feehan, Franklin, and
178 Slusky 2021). When using cross validation to evaluate newer (intermediate) iterations of
179 MAHOMES, we found seemingly-incorrect predictions were often actually the sites our
180 data generation pipeline mislabeled. We therefore intentionally used ML to hunt for
181 mislabeled sites in our dataset via cross-validation.

182 Cross-validation is an ML method that leaves out a fraction of the dataset during training
183 so that it can be used to assess the model's predictive performance. The left-out

184 fraction is iterated over the entire dataset, meaning a model makes predictions for each
185 site in the training dataset. We manually examined non-enzymatic dataset sites that
186 were predicted to be enzymatic during cross validation (see methods for more details).
187 Because manual inspection during work on MAHOMES of 50 random dataset enzyme
188 sites revealed an ~0% enzyme labeling error (Feehan, Franklin, and Slusky 2021), we
189 did not examine enzymatic sites that were predicted to be non-enzymatic.

190 We used the available literature (structure publications, RCSB (Burley et al. 2019), and
191 UniProt (UniProt 2019)) to investigate 225 sites that were previously labeled non-
192 enzymatic but were classified during this cross validation as enzymatic. 94 of those
193 sites had definitive literature support of catalytic activity (mislabeled) and 26 PDBs were
194 removed from our set due to inconclusive evidence. Our previous estimate of 6%
195 mislabeled non-catalytic sites implied approximately 158 mislabeled sites in the dataset.
196 Therefore, we estimate that finding 94 mislabeled sites reduces our site mislabeling by
197 60%.

Figure 2: Cross-validation performance by algorithm. Each dot represents one of the 1,792 models assessed in this work. The dots are colored to represent the type of ML algorithm the model uses: support vector machines = purples, decision-tree ensemble methods = blues, linear models = reds, discriminant analysis=greens, naive Bayes = yellow, nearest neighbor = orange, and neural network = brown. Better performing classifiers should have higher precision, Mathews correlation coefficient (MCC), true negative rate (TNR), and recall, meaning better classifiers will be close to the upper right corner. The black boxes with numbers show CV performance of: (1) the previously reported MAHOMES, (2) MAHOMES recalculated with the updated data labels, (3) MAHOMES retrained on updated labels, and (4) MAHOMES II – updated labels, trained on updated labels, and using new features. Right panels are zoomed in views of blue boxes in left panels.



198 2.3 ML model assessment

199 We generated 1,792 different ML models (Figure 2) using the following steps: feature
 200 standardization, feature selection, and fourteen ML classification algorithms using one
 201 of four optimization scoring terms. Since ML algorithms require or are greatly aided by

202 standardization of feature values in order to make comparable scales between the
203 values of different features, we tested four different standardization techniques (see
204 methods). Additionally, large numbers of input features can be detrimental to certain ML
205 algorithms. To decrease the number of features with minimal information loss, we
206 identified four feature subsets each using a different cut off to remove correlated
207 features (see methods). In total we tried six feature sets (four low correlation subsets, all
208 features, and a manually curated set) The six standardized feature sets were then used
209 as inputs to ML classification algorithms which include: linear regression, decision-tree
210 ensemble methods, support vector machines , nearest neighbors, Bayesian
211 classification, and simple neural networks (see methods).

212 Selecting the best variation of the ML algorithm on the same data used to access a
213 model can inflate performance metrics. To avoid inflated model assessment metrics, we
214 used nested cross validation using an inner loop and an outer loop. During the inner
215 loop, the ML algorithm was fine-tuned for a particular scalar and feature set using one of
216 four different scoring metrics— accuracy, precision, Matthews correlation coefficient
217 (MCC)(Matthews 1975), or a multi-score combination of accuracy, MCC, and Jaccard
218 index(Jaccard 1907). Among our hyperparameter search space, each of the top three
219 ranking ML algorithm variations were used to make models that were accessed using
220 the outer loop. In total, we attempted 4,032 machine learning combinations (14
221 algorithms x 6 feature sets x 4 standardization techniques x 4 optimization terms x 3 top
222 algorithm variations). Due to convergence during model optimization, this process
223 resulted in 1,792 different ML models.

224 The vast majority of all attempted ML models in this study outperformed the previous
225 reported MAHOMES cross validation metrics (Figure 2, black box 1) because the
226 training set was substantially corrected for all the new models. In order to make a more
227 fair comparison between MAHOMES and the new models, we re-calculated MAHOMES
228 cross validation performance metrics using the corrected enzyme/non-enzyme labels
229 (Figure 2, black box 2). The number of MAHOMES cross validation false positives
230 dropped from 182 to 90, which increased the precision by nearly 10% (Figure 2, top
231 row) but the rest of the performance metrics remained far below those of our new
232 models.

233 To assess if the increase in performance was purely due to corrected data labels, we
234 assessed an intermediate model, which retrained MAHOMES using the corrected data
235 but using the old MAHOMES features (Figure 2, black box 3). Despite an increase in
236 recall, the retrained MAHOMES still identified significantly fewer enzyme sites than
237 similar ML models that used the new features (Figure 2, CV blue). Thus, our ML
238 benefitted from the improvement of both the quality of training labels and the improved
239 features.

240 2.4 MAHOMES II performance

241 To evaluate if these metrics are inflated from overtraining despite cross validation, we
242 also predicted sites in an updated hold-out test set. In addition, we developed a new set
243 derived from the hold-out test set to evaluate the reliability of the models. This set, the
244 T-metal-sites¹⁰, includes ten different minimized structures for each site in T-metal-
245 sites. The sub-angstrom variations for each site allowed us to calculate two

246 reproducibility metrics. First, we calculated the divergence frequency (equation 4,
247 methods), which is the percent of test-set sites that received both an enzyme and non-
248 enzyme prediction. Then, we calculated the divergence score (equation 5, methods), a
249 measurement of the severity of divergent predictions. The divergence score ranges
250 from 0 (the site receives the same prediction for every structure) to 1 (the site is
251 predicted enzymatic for half of the structures and non-enzymatic for the other half).

252 **Table 1.** ML model performance evaluations. Predictive performance of MAHOMES,
253 retrained MAHOMES with corrected labels, and MAHOMES II on the holdout test-set, T-
254 metal-sites10, and the quality AlphaFold set, which is the subset of generated sites with
255 confidence scores recommended to characterize binding ($pLDDT > 90$) within 15 Å of the
256 metal. TNR is the true negative rate and MCC is the Mathews correlation coefficient.
257 Descriptions of performance metric calculations in methods. *Evaluation using T-metal-
258 sites, which includes ten incorrectly labeled sites and eight undeterminable sites which
259 were removed from T-metal-sites10.

ML model	Evaluation	Accuracy	Precision	Recall	TNR	MCC	div. freq.	div. score
MAHOMES*	T-metal-sites	94.2	92.2	90.1	96.2	0.87	-	-
Recalculated MAHOMES	T-metal-sites10	92.6	94.2	85.3	96.9	0.84	6.6	0.53
	Quality AlphaFold set	92.7	94.1	66.7	99.0	0.75	-	-
Retrained MAHOMES	T-metal-sites10	93.4	91.9	90.0	95.4	0.86	5.9	0.55
MAHOMES II	T-metal-sites10	94.9	94.1	92.1	96.6	0.89	5.0	0.47
	Quality AlphaFold set	97.6	95.7	91.7	99.0	0.92	-	-

260

261 For MAHOMES II, we selected a GradBoost model that used FeatureSet4. We selected
262 that as our final model because of its high cross validation metrics. However,
263 ExtraTrees models, which is the algorithm used by the previous MAHOMES model, had

264 the lowest divergence frequency (Figure S2). So, we further refined hyperparameters,
265 which were too computationally expensive to optimize for GradBoost during our inner
266 cross validation using GridSearch optimization to mimic those favored by the
267 ExtraTrees models (supplemental methods MAHOMES II, fine tuning). The final
268 MAHOMES II model had a cross validation MCC higher than any other ML model
269 (Figure 2 black box 4). Though this could have indicated an overfit model, our final
270 performance evaluation on the hold-out test set T-metal-sites10 (Table 1), which we
271 only used for reproducibility metric calculations during optimization, fell within the
272 projected performance of the CV assessment (Sup. Table S1), thereby supporting the
273 dependability of both the CV assessment and final evaluation of MAHOMES II
274 performance.

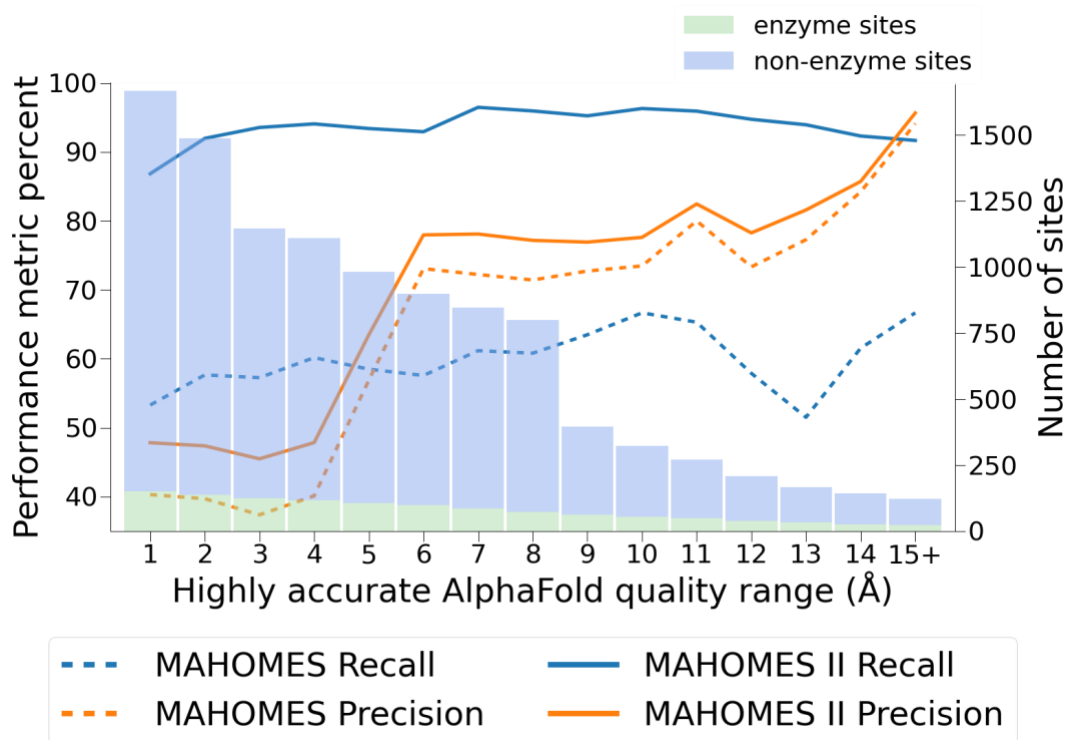
275 In addition to improved performance, our aim was to lessen the effects of sub-angstrom
276 deviations in input structures. Retraining MAHOMES with corrected labels decreased
277 the divergency frequency but increased the divergence score (Table 1). Updated
278 features in MAHOMES II further decreased the divergence frequency *and* decreased
279 the divergence score, demonstrating that our feature improvements were effective at
280 improving reproducibility.

281 To test our hypothesis that upgraded features and improved training data can be used
282 to successfully predict enzyme activity for predicted structures, we tested MAHOMES II
283 on a set of AlphaFold generated structures (Tunyasuvunakool et al. 2021; Jumper et al.
284 2021). However, AlphaFold generated structures do not have ligands such as metals.
285 To create the AlphaFold set of protein structures with metals we queried UniProt
286 (UniProt 2019) for proteins with known metal coordinating residues and no solved

287 crystal structure and filtered for metal ions that could be mapped to AlphaFoldDB
288 structures. Benchmarking our metal method using dataset sites revealed sub-angstrom
289 placement accuracy.

290 AlphaFold predictions have a confidence metric associated with each residue. The
291 AlphaFold authors recommend using residues with high confidence (pLDDT >90) for
292 characterizing binding sites. Very few sites in our AlphaFold set had high confidence for
293 all residues used to calculate the MAHOMES II features (residues within 15 Å of the
294 metal). So, we made MAHOMES II predictions on the entire non-homologous,
295 metalloprotein AlphaFold set and made multiple performance evaluations by requiring

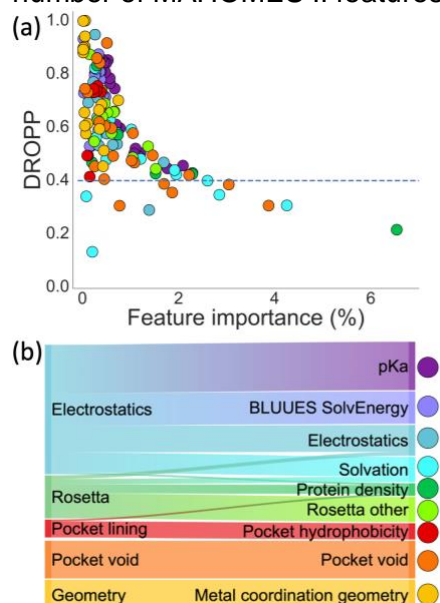
Figure 3. AlphaFold set performance evaluation. The number of enzyme (green bar) and non-enzyme (blue bar) AlphaFold set sites containing only highly confident residues within X Å, where X ranges from 1 to 15. The recall (blue lines) and precision (orange lines) of MAHOMES (dotted lines) and MAHOMES II (solid lines) are shown for the AlphaFold set sites at each cutoff.



296 residues within $X \text{ \AA}$ of the site to be high quality (Figure 3, Table 1 and S2). For the

297 entire AlphaFold set, MAHOMES II was able to

Figure 4. Feature importance and DROPP. (a) Each dot represents MAHOMES II feature and is colored by physicochemical group. The y-axis is the feature's DROPP, or overlap between values for enzyme and non-enzyme dataset sites. The x-axis is feature importance for MAHOMES II, which is a measurement of the mean decrease of impurity by a feature during training. The blue dotted line represents the lowest feature DROPP from MAHOMES. (b) Sankey diagram of feature distribution between feature categories and feature groups, where width is representative of number of MAHOMES II features.



correctly identify 87% of the enzyme sites (recall) and 90% of the non-enzyme sites (true negative rate)(Sup Table S2). As we removed structures with low quality residue predictions close to the metal, MAHOMES II performance increases up to an accuracy of 97.5% an improvement even over our test-set metrics (Figure 3).

Interestingly, the coordinating residues' quality was not the most important, as MAHOMES II performance increases the most as the quality range increases from 4 \AA to 6 \AA (Figure 3). MAHOMES II enzyme recall (predicting which protein sites are catalytic) was very stable over all confidence regions and vastly outmatched the previous MAHOMES model (Figure 3).

2.5 Feature importance

314 Because MAHOMES II uses a decision-tree based
 315 gradient boosting algorithm, we can measure each feature's importance via the feature
 316 contribution to the decrease in impurity on the training data. As previously shown
 317 (Feehan, Franklin, and Slusky 2021), features with high importance had low DROPP

318 (overlap between enzyme site and non-enzyme site feature values). The five most
 319 important features for MAHOMES II had lower DROPP than any MAHOMES feature
 320 (Figure 4). However, the feature with the lowest DROPP, the minimum solvent exposure
 321 parameter for outer sphere (3.5 – 9 Å) ionizable residues, was not important to
 322 MAHOMES II—it ranked 116th in feature importance for MAHOMES II (Sup Table S3).
 323 Hence, though quantitative differences, such as those measured by DROPP, can
 324 indicate potentially important features, MAHOMES II is learning more than just these
 325 numerical differences in order to successfully differentiate between enzyme and non-
 326 enzyme sites.

327 **Table 2.** Feature group importance. Each feature group is described by its number of
 328 included features (num total), the percent of MAHOMES II feature space accounted for
 329 by the group, the total feature importance for all group features, the mean feature
 330 importance of features in the group, the rank of the most important feature in the group,
 331 and the mean DROPP of features in the group.

Feature group	Number of features	MAHOMES II feature space	Feature importance			DROPP mean
			total	mean	Max (rank)	
Local protein density	7	4.9%	14%	2.0%	6.5% (1)	0.48
solvation	14	9.7%	18%	1.3%	4.3% (2)	0.46
Pocket void	21	14.6%	22%	1.1%	3.9% (3)	0.54
pKa	27	18.8%	19%	0.7%	2.1% (10)	0.69
Rosetta	14	9.7%	9%	0.6%	1.5% (17)	0.62
Electrostatics	17	11.8%	9%	0.5%	1.7% (16)	0.63
Pocket hydrophobicity	9	6.2%	2%	0.2%	0.4% (75)	0.64
BLUUES SolvEnergy	18	12.5%	4%	0.2%	0.5% (59)	0.75
Metal coordination geometry	17	11.8%	3%	0.2%	0.7% (34)	0.74

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333

334 Since the original feature categories were based on calculation method, we transitioned
335 to feature groups (Fig. 1B) for analyzing which physicochemical properties were the
336 most important for identifying catalytic activity. For example, the Coulombic electrostatic
337 potential RBF feature had been in the Rosetta category but was a better fit for the
338 electrostatic group. Due to differences in feature importance, we split features
339 describing solvation into two groups. The BLUUES SolvEnergy group includes features
340 calculated directly from the BLUUES solvation energy output. We placed other solvation
341 related features in the Solvation group.

342 The three most important feature groups are local protein density, solvation, and pocket
343 void (Table 2). Despite only making up 29% of MAHOMES II feature space, these
344 groups account for 55% of what the model learned during training. These feature
345 groups also have the lowest average DROPP. Using the DROPP plots for features in
346 these groups, we identify specific subgroups that were fundamental to MAHOMES II
347 distinguishing between enzyme and non-enzyme sites.

348 The local protein density feature group (seven features) has the highest average feature
349 importance and includes Lennard-Jones energies and the number of residues within a
350 certain distance of the site. This group includes the most important MAHOMES II
351 feature, the number of residues within 15 Å (Figure 1B), which is more important than
352 the 44 least important features combined (Table S3).

353 The next most important group, the solvation feature group (fourteen features), includes
354 Rosetta solvation features and BLUUES generalized Born features. The second most
355 important MAHOMES II feature is the average BLUUES solvent exposure parameter for

356 second shell (3.5-9Å) ionizable residues. The DROPP plot for this feature shows that
357 most second shell ionizable residues are buried for enzyme sites and relatively exposed
358 for non-enzyme sites (Figure S3b). This group also contains the sixth most important
359 feature, the maximum second shell generalized Born radius, which measures an atom's
360 shielding from high solvent dielectric (Figure S3f). Enzyme sites also have higher
361 Rosetta solvation features that rank fifth, twelfth, and twenty-first in feature importance
362 (Figure S3e, Table S3), which corresponds with the energetic cost associated with
363 buried charged residues.

364 The third most important feature group is the pocket void group (twenty-one features).
365 The pocket void group has features that describe the pockets' location, shape, and size.
366 The third most important MAHOMES II feature describes the slice of the pocket closest
367 to the metal as being larger for enzymes (Figure S3c). The fourth most important
368 feature is the shortest distance between a metal and pocket grid point, which is smaller
369 for enzyme sites (Figure S3d). These features combine to make a subgroup describing
370 site accessible pockets.

371 **3. Discussion**

372 Our previous classifier, MAHOMES, outperformed available, alternative methods for
373 classifying enzymes or non-enzymes. MAHOMES II, outperforms its predecessor with
374 increased reliability thanks to both upgraded features and reduced training data error.
375 MAHOMES II's performance generalize to new, unseen metalloproteins. Moreover,
376 MAHOMES II learned physicochemical properties related to our current understanding
377 of enzyme function.

378 3.1 MAHOMES II learned general enzyme activity

379 A key question of any classifier is if it has learned beyond its training, i.e. can it predict
380 for examples it has never seen before. For MAHOMES II, training on solved, crystal
381 metalloproteins structures could limit its performance to the 0.056% of proteins with
382 experimentally determined structures (UniProt 2019). Our evaluation using the newly
383 curated AlphaFold set finds that MAHOMES II generalizes to new enzyme reactions
384 and even generalizes to very unrelated proteins.

385 Alternative tools that can be used to identify enzymatic activity (Zou et al. 2019; Kumar
386 and Skolnick 2012) are less successful than MAHOMES II at predicting if our set of
387 metalloproteins are catalytic (Table S4). Despite using ML, these enzymatic activity
388 classifiers and catalytic residues predictors (Somarowthu and Ondrechen 2012; Song et
389 al. 2018) rely on homology-based features causing their performance to not be
390 transferable to catalysis more generally or be applicable for novel or designed enzymes.

391 To make our training data different enough from our testing data to facilitate
392 generalizability, our training datasets and test-sets in both in this work and our previous
393 work (Feehan, Franklin, and Slusky 2021) remove redundancy using local similarity. We
394 only kept sites with dissimilar surrounding amino acid identities preventing training and
395 evaluation of repeated sites among homologs and rare cases of similar active sites on
396 different structural folds (Parasuram et al. 2016).

397 Using the AlphaFold data set, we determined that our model was extremely
398 generalizable and was not implicitly using homology trends. The extensive quantity of
399 AlphaFold structures and experimental data from UniProt for enzyme labeling (instead

400 of homology) allowed us to use a very high E-value of 1, i.e. only proteins with no
401 evolutionary relationship, for creating our AlphaFold set. In comparison, only 17% of our
402 previous test set, T-metal-sites10, sequences have no detectable homology to
403 metalloproteins used for training MAHOMES II (E-value > 1). Furthermore, only seven
404 of the 46 biochemical reactions included in the AlphaFold set are also included in the
405 dataset used to train MAHOMES II. Despite the use of computationally generated
406 structures and strict redundancy removal, MAHOMES II's 90-97.5% accuracy on the
407 AlphaFold set was similar to its CV and T-metal-sites10 evaluations. Therefore, we
408 believe our assessment of MAHOMES II performance will remain true for any natural
409 metalloprotein structure uploaded by the community on the webserver, even if it is for a
410 novel enzyme reaction. However, due to a lack of available structures, we remain
411 uncertain if MAHOMES II performance transfers to *de novo* metalloproteins.

412 3.2 The less important first shell

413 Frequently, enzyme bioinformatics focuses on the active site's first shell, which is the
414 residues interacting directly with substrate(s) or cofactor(s), such as metal ion(s) (Bartlett
415 et al. 2002; Furnham et al. 2016; Ribeiro et al. 2018). The crucial roles played by first
416 shell residues are well supported by conservation and experimental studies (Morley and
417 Kazlauskas 2005; Ribeiro et al. 2020). MAHOMES II has 60 features that describe only
418 first shell properties, covering coordination geometry, inner shell electrostatics (< 3.5 Å
419 from metal), and pocket lining. Despite making up 42% of MAHOMES II's feature space,
420 first shell features account for only 18% of feature importance. Since the same metal
421 and coordinating residues are found to participate in enzyme and non-enzyme functions
422 (Lee et al. 2019), it makes sense that first shell features are largely incapable of

423 differentiating enzyme and non-enzyme sites in metalloproteins since in both the first
424 shell coordinates metals. Consequently, despite the well-known critical roles of the first
425 shell, distinction between metallo-enzymes and metallo-proteins is driven by more
426 distant physicochemical properties.

427 3.3 Comparing important MAHOMES II subgroups to current enzyme paradigms

428 The physicochemical features most important to MAHOMES II success can be
429 considered as three groups/subgroups—1) local protein density, 2) second shell
430 ionizable residue burial, and 3) site accessibility of pockets (in the pocket void feature
431 group) – align with the current paradigm of the enzyme function, which also consists of
432 three features: 1) local environment control of functional sites through control of water
433 access, 2) networks of residue interactions spanning from functional residues, and 3)
434 conformational dynamics (Mazmanian, Sargsyan, and Lim 2020; Agarwal 2019).

435 *Control of water access:* MAHOMES II captures local environmental control through
436 water access with two of the important MAHOMES II feature subgroups: the local
437 protein density group and site accessibility of pockets feature subgroup. The local
438 protein density features, detect the dense packing of enzyme sites which protects them
439 from high external dielectrics of bulk water, enhancing the local electrostatic effects from
440 hydrogen-bonding and charge-charge interactions. The site accessibility of pockets
441 subgroup identifies close pockets with large openings adjacent to the site that can
442 enable access by individual water molecules, which commonly participate as
443 nucleophiles, to form hydrogen-bonding networks, and to facilitate the release of

444 products. Hence, MAHOMES II can detect the control of water access to enzyme sites
445 by combing local protein density and site accessibility of pockets.

446 *Networks of connected interactions:* Distal residues that interact with catalytic residues
447 or as part of networks connecting to the catalytic site are essential for fine tuning and
448 optimization of enzyme activity(Dudev et al. 2003; Somarowthu, Brodtkin, et al. 2011;
449 Parasuram et al. 2018; Brodtkin et al. 2015; Tiwari et al. 2014; Coulther, Ko, and
450 Ondrechen 2021; Coulther et al. 2021; Ngu et al. 2020). The MAHOMES II burial of
451 ionizable residues feature subgroup differentiates enzyme sites based on buried second
452 shell polar and charged residues, which would be a direct result of crucial coupled
453 interactions that enhance enzyme activity. In addition, the MAHOMES II local protein
454 density group uses the density of residues surrounding the active site to provide the
455 most basic description of networks of interactions connected to enzyme sites with the
456 potential to promote activity. The combination of these two subgroups therefore seems
457 to accurately estimate connected networks.

458 *Conformational flexibility:* Although we did not design any MAHOMES II features to
459 directly describe conformational dynamics, the final aspect of the current enzyme
460 function paradigm, all of the three most important physicochemical subgroups describe
461 properties that affect conformational stability. Local protein density describes tight
462 packing that increases backbone hydrogen-bonding which increases stability and
463 rigidity. Burial of charged residues amongst nonpolar sidechains makes for an
464 energetically unfavorable conformation that will promote destabilization and flexibility.
465 Moreover, interactions between charged sidechains will also increase or decrease
466 stability of various active site conformations depending on the charges. Finally, the site

467 accessibility of pockets enables active site interactions with cofactors, substrates, and
468 solvent that will change the flexibility or rigidity of an active site. Therefore, all three
469 important subgroups contribute to the conformational changes required for enzyme
470 activity, such as the shifting from the ground state to transition state(s).

471 3.4 Machine learning lessons for metalloenzyme design

472 Considering our training dataset covers all enzyme reaction types(Feehan, Franklin,
473 and Slusky 2021), the physicochemical properties highlighted by MAHOMES II gives us
474 insight for making better metalloenzyme designs. Their feature importance indicates a
475 fundamental blueprint that is harnessed by a range of known catalysis mechanisms
476 performed by nature. To this point, recent work exploring the functional space of non-
477 metallo TIM barrel enzymes has also highlighted the importance of local atomic
478 density(Lipsh-Sokolik et al. 2023). *De novo* enzyme designs on non-enzyme protein
479 backbones could benefit from selecting densely surrounded positions with large pocket
480 openings. Furthermore, lower solvation penalties for buried for ionizable residues might
481 also help design active sites that more closely resemble those in nature. We anticipate
482 that dense protein regions with buried ionizable residues can improve the success rate
483 of designed enzymes and limit additional steps that are currently necessary to reach
484 native enzyme reaction rates, such as directed evolution (Yang, Wu, and Arnold 2019).

485 **4. Conclusion**

486 Our ML classifier, MAHOMES II (<https://mahomes.ku.edu>), uses protein structure-based
487 features describing the local site to distinguish between enzyme and non-enzyme metal
488 ion sites on proteins with 94% precision and 92% recall. We demonstrated that

489 MAHOMES II can make quality predictions for computationally generated structures,
490 which greatly expands its utility when combined with the structure prediction tool
491 AlphaFold. Additionally, the similarity among performance metrics for our cross-
492 validation, holdout test-set, and evolutionarily unrelated AlphaFold set supports that
493 MAHOMES II evaluation is not bias, overfit, or the result of off-target learning. Finally,
494 we were able to identify that MAHOMES II was making successful predictions due to its
495 use of physicochemical features related to densely packed active sites, burial of second
496 shell ionizable residues, and site accessible pockets.

497 **5. Methods**

498 5.1 Metal ion dataset and T-metal-sites

499 The data developed to train and evaluate MAHOMES (Feehan, Franklin, and Slusky
500 2021) is, to our knowledge, the largest non-redundant dataset of enzymatic and non-
501 enzymatic labeled protein bound metal ions. Briefly, protein structures containing
502 transition metals were filtered to remove poor quality structures and structures dissimilar
503 to metalloenzymes. Metal ion sites bound to multiple chains were removed to avoid
504 labeling partial enzyme active sites during our homology-based enzyme labeling.
505 Metalloenzymes were identified using explicit enzymatic annotations and homology to
506 entries in the Mechanism and Catalytic Site Atlas (M-CSA)(Ribeiro et al. 2018), a
507 database of enzyme active sites. Alignment with M-CSA homolog structures was used
508 to label metal sites on metalloenzymes as enzyme or non-enzyme. Metals on
509 metalloproteins that lacked explicit enzymatic annotations and had no M-CSA homolog
510 were labeled as non-enzyme sites. Finally, sequence and structural homology were
511 used to remove redundancy. Sites on structures deposited in the Protein Data Bank
512 (PDB)(Berman et al. 2000) prior to 2018 were placed in the dataset, which was used for
513 training ML models, ML optimization, and model selection. Sites on structures deposited
514 in the PDB in 2018 or later were placed in a holdout test-set, called T-metal-sites, which
515 was used for final model evaluation.

516 All the metalloprotein structures in the dataset and T-metal-sites were relaxed using
517 Rosetta (Conway et al. 2014) using a previously provided RosettaScript (Feehan,
518 Franklin, and Slusky 2021). To remove loosely bound metals that are less likely to be

519 physiologically relevant, i.e. crystal artefacts, we removed 729 sites that moved more
520 than 3 Å from the aligned crystal structure. We also removed 179 sites that failed
521 MAHOMES feature calculations since this was commonly due to issues like lack of
522 multiple coordinating atoms. New sites were defined as any metals within 5 Å of each
523 other in a relaxed structure. Since the original dataset defined sites using the crystal
524 structures, the revised set slightly differs in the number of sites. All sites containing a
525 metal atom that was previously a part of an enzyme site were labeled enzyme. Any
526 remaining site with a metal atom that was previously included in a non-enzymatic site
527 was labeled non-enzymatic. All other sites on these structures were discarded. We
528 found that MAHOMES was susceptible to making different predictions for the same site
529 on different relaxed structures. To check model prediction reproducibility, we included
530 the ten relaxed structures with the lowest Rosetta energy units for each metalloprotein
531 in T-metal-sites, making T-metal-sites10. We repeated the labeling procedure for the T-
532 metal-sites10 sites.

533 We removed sites that were flagged by our automated feature process as problematic
534 or that had extreme outlier feature values (greater than ten standard deviations from the
535 dataset mean). Manual examination of sites with incorrect ML predictions identified a
536 significant number of incorrect non-enzyme labels by our pipeline for sites in both the
537 dataset and T-metal-sites10 (Table S5). Sites found to actually be enzymatic were
538 relabeled and sites with undeterminable enzyme activity were removed. At the end of
539 this work, the MAHOMES II dataset contained 957 enzyme sites and 2,467 non-enzyme
540 sites. The final T-metal-sites10 consisted of 1,895 enzyme entries and 3,277 non-

541 enzyme entries, which were representative of 189 enzyme sites and 328 non-enzyme
542 sites.

543 5.2 Feature engineering

544 For machine learning input features, we calculated physicochemical properties for five
545 categories – Rosetta energy terms, pocket void, pocket lining, electrostatics, and
546 coordination geometry. A complete feature list with descriptions can be found in Table
547 S6. For exact calculations, please see our available github code (Feehan et al. 2022).

548 5.2.1 Rosetta energy terms

549 Rosetta 3.13 was used to score all residues in a metalloprotein structure for all energy
550 terms in the energy function *beta_nov16* with all weights set to one (Alford et al. 2017).
551 For each energy term, E , a squared inverse radial basis function (Eq. 1) was used to
552 calculate the energy for a given site, \mathbf{s} .

$$553 \quad E(\mathbf{s}) = \sum_r \frac{E(r)}{d(r)^2} \quad (\text{Eq. 1})$$

554 where r is a residue with a distance $d(\mathbf{r}) < 15 \text{ \AA}$ from the site center. We included the
555 number of residues used for these calculations as a feature. Our Rosetta energy terms
556 category included 25 features in total.

557 5.2.2 Pocket void terms

558 We used GHECOM (Kawabata 2019) – a program for detecting multiscale pockets via
559 grid representations and probes – to identify all pockets for a given metalloprotein.
560 Then, for each site on the metalloprotein, we identify all adjacent pockets – pockets with

561 a grid point within 5Å of the site's center. For sites with multiple adjacent pockets, we
562 select the closest adjacent pocket with a volume greater than 100 Å³ as its pocket. In all
563 other cases, the GHECOM pocket with the closest grid point is selected.

564 We used the selected pocket to calculate the pocket void features previously used by
565 MAHOMES (Feehan, Franklin, and Slusky 2021), which include volume, Euclidean and
566 Manhattan distance between pocket centroid and site center, terms describing the size
567 and shape of three pocket slices at the site center, pocket center, and midpoint of site
568 center and pocket center.

569 We added pocket void features output by GHECOM for the relative rank among all the
570 metalloprotein's detected pockets and terms that describe the pockets shallowness and
571 openness of the pocket. Then, we rotated the pocket so that the z-axis runs from the
572 protein centroid to the pockets centroid and calculate its height, max z – min z, and
573 depth, the Euclidean distance between the grid points with the max z and min z. Finally,
574 we calculate the site's height and depth in the pocket using the Euclidean distance
575 between the site center and the max z grid point or min z grid point respectively.

576 5.2.3 Pocket lining

577 The selected GHECOM pocket was used to identify pocket adjacent residues (within 3.0
578 Å). We identified pocket lining residues as pocket adjacent residues with a sidechain
579 distance of less than 2.2 Å or where the sidechain was closer to the pocket than the
580 backbone. The pocket lining residues were used to calculate the average, minimum,
581 maximum, skew, and standard deviation of the hydrophobicity for both Eisenberg
582 (Eisenberg et al. 1984) and Kyte-Doolittle (Kyte and Doolittle 1982). We also calculated

583 the sum of the pocket lining residues van der Waals sidechain volumes, the volume of
584 the pocket without the lining residues' sidechains, and the percent of that volume
585 occupied by the sidechains. Finally, the number of pocket lining residues and the
586 number of backbone adjacent residues (pocket adjacent but not considered pocket
587 lining) were included as features.

588 5.2.4 Electrostatic terms

589 Our previous electrostatics features were based on the use of theoretical titration curves
590 by THEMATICS (Ondrechen, Clifton, and Ringe 2001; Somarowthu, Yang, et al. 2011;
591 Ko et al. 2005). To calculate these, we used the generalized Borne program, BLUUES
592 (Fogolari et al. 2012a). For the first derivative of the theoretical titration curve, we
593 calculated the second, third, and fourth moment of each ionizable residue using SciPy's
594 (Virtanen et al. 2020) variation, skew, and kurtosis functions respectively. The mean,
595 standard deviation, maximum, minimum, and range was calculated for two shells. The
596 first, inner shell included ionizable residues within 3.5 Å of a site's metal atom(s). The
597 second, outer shell included ionizable residues within 9 Å of a site's metal atom(s),
598 excluding residues that are in the first shell. For each moment calculation, the Z-score
599 was calculated (Eq. 2), where x is the residue's moment value, μ is the moment's average
600 for all of the structure's ionizable residues, and σ is the moment's standard deviation. We
601 turned this into a site feature by counting the number of residues with a Z-score greater
602 than 1.

$$603 \quad z = \frac{x - \mu}{\sigma} \quad (\text{Eq. 2})$$

604 All residues from both shells were used to calculate an environmental feature for each
605 moment using a squared inverse radial basis function (Eq. 1). The number of residues
606 used for the inner shell, outer shell, and environmental feature calculations were also
607 saved to be used as features.

608 The inner shell, outer shell and environmental features were also calculated for additional
609 BLUUES outputs, which included: generalized Born radius, residuals for the deviation of
610 the theoretical titration curve from a sigmoidal curve, pKa shift from ideal amino acid
611 values, pKa shift due to desolvation, pKa shift due to the interaction with other charges in
612 the molecule with all titratable sites in their neutral state, pKa shift due to the interaction
613 between titratable sites, solvation energies, and solvent exposure parameter. All of the
614 structure's residues were sorted by BLUUES solvation energy and placed into five bins;
615 destabilizing ranks were assigned from highest to lowest solvation energy and stabilizing
616 ranks were assigned from lowest to highest solvation energy. The inner shell, outer shell
617 and environmental features were calculated for both destabilizing and stabilizing ranks.
618 Overall, there are 152 electrostatic features.

619 5.2.5 Coordination geometry terms

620 We used FindGeo (Andreini, Cavallaro, and Lorenzini 2012) to determine the
621 coordination geometries of the site's metal atom(s). First, we record the total number of
622 ligand N, O, S, and other atoms used as input for FindGeo (within 3.5 Å of any site
623 metal). Then, we record if the metal atom(s) were identified as a regular, distorted, or
624 irregular geometry. If the geometry is regular or distorted, we use the RMSD from the
625 idealized geometry, the number of coordinating atoms for the geometry, and if it is

626 completely or partially filled. To prevent issues with ML algorithms and categorical
627 features, the number of coordinating geometries are one hot key encoded, giving us a
628 total of 20 coordination geometry features.

629 5.2.6 Feature analysis

630 DROPP was calculated as previously described for feature similarity (Feehan, Franklin,
631 and Slusky 2021). For discrete features, we used the Jaccard index between the
632 proportions observed in the enzymatic and non-enzymatic sites (Jaccard 1907). For
633 continuous features, we calculated the overlap of the kernel density estimators for the
634 values of the enzymatic and non-enzymatic sites. To prevent extreme outliers from
635 having large influence on DROPP, values greater than ten standard deviations the
636 mean were ignored. Only dataset sites were used for calculating DROPP. The code for
637 the DROPP calculation can be found in the MAHOMES II repository file
638 FeatureCalculations/CalcFeatureDROPP.py (Feehan et al. 2022).

639 Any feature that had the same value calculated for the entire dataset was discarded,
640 leaving a total of 250 features. To decrease feature space with minimal information loss,
641 additional subsets of features were identified for ML input using maximum correlation
642 cutoffs between features in the same category (Sup. Figure S1). For highly correlated
643 features, the feature with the higher DROPP was removed. FeatureSet2, FeatureSet3,
644 and FeatureSet5 used correlation cutoffs of 0.99, 0.9, and 0.75 respectively. Due to the
645 dramatic increase of electrostatic features, FeatureSet4 used a correlation cutoff of 0.75
646 for electrostatic features and 0.9 for other categories. Finally, we manually selected
647 features for FeatureSet6.

648 5.2.7 Preparation for ML

649 ML algorithms require or are greatly aided by standardization of feature values to take
650 them close to zero and make comparable scales between the values of different
651 features. We selected four different standardization techniques available in scikit-learn
652 (Pedregosa et al. 2011) to use during model optimization and selection. The
653 StandardScaler removes the mean and divides by the features standard deviation. The
654 RobustScaler removes the median and divides by range between the 20th and 80th
655 quantile to mitigate the effect of extreme outliers. We also examined uniform and
656 gaussian QuantileTransformers which use non-linear transformations to map feature
657 values to uniform or gaussian distributions respectively. All scalars include an imputer to
658 fill missing feature values with the dataset sites' average feature values. MAHOMES II
659 used the uniform QuantileTransformer.

660 5.3 Machine Learning

661 5.3.1 Classification performance metric calculations

662 To calculate predictive performance metrics, a prediction is counted as a true positive
663 (TP) if it is an enzyme prediction for an enzyme labeled site. It is considered a false
664 positive (FP) if it is an enzyme prediction for a non-enzyme labeled site. A true negative
665 (TN) is a non-enzyme prediction for a non-enzyme labeled site. Finally, a false negative
666 (FN) is a non-enzyme prediction for an enzyme labeled site. The TP, FP, TN, and FN
667 counts are then used to calculate a model's accuracy, precision, recall, true negative
668 rate (TNR), and Matthews correlation coefficient (MCC)(Matthews 1975).

669
$$accuracy = \frac{TP+TN}{TP+FP+TN+FN} \quad (\text{Eq. 3})$$

670
$$precision = \frac{TP}{TP+FP} \quad (\text{Eq. 4})$$

671
$$recall = \frac{TP}{TP+FN} \quad (\text{Eq. 5})$$

672
$$TNR = \frac{TN}{TN+FP} \quad (\text{Eq. 6})$$

673
$$MCC = \frac{(TP \times TN) - (FP \times FN)}{\sqrt{(TP+FP)(TP+FN)(TN+FP)(TN+FN)}} \quad (\text{Eq. 7})$$

674 5.3.2 Optimization of ML model(s)

675 Since the best performing hyperparameters for ML classification algorithm can change
676 depending on input feature subset and standardization technique, a nested cross
677 validation (CV) strategy was used to find the optimal ML models. The outer CV used
678 stratified k-fold and the inner loop used stratified shuffle split. During the inner loop,
679 different hyperparameters sets were attempted and scored using one of four terms -
680 accuracy, precision, MCC, or a multi-score combination of accuracy, MCC and Jaccard
681 index. The hyperparameter sets were ranked according to the average of the scoring
682 metric. Our multi-score optimization ranked each scoring metric and then averaged the
683 rankings. Due to the potential for ties, the top three were selected as the optimized ML
684 models. Depending on the convergence of the different scoring terms, ML algorithm,
685 feature subset, and standardization could have between three and twelve optimized ML
686 models. To reliably compare optimized ML models, we used the average performance
687 metrics during stratified k-fold CV with ten repetitions during each iteration using
688 different random state hyperparameters for the classifier when applicable.

689 5.3.3 Evaluating a model's reproducibility

690 For considering a model's reproducibility, different minimized structure inputs in T-
691 metal-sites10 were used to make a set of predictions, \mathbf{p} , for the same site, s . The site's
692 divergence, $d(s)$, was calculated using Equation 8, where p_i is either 1 (enzyme
693 prediction) or -1 (non-enzyme prediction) and n is the number of minimized input
694 versions for s .

$$695 \quad d(s) = 1 - \left| \frac{\sum_{i=1}^n p_i}{n} \right| \quad (\text{Eq. 8})$$

696 Therefore, $d(s)$ ranges from 0 to 1, where 0 is a site with the same prediction for all
697 minimized inputs and 1 is a site with five enzyme predictions and five non-enzyme
698 predictions. Using the set of all sites in T-metal-sites10, \mathbf{T} , and the subset of divergent
699 sites, $\mathbf{D} = \{s \mid s \in \mathbf{T} \text{ and } d(s) > 0\}$, we calculated our reliability metrics. The divergence
700 frequency is the percent of sites in T-metal-sites10 that were divergent (Eq. 9).

$$701 \quad \text{divergence frequency} = \frac{n(\mathbf{D})}{n(\mathbf{T})} = \frac{n(\{s \mid s \in \mathbf{T} \text{ and } d(s) > 0\})}{n(\mathbf{T})} \quad (\text{Eq. 9})$$

702 The divergence score is the average site divergence of the divergent sites (Eq. 10).

$$703 \quad \text{divergence score} = \frac{\sum\{d(s) \mid s \in \mathbf{D}\}}{n(\mathbf{D})} = \frac{\sum d(s)\{s \mid s \in \mathbf{T} \text{ and } d(s) > 0\}}{n(\{s \mid s \in \mathbf{T} \text{ and } d(s) > 0\})} \quad (\text{Eq. 10})$$

704 Since only divergent sites are considered, the lowest divergence score is 0.2.

705 5.3.4 ML guided dataset manual annotation

706 We optimized decision tree-based algorithms (ExtraTrees, GradBoost, and
707 RandomForest) using FeatureSet4 and selected an ExtraTrees model with high MCC
708 and high recall. We manually checked non-enzyme sites that were predicted to be
709 enzyme sites during the k-fold CV using available structure publications, RCSB(Burley
710 et al. 2019), and UniProt(UniProt 2019). We changed the labels for 73 sites that were
711 actually enzyme sites and removed all sites from 16 metalloproteins that were
712 undeterminable, for reasons such as lack of publication and enzymatic homologs. We
713 repeated the process without ExtraTrees models to minimize redundancy of
714 mispredicted sites. We fixed 19 additional site labels found during the second iteration,
715 removed all sites from eight undeterminable metalloproteins. We also removed two sites
716 that were located on the edge of active sites, meaning they could be correctly labeled
717 as both enzymatic and non-enzymatic.

718 5.3.5 Recalculated MAHOMES and Retrained MAHOMES

719 Since we were able to identify a significant amount of labeling error in the data used for
720 previously reported MAHOMES performance evaluations, we made updated
721 performance evaluations to enable more fair comparisons between MAHOMES and
722 MAHOMES II. We recalculated the k-fold performance for the MAHOMES predictions
723 using the corrected dataset labels for what was and wasn't an enzyme. Since we made
724 new relaxed structures for T-metal-site10 (the MAHOMES II test set), new MAHOMES
725 predictions were made for T-metal-sites10. Both variations of the test-set received
726 nearly the same predictions, but the performance evaluation changes significantly due
727 to reduced labeling error in T-metal-sites10.

728 The recalculated MAHOMES performance evaluations were still from a model that was
729 trained using dataset sites which have since been identified as mislabeled. So, we
730 made a retrained MAHOMES model, which differs from recalculated MAHOMES in two
731 ways. The first difference is that the fixed dataset with updated labels and removed
732 undeterminable sites were used during training. The number of enzyme sites increases
733 by 10% when the dataset labels are fixed, which prevents under-sampling at a ratio of 3
734 non-enzyme:1 enzyme site during training. So, the retrained MAHOMES model under-
735 samples by randomly removing 10% of the enzyme sites, followed by random removal
736 of non-enzyme sites until the ratio of training data is 3 non-enzyme:1 enzyme sites.
737 Otherwise, retrained MAHOMES model uses the same methods as the recalculated
738 MAHOMES model, including calculated feature values, algorithm, and optimized
739 hyperparameter set.

740 5.3.6 Model selection and performance evaluation

741 Despite the favorable performance of decision tree-based classifiers during work on
742 MAHOMES(Feehan, Franklin, and Slusky 2021), we tested fourteen ML classification
743 algorithms from Scikit-learn(Pedregosa et al. 2011) for MAHOMES II, which include:
744 linear regression, decision-tree ensemble methods, support vector machines , nearest
745 neighbors, Bayesian classification, and simple neural networks. We decided to attempt
746 these various algorithms because decision tree ensemble-based classifiers are known
747 to be robust against mislabeled data, large feature spaces, and outlier feature values.
748 So, our upgraded features and reduced training label error does not affect decision tree
749 ensemble-based algorithms as much as it affects alternative ML classification
750 algorithms.

751 In total, we assessed 4,032 machine learning combinations (14 algorithms x 6 feature
752 sets x 4 standardization techniques x 4 optimization terms x 3 top hyperparameter sets).
753 However, we only ended up with 1,792 unique ML models due to convergence during
754 model optimization. The specific code used for the algorithms, standardization
755 techniques, and hyperparameters can be found in the MAHOMES II repository file
756 MachineLearning/GeneralML.py (Feehan et al. 2022).

757 We selected a model that used a gradient boosting classifier with FeatureSet4 and a
758 uniform QuantileTransformer because it had the highest recall for models with greater
759 than 0.845 MCC and 88.5% precision. For MAHOMES II, we further refined this model
760 to improve both its cross validation MCC, divergence frequency and divergence score
761 by adjusting hyper-parameters that were too computationally expensive to optimize
762 during our inner cross validation using GridSearch optimization (Sup. methods).

763 5.3.7 Feature importance

764 For algorithms using decision-tree classifiers, sci-kit learn has a built in feature
765 importance output that measures the mean decrease in impurity that a feature was
766 responsible for during training. The MAHOMES II feature importance is the average of
767 feature importance output of the models trained during k-fold cross validation.

768 5.4 AlphaFold set

769 5.4.1 AlphaFold set generation

770 To make the AlphaFold set, we queried UniProt(UniProt 2019) for reviewed entries with
771 no solved crystal structure, an AlphaFold model (as of February 15, 2022), and metal

772 binding data. Entries with no EC number and no catalytic activity annotation of any kind
773 were labeled as non-enzyme. Entries with annotated with experimental catalytic activity
774 were labeled as enzyme. Remaining unlabeled entries were removed.

775 Since each entry is a protein sequence at this point, we chose to remove homology with
776 training and evaluating data next. We used PHMMER (Eddy 2011) to search each
777 protein sequence against all sequences in the PDB as of May 21, 2020. Entries with
778 detected homology, using an E-value < 1 , to any protein sequence in the dataset or
779 test-set were removed.

780 To go from sequence to the site level data, we retrieved all available metal binding data
781 from UniProt for each of the remaining entries. We removed data for metals other than
782 Copper, Iron, Magnesium, Manganese, Zinc, and Nickel. To ensure that the labels were
783 accurate at the site level, we removed enzyme labeled entries that did not include
784 'catalytic' annotations for the metal binding site. Due to automatic metal site annotations
785 or lack of EC coverage, non-enzyme labeled entries with 'catalytic' metal binding
786 annotations also had to be removed. Entries with only one or two listed metal binding
787 residue(s) were removed. We did not relax or perform any additional structure
788 minimization. The resulting AlphaFold set contains 1740 computationally generated
789 structures with 1583 non-enzyme sites and 157 enzyme sites.

790 We placed metals in sites using the average coordinates of the atoms binding to the
791 metals. For hydrophilic amino acids, we used the coordinate of the sidechain atom
792 capable of binding a metal ion (N, O, or S). For amino acids with multiple sidechain
793 atoms capable of binding metal residues (GLU, ASP, GLN, ASN), we used the average

794 of these atomic coordinates. For GLY, we used the average coordinate of the backbone
795 N and O. Some entries also listed other non-polar amino acids as coordinating residues.
796 We found the average coordinate of sidechain carbons worked best for placing these
797 metals without any steric clash. Since some entries with multiple metal binding sites did
798 not differentiate different bound metal sites, we removed any entries if the coordinating
799 residues were more than 12 Å from each other. The resulting 1,675 metalloprotein
800 structures and enzyme/non-enzyme labels are available on Zenodo (Feehan 2023).

801 5.4.2 AlphaFold set metal ion placement accuracy

802 We evaluated the accuracy of the AlphaFold set metal placement by adding a metal to
803 the sites in our dataset and test-set using UniProt data and comparing it to the metal
804 location in the relaxed crystal structures.

805 To find appropriate crystallographically-resolved sites to compare with the AlphaFold set
806 metal placement, we retrieved available binding site data for 2,608 of the 2,643 UniProt
807 entries in our dataset and test-set. However, only 1,207 entries included data for relevant
808 metal binding sites -- CHEBI ids: 29105 (Zn²⁺), 29033 (Fe²⁺), 29034 (Fe³⁺), 29035
809 (Mn²⁺), 29036 (Cu²⁺), 49552 (Cu⁺), 18420 (Mg²⁺), or 49786 (Ni²⁺). To create the
810 dataset for benchmarking, we removed UniProt entries and PDBs with multiple sites.
811 Also, entries with different metals in the PDB and UniProt binding data were removed. To
812 accurately depict the placement of sites in our AlphaFold set, we removed sites with fewer
813 than three coordinating residues. Finally, coordinating residues had to be among the
814 previously described amino acid types, resolved in the PDB, and indexed with the same

815 numbers in UniProt and the PDB. These filtering steps resulted in a total of 103 sites
816 remaining for benchmarking.

817 The final benchmark set consists of 103 successfully placed sites. The average distance
818 between the placed metal and the metal in the relaxed crystal structure was 0.87 Å (Fig.
819 S4). For comparison, the same 103 sites moved an average of 0.54 Å during minimization
820 of the crystal structure with Rosetta relax. Moreover, 56% of sites were placed within 1 Å,
821 96% were placed within 2 Å, and only one was more than 3 Å from its respective
822 experimentally resolved location in the PDB structure.

823 The UniProt metal binding annotations were converted to binding site annotations
824 (Coudert et al. 2023). This data conversion occurred after we created the AlphaFold set
825 but before we benchmarked our metal binding site placement method. This conversion
826 therefore required different data retrieval scripts for the benchmarking set than for the
827 AlphaFold set.

828 5.5 webserver

829 The MAHOMES Web Server was implemented in Python 3 on the back end using the
830 Flask framework with Jinja for templates in creating the HTML client-side interfaces.
831 When a PDB file and email are submitted to MAHOMES, metadata about the job is
832 stored in a JSON file. The information necessary to schedule the job for processing is
833 placed into an SQLite3 database.

834

835 The job execution program, which is written in Python 3, monitors the SQLite database
836 for new user submissions, and then handles executing the job, monitoring the job
837 execution, and then sending an email to the user with a link to the results page.

838

839 The jobs and the web application are run on the Slusky Lab web server, which is a
840 virtual machine running in the University of Kansas's enterprise data center. Running
841 this service as a virtual machine has allowed us to scale up the hardware backing the
842 instance as we have needed additional resources while working to control the long-term
843 costs associated with running the MAHOMES service.

844

845 **6. Supplementary material description**

846 *Figure S1.* Comparison of feature sets.

847 *Figure S2.* ML models reproducibility.

848 *Figure S3.* Top 6 feature DROPP plots

849 *Figure S4.* Metal binding site placement accuracy

850 *Table S1.* Performance evaluations.

851 *Table S2.* AlphaFold set evaluations.

852 *Table S3.* MAHOMES II feature importance

853 *Table S4.* Comparison of MAHOMES II performance to similar tools that make
854 enzymatic and non-enzymatic predictions

855 *Table S5. Manually annotated sites*

856 *Table S6. Feature details*

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862 **8. Data and code availability**

863 The data and code used to train and evaluate MAHOMES II can be found at
864 <https://github.com/SluskyLab/MAHOMES-II> (Feehan et al. 2022). The AlphaFold set
865 metalloprotein structures and enzyme/non-enzyme labels can be downloaded from
866 <https://doi.org/10.5281/zenodo.7703098> (Feehan 2023).

867 **8. Conflicts of interests**

868 The authors declare no conflicts of interests.

869 9. References

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