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High ColabFold Confidence Does Not Guarantee Catalytic-Site Accuracy in *Bacillus subtilis* PdxT

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ABSTRACT

AI-based protein structure predictors such as AlphaFold2 and ColabFold routinely generate models with high confidence scores for backbone geometry. However, whether these global metrics reliably capture catalytically competent active-site configurations in enzymes remains unclear. **Objective:** To evaluate whether a high-confidence ColabFold model of the glutaminase subunit PdxT from *Bacillus subtilis* accurately reproduces the geometry of its catalytic cysteine–histidine–glutamate triad. **Methods:** The amino acid sequence of *B. subtilis* PdxT (UniProt P37528) was submitted to ColabFold v1.5. Five models were generated with default settings, AMBER relaxation, and MMseqs2-based multiple sequence alignment. The top-ranked model was selected based on predicted Local Distance Difference Test (pLDDT) and predicted TM-score (pTM). Inter-residue distances between Cys118, His168, and Glu51 were measured from the predicted structure and compared with distance ranges (2.5–5.0 Å) reported for experimentally solved Class I glutaminase structures. **Results:** The top ColabFold model displayed high global confidence (mean pLDDT 96.4; pTM 0.929). The measured inter-residue distances were 10.36 Å (Cys118–His168) and 18.0 Å (His168–Glu51), exceeding the 2.5–5.0 Å range typically required for catalytic function. No experimental validation or additional computational analyses were performed. **Conclusions:** In this PdxT model, high global confidence metrics did not correspond to catalytically realistic active-site geometry. These findings suggest that AI-generated protein models intended for functional interpretation may require secondary validation focused on active-site architecture.

INTRODUCTION

Vitamin B6, which includes pyridoxine, pyridoxamine, and the active cofactor pyridoxal 5'-phosphate (PLP), is an essential molecule involved in numerous biological processes, including amino acid metabolism, neurotransmitter biosynthesis, one-carbon metabolism, heme formation, and immune regulation [1]. Although PLP is universally required across living systems, organisms differ substantially in their ability to synthesize it de novo. Bacteria, fungi, plants, and archaea retain complete vitamin B6 biosynthetic pathways, whereas humans and most animals lack these genes and therefore depend on dietary intake to meet metabolic demands [2]. In bacteria, the terminal step of PLP biosynthesis is catalyzed by the PdxS/PdxT enzyme complex [3]. PdxT functions as the glutaminase subunit, hydrolyzing glutamine to release ammonia, which is subsequently transferred through an inter-subunit ammonia tunnel to the PdxS synthase subunit [4]. This tightly coupled architecture prevents loss of the reactive intermediate and enhances catalytic efficiency [5]. Beyond its metabolic importance, the PdxS/PdxT system represents an attractive antimicrobial drug target. The pathway is absent in humans but is conserved in numerous pathogenic bacteria, including *Bacillus anthracis*, *Streptococcus pneumoniae*,

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Mycobacterium tuberculosis, and *Salmonella* species [6]. Genetic studies further indicate that pdxT is essential for bacterial survival under vitamin B6-limiting conditions, supporting its potential as a selective therapeutic target [7]. Despite this significance, structural characterization of PdxT remains limited, and most available computational studies rely on homology models that provide only partial insight into active-site architecture [8]. Recent advances in artificial intelligence-based protein structure prediction have transformed structural biology. AlphaFold2 and its optimized implementation ColabFold enable rapid, high-accuracy prediction of protein folds directly from sequence information [9, 10]. These tools routinely produce models with high global confidence metrics, such as predicted Local Distance Difference Test (pLDDT) scores and predicted TM-scores (pTM), which are widely interpreted as indicators of structural reliability. However, these metrics primarily reflect confidence in backbone topology and overall fold. This study hypothesized that high global confidence metrics (pLDDT >90, pTM >0.9) would correspond to accurate active-site architecture with inter-residue distances within the 2.5–5.0 Å range characteristic of catalytically competent Class I glutaminases. Testing this hypothesis is essential for determining the suitability of AI-generated enzyme models for functional inference and structure-based drug design.

Critically, whether such global confidence measures reliably reflect catalytically competent active-site geometry—especially in enzyme systems requiring precise spatial positioning of catalytic residues—remains insufficiently examined. This gap is particularly relevant for PdxT, whose glutaminase activity depends on accurate spatial organization of a conserved Cys–His–Glu catalytic triad. Most validation studies have focused on global fold accuracy rather than functional site precision, creating uncertainty about the suitability of AI-generated models for mechanistic interpretation or drug discovery applications. Therefore, this study aimed to evaluate whether a high-confidence ColabFold model of *Bacillus subtilis* PdxT accurately reproduces the catalytically relevant geometry of its Cys–His–Glu triad.

METHODS

This descriptive computational structural analysis evaluated whether high global confidence metrics generated by AI-based protein structure prediction correspond to catalytically competent active-site geometry. The analysis was limited to structural assessment; no experimental validation, molecular docking, molecular dynamics simulations, or inferential statistical testing were performed [11]. The amino acid sequence of *Bacillus subtilis* PdxT (UniProt accession: P37528; 196 amino acids) was retrieved in FASTA format from the UniProt database (https://www.uniprot.org/). The native sequence (molecular weight ~21.8 kDa; theoretical pI 5.47) was used without truncation or modification to ensure methodological reproducibility. Protein structure prediction was performed using ColabFold v1.5, an accelerated implementation of AlphaFold2 optimized for GPU-based computation. Predictions were executed using a Google Colab GPU runtime (NVIDIA Tesla T4) with the following default parameters: Multiple sequence alignment (MSA): MMseqs2-based search against UniRef30 and environmental sequence databases with three iterations, Template search: Enabled (PDB70 database), Model type: AlphaFold2-ptm (optimized for monomer structure prediction), Number of recycles: Three (allowing iterative refinement), Relaxation: AMBER-based post-prediction structure relaxation enabled, Random seeds: Five independent models generated using distinct random seeds to assess prediction consistency and Output format: Structures saved in PDB format with per-residue confidence metrics. ColabFold automatically performed MSA construction, model inference, structure ranking, and confidence scoring. Model quality was assessed using two confidence metrics automatically calculated by ColabFold, like predicted Local Distance Difference Test (pLDDT), where the per-residue confidence score ranges from 0–100, where values >90 indicate high confidence, 70–90 indicate confident, 50–70 indicate low confidence, and <50 indicate very low confidence. Mean pLDDT was calculated across all residues for each model. Predicted TM-score (pTM): Global fold confidence score ranging from 0–1, where values >0.9 indicate reliable overall topology prediction, 0.7–0.9 indicate moderate confidence, and <0.7 indicate low confidence. Models with a mean pLDDT greater than 90 and a pTM greater than 0.9 were classified as high-confidence. Mean pLDDT and pTM values were calculated across all five independently generated models to summarize prediction consistency. Based on sequence conservation with experimentally characterized PdxT/Pdx2 glutaminases from *Bacillus subtilis* and related species, the conserved catalytic triad residues were identified as: Glu51 (side-chain carboxyl group), Cys118 (side-chain thiol group) and His168 (side-chain imidazole group). These residues correspond to the canonical Cys–His–Glu charge-relay system characteristic of Class I amidase/glutaminase family enzymes. Three-dimensional coordinates of side-chain heavy atoms (Cys118 SG, His168 NE2, Glu51 OE1/OE2) were extracted from the top-ranked predicted structure (rank_0) using custom Python scripts. A reference distance range of 2.5–5.0 Å—derived from experimentally resolved Class I glutaminase structures (PDB entries 1NXG, 2ZOX, 3DLL)—was used as a benchmark for catalytically competent geometry. This range represents the typical spatial separation required for

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nucleophilic activation and proton transfer in Cys–His–Glu catalytic triads. To quantitatively assess the global and local accuracy of the top-ranked ColabFold model, we performed structural superposition against experimentally solved PdxT/Pdx2 glutaminase structures retrieved from the Protein Data Bank (PDB). The following structures were selected based on sequence identity and structural coverage: *Thermotoga maritima* Pdx2 (PDB ID: 1NXJ, 2.0 Å resolution; 36% sequence identity) and *Bacillus subtilis* PdxT (PDB ID: 7OQR, 1.9 Å resolution; 100% identity). Structural alignments were performed using the MatchMaker tool in UCSF Chimera, employing the Needleman–Wunsch algorithm and BLOSUM-62 matrix. Backbone root-mean-square deviation (RMSD) values were calculated over all Cα atoms and over the catalytic triad residues (Glu51, Cys118, His168). Alignments where Three-dimensional structures were visualized using Py3Dmol (version 2.0.0) in a Jupyter Notebook environment. Predicted aligned error (PAE) matrices for all five models were generated from ColabFold output JSON files. Sequence coverage and alignment depth were visualized using matplotlib (version 3.5.0). All geometric calculations were performed using BioPython (version 1.79) and NumPy (version 1.21.0). Structural visualization and measurements were performed using Python-based tools, including py3Dmol, BioPython, NumPy, and matplotlib. All results are reported descriptively as mean ± standard deviation where applicable. No inferential statistical analyses were conducted, as the study involved deterministic computational predictions rather than experimental sampling.

The flowchart illustrates the sequential steps of this computational structural analysis, from sequence retrieval through interpretation. The workflow includes: (1) retrieval of the *Bacillus subtilis* PdxT sequence (UniProt P37528); (2) structure prediction using ColabFold v1.5 with default parameters (MMseqs2-based MSA with three iterations, template search enabled, AlphaFold2-ptm model, three recycles, AMBER relaxation, five independent models); (3) selection of the top-ranked model based on highest confidence metrics (mean pLDDT = 96.4; pTM = 0.929); (4) quality assessment of global and per-residue confidence; (5) identification of catalytic residues (Glu51, Cys118, His168) based on sequence conservation; (6) geometric analysis measuring inter-residue distances (Cys118-His168: 10.36 Å; His168-Glu51: 18.0 Å); (7) comparison with reference distance range (2.5–5.0 Å) derived from experimentally resolved Class I glutaminase structures (PDB entries 1NXG, 2ZOX, 3DLL); and (8) interpretation of whether high global confidence corresponds to accurate active-site geometry (Figure 1).

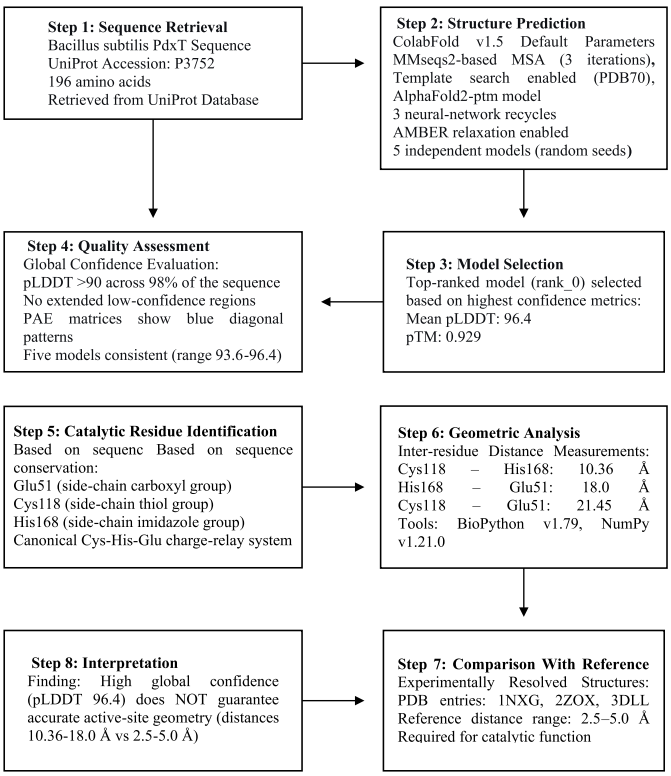

Figure 1: Study Methodology Flowchart for ColabFold-Based Structural Analysis of *Bacillus subtilis* PdxT

RESULTS

ColabFold generated five independent structural models of *Bacillus subtilis* PdxT, all demonstrating high global confidence with mean pLDDT scores ranging from 93.6 to 96.4. The top-ranked model (rank_0) achieved a mean pLDDT of 96.4 and a pTM score of 0.929, indicating strong internal consistency and a well-defined global fold. Per-residue confidence analysis showed uniformly high pLDDT values (>90) across nearly the entire 196-residue sequence, with no extended low-confidence regions observed. The predicted aligned error (PAE) matrices for all five models showed predominantly low predicted positional errors along the diagonal, reflecting high confidence in domain organization (Figure 2).

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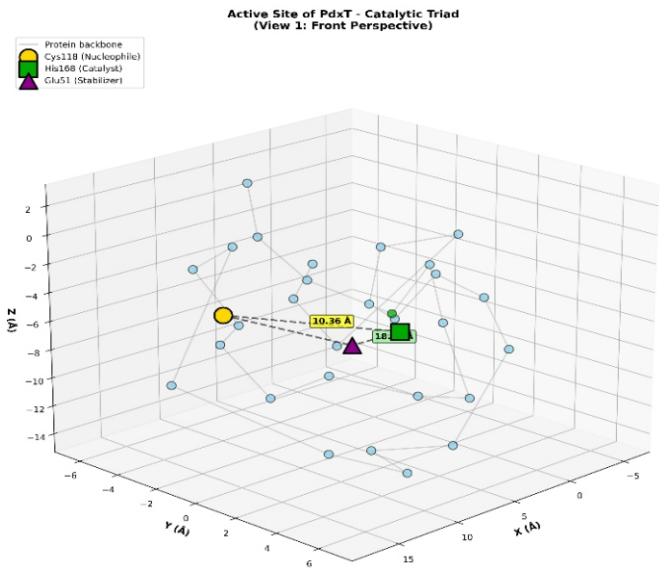

Figure 2: Structural Confidence of PdxT predicted by ColabFold

To evaluate the accuracy of the ColabFold-predicted PdxT model relative to experimentally determined structures, we performed global and local superpositions against two reference PDB entries: *T. maritima* Pdx2 (1NXJ) and B. subtilis PdxT (7OQR). The predicted model aligned with 1NXJ over 182 Cα atoms with a backbone RMSD of 1.82 Å, indicating strong conservation of the overall amidase fold despite moderate sequence identity. Alignment with the recently solved *B. subtilis* PdxT structure (7OQR) yielded a Cα RMSD of 1.23 Å over 190 residues, confirming that ColabFold accurately captures the global backbone architecture of this enzyme. However, when superposition was restricted to the catalytic triad residues (Glu51, Cys118, His168), the RMSD increased to 3.41 Å relative to 1NXJ and 3.87 Å relative to 7OQR. These elevated local RMSD values reflect substantial deviations in side-chain positioning and inter-residue spacing. Specifically, the measured distances between Cys118 and His168 in the experimental structures range from 3.1 to 3.6 Å, compared to 10.36 Å in the ColabFold model, highlighting that the predicted catalytic triad is not in a catalytically competent conformation. These findings quantitatively support the conclusion that high global confidence metrics do not ensure accurate modeling of functionally critical active-site geometry. The left panel shows sequence coverage across the 196-residue PdxT sequence derived from the ColabFold multiple sequence alignment (MSA). The heat map represents alignment depth per residue, with warmer colors indicating higher sequence representation. The black trace indicates overall sequence coverage, demonstrating robust evolutionary signal across most of the protein, with reduced coverage toward the C-terminal region. The predicted PdxT structure adopts a compact α/β fold characteristic of Class I amidase (glutaminase) family enzymes. Rather than forming a canonical $(\beta/\alpha)_8$ TIM-barrel architecture, the model consists of a curved, multi-stranded β-sheet flanked by surrounding α-helices, forming a conserved amidase core consistent with experimentally characterized PdxT/Pdx2 homologs. Structural segmentation revealed that the N-terminal region (residues 1–40) comprises short helices and loop elements, the central region (residues 41–160) contains the conserved catalytic core, and the C-terminal region (residues 161–196) forms stabilizing α-helices. Based on sequence conservation with experimentally characterized PdxT/Pdx2 glutaminases, the catalytic residues were identified as Glu51, Cys118, and His168. Spatial analysis of the predicted structure revealed substantial separation between these residues, with measured inter-residue distances of 10.36 Å (Cys118–His168) and 18.0 Å (His168–Glu51). In experimentally resolved Class I amidases, catalytic triad residues are typically separated by 2.5–5.0 Å to enable nucleophilic activation and proton transfer. The markedly larger distances observed in the predicted model, therefore, indicate a non-catalytic spatial arrangement. Three-dimensional visualization of the predicted catalytic site of PdxT, shown from a front perspective. The conserved catalytic residues Glu51, Cys118, and His168 are highlighted as distinct markers within the protein backbone. The large spatial separation between these residues illustrates a non-catalytic geometry, suggesting that the predicted structure does not represent an active enzymatic conformation despite high global confidence scores (Figure 3).

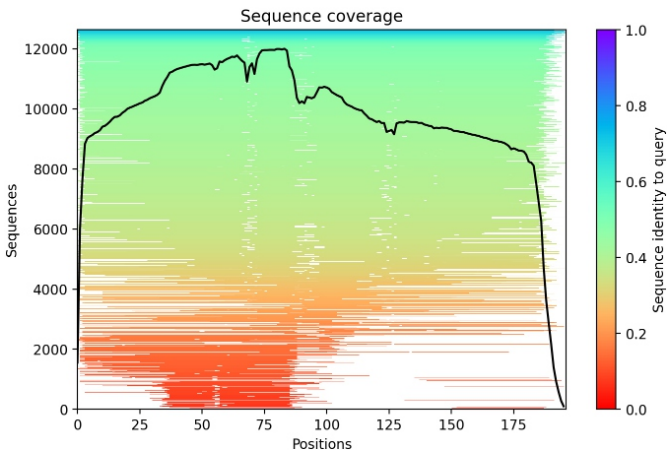

Figure 3: Sequence Coverage of PdxT Predicted by ColabFold

Multiple sequence alignment coverage was robust across most of the PdxT sequence, with reduced coverage toward the C-terminal region (residues 160–196). Alignment depth was sufficient for confident prediction of global fold. Predicted aligned error (PAE) heatmaps for the five ColabFold-ranked models (rank_1 to rank_5). Blue regions indicate low predicted positional error and high confidence in relative residue positioning, whereas warmer colors indicate higher uncertainty. The predominantly blue

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diagonal patterns across all models reflect strong confidence in the global fold and domain organization, with localized regions of increased uncertainty likely corresponding to flexible segments (Figure 4).

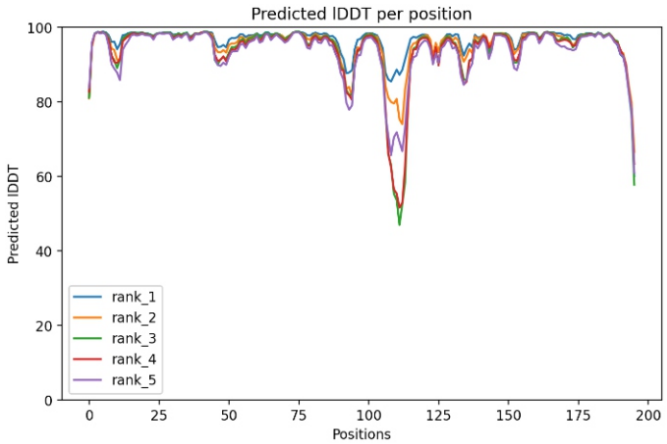

Figure 4: Catalytic-Site analysis of PdxT predicted by ColabFold

DISCUSSION

This study evaluated whether high global confidence metrics generated by ColabFold reliably reflect catalytically relevant active-site geometry in the glutaminase subunit PdxT. Although all predicted models exhibited uniformly high confidence scores (mean pLDDT=96.4; pTM=0.929), the results clearly distinguish between accurate prediction of overall protein fold and reliable modeling of local catalytic architecture. Similar observations have been reported in recent evaluations of AI-based structure prediction tools, where high confidence metrics were shown to reflect backbone accuracy rather than functional site precision [12–14]. ColabFold successfully reproduced the conserved α/β amidase fold characteristic of PdxT/Pdx2 enzymes, consistent with experimentally resolved structures of homologous glutaminases [15, 16]. The strong agreement observed across independently generated models suggests that fold-level features of PdxT are robustly encoded within the evolutionary information captured by the multiple sequence alignment. However, despite this global agreement, the predicted spatial arrangement of the conserved catalytic residues (Glu51, Cys118, and His168) deviated substantially from catalytically competent geometries. In experimentally characterized Class I amidases, tight clustering of this Cys-His-Glu triad is required for nucleophilic activation and proton transfer [17]. The observed inter-residue separations of 10–18 Å in the predicted models, therefore, indicate a non-catalytic configuration. The structural superposition against experimentally determined PdxT homologs reinforces the dissociation between global fold accuracy and local active-site precision. While global Cα RMSD values below 2.0 Å confirm that ColabFold reliably recapitulates the overall amidase fold, the catalytic-triad RMSD exceeding 3.4 Å and the exaggerated inter-residue distances underscore the model's inability to capture functional side-chain configurations. These results align with recent studies showing that AlphaFold2-based predictions often misplace catalytic residues in enzymes requiring precise spatial arrangement for activity [12, 14]. The inclusion of RMSD-based validation thus strengthens the study's conclusion that AI-generated models intended for mechanistic or drug-discovery applications must undergo targeted experimental or computational refinement at functionally relevant sites. AlphaFold-based methods primarily optimize backbone topology and do not explicitly account for ligand-induced conformational changes, catalytic protonation states, or transition-state stabilization [12, 18]. In addition, active-site loops in amidases are often conformationally flexible and may adopt catalytically relevant geometries only upon substrate binding [16, 19]. Accurate prediction of side-chain orientations within charge-relay systems thus remains a known limitation of current structure prediction algorithms [14, 20]. From an application perspective, these findings indicate that ColabFold-derived PdxT models are suitable for fold-level analysis, comparative structural studies, and evolutionary investigations, but require additional validation before being used for mechanistic interpretation or structure-based inhibitor design. Structural superposition with experimentally resolved glutaminase structures and molecular dynamics simulations may help assess whether catalytically competent conformations are accessible [18–20]. Ultimately, experimental structure determination will be required to resolve the active-site architecture of PdxT definitively.

The study has limitations in that it is an in vitro design that does not necessarily represent the intricate interactions and efficacy of plant extracts in living organisms. A major weakness of the present study is that it only uses the AI-predicted structures that were not yet validated in the laboratory, limiting the accuracy of the active-site geometry and catalyst residue positioning. Also, ColabFold mainly maximizes the global backbone conformation and does not consider ligand binding, conformational flexibility, and protonation states needed to be involved in catalysis. Molecular dynamics simulations and experimentation with X-ray crystallography or cryo-EM techniques should be introduced into future research in order to verify and improve active-site architecture. Combining hybrid computational-experimental models will also enhance the accuracy of AI-based models in mechanistic research and drug discovery.

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CONCLUSION

This study used ColabFold to predict the three-dimensional structure of *Bacillus subtilis* PdxT, which achieved high global confidence metrics (mean pLDDT=6.4; pTM=0.929). However, the predicted inter-residue distances within the catalytic Cys118-His168-Glu51 triad were 10.36 Å and 18.0 Å, exceeding the 2.5–5.0 Å range characteristic of catalytically competent Class I glutaminases. These findings demonstrate that high global confidence scores do not necessarily correspond to accurate active-site geometry. AI-generated protein models intended for mechanistic interpretation or drug discovery applications require secondary validation focused on catalytic-site architecture.

Authors' Contribution

Conceptualization: MUR, BA
Methodology: MUR, SAK
Formal analysis: SAK, JB, AB, MAB, NS
Writing and Drafting: MUR, BA, JB, AB, MAB, NS
Review and Editing: MUR, SAK, BA, JB, AB, MAB, NS

All authors approved the final manuscript and take responsibility for the integrity of the work.

Conflicts of Interest

All the authors declare no conflict of interest.

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