Article

Undergraduates Improve upon Published Crystal Structure in Class Assignment[S]

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Abstract

Recently, 57 undergraduate students at the University of Michigan were assigned the task of solving a crystal structure, given only the electron density map of a 1.3 Å crystal structure from the electron density server, and the position of the N-terminal amino acid. To test their knowledge of amino acid chemistry, the students were not given the protein sequence. With minimal direction from the instructor on how the students should complete the assignment, the students fared remarkably well in this task, with over half the class able to reconstruct the original sequence with over 77% sequence identity, and with structures whose median ranked in the 91st percentile of all structures of comparable resolution in terms of structure quality. Fourteen percent of the students' structures produced Molprobity steric clash validation scores even better than that of the original structure, suggesting that multiple students achieved an improvement in the overall structure quality compared to the published structure. Students were able to delineate limiting case chemical environments, such as charged interactions or complete solvent exposure, but were less able to distinguish finer details of hydrogen bonding or hydrophobicity. Our results prompt several questions: why were students able to perform so well in their structural validation scores? How were some students able to outperform the 88% sequence identity mark that would constitute a perfect score, given the level of degenerate density or surface residues with poor density? And how can the methodology used by the best students inform the practices of professional X-ray crystallographers? © 2014 by The International Union of Biochemistry and Molecular Biology, 42(5):398–404, 2014.

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Introduction

Protein structure dictates function, and thus, discussion of the fundamentals of structural biology has long constituted an important component of biochemistry and molecular biology education. The explosion of crystal structures, and accompanying data, available in the protein data bank (PDB) over the past decade may merit re-evaluating how we teach protein structure to students. In a course entitled "Introduction to Protein Structure and Function MCDB 411" at the University of Michigan, a group of fourth year undergraduate biology and biochemistry students (75% premedicine) were given the assignment of resolving a published crystal structure from scratch using publicly available deposited data from the electron density server (EDS) [1]. As an added challenge, students were not provided with the protein sequence, requiring them to determine the sequence of the protein by solving its structure. The purpose of this assignment was twofold: to teach students about X-ray crystallography and protein model building, and to also provide a form of hands-on learning about protein structure.

Training in crystallography model building is typically reserved for graduate students and postdoctoral fellows,

[S] *Additional Supporting Information may be found in the online version of this article.*

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and usually taught on-the-fly during the course of laboratory research. However, the increased availability of electron density data from (EDS), we reasoned might provide a facile entry-point for educating undergraduate students on crystallography. Also, the popular model building program Coot [2] has been under continual development for a decade, and now provides an easy and free user interface for protein model building. As such, the tools to teach protein structure through crystallographic model building are now freely available and accessible to anyone with basic knowledge of biochemistry.

The students approached the assignment with enthusiasm, with many noting unprompted that they enjoyed the assignments in a subsequent questionnaire. The student's structure solutions, perhaps as a consequence of their enthusiasm, were of remarkably high quality, ranking above the 90th percentile in both steric clashes and overall structure quality when compared with other structures of similar resolution. Fourteen percent and nine percent of the class even bettered the published crystal structure in terms of steric clashes and overall model quality, respectively. In the future, the results and analysis of this assignment may be useful in helping design curricula to improve students' understanding of amino acid and protein structure.

Assignment

Prior to the assignment, students in MCDB411 were given a one and a half hour lecture on protein crystallography. This lecture only briefly touched on model building and refinement. The day after the assignment was posted, students participated in a 1.5 hour lab session in three groups of ~20 students each. This session began with a 20 min demonstration on how to use the program Coot (version 0.7.1) to build a protein chain into electron density, followed by an hour to begin the assignment with the instructor, and an assistant familiar with the assignment present to answer questions. Specifically, students were instructed on the use of the following Coot tools: real space refine zone, rotate/translate zone, simple mutate, rotamers, add terminal residue, delete, and undo. One week later, the students had a second 1.5 hour lab section that began with a 10 min demonstration of tools within Coot that could assist the students in finishing their structure, followed by another hour and 10 min to work on the assignment with the instructor and assistant present to answer any questions. During this session, students were instructed on the use of Coot's regularize zone, geometry analysis, and density fit analysis features. The Coot validation tools (geometry analysis and density fit analysis) are graphical representations that show the quality of the model for the parameter being checked at single amino acid resolution, and thus provide a means to easily find and tweak incorrect amino acids. The students were, then, given an additional 16 days to complete the crystal structure on their own, with continued aid available through 4 office hours as well as email correspondence with the instructor. Approximately 10 students attended office hours and 20 email enquiries were submitted during this time. Students were allowed to discuss their project between each other, and use any outside computational resources they knew of, so long as each turned in his or her own independent assignment.

All students were assigned to solve the same crystal structure, a 95 amino acid, single chain protein with no bound ligands and five disulfide bonds that diffracted to 1.3 Å resolution [3], at which resolution the identity of most amino acids should be evident using only the electron density as a guide. This structure was chosen based, in large part, on its high quality and its publication in Protein Science, a peer-reviewed journal, as opposed to being submitted directly to the PDB without an accompanying publication. Students were provided with a 2Fo-Fc electron density map downloaded from the EDS, which has served as the repository for data used to solve crystal structures in the PDB since 2004 [1]. The 2Fo-Fc map primarily depicts the electron density of the structure that has already been modeled. As such, when the published crystal structure is matched with its final 2Fo-Fc map, the structure should mask the map. As is customary with electron density maps, a Fo-Fc map was also made available to the students, but they were instructed that it would not be of use in this assignment as it usually does not mask the finished protein model, and thus, they should not plan on building into it.

In addition to the 2Fo-Fc and Fo-Fc maps, students were given the N-terminal amino acid (glycine) of the protein of interest modeled into its correct position as a starting point, but no other sequence or structural information. The students were given the assignment of completing the crystal structure, using the electron density map as well as their knowledge of protein structure and chemistry. The students were instructed not to model water molecules into the structure, and were not informed that the structure contained disulfide bonds.

Student questions during these sessions covered many topics, but mainly focused on which amino acids to model into particularly difficult areas of density (especially those involving proline), specifics of the Coot user interface, and how to choose which amino acid to model into degenerate density (i.e., how to choose between two amino acids that have the same shape). Most of the problems due to difficult density were easily solved by carefully working through trouble spots with the students by suggesting which tool to use for a particular modeling problem. A common example of the degenerate density problem was how to tell the difference between asparagine and aspartic acid, considering that these amino acids have the same shape. Degenerate density issues were addressed by instructing the students to think about the chemistry of the amino acid (i.e.,

hydrophobicity, hydrogen bond accepting/donating capability, charge, etc.) and to use these properties to choose between the candidates that could fit into the density while considering its chemical environment. The students were only given instruction on degenerate density upon their individually asking questions about specific instances of degenerate density. Many students also asked questions pertaining to the disulfide bonds. Students were never explicitly informed that disulfide bonds existed in this structure. Instead, the instructor reminded the students that there is a single amino acid that is capable of covalent bonded side-chain branching, both verbally in an announcement to the entire class, and also in response to individual questions.

Students were informed that they would be graded using a combined approach to evaluate both the fit of their modeling to the electron density, as well as the quality of the geometry of their structure. The structures were evaluated using sequence alignments of their fit structure to that of the original structure, as well as visual inspection of the Coot density fit validation tool for each structure. These metrics were used in place of *R*-factors, as the students did not use a refinement strategy that incorporated temperature factor or occupancy refinement. The geometry was validated both by using the Coot geometry validation tool and by using the Molprobity structural validation server [4, 5], which is commonly used to judge geometry parameters in addition to steric clashes. Note that lower scores from the Molprobity server are used to denote better structural models. In the final evaluation, only sequence alignments and Molprobity scores were used in grading, as they correlated well with the Coot validation checks, but are more quantifiable.

After completing the assignment, the students were given a short questionnaire in which they were asked to describe their procedure for completing the assignment, whether they used BLAST or other online tools or databases to aid in the assignment, to rate the difficulty of the assignment, and provide any suggestions or comments to help in improving the assignment (Supporting Information).

Results

The 1.3 Å resolution structure of antiviral lectin scytovirin (2QT4.pdb) [3] was chosen for re-solving by the students, in large part due to its high quality. Analyzing the structure from the PDB, this published structure was found, in comparison to other structures of comparable resolution, to be in the 90th percentile in terms of steric clashes as evaluated by Molprobity (clashscore), and 94th percentile in terms of the overall structure quality (Molprobity score), which combines evaluations of steric clashes, rotamers, and Ramachandran space into a single score. Given these statistics, the structure's high resolution, and that the protein consisted of a single chain with no bound ligands, it seemed an appropriate choice as a target for students to try and re-solve from scratch. Furthermore, the presence of five disulfide bonds presented an added challenge not present in other comparable structures.

Given the lack of crystallography experience of the class, the students achieved remarkable success in creating quality protein models, as evaluated by the Molprobity structure validation server (Fig. 1*a*), especially considering that the students were not given the protein sequence. Note that for both Molprobity score and clashscore, smaller values denote better quality models. Student structures ranged from 0 to 202 and 0.53 to 4.48 in Molprobity score and clashscore, respectively. For comparison, the initial structure was first stripped of waters before calculating its Molprobity score (1.00) and clashscore (2.27) before comparing with students' structures. The students' median Molprobity score (1.31) and clashscore (4.99) would score in the 91st and 87th percentile of all structures, respectively, within the comparable resolution range, without any editing of the students' structures. Evaluating the students' structures using Molprobity, eight students (14%) achieved a better clashscore than the published structure, and five students (9%) achieved a better Molprobity score, thereby ranking in at least the 99th percentile of all structures in this resolution range. The best clashscore (0.00) and Molprobity score (0.53) ranked in the 100th percentile of all structures in the same resolution range. This achievement is extraordinary considering that the students did not use many standard refinement techniques that are designed to aid in geometry optimization, and were limited to mainly real-space refinement in Coot. Moreover, based on the subsequent questionnaire turned in by all students, none of the students used outside tools that would have aided their assessment by the Molprobity server.

Students were not provided with the protein sequence to challenge them to interpret the electron density maps more closely, and to require them to choose between amino acids that have degenerate electron density (due to similar molecular shape), but differing chemical properties that could be interpreted from examining the amino acid's chemical environment within the protein. In the original electron density map, six residues had poor electron density (primarily surface lysines), in addition to 10 surface-exposed glutamine, asparagine, or serine residues that would be difficult to correctly assign due to a lack of protein contacts. Assuming that the students have a negligible chance of correctly assigning the residues with poor density, and only a 50% chance of correctly assigning the surface-exposed glutamines and asparagines (and a single serine in multiple conformations), the highest expected sequence identity expected would be 88%. There were 14 other residues (threonines, asparagines, aspartates, and glutamines) for which the density was degenerate between the correct choice and at least one other possible amino acid, but for whom the immediate chemical environment

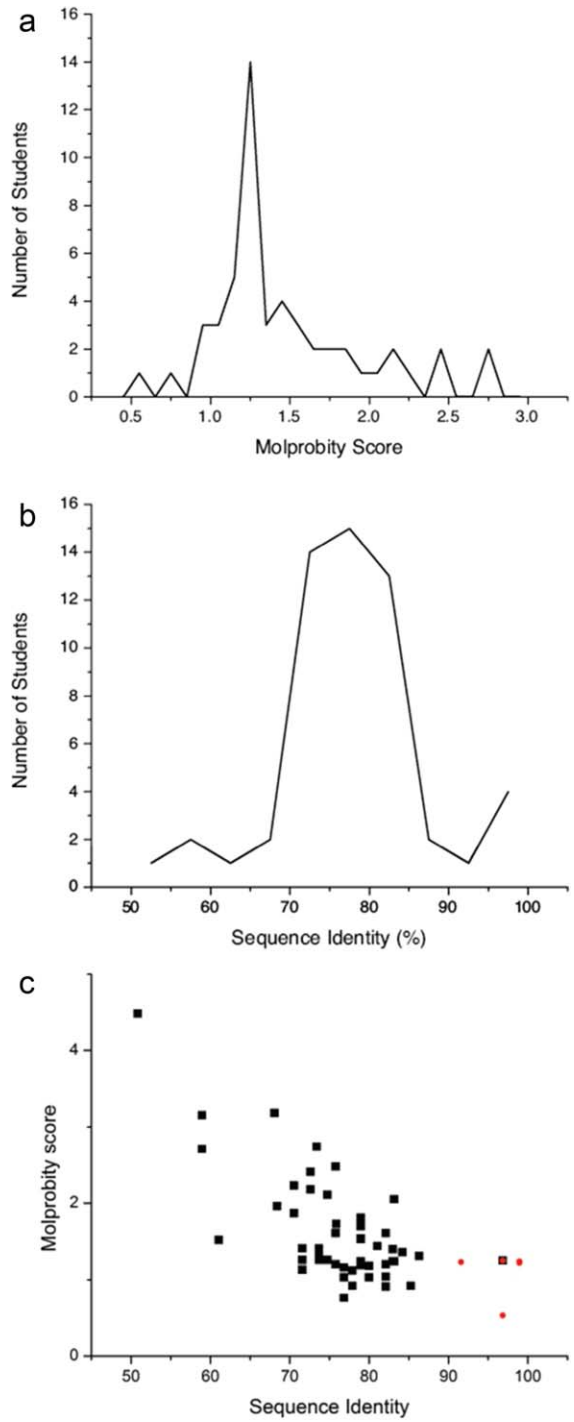

FIG 1

Evaluation of students' structures. (a) Structural quality as determined by Molprobity score (lower score means better quality structure). Median score was 1.31, or in the 91st percentile of all structures of the same resolution. The Molprobity score of the original structure was 1.00. (b) Sequence identity of student structures to original published structure. Median sequence identity was 78%. (c) Molprobity score is loosely correlated with sequence identity among students' structures. Structures solved with the aid of BLAST to aid in sequence determination are depicted with red dots. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

should provide enough information to choose the correct amino acid. Taking into account these 14 amino acids, if a student did not have chemical knowledge of the nearby area, but instead guessed as to the residue based entirely on density each time, the student could achieve as high as 81% sequence identity.

Given these expectations, the students performed very satisfactorily at recreating the sequence of the test protein. For the 56 students who completed the full assignment, the mean sequence identity was 77%, and median sequence identity 78% (Fig. 1*b*). Of note, sequence identity and Molprobity scores were loosely correlated (Fig. 1*c*). Examining the sequence alignments (Table 1 and Supporting Information Table S1) showed that the most common errors were in lysine or asparagine residues for which the electron density was poor, with few students correctly assigning these residues. Similarly, those residues with degenerate density proved challenging, with nine of the fourteen being answered incorrectly at high rates (Supporting Information Table S1). These residues were primarily asparagine or threonine residues that could be identified based on their hydrogen bonding patterns. Of the five degenerate cases that students assigned correctly (75% of the time), four of these were aspartate or glutamate residues that form a salt bridge with an arginine, clearly defining the impetus for a negatively charged residue. The one remaining example that was interpreted correctly (65% of the time) was Thr5, which is solvent exposed. Thus, while the vast majority of students were able to correctly choose when a charged residue was most appropriate to model, more nuanced details of hydrophobicity, and the differential hydrogen bonding pattern of asparagine and aspartate eluded many students. Of those cases where the students had to use knowledge of hydrogen bonding donor and acceptor properties and hydrophobicity, with the exception of Thr5, the students only managed to assign these residues correctly 42% of the time, the same rate as those from those where no useful chemical information was available. Not surprisingly, students whose sequences aligned poorly with the test protein were substantially more likely to have consistently made these errors (Table 1). For example, Thr79 is on the surface of the protein, and its nearest spatial neighbor is a glutamine, but 53% of the students inserted the similarly shaped valine instead. However, when examining this effect as a function of overall sequence identity, it becomes clear that the students who correctly chose threonine in this case were far more likely to have produced high sequence identity scores. Of the 29 students who correctly chose threonine, 16 were in the top 17 overall alignments, and 22 in the top 30 overall alignments. Therefore, of the remaining 25 students whose alignments formed the bottom of the sequence identity distribution, only eight correctly chose threonine over valine. A similar trend was observed for the disulfide bonds, which were correctly modeled by the majority of students, but primarily by those who scored

TABLE 1 ***Student sequences aligned with that of original crystal structure ranked by % identity to the actual antiviral lectin scytovirin sequence.***

Residue	9	10	11	12	13
sequence identity (%)	N	E	A	N	N
99.0	.	.	.	.	.
99.0	.	.	.	.	.
96.8	D	.	.	.	.
96.8	.	.	.	.	.
91.6	.	.	.	.	.
86.3	D	.	.	S	.
85.3	D	.	.	S	.
84.2	.	.	.	S	.
83.2	.	.	.	S	D
83.2	.	.	.	S	D
83.2	.	.	.	S	L
83.0	.	.	.	S	.
83.0	.	.	.	S	D
82.1	.	.	.	S	D
82.1	D	.	.	S	D
82.1	D	.	.	C	D
82.1	D	.	.	S	D
81.1	.	Q	.	S	.
80.0	D	.	.	S	.
80.0	.	.	.	C	.
79.0	D	.	.	S	D
79.0	D	.	.	S	D
79.0	.	.	.	C	D
79.0	.	Q	.	S	.
79.0	.	.	.	L	D
79.0	.	.	.	C	D
77.9	.	.	.	C	D
77.9	.	.	.	C	D
76.8	D	.	.	S	D
76.8	D	.	.	S	D
76.8	.	.	.	C	D
75.9	L	.	.	G	L
75.8	D	.	.	S	D
75.8	.	.	.	C	D
75.8	.	Q	.	S	.
74.7	.	.	.	C	.
74.7	D	.	.	A	.
73.7	.	.	.	C	D
73.7	.	.	.	C	D
73.7	.	.	.	S	D
73.7	D	Q	.	C	D
73.4	L	.	.	S	L
72.6	L	.	.	C	.
72.6	L	.	.	C	D
71.6	L	.	.	C	D
71.6	.	.	.	C	D
71.6	C	.	.	S	D
70.5	.	Q	.	C	.
70.5	D	.	.	C	L
68.4	D	C	.	S	D
68.1	L	.	.	S	L
61.1	L	.	.	S	L
59.0	D	Q	.	S	D
59.0	D	.	.	L	D
50.9	L	.	.	A	L
Correct (%)	53	88	100	12	35

A short stretch of the sequence of scytovirin is shown encompassing Residues 9—13. Representative residues of interest are color coded: easy to assign density (unshaded), ambiguous density (green), poor density in which students were not expected to be able to correctly assign residue (blue), degenerate density in which students could use chemical environment to determine correct assignment and did so in above 60% (yellow) or below 60% (red) of the cases. The complete table with all amino acids is included in Supporting Information Table S1. [Color table can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

well overall (Supporting Information Table S1). This trend suggests that while some students displayed a firm grasp of the ability to correctly choose the amino acid based on chemical environment, much of the class did not display mastery of this concept.

Discussion

A class of fourth year undergraduate biology and biochemistry majors was given the assignment of solving a protein crystal structure from scratch given the electron density maps and the N-terminal amino acid, but with no sequence provided. The two purposes of this assignment were to introduce the students to the technique of X-ray crystallography, and provide a hands-on learning activity focused on protein structure and amino acid properties. The surprisingly high validation scores and sequence identity reconstruction prompted us to give the students a questionnaire regarding the assignment that provided insight into how the students approached their structures.

How students managed to perform so well in Molprobity scores is difficult to fully analyze, given the wide range of sequence variation. It is conceivable that not providing the sequence actually aided the students, as they were forced to attempt to fit more rotamers and conformations to the structure to find the best possible fit. For instance, Asn13, which is likely flipped 180° from that in the published structure based on its hydrogen bonding pattern, was chosen to be in the opposite side-chain conformation by nearly all of the 35% of students who correctly identified this residue as an asparagine. Additionally, the high Molprobity scores are likely partially due to the nature of the protein and dataset used in this assignment. The students had the advantage of using the same high-quality data and maps as the original publication to solve their structures. Also, by not modeling in some large side chains (such as the surface-exposed lysines that were often not modeled due to incomplete density), it is possible that the students naturally lessened steric clashes. Although students with high sequence identity scores tended to do better in Molprobity scores, the correlation is not particularly strong. Even with these advantages, that several students outscored the original structure in Molprobity score and clashscore, and therefore scored at or above the 99th percentile of structures of the same resolution, is a testament to the skill and hard work of these students, and underscores the importance of attention to detail in crystallographic model building. Responses to the survey showed that students who achieved the highest validation scores all made extensive and almost exclusive use of both real-space refinement and the geometry analysis tool in Coot, suggesting that used properly, these tools are enough to build a high quality structure without utilizing the advantages of structure validation or additional refinement techniques.

To evaluate how some students managed to achieve such high sequence identity scores, we analyzed the students' responses to the questionnaire that was given to them subsequent to the assignment and was completed by all students. Notably, four students used BLAST to help them solve the structures. These students first completed the structure by hand, extracted the resulting sequence, and then used BLAST to find the protein used in the assignment. Then, the students compared the sequences to see positions where they likely made mistakes, and then went back to correct their original structures. Not surprisingly, all four of these students broke the 88% identity barrier. However, one student, who did not use BLAST but instead used PubMed to attempt to determine what sorts of amino acids were more likely to exist in certain interactions, scored a 97% sequence identity. Based on the student's reading, asparagines were more likely than aspartates in most circumstances, and degenerate and surface-exposed residues were often chosen to be asparagines as a result.

By assessing the most common problems encountered by the students, we were able to assess some of the successes and deficiencies of biochemistry education *vis-à-vis* protein structure determination. The ability of most students to successfully distinguish when a salt bridge was appropriate shows a basic grasp of intermolecular interactions. Hydrophobicity, however, was less well understood, as demonstrated by the students assigning over 50% of solvent exposed threonines as valines. The more advanced concept of hydrogen bond donor and acceptor analysis based on the ability to correctly choose between aspartate and asparagine was apparently only mastered by a small number of students. Still, the top students displayed an excellent knowledge both of hydrophobicity and intermolecular interactions.

The students were also asked in the survey for comments or suggestions to help improve the assignment. When asked to "Please list any suggestions/comments that you feel could improve this assignment for future classes," a very satisfying percentage (38%) of the students volunteered that they found the assignment especially enjoyable or interesting. The other most common response, also at 38%, was the useful suggestion that additional instruction on the usage of Coot, especially in either video or written format, would have been helpful. The next most common comment, made by 10% of the students, was that 95 amino acids were more than was necessary to learn the concepts of the assignment. One especially interesting comment suggested that the assignment should be broken into two parts to better test the student's skill at model building: one take-home assignment as was given, followed by an in-class timed test using a different protein. Thus, students who were able to independently complete a protein structure would be rewarded for mastering this skill.

Combining the information from the students' assignments and the questionnaire, it becomes clear that the most useful improvement for this assignment would be permanent material, either a document or video, that not only covers the basics of Coot function but also explicitly reminds students to take into account amino acid properties, such as hydrophobicity, as well as hydrogen bonding, in building their structure. As the structure and properties of amino acids are covered in classes the students have taken previously, a reminder in writing should be sufficient to improve recall of these concepts and, therefore, improve the performance on this part of the assignment. Also, it should be considered whether the students should be allowed to use BLAST, and if a protein should be chosen with a less unique sequence that would be more difficult to discover in this fashion.

Finally, the results of this assignment have some rather strong implications for the field of X-ray crystallography. The positive response of the students in the survey to the assignment indicates that perhaps it should be used earlier on in the undergraduate students' career, at which point they will be more likely open to structural biology as a future career. This sentiment was echoed by one student in his suggestions/comments response, noting, "It was a very fun assignment and it opened my mind to this area of study." Given the recent success of Project CRYSTAL [6], which introduces protein crystallography to middle school students, moving crystallography training earlier in undergraduate education is both feasible and could help to reinforce biochemistry training in earlier classes. As the students in this class were able to learn how to use Coot with minimal guidance, we anticipate that experts in biochemistry can learn to use Coot by following the aggregated tutorials included here in the Supporting Information, even with little to no prior crystallography experience. As such, little barrier remains to incorporating model building as a part of a biochemistry curriculum. On a separate note, this assignment emphasizes the need to maintain the EDS for the foreseeable future. Although it is now possible to easily convert most structure factors from the PDB into electron density maps using programs such as Phenix [7], it is imperative that easily accessible electron density maps remain available to the scientific community to allow nonexperts to learn about model building, both in the classroom and in the laboratory. And most importantly, the ability of 14% of the students to outperform the published structure, and in some cases substantially so, serves a strong reminder about what we can learn from our students.

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