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Comment on Three X-ray Crystal Structure Papers

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Letters to the Editor

The Journal of Immunology

Comments from the Editor-in-Chief

The correspondence below captures a lively scientific debate on the best practices for accurate analysis of x-ray crystallography data. In the course of handling this correspondence, I solicited comments from a number of external reviewers who generously helped me navigate these waters and I wish to thank them for their time and efforts. The consensus of these experts is clear that the quality of the data and the level of noise within the electron density map in the Salunke study preclude tracing peptide residues within the x-ray crystal structures.

This debate has underscored the need to improve the rigor of the review process for manuscripts that include x-ray crystallography data. For many years, *The Journal of Immunology* has required that authors of manuscripts containing high-resolution structural data deposit those data in the Protein Data Bank (PDB) at the time of manuscript submission, and to include the PDB accession number in the published article. As a result of the correspondence below, *The Journal of Immunology* now requires that the PDB Summary Validation Report (available only recently) be included with submission of the manuscript so that it is available to editors and reviewers during the review process. This additional requirement will allow a thorough vetting of the authors' interpretation of x-ray crystallographic data by expert reviewers, with the goal of eliminating ambiguity in the protein structures contained within published articles in the future.

We hope that publishing this correspondence and the journal's response to the issues it exposes will stimulate discussion in the community of structural biologists and lead to constructive changes in the way crystallographic data are reviewed and interpreted in the future.

Pamela J. Fink, Ph.D.
Editor-in-Chief

Abbreviation used in this article: PDB, Protein Data Bank.

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Comment on Three X-ray Crystal Structure Papers

Three recent papers published in *The Journal of Immunology* by Dr. Salunke and his coworkers at the National Institute of Immunology in New Delhi have attracted our attention (1–3). We regret to inform you that our findings cast serious doubt on the validity of the structural data presented in these publications. A paper published by the same group elsewhere exhibits similar problems (4).

To summarize, the publications in question attempt to address the role of plasticity in molecular recognition by Abs. Specifics vary from publication to publication, but the overall approach includes determination of multiple crystal structures of corresponding Abs in complex with various peptides. Based on the refined structure models, details of molecular recognition are then derived at an atomic level, and conclusions are presented regarding mechanisms of such recognition.

Unfortunately, in each of these publications describing peptide–Ab complexes determined by x-ray crystallography, the very central claim of a peptide actually bound to an Ab is not supported by evidence. The necessary evidence in the form of electron density is absent and the analyses of the experimental data are systematically flawed when examined according to accepted professional and scientific standards. Deposition of experimental data in the Protein Data Bank (PDB) has been mandatory since 2008, and availability of such data allows us (as well as anyone trained in protein crystallography) to verify the claims presented in the publications of Dr. Salunke. We inspected electron density maps for every protein–peptide complex structure associated with these papers, and found that the required primary experimental evidence, positive omit electron density in support of presented claims, is lacking. No electron density exists for the peptides in the purported protein–peptide complexes.

Our goal here is to correct the scientific record and preserve the integrity of the PDB as a valid database (5, 6). It should also be noted that the electron density map calculation required to visualize these problems requires access to the coordinates and structure factors. Consequently, editors or reviewers are not at fault that these problems were overlooked during the editorial review process, as they likely did not have access to those data at the time. The importance of inspection of electron density fit as a primary means of local validation has been repeatedly pointed out (7–9).

Below we describe the accepted standard validation procedure and compare results of its application to test cases selected from the literature and to seven structure models

Table I. Average B-factors of the peptide ligands compared with those of surrounding atoms in crystal structures from (1–3)

	Deposited Model		Rerefined Model	
PDB ID	$B_{peptide}$	$B_{protein}$	$B_{peptide}$	$B_{protein}$
2XZQ	64.9	24.9	102.8	29.1
2Y06	71.7	65.4	145.6	87.7
2Y07	37.6	41.4	111.4	61.1
2Y36	66.4	39.3	139.0	39.8
4BH7	64.5	26.8	132.3	30.8
4BH8	83.1	31.4	98.7	36.9
4H0H	91.4	27.7	105.5	31.9

Values are shown for both original models as deposited in the PDB and upon rerefinement in BUSTER-TNT (10).

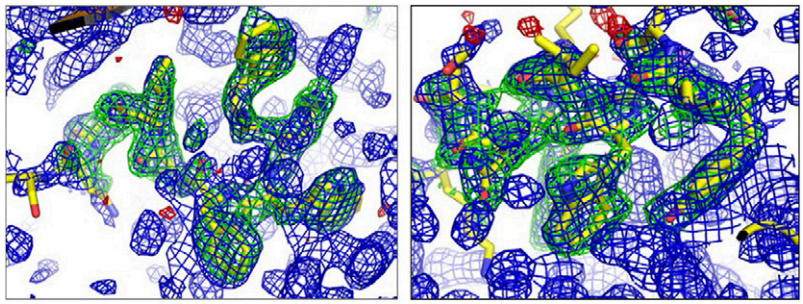

FIGURE 1. Omit electron density maps for the Ab-bound peptides from PDB entries 3FN0 (*left panel*) and 3GGW (*right panel*). $2fo\text{-}f_c$ map (blue) contoured at 1σ and $f_o\text{-}f_c$ map (green/red) contoured at ± 3 σ are shown. Peptide model is shown (yellow sticks). This figure and Fig. 2 were rendered using PyMOL (http://www.pymol.org).

deposited by Dr. Salunke et al. [2XZQ/2Y06/2Y07/2Y36 (1); 4BH7/4BH8 (2); 4H0H (3)].

Validation procedure

We use an established validation procedure to verify the presence (or absence) of peptide electron density. The procedure includes following steps:

1) Rerefine the deposited structure models against the deposited experimental data. The refinement results described below were obtained using BUSTER-TNT software (10). Using other modern crystallographic refinement programs [e.g., REFMAC5 (11) or phenix.refine (12)] yields the same results and conclusions.
2) Compare the average *B*-factor of the peptide molecule to that of the set of atoms in immediate contact with the peptide (we use a 4 Å interatomic distance cutoff to define the molecular neighborhood). It is expected for a genuine protein–peptide complex that these two sets of *B*-values will be close. Large discrepancy indicates that the peptide molecule is either present at partial occupancy or that its presence is not supported by electron density.
3) Remove the peptide molecule from the deposited structure model and rerefine the model without peptide—the omit-map procedure (13, 14). The term omit map here refers to the fact that the model component in question, i.e., the peptide in this case, is omitted from the model refinement to reduce model bias in the electron density map. If the peptide molecule in question is present, the shape of the resulting difference electron density will provide corresponding evidence. The standard approach according to modern practice (15, 16) is to inspect the difference electron density omit map contoured at 3 σ. We list two positive control examples and the negative results for the seven PDB entries from the papers in question.

Positive controls

In order to demonstrate the expected behavior, we have applied the same analysis to PDB entries 3FN0 (17) and 3GGW (18): both are genuine and validated Fab/peptide complexes (17, 18). For these two structures, rerefinement of the original model results in similar *B*-factors for the bound peptides and the neighboring Ab atoms (36.0/37.7 $Å^2$ and 37.6/27.3 $Å^2$ for 3FN0 and 3GGW, respectively). As expected in the case of bound ligands, the degree of combined dynamic and static disorder observed as expressed by the average *B*-factor in the peptide molecule and the surrounding protein atoms is very similar, given that these atoms are in direct contact and interact via a number of hydrophobic interactions and hydrogen bonds. As ultimate consequence and as the necessary proof positive, the peptide molecules are clearly evident in the omit electron density map (see Fig.1).

These results serve to establish expected positive proof for the presence of the peptides when comparing them to the structure models in question.

Negative results

2XZQ/2Y06/2Y07/2Y36 (1), 4BH7/4BH8 (2), 4H0H (3): average *B*-factors for the peptides and surrounding atoms are shown in Table 1. The discrepancies between the deposited models and the results of rerefinement are discussed below. In all seven cases, upon rerefinement of the original models, *B*-factors of peptide ligands significantly exceed that of their immediate molecular neighborhood. These significant discrepancies provide clear indication of a lack of scattering contribution originating from the purported peptide molecules.

In agreement with these findings, the difference omit maps in all seven cases show no evidence of peptides when contoured at the 3 σ level (see Fig. 2).

Inconsistencies between refinement results and tables in publications

Table I shows the comparison of relative *B*-factors for the peptides in the structures discussed above as they appear after a cycle of rerefinement to the same values as reported in the corresponding models deposited in the PDB. The tabulated values for $B_{peptide}$ are unexpectedly and inexplicably lower than the actually refined $B_{peptide}$ values, while the $B_{protein}$ values are much closer. The average *B*-factor of a structure model may vary when it is refined with different programs and/or a different set of parameters, but it has never been observed that a different refinement would produce much higher *B*-factors for specific groups of atoms such as the purportedly bound peptide relative to the protein to which it is bound. In one case (4H0H), the occupancy of the peptide was set to 0.8, which can only be justified if that brings *B*-factors of peptide and Ab into agreement (not the case here despite the reduced occupancy). We assume that Dr. Salunke and his coauthors are aware that the overall *B*-factors of neighboring components in a crystal structure cannot differ drastically.

Summary

High relative *B*-factors of the purported peptides serve as a first indication of problems with the peptide models. The absence of the peptides is firmly supported by the clear absence of positive omit difference density. Tabulated values for

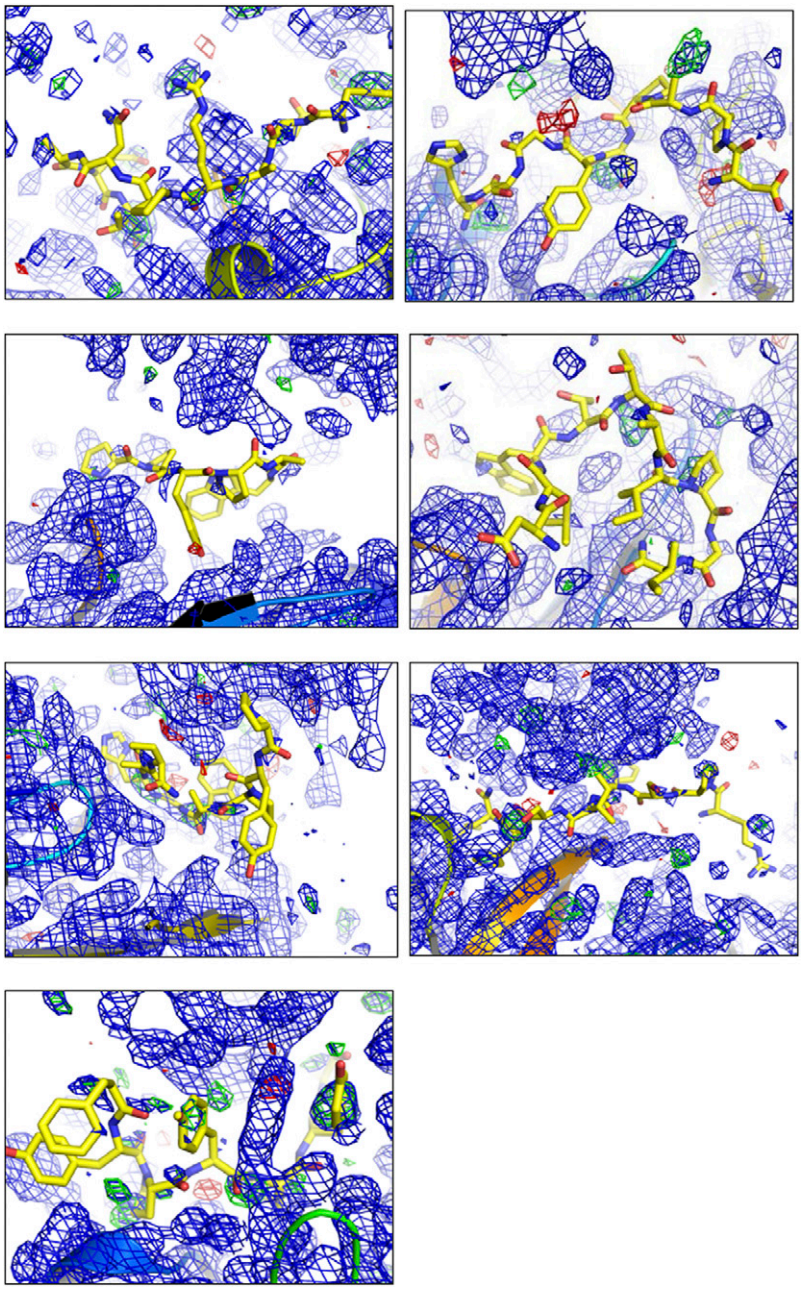

FIGURE 2. Omit electron density maps calculated using experimental data from (1–3). $2fo\text{-}f_c$ maps (blue) contoured at 1σ and $f_o\text{-}f_c$ maps (green/red) contoured at ± 3 σ are shown. Panels correspond to the following PDB entries: 2XZQ/2Y06 (*first row*), 2Y07/2Y36 (*second row*), 4BH7/4BH8 (*third row*), 4H0H (*bottom* row). Deposited peptide model is shown (yellow sticks).

mean peptide *B*-factors in the deposited PDB files are unexplainably lower and inconsistent with those obtained by standard crystallographic refinement practice and cannot be reconciled with the demonstrated absence of electron density. The genesis of these structure models is unknown to us, but in our opinion, it is abundantly clear that they are erroneous and do not support the conclusions of the corresponding papers.

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Abbreviation used in this article: PDB, Protein Data Bank.

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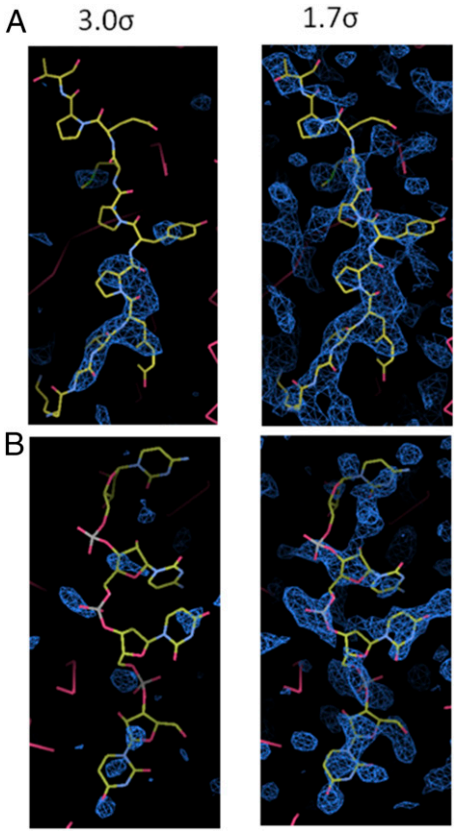

FIGURE 1. Delete-refine *Fo–Fc* maps for ligands interpretable at less than 3 σ as positive controls. (**A**) PDB: 1PWW (5); (**B**) PDB: 1UVI (4), contoured at 3.0 σ and 1.7 σ, respectively. Ligands are shown as sticks in elemental colors, whereas macromolecules are depicted as Cα traces. Delete-refine *Fo–Fc* maps are shown as blue mesh.

Response to Comment on Three X-ray Crystal Structure Papers

Drs. Pozharski, Rupp, and Stanfield have raised concerns about several recent publications from our group. Contrary to what they state, we have adequate direct and supporting evidence to confirm the presence of bound peptide ligands in all of the structures under discussion. Therefore, we reject the conclusions drawn by Pozharski et al. As we show below, by correlations with several published works, our structural data are consistent with accepted scientific and professional standards.

It is incorrect to state that primary experimental evidence (positive omit electron density) necessary to support the protein–peptide structure models in each of the three articles published by our group in *The Journal of Immunology* (1–3) is not present in the corresponding Protein Data Bank (PDB) depositions. In fact, when observed at the contour levels in the σ cutoff range routinely used by practicing macromolecular crystallographers (4–6) (see Fig. 1), the positive delete-refine *Fo–Fc* electron density is evident in each of our structures (Fig. 2). The *B*-factors reported for the structures determined by us are well within the range reported in many other published works (4–10) (Table I). Also, no actual differences exist in the *B*-factors reported in our papers and those deposited by us in the PDB. The perceived anomalies are merely a consequence of differences in the refinement strategies involving temperature factors (*B*-individual versus *B*-group) and criteria for evaluating structures.

Additional strong support for the existence of the bound ligands can be seen in the structural analyses of germline Ab BBE6.12H3 in ligand-free state and in the states bound to four different peptide ligands (1). Abs often have induced fit on binding of the Ag, reflected in terms of structural changes in the Ab, particularly in elbow angle and conformations of the CDRs (11–14). It is evident that the Ab BBE6.12H3 shows significant changes in the conformations of CDRs when bound to different ligands. Also, elbow angles of the ligand-bound Abs are significantly different from the elbow angle of the unliganded Ab (Table II). This is particularly common in the crystal structures involving conformational flexibility of ligand/receptor.

We disagree also with other statements made by Pozharski et al. It is to be noted that PDB ensures the quality of deposited data by implementing various validation mechanisms. Likewise, it is incorrect to state that the editors or reviewers have no access to the data during the review process. The authors' obligation to make available the primary data at any stage of the review process is well recognized.