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REPLY TO KISER:
Dioxygen binding in NOV1 crystal structures

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In PNAS (1) Kiser has expressed some skepticism about the identity of the active-site dioxygen molecule and suggests that the density is better modeled with two water molecules at partial occupancy. In response, we have performed an extended analysis with the following results. There are currently 321 entries for proteins containing dioxygen (PDB OXY) in the Protein Data Bank (PDB). As might be expected, the electron density ranges from highly symmetric to highly irregular. For example, naphthalene 1,2-dioxygenase [PDB ID code 1O7N (2)], cited by Kiser as a comparable enzyme, has a dioxygen bound in a side-on manner to an iron in almost exactly the same orientation as the NOV1 structures with and without substrate (3). Like NOV1, the density surrounding the dioxygen is not completely symmetric, and there are many more examples in the PDB. Oxygen atoms from a dioxygen species that are in identical chemical environments have more regular density and similar B-factors, although the opposite is also true. In apo-NOV1 (PDB ID code 5J53), a solvent molecule is 2.4 Å from the oxygen atom that is most closely bound to the iron, and it may perturb the oxygen's position. This may also address Kiser's second critique, which is that the two oxygens have different B-factors and the oxygen closer to the iron has the high B-factor. Naphthalene 1,2-dioxygenase (PDB ID code 1O7N) also has a higher B-factor for the oxygen bound more closely to the iron, and a survey of OXY PDB entries shows that the B-factors are often quite different. Finally, Kiser reports that refinement of individual oxygen atoms or an unrestrained dioxygen results in O–O distances of ∼1.8 Å, which is longer than the expected 1.2 Å. However, as has been pointed out by others, unrestrained refinement should only be used for ultra-high-resolution structures with observations to parameters ratios of 10 or greater (4). Below that ratio, the uncertainty will be very large, and the results may be meaningless. To demonstrate this, we refined a number of related dioxygen-bound structures this way (Table 1). Naphthalene 1,2-dioxygenase (PDB ID code 1O7N) (2) and homogentisate 1,2-dioxygenase (PDB ID code 3ZDS) (5) both bind dioxygen similarly to NOV1. They refine to distances that are much higher (∼1.8 Å) or lower (∼0.9 Å) than dioxygen. When we performed the same refinement on apo-NOV1 (PDB ID code 5J53), we did not obtain the distances reported by Kiser. They were only slightly larger than standard dioxygen (Table 1), and we note that when the occupancy of the dioxygen in apo-NOV1 (PDB ID code 5J53) is lowered the distances converge on 1.2 Å (Table 1) and the B-factors also decrease. The occupancies of the dioxygen were refined in both NOV1 structures, but at the data resolutions involved B-factors and occupancies are highly correlated (4). Therefore, it is possible that the dioxygen could be modeled with an occupancy slightly lower than the refined value of 0.9. In summary, our original paper and the analyses presented here show that Kiser's statements in no way preclude the presence of dioxygen in the active site.

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Table 1. Distances between either two waters with repulsive nonbonding interactions removed or two oxygen atoms from dioxygen without bond or metal coordination restraints as refined by phenix.refine version dev-274 (6)

PDB ID code	Waters, Å	Unrestrained dioxygen, Å
1O7N	1.7	1.9
3ZDS	0.7	1.0
5JRR	1.1	1.2
5FLJ	0.8	0.9
4F0H	1.8	1.7
4QMA	1.4	1.5
NOV1 (5J54)	1.7	1.7
NOV1 (5J53)	1.3	1.5
NOV1 (5J53) Occ. 0.8	—	1.4
NOV1 (5J53) Occ. 0.7	—	1.3
NOV1 (5J53) Occ. 0.6	—	1.2

NOV1 (PDB ID code 5J53) with reduced dioxygen occupancy was also tested.

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