



Protonation states of hen egg-white lysozyme observed using D/H contrast neutron crystallography

Toshiyuki Chatake,^{a,*} Ichiro Tanaka,^{b,c} Katsuhiko Kusaka^c and Satoru Fujiwara^d

Received 7 February 2022

Accepted 29 April 2022

Edited by P. Langan, Oak Ridge National Laboratory, USA

Keywords: neutron crystallography; D/H contrast maps; protonation; lysozyme.

PDB references: lysozyme, X-ray structure, D₂O solvent, 7fcu; X-ray structure, H₂O solvent, 7fcw; neutron structure, D₂O solvent, 7vei

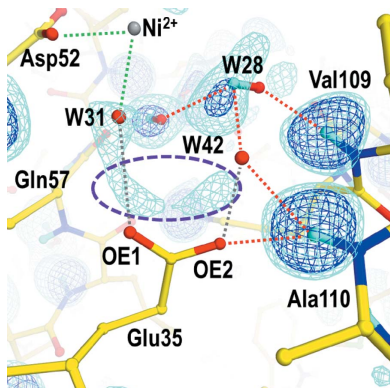
Supporting information: this article has supporting information at journals.iucr.org/d

^aInstitute for Integrated Radiation and Nuclear Science, Kyoto University, Asashironishi 2, Kumatori, Osaka 590-0494, Japan, ^bDomain of Materials Science and Engineering, Graduate School of Science and Engineering, Ibaraki University, Nakanarusawa 4-12-1, Hitachi, Ibaraki 316-8511, Japan, ^cDivisions of Neutron Beamline and Research, Frontier Research Center for Applied Atomic Sciences, Shirakata 162-1, Tokai, Ibaraki 319-1106, Japan, and ^dInstitute for Quantum Life Science, National Institutes for Quantum Science and Technology, Shirakata 2-4, Tokai, Ibaraki 319-1106, Japan. *Correspondence e-mail: chatake.toshiyuki.6x@kyoto-u.ac.jp

Hen egg-white lysozyme (HEWL) is an enzymatic protein with two acidic amino acids, Glu35 and Asp52, in its active site. Glu35 acts as a proton donor to the substrate and Asp52 interacts with the positively charged substrate, suggesting different protonation states of these residues. However, neutron crystallographic studies thus far have not provided a consistent picture of the protonation states of these residues. Only one study succeeded in observing the active protonation states of Glu35 and Asp52 in the triclinic crystal system. However, their active states in the most widely studied tetragonal crystal system are still unknown. The application of the D/H contrast technique in neutron crystallography improves the ability to locate exchangeable D/H atoms in proteins. In the present study, D₂O and H₂O solvent crystals were prepared. Each neutron data set was collected for only five days by combining a time-of-flight diffractometer (iBIX) and the spallation neutron source at the Japan Proton Accelerator Research Complex. The D/H contrast map provided better visualization of the D/H atoms in HEWL than the conventional neutron scattering length density map. The neutron D/H contrast map demonstrated the alternative protonation of the OE1 and OE2 atoms in the carboxyl group of Glu35. This alternative protonation occurs in the absence of a substrate, where high selectivity of the protonation site does not occur. In this case, only the OE1–HE1 bond attacks the substrate in an equilibrium between OE1–HE1 and OE2–HE2, or the H⁺ ion of the OE2–HE2 bond moves to the OE1 atom just before or after substrate binding to initiate the catalytic reaction. In contrast, the carboxyl group of Asp52 is not protonated. Protonation of the carboxyl group was not observed for other Asp and Glu residues. These results are consistent with results from NMR spectroscopy and explain the protonation states at the active site in the apo form of HEWL.

1. Introduction

Hydrogen (H) atoms account for approximately one half of all atoms in proteins. Changes in the charge states of amino and carboxyl groups by protonation/deprotonation regulate the electrostatic surface, and water molecules surrounding the proteins interact with the protein surfaces. They cooperatively mediate various biological processes, including protein folding, enzymatic reactions and molecular recognition. Thus, determining the positions of H atoms, especially those in polar functional groups, has been an important topic since the genesis of protein crystallography. However, synchrotron X-ray crystallography alone is insufficient to determine all H atoms in proteins (Niimura, 1999; Habash *et al.*, 2000). Since X-rays are scattered by electrons, H atoms, containing only



one electron, have a much weaker scattering power than other atoms found in proteins. Indeed, high-resolution X-ray structures ($d_{\min} < 1 \text{ \AA}$), which can include a considerable number of H atoms, compose $\sim 0.5\%$ of Protein Data Bank (PDB) entries (as of 23 July 2021). Furthermore, H^+ ions, which contain no electrons, are invisible in X-ray electron-density maps. This causes serious problems in determining the protonation states of amino acids in proteins. Moreover, H atoms in water molecules show high mobility, induced by a combination of translation and rotation of each water molecule, rendering them invisible even at ultrahigh resolution.

Neutron crystallography is a powerful technique for directly locating H atoms in proteins (Schoenborn & Nunes, 1972; Blakeley, 2009; Blakeley & Podjarny, 2018; Helliwell, 2020). The neutron scattering lengths of H and its isotope deuterium (D) ($b_{\text{H}} = -3.74 \text{ fm}$ and $b_{\text{D}} = 6.67 \text{ fm}$) are comparable to those of other atoms found in proteins (for example, b_{C} , b_{N} and $b_{\text{O}} = 6.65, 9.36$ and 5.80 fm , respectively). Densities for H atoms in the neutron scattering length density map (neutron map) can be observed even at medium resolution ($d_{\min} \simeq 2.0 \text{ \AA}$). Diffraction instruments equipped with advanced two-dimensional neutron detectors (Tanaka *et al.*, 2002; Ostermann & Schrader, 2015; Blakeley *et al.*, 2010), as well as the utilization of spallation neutron sources (Langan & Greene, 2004; Coates *et al.*, 2010; Tanaka *et al.*, 2010; Kusaka *et al.*, 2013), enable the collection of diffraction data with reasonable measurement times; hence, the number of protein structures including H atoms and hydration structures determined using neutron protein crystallography is increasing. However, as in X-ray crystallography, the problem of accuracy due to limited resolution cannot be avoided in neutron crystallography, particularly because of the low flux of the neutron beams. To improve the signal to noise in neutron diffraction data, crystals containing D_2O solvent are usually employed, since they significantly reduce the background noise due to the high incoherent neutron scattering of H atoms (inc_{H} and $\text{inc}_{\text{D}} = 80.26$ and 2.05 barns, respectively). Significant effort is put into obtaining large crystals to compensate for the low flux of neutron beams. Although crystals of $0.1\text{--}0.3 \text{ mm}^3$ are acceptable for current neutron protein crystallography (Blakeley & Podjarny, 2018; Budayova-Spano *et al.*, 2020), these are still larger than the crystals used in X-ray protein crystallography. It is difficult to obtain high-resolution diffraction data in neutron crystallography. Therefore, further improvement is necessary to enhance the diffraction signals in order to determine all H atoms.

The different scattering properties of H and D (due to the opposite signs of their scattering lengths) can be utilized to improve the visibility of H atoms in Fourier maps. The 'difference' diffraction measurements of a sample containing H atoms and one in which these atoms are replaced with D atoms could enhance the signals from the replaceable H atoms [$6.67 - (-3.74) = 10.41 \text{ fm}$], while also canceling out the signals from other atoms. In D/H contrast neutron protein crystallography, data sets from both deuterated and hydrogenated crystals are required. This has been a problem in D/H contrast neutron protein crystallography, in which it takes a

relatively long time to collect each of the two data sets. In particular, smaller crystals sometimes require an even longer data-collection time. Nevertheless, the D/H contrast technique is promising for neutron protein crystallography since it is difficult to improve the resolution limit ($d_{\min}$) in neutron diffraction, which is directly related to the detectability of H atoms. The D/H contrast technique provides high detectability, albeit with an extended data-collection time. The D/H contrast technique in neutron crystallography was first developed in pioneering work by Kossiakoff and coworkers (Kossiakoff, 1985; Shpungin & Kossiakoff, 1986; Kossiakoff *et al.*, 1990, 1992). However, the D/H contrast map was calculated in reciprocal space and thus is unsuitable for conventional structure determination, which involves iteration of model building and refinement of atomic parameters. We have developed a new methodology to calculate the D/H contrast map by subtracting in real space instead of in reciprocal space. This technique was incorporated into the conventional analysis protocol (Chatake & Fujiwara, 2016). This real-space D/H contrast neutron crystallography was applied to crystals of bovine ribonuclease A using the BIX-3 diffractometer (Tanaka *et al.*, 2002) at a neutron reactor source (Chatake & Fujiwara, 2016). The two previous D/H contrast neutron crystallographic experiments were conducted at neutron reactors (Kossiakoff, 1985; Chatake & Fujiwara, 2016), while the present D/H contrast neutron crystallographic experiment was performed at a spallation neutron source. Each data set for hen egg-white lysozyme (HEWL) crystals was collected with $d_{\min} = 1.8 \text{ \AA}$ using the iBIX time-of-flight diffractometer (Tanaka *et al.*, 2010; Kusaka *et al.*, 2013). The preliminary results of these experiments have been reported in Tanaka *et al.* (2020).

Lysozyme is an enzymatic protein that hydrolyses glycosidic bonds in peptidoglycan, which is present in bacterial cell walls. The active site of HEWL contains two important polar functional groups: the carboxyl groups of Glu35 and Asp52. Glu35 is a proton donor to the substrate (Phillips, 1967), while Asp52 interacts with the positively charged substrate. Possible interactions are electrostatical stabilization of the intermediate carbenium ion (Phillips, 1967) and a direct bond to the intermediate (Vocadlo *et al.*, 2001). In order to perform the chemical reaction, the protonation states of these residues should be different. Numerous studies, including structural studies of HEWL, have been carried out to reveal the catalytic mechanism of lysozyme by elucidating the protonation states at the catalytic site. However, the diffraction resolution limits of HEWL crystals are usually in the medium-resolution range and are not sufficient to locate H atoms by X-ray diffraction. There are only two structures of tetragonal HEWL crystals at a resolution of better than $1 \text{ \AA}$ in the PDB (as of 23 July 2021): 0.94 and $0.98 \text{ \AA}$ for PDB entries 1iee (Sauter *et al.*, 2001) and 7bcu (Chiniadis *et al.*, 2021), respectively. Although some densities corresponding to H atoms were observed in these X-ray Fourier maps, the H atoms were not included in the PDB files. HEWL structures in other crystal forms [PDB entries 7ave ($0.98 \text{ \AA}$, pH 4.5; Ramos, Laux, Haertlein, Boeri Erba *et al.*, 2021), 2vb1 ($0.65 \text{ \AA}$, pH 4.7; Wang *et al.*, 2007) and

7p6m (0.89 Å; pH 4.5; Ramos, Laux, Haertlein, Forsyth *et al.*, 2021) in the triclinic form and PDB entries 6f1o (0.96 Å; pH 4.5; Plaza-Garrido *et al.*, 2018) and 6sye (0.97 Å; pH 7.0; Plaza-Garrido *et al.*, 2020) in the orthorhombic form] included H atoms. All pH values were acidic except that for PDB entry 6sye. However, the X-ray analyses could not identify an H atom at the carboxyl group of Glu35 in the active site. Neutron crystallography was performed to locate H atoms, and three medium-resolution structures were obtained (Niimura *et al.*, 1997; Bon *et al.*, 1999; Kita & Morimoto, 2020): structures from tetragonal crystals at pH 7.0 (PDB entry 1io5; Niimura *et al.*, 1997) and pH 4.6 (PDB entry 6k8g; Kita & Morimoto, 2020) determined at 2.0 Å resolution and a structure from a triclinic crystal at pH 4.7 determined at 1.7 Å resolution (PDB entry 1lzn; Bon *et al.*, 1999). These structures showed the different protonation states of HEWL, particularly at Glu35 and Asp52. Compared with these conventional analyses, our analysis using the D/H contrast technique at 1.8 Å resolution should provide the best pictures of Glu35 and Asp52 at an acidic pH (4.5). Here, we present the results of our D/H contrast analysis, juxtaposed with previous structures, to discuss the relationship between protonation and the enzymatic reaction of HEWL.

2. Materials and methods

2.1. Sample preparation

HEWL (Hampton Research) was crystallized in an H₂O solution at 293 K using the concentration-gradient technique (Ataka & Katsura, 1993). H₂O-solvent crystals (*h*-crystals) were grown from a solution consisting of 15–25 mg ml^{−1} HEWL, 50 mM sodium acetate pH 4.5, 0.1 g ml^{−1} NiCl₂·6H₂O. The crystals reached their maximum size (~10 mm³) within 3–6 months. D₂O-solvent crystals (*d*-crystals) were obtained from *h*-crystals by changing the solvent from H₂O to D₂O using the vapor-equilibrium method for three months. The pH meter reading of the D₂O solution was adjusted to 4.1 owing to the pH shift of 0.4 units for D₂O solutions. This mild method was used to maintain isomorphism between the *h*-crystals and *d*-crystals. The crystals for the X-ray and neutron experiments were sealed in thin glass capillaries and quartz tubes, respectively.

2.2. X-ray experiments

X-ray diffraction experiments were performed on the *d*-crystal to build a starting model for neutron structure determination. An X-ray diffraction experiment was also carried out on the *h*-crystal to confirm that the difference in the structures of the *h*- and *d*-crystals is small. X-ray diffraction data sets for the two crystals were collected at ~293 K on the BL5A beamline at the Photon Factory and were processed using *XDS* (Kabsch, 2010). *Phenix* (Liebschner *et al.*, 2019) and *Coot* (Emsley *et al.*, 2010) were used for structure determination. The initial phases of the *d*-crystal were determined by the molecular-replacement method using PDB entry 1iee (Sauter *et al.*, 2001) as the starting model. To determine the structure of the *h*-crystal, the structure of the *d*-crystal was

Table 1

Statistics for X-ray experiments.

Values in parentheses are for the highest resolution shell.

	<i>d</i> -crystal	<i>h</i> -crystal
Data collection		
$d_{\min}$ (Å)	1.42	1.43
Space group	<i>P</i> 4 ₃ 2 ₁ 2	<i>P</i> 4 ₃ 2 ₁ 2
$a = b$ (Å)	79.14	79.23
c (Å)	37.78	37.82
Unique reflections	23269 (1130†)	22878 (1136‡)
Multiplicity	14.8 (15.0†)	14.7 (14.6‡)
Completeness (%)	100.0 (100.0†)	100.0 (100.0‡)
$\langle I/\sigma(I) \rangle$	36.6 (5.1†)	26.1 (4.5‡)
$R_{\text{p.i.m.}}$	0.015 (0.236†)	0.023 (0.251‡)
Wilson B factor (Å ²)	18.1	18.7
Structure determination		
$d_{\min}$ in refinement (Å)	1.42	1.43
R_{work}	0.163 (0.221§)	0.167 (0.233¶)
R_{free}	0.189 (0.260§)	0.186 (0.254¶)
R.m.s.d., bond lengths (Å)	0.010	0.006
R.m.s.d., angles (°)	1.05	1.02
PDB code	7fcu	7fcw

† Highest resolution shell 1.44–1.42 Å. ‡ Highest resolution shell 1.45–1.43 Å. § Highest resolution shell 1.471–1.420 Å. ¶ Highest resolution shell 1.481–1.430 Å.

used as the starting model. Structure determination of the *h*-crystal was carried out in the same manner as that for the *d*-crystal. The differences in the unit-cell parameters a , b and c between the *d*- and *h*-crystals were ~0.11%. The root-mean-square difference (r.m.s.d.) of all atoms between the two crystals was 0.032 Å on superimposing the two crystal structures. In addition, the overall B factors of the two crystals were approximately the same (18.1 and 18.7 Å² for the *d*- and *h*-crystals, respectively). These results indicate that the isomorphism between the *d*- and *h*-crystals was sufficiently high to guarantee the feasibility of the D/H contrast technique. The statistics of the X-ray experiments are summarized in Table 1.

2.3. Neutron experiments

Neutron diffraction data sets were collected from the two crystals at ~293 K using the IBARAKI biological crystal diffractometer (iBIX; Tanaka *et al.*, 2010; Kusaka *et al.*, 2013) installed at BL03 of the Material and Life Science Facility (MLF) at the Japan Proton Accelerator Research Complex (J-PARC). A neutron wavelength (λ) bandwidth of between 2.3 and 6.3 Å was used. For each crystal, 21 diffraction patterns with different orientations were collected using 30 detectors, with a measurement time of approximately 5 h 40 m. The total measurement time for the two crystals was approximately ten days. Diffraction data sets were processed to 1.8 Å resolution using the *STARGazer* data-processing software (Yano *et al.*, 2018). Diffraction spots for $\lambda = 2.93$ –5.57 Å were indexed and integrated. Structure determination using the D/H contrast technique was performed as described previously (Chatake & Fujiwara, 2016). Atomic parameter refinement and Fourier map calculations were performed using *Phenix* (Liebschner *et al.*, 2019). The effective resolution used in the refinement was 2.0 Å since the intensities in the high-resolution shell were

Table 2

Neutron structure determination.

mc and sc are main and side chains, respectively.

	$R_{\text{work}}/R_{\text{free}}$ (%)		H	H/D		D
	Neutron	X-ray		mc	sc	Water
X-ray structure		16.0/18.9	0	0	0	0
Add H atoms of C—H and N—H in mc	25.8/30.1	15.3/18.1	822	0	0	0
Estimate D/H exchange ratio of N—H in mc	19.7/24.1	15.2/18.1	696	126	0	0
First modeling step and refinement	17.1/21.8	15.2/18.1	696	126	80	93
Second modeling step and refinement	17.0/21.5	15.1/17.9	694†	126	84‡	94
Third modeling step	16.9/21.6	15.1/18.0	694†	126	84‡	94
Correction for D ₂ O at special position	17.0/21.5	15.4/18.1	694†	126	84‡	94

† The HD2 and HE1 atoms of His15 were excluded. ‡ The DE1 atoms of Trp28 and Trp111 were included as D/H atoms.

Table 3

Statistics of neutron experiments.

Values in parentheses are for the highest resolution shell.

	<i>d</i> -crystal	<i>h</i> -crystal
Data collection		
d_{min} (Å)	1.8	1.8
Space group	$P4_32_12$	$P4_32_12$
$a = b$ (Å)	79.14	
c (Å)	37.78	
Unique reflections	11376 (1072†)	11264 (1004†)
Multiplicity	7.4 (4.9†)	6.6 (4.2†)
Completeness (%)	97.9 (95.4†)	97.0 (89.5†)
$\langle I/\sigma(I) \rangle$	12.2 (1.5†)	10.4 (1.5†)
$R_{\text{p.i.m.}}$	0.071 (0.284†)	0.076 (0.418†)
Wilson B factor (Å ²)	21.2	18.1
Structure determination		
d_{min} in refinement (Å)	2.0	
R_{work}	0.169 (0.170‡)	
R_{free}	0.216 (0.217‡)	
R.m.s.d., bond lengths (Å)	0.008	
R.m.s.d., angles (°)	1.12	
PDB code	7vei	

† Highest resolution shell 1.86–1.80 Å. ‡ Highest resolution shell 2.125–2.000 Å.

weak. In contrast, all reflections to 1.8 Å resolution were used in the neutron Fourier map calculations. The D/H contrast maps were calculated from the $2m|F_o| - D|F_c|$ maps (CNS/*X-PLOR* format) of the *d*- and *h*-crystals using a previously described algorithm (Chatake & Fujiwara, 2016). The structure-determination workflow is summarized in Table 2. The starting model was the X-ray structure of the *d*-crystal. Before calculating the first D/H contrast maps, unexchangeable H atoms in C—H bonds were generated stereochemically in the X-ray structure. Peaks higher than 1.5σ were assigned to exchangeable D/H atoms in the D/H contrast $2m|F_o| - D|F_c|$ map. The atomic parameters were optimized using joint neutron/X-ray refinement. The neutron and X-ray data sets of the *d*-crystal were used in the refinement. The statistics of the neutron experiments are summarized in Table 3.

3. Results and discussion

3.1. Overview

The D/H atoms of HEWL are summarized in Table 4. We identified 70% (44 of 63) of the functional groups as

containing exchangeable D/H atoms. Most unidentified groups are not well ordered; they have large B factors ($>40 \text{ Å}^2$) and/or adopt multiple conformations. If groups that were not well ordered were excluded from the statistics, the percentage of identified groups was 93% (39 of 42), indicating the high detectability of D/H atoms in D/H contrast neutron crystallography. Moreover, most of the identified D/H atoms (80 of 84) were found during the first modeling step in the structure determination (Table 2). The detect-

ability of D/H atoms by the D/H contrast technique is higher than that of conventional neutron analysis. Our conventional neutron analyses of the rubredoxin mutant and deoxy-hemoglobin show that 64% of groups were identified at 1.6 Å resolution (Chatake *et al.*, 2004) and 57% at 2.0 Å resolution (Chatake *et al.*, 2007), even though all functional groups have neither large B factors nor multiple conformations in these structures. The protonation states of Glu35 and Asp52 at the active site could be observed in the contrast map of the tetragonal HEWL crystal. These protonation states have previously been observed in the conventional neutron map of the triclinic HEWL crystal (Bon *et al.*, 1999). In contrast, tetragonal crystals of HEWL tend to diffract more weakly than triclinic crystals, resulting in a lack of observable protonation states in tetragonal crystals. The present HEWL model obtained using the D/H contrast technique contains 88 water molecules. This is approximately one third of the 255 water molecules in the X-ray structure of the tetragonal HEWL crystal determined at the highest resolution (Sauter *et al.*, 2001). This difference is likely to arise from the dynamic disorder of water molecules and the resolution limitation. While the neutron measurements were carried out at $\sim 293 \text{ K}$ with $d_{\text{min}} = 1.8 \text{ Å}$, the X-ray measurements were performed at low temperature with $d_{\text{min}} = 0.94 \text{ Å}$. The densities for water molecules observed in neutron crystallography are classified into the following three types, depending on the mobility of the molecules (Chatake *et al.*, 2003): (i) densities for two D/H atoms, indicating the orientation of the water molecule (D—O—D type), (ii) density for only one D/H atom, indicating that another D/H atom is rotationally disordered around the observable O—D/H bond (O—D type), and (iii) no density, indicating rotational disorder (O type). The water molecules in the present model comprised 27 molecules of the D—O—D type, 31 molecules of the O—D type and 31 molecules of the O type.

3.2. Protonation states of Glu and Asp residues

Fig. 1 shows the neutron D/H contrast $2m|F_o| - D|F_c|$ map around the carboxyl groups of Glu35 and Asp52, which are included in the active site. In the D/H contrast map, only exchangeable D/H atoms were observed, as the densities of all other atoms were canceled out. Fig. 1(a) shows the region

Table 4

D/H atoms in the present tetragonal HEWL crystal.

For the underlined residues, the B factors of the atom at the protonation site (the NE atom of Arg) are higher than $40 \text{ \AA}^2$.

Functional groups	Residues
Assigned atoms	
–ND ₃ ⁺	Nter, <u>Lys1</u> , <u>Lys13</u> , Lys33, Lys96, Lys116
–ND ₂	<u>Asn19</u> , <u>Asn27</u> , <u>Asn37</u> , <u>Asn39</u> , <u>Asn44</u> , <u>Asn46</u> , <u>Asn59</u> , <u>Asn74</u> , <u>Asn93</u> , <u>Asn106</u> , <u>Asn113</u> , <u>Gln41</u> , <u>Gln57</u>
–ND	Trp28†, Trp63, Trp108, Trp111†, Trp123
–ND–C–(ND ₂) ⁺	Arg5, Arg45, Arg68, Arg114
–OD	Ser24, Ser36, Ser50, Ser60, Ser72, Ser81, Ser91, Thr40, Thr43, Thr51, Thr69, Thr89, Thr118, Tyr23, Tyr53
–COOD	Glu35
Unassigned atoms	
–ND ₃ ⁺	<u>Lys97</u>
–ND ₂	<u>Asn65</u> , <u>Asn77</u> , <u>Asn103</u> , <u>Gln121</u>
–ND	<u>Trp62</u>
–ND–C–(ND ₂) ⁺	<u>Arg14</u> , <u>Arg21</u> ‡, <u>Arg61</u> , <u>Arg73</u> , <u>Arg112</u> ‡, <u>Arg125</u> , <u>Arg128</u> ‡
–OD	Ser85, Ser86‡, Ser100, <u>Thr47</u> , Tyr20
Imidazole	<u>His15</u>

† The D/H exchange ratio was calculated. ‡ The side chains have alternative conformations.

around Glu35. Neutron scattering length densities at the 2σ level were observed near the OE1 and OE2 atoms of the carboxyl group. Two water molecules (W31 and W42) were also located near each of these densities. These 2σ densities were located near-centrally between the OE1 (Glu35) and O (W31) atoms and between the OE2 (Glu35) and O (W42) atoms, respectively. These densities indicate that the OE1 and OE2 atoms can be alternately protonated. The density peak between the OE1 (Glu35) and O (W31) atoms was located 0.89 and $1.84 \text{ \AA}$ from the OE1 (Glu35) and O (W31) atoms, respectively, suggesting that most of the density belongs to the OE1–H bond. In the case of the OE2 atom, careful consideration was required to assess the protonation. The distance between the OE2 (Glu35) and O (W42) atoms ($2.50 \text{ \AA}$) is

considerably shorter than a regular hydrogen bond. The density peak is located at the middle point between the OE2 (Glu35) and O (W42) atoms (1.34 and $1.33 \text{ \AA}$ from OE2 and O, respectively). The observed density may be a summation of the densities for OE2–H (Glu35) and O–H (W42). In addition, the low occupancy of W42 supports protonation of the OE2 atom. As summarized in Table 5, the W42 molecule was observed in the highest resolution X-ray structure (Sauter *et al.*, 2001) and in one of the neutron structures (Niimura *et al.*, 1997), while the W31 molecule was conserved in all of the neutron structures (Niimura *et al.*, 1997; Bon *et al.*, 1999; Kita & Morimoto, 2020) and in the two highest resolution X-ray structures (Sauter *et al.*, 2001; Chiniadis *et al.*, 2021). In the highest resolution X-ray structure, the occupancies of W31 and W42 were 0.78 and 0.46 , respectively (Sauter *et al.*, 2001). In the present structure, the electron densities for W31 and W42 in the X-ray $2m|F_o| - D|F_c|$ map were at the 3.39σ and 1.72σ levels, respectively. These observations suggest that the occupancy of W42 would be approximately 0.5 . While W42 was observed in three crystals obtained using different pH values (4.5 for PDB entries 7vei and 1lee and 7.0 for PDB entry 1io5) and salts (NaCl for PDB entry 1lee, NiCl₂ for PDB entries 7vei and 1io5), it was not observed in two crystals containing the same salt (NaCl) at the similar pH value of 4.6 (PDB entries 7bcu and 6k8g). This low occupancy might be why a water molecule was not modeled at the W42 position in the other structures. Considering the low occupancy of W42, the density between the OE2 and O (W42) atoms is for the OE2–H bond. Generally, exchangeable D/H atoms with low occupancy are difficult to observe. In the present structural analysis, the D/H atoms of functional groups with alternative conformations could not be observed, as shown in Table 4. The contrast density of the D/H atoms of the water molecule (W42) would be further decreased due to the observed dynamic behavior, suggesting that the density would correspond to protonation of the OE2 atom. Thus, it can be concluded that the OE2 atom would be partially protonated.

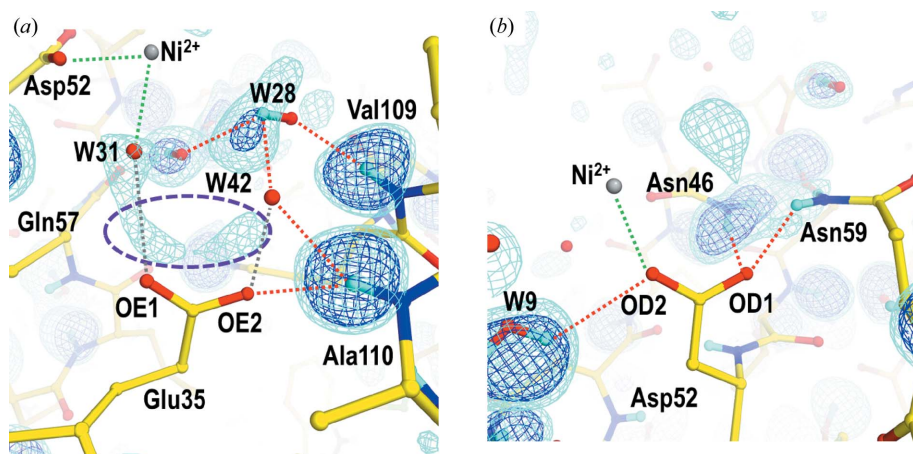


Figure 1

The D/H contrast $2m|F_o| - D|F_c|$ maps near the carboxy groups of (a) Glu35 and (b) Asp52 in the active site. Blue and cyan contours represent $+3.0\sigma$ and $+2.0\sigma$ densities, respectively. Red, green and gray broken lines are hydrogen bonds, coordinate bonds and possible hydrogen bonds, respectively. The ball-and-stick models and Fourier maps were drawn using CUEMOL2 (Molecular Visualization Framework; <http://www.cuemol.org/>).

Fig. 1(b) shows the region around Asp52. There was no significant density near the two O atoms of the carboxyl group of Asp52, indicating that the carboxyl group was deprotonated and had a negative charge: $-\text{COO}^-$. The negative charge of Asp52 is neutralized by a direct coordinate bond between the Ni^{2+} cation and the OD2 atom. In addition, the other O atom in the carboxyl group (OD1) forms a bifurcated hydrogen bond to the $-\text{NH}_2$ amide groups of Asn46 and Asn59. The deprotonated (negatively charged) Asp52 would participate in the negatively charged pocket to interact with positively charged intermediates. Both of the enzymatic reaction models (Phillips, 1967; Vocadlo *et al.*, 2001)

Table 5

Water molecules that interact with the carboxyl group of Glu35.

The values are the distances between the O atoms of the water and the carboxyl group.

PDB code	pH	Salt	W31 position	W42 position	Comment	Reference
7vei	4.5	NiCl ₂	2.70 (3.39†)	2.50 (1.72†)	Present analysis	
1iee	4.5	NaCl	2.62 (0.78‡)	2.51 (0.46‡)	0.94 Å resolution X-ray analysis	Sauter <i>et al.</i> (2001)
7bcu	4.6	NaCl	2.58	—	0.98 Å resolution X-ray analysis	Chiniadis <i>et al.</i> (2021)
1lzn	4.7	NaNO ₃	3.21	—	Triclinic, neutron analysis	Bon <i>et al.</i> (1999)
lio5	7.0	NiCl ₂	2.66	3.11	Tetragonal, neutron analysis	Niimura <i>et al.</i> (1997)
6k8g	4.6	NaCl	2.59	—	Tetragonal, neutron analysis	Kita & Morimoto (2020)

† The values in parentheses are density heights (σ level at the peak) of the $2m|F_o| - D|F_c|$ X-ray Fourier map. ‡ The values in parentheses are occupancies of the O atoms of the water molecules.

Table 6

Protonation states of Glu35 and Asp52 observed in neutron crystallography.

	pK _a †	Present	PDB entry 1lzn‡	PDB entry lio5‡	PDB entry 6k8g‡
Crystal system		Tetragonal	Triclinic	Tetragonal	Tetragonal
Precipitant		NiCl ₂	NaNO ₃	NiCl ₂	NaCl
pH		4.5	4.7	7.0	4.6
<i>d</i> _{min} (Å)		2.0 (1.8)§	1.7	2.0	2.0
Glu35	6.20	Protonated	Protonated	Deprotonated	¶
Asp52	3.68	Deprotonated	Deprotonated	Deprotonated	Deprotonated

† pK_a value determined by two-dimensional ¹H-NMR (Bartik *et al.*, 1994). ‡ References for PDB entries 1lzn, lio5 and 6k8g are Niimura *et al.* (1997), Kita & Morimoto (2020) and Bon *et al.* (1999), respectively. § Atomic refinement and map calculation were conducted at 2.0 and 1.8 Å resolution, respectively. ¶ 'The protonation state of Glu35 was not determined in this experiment' (Kita & Morimoto, 2020).

work on the assumption that Asp52 interacts with the positively charged intermediate either indirectly (Phillips, 1967) or directly (Vocadlo *et al.*, 2001). In the present structure, the positively charged Ni²⁺ ion interacts with Asp52 instead of the intermediate in the absence of the substrate.

The protonation states of Glu35 and Asp52 observed in the present analysis were compared with those in previous neutron structures (Niimura *et al.*, 1997; Bon *et al.*, 1999; Kita & Morimoto, 2020), as summarized in Table 6. The neutron structure analysis of the tetragonal HEWL crystal at pH 7.0 (Niimura *et al.*, 1997) showed no protonation of Glu35 and Asp52. The neutron structure analysis of the triclinic HEWL crystal at pH 4.7 (PDB entry 1lzn; Bon *et al.*, 1999) showed that Glu35 is protonated at the OE1 atom, while Asp52 is deprotonated. The neutron structure analysis of a tetragonal crystal of HEWL at pH 4.6 (PDB entry 7k8g; Kita & Morimoto, 2020) showed that Asp52 is deprotonated; however, the protonation of Glu35 could not be confirmed (see the supporting information in Kita & Morimoto, 2020). Previous two-dimensional ¹H-NMR spectroscopy (Bartik *et al.*, 1994) studies reported that Glu35 has a significantly higher pK_a (6.20) than Asp52 (3.68). These values are consistent with the Phillips model of the chemical reaction of lysozyme (Phillips, 1967), in which Glu35 donates an H⁺ ion to the substrate and Asp52 accepts an H⁺ ion from the substrate. In this model, Glu35 and Asp52 are expected to be protonated and deprotonated, respectively, in the active state of HEWL. Thus, the neutron structure of the tetragonal crystal at pH 7.0 (Niimura *et al.*, 1997) indicates an inactive state, while that of the triclinic crystal at pH 4.7 (Bon *et al.*, 1999) and our present structure indicate an active state. Our present neutron struc-

ture analysis, combined with the D/H contrast technique and a time-of-flight neutron experiment, successfully identifies the protonation state of active HEWL in the tetragonal crystal form.

The protonation state of Glu35 in the present structure differs from that in the previous structure of the triclinic crystal. While Glu35 is only protonated at OE1 in the previous structure (PDB entry 1lzn; Bon *et al.*, 1999), both the OE1 and OE2 atoms are alternatively protonated in the present structure, suggesting that

both atoms could potentially accept an H⁺ ion. While Glu35 is neutrally charged in both crystal structures, its protonation sites are different. The reason for this difference is unclear. The three-dimensional structures of Glu35 and its neighboring residues are similar to each other. We observed minor differences in the pH in the present (PDB entry 7vei) and previous (PDB entry 1lzn) structures, which were 4.5 and 4.7, respectively. The cation coordination of PDB entry 7vei was an Ni²⁺ ion coordinated to Asp52. In contrast, an Na⁺ ion is coordinated to the main-chain C=O bonds of Ser36 and Asn37. The crystal packings of PDB entries 7vei (P4₃2₁2) and 1lzn (P1) were also different, but this difference did not affect the structure of the active site. In the hydrolysis of HEWL, Glu35 and Asp52 sandwich the substrate (see the locations of Glu35 and Asp52 in Fig. 1a). When Glu35 acts as a proton donor during hydrolysis, selectivity of the protonation sites might be important for attacking the glycosidic bond in peptidoglycans. In this case, the OE1–HE1 bond (Fig. 1a) is preferable to the OE2–HE2 bond (Fig. 1b) for attacking the substrate. Indeed, previous neutron analysis of the triclinic crystal at pH 4.5 showed protonation of the OE1 atom (PDB entry 1lzn; Bon *et al.*, 1999). On the other hand, in the absence of a substrate the selectivity of the protonation site does not seem to be high when the environment is considered. The carboxyl group of Glu35 is exposed to the solvent region, and the OE1–HE1 or OE2–HE2 bond only forms a hydrogen bond to a water molecule. Hence, an alternative protonation site is appropriate when the substrate is not bound. If this is the case, only Glu35 with a protonated OE1 atom can attack the substrate during the equilibrium between protonation of the OE1 and OE2 atoms; otherwise, the protonation site shifts

to the OE1 atom immediately before or after substrate binding to attack the substrate. The H^+ ion moves from the OE2 atom to the OE1 atom, or the carboxyl groups rotate by 180° around the CD—CG bond.

Fig. 2 shows the neutron D/H contrast maps around the Glu and Asp side chains, except for Asp101 and Asp119, which were excluded from consideration because their side chains were not well ordered ($B > 50 \text{ \AA}^2$). There are no densities at the possible protonation sites of Glu7, Asp18, Asp48, Asp66 and Asp87, as shown in Figs. 2(a)–2(e), respectively. All carboxyl groups were exposed to the solvent regions, and at least one of the O atoms of the carboxyl groups appeared to directly contact a water molecule. Nevertheless, no obvious densities (higher than 3.0σ) were observed in the D/H contrast $2m|F_o| - D|F_c|$ Fourier map. Suspicious densities were observed around Glu7 (Fig. 2a) and Asp48 (Fig. 2c). However, the density near the OE1 atom of Glu7 is likely to be one of the D/H atoms of W78, which links Glu7 and the main chain via a hydrogen-bonding network. The density around Asp48 is likely to be that of the O atom of W71 because its density is closer to that of the OD2 atom. Thus, it appears that these side chains are deprotonated. These results are consistent with the previous NMR analysis (Bartik *et al.*, 1994), which showed that the estimated pK_a values of Glu and Asp listed in Fig. 2 were

lower than 3.0 (except for Glu35, with a pK_a of 6.20) and that Glu7 had the lowest pK_a (2.85).

3.3. Other D/H atoms

Fig. 3 shows the D/H contrast $2m|F_o| - D|F_c|$ maps around the indole rings of Trp in HEWL. An H atom bound to the NE1 atom of each Trp is exchanged with a D atom from the D_2O solvent; thus, a positive density should be found in the D/H contrast map. Trp63, Trp108 and Trp123 indeed have significant positive densities at the HE1 position of the atom, indicating complete D/H exchange at these sites. In contrast, Trp28, Trp62 and Trp111 did not show significant positive densities for their HE1 atoms. However, the side chain of Trp62 was not well ordered and was excluded from the discussion. The most plausible reason for the lack of positive densities in Trp28 and Trp111 is that the exchange rates from the HE1 atom to the DE1 atom of the NE1—H bonds were so slow that the exchange was not completed. Neutron protein crystallography has shown that some N—H bonds that are inside a protein and/or tightly interact with functional groups remain unexchanged, and some unexchanged H atoms are observed in the main chain of the protein (Niimura & Podjarny, 2011). Trp28 and Trp111 are in a similar state, where

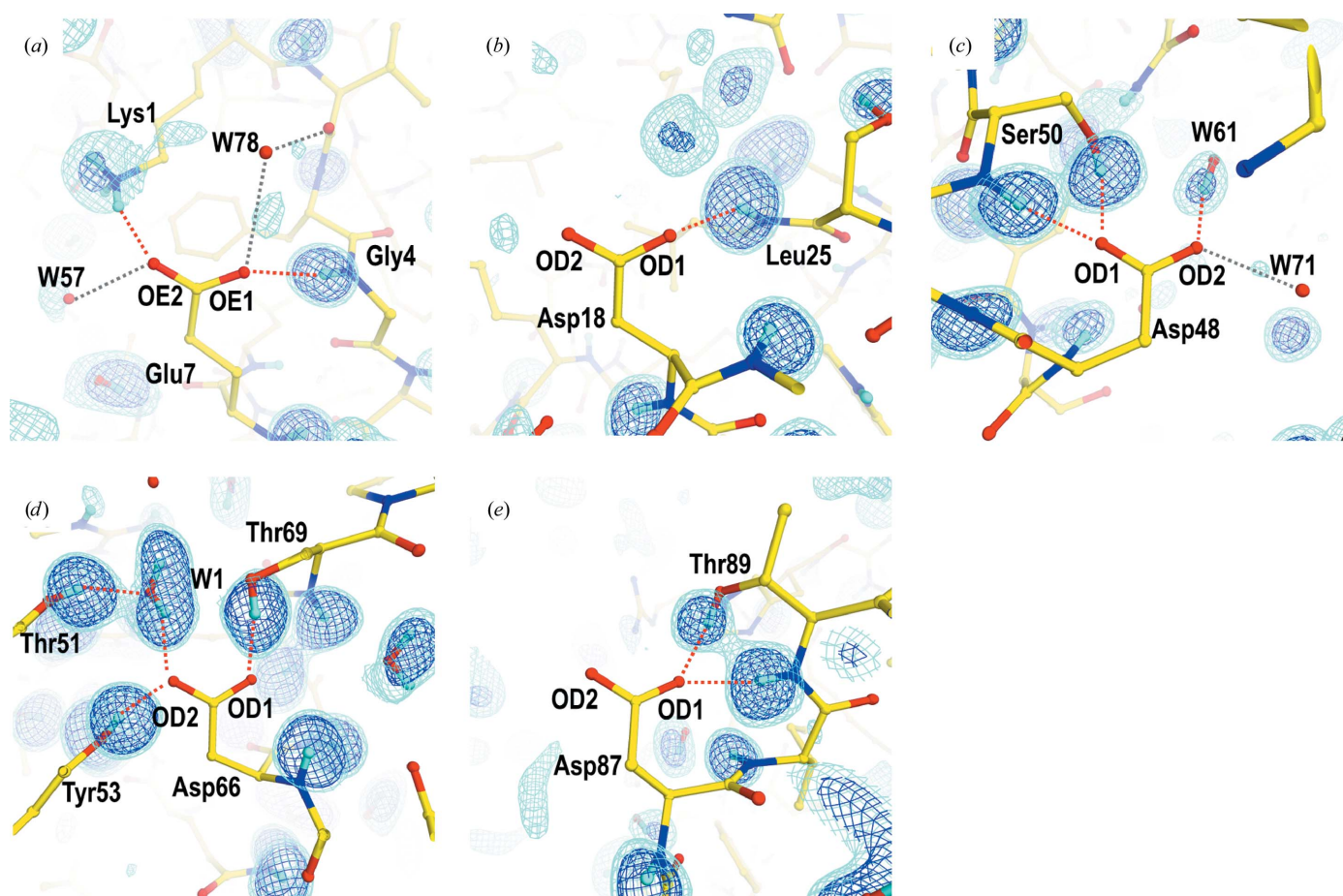


Figure 2

The D/H contrast $2m|F_o| - D|F_c|$ maps near the carboxy groups of (a) Glu7, (b) Asp18, (c) Asp48, (d) Asp66 and (e) Asp87. The ball-and-stick models and Fourier maps are drawn in the same manner as in Fig. 1.

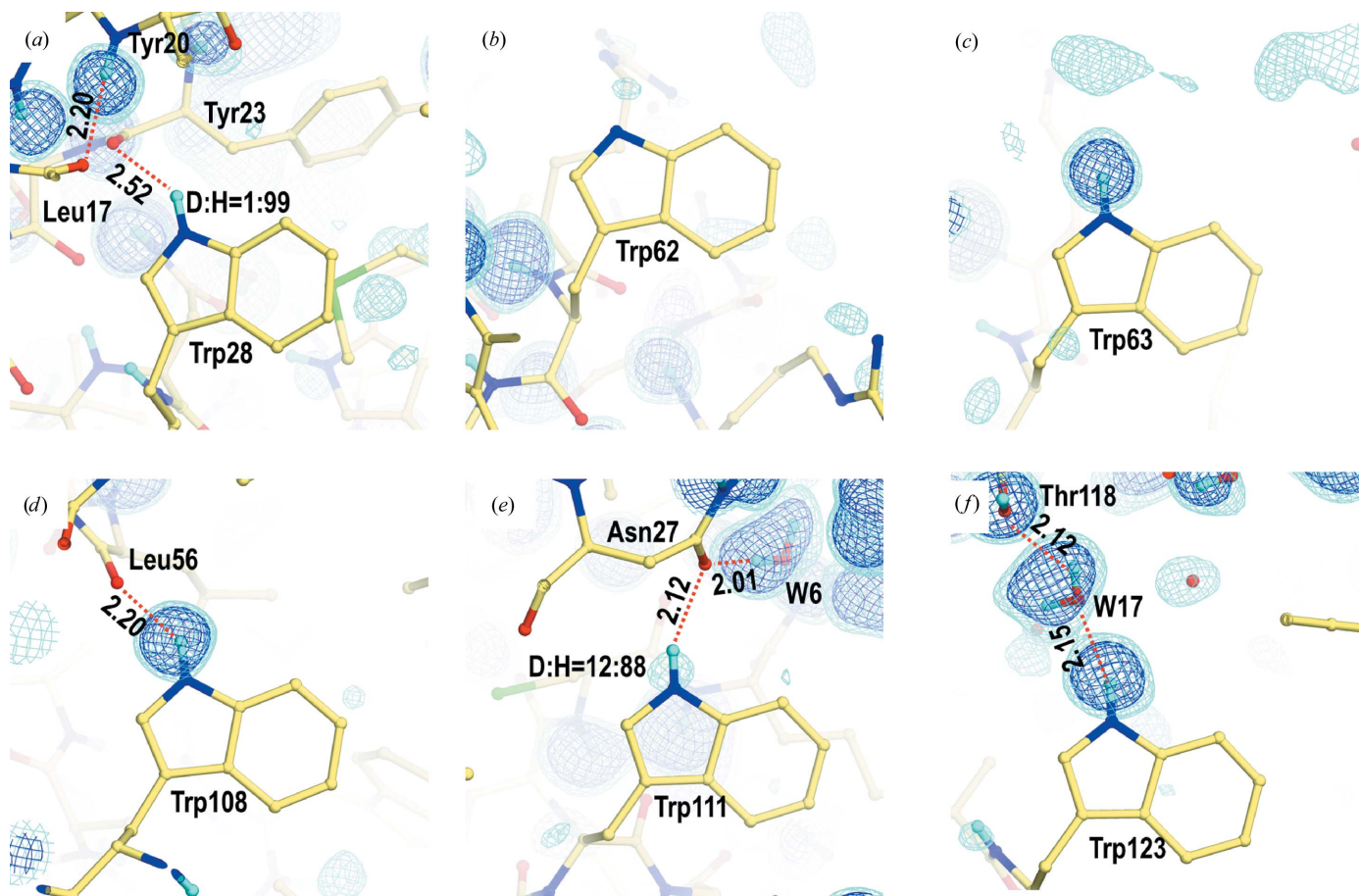


Figure 3

The D/H contrast $2m|F_o| - D|F_c|$ maps around the indole rings of (a) Trp28, (b) Trp62, (c) Trp63, (d) Trp108, (e) Trp111 and (f) Trp123. The ball-and-stick models and Fourier maps are drawn in the same manner as in Fig. 1.

the NE1 atoms of Trp28 and Trp111 interact with the main-chain O atoms of Tyr23 and Asn27, respectively. Previous neutron crystallographic analyses of HEWL showed that the NE1–H bond of Trp28 is not D/H exchanged (Bon *et al.*, 1999) or has a relatively lower D occupancy (0.12) than the average for D atoms (0.70; Kita & Morimoto, 2020). In addition to Trp28, the NE1–H bond of Trp111 is not D/H exchanged. The D:H ratios of the D/H atoms were optimized in the refinement, and the D:H ratios of Trp28 and Trp111 were 1:99 and 12:88, respectively, suggesting that the D/H exchange of Trp111 is faster than that of Trp28. The unexchanged NE1–H bond of Trp111 was first observed in the present study, and D/H exchange occurred by the vapor-diffusion technique in the solvent exchange from H₂O to D₂O that was employed to maintain isomorphism between the *h*- and *d*-crystals; hence, the H atoms would remain in the NE1–H bond of Trp111 in the mild D₂O replacement. The same situation only occurred for His15 in HEWL, for which the D/H atoms of the imidazole ring could not be determined.

4. Conclusion

Neutron D/H contrast crystallography of tetragonal HEWL crystals was performed. The combination of the time-of-flight

neutron diffractometer and a spallation neutron source made it possible to measure the diffraction data within a reasonably short time. The D/H contrast map provided better visualization of D/H atoms in HEWL than the conventional neutron map, thereby unambiguously providing the protonation states. In particular, the active protonation states of Glu35 and Asp52 were observed in the tetragonal crystal. The alternative protonation of OE1 and OE2 in the carboxyl group of Glu35 suggests that both atoms have the potential to accept an H⁺ ion. The results obtained in the present study demonstrate the usefulness of the D/H contrast technique in neutron crystallography to locate H atoms in proteins.

Acknowledgements

X-ray data were collected using the BL5A beamline at the Photon Factory (2017G650). Neutron data were collected at iBIX in MLF, J-PARC (2018PX0018 and 2019PX2006) in the Ibaraki Prefecture.

Funding information

The following funding is acknowledged: Japan Society for the Promotion of Science (16K05014 to TC).

References

- Ataka, M. & Katsura, T. (1993). *Neutrons in Biology*, edited by S. Funahashi & N. Niimura, p. 61. Tokai: Japan Atomic Energy Research Institute.
- Bartik, K., Redfield, C. & Dobson, C. M. (1994). *Biophys. J.* **66**, 1180–1184.
- Blakeley, M. P. (2009). *Crystallogr. Rev.* **15**, 157–218.
- Blakeley, M. P. & Podjarny, A. D. (2018). *Emerg. Top. Life Sci.* **2**, 39–55.
- Blakeley, M. P., Teixeira, S. C. M., Petit-Haertlein, I., Hazemann, I., Mitschler, A., Haertlein, M., Howard, E. & Podjarny, A. D. (2010). *Acta Cryst.* **D66**, 1198–1205.
- Bon, C., Lehmann, M. S. & Wilkinson, C. (1999). *Acta Cryst.* **D55**, 978–987.
- Budayova-Spano, M., Koruza, K. & Fisher, Z. (2020). *Methods Enzymol.* **634**, 21–46.
- Chatake, T. & Fujiwara, S. (2016). *Acta Cryst.* **D72**, 71–82.
- Chatake, T., Kurihara, K., Tanaka, I., Tsyba, I., Bau, R., Jenney, F. E., Adams, M. W. W. & Niimura, N. (2004). *Acta Cryst.* **D60**, 1364–1373.
- Chatake, T., Ostermann, A., Kurihara, K., Parak, F. G. & Niimura, N. (2003). *Proteins*, **50**, 516–523.
- Chatake, T., Shibayama, N., Park, S.-Y., Kurihara, K., Tamada, T., Tanaka, I., Niimura, N., Kuroki, R. & Morimoto, Y. (2007). *J. Am. Chem. Soc.* **129**, 14840–14841.
- Chiniadis, L., Giastas, P., Bratsos, I. & Papakyriakou, A. (2021). *Inorg. Chem.* **60**, 10729–10737.
- Coates, L., Stoica, A. D., Hoffmann, C., Richards, J. & Cooper, R. (2010). *J. Appl. Cryst.* **43**, 570–577.
- Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. (2010). *Acta Cryst.* **D66**, 486–501.
- Habash, J., Raftery, J., Nuttall, R., Price, H. J., Wilkinson, C., Kalb (Gilboa), A. J. & Helliwell, J. R. (2000). *Acta Cryst.* **D56**, 541–550.
- Helliwell, J. R. (2020). *Methods Enzymol.* **634**, 1–19.
- Kabsch, W. (2010). *Acta Cryst.* **D66**, 125–132.
- Kita, A. & Morimoto, Y. (2020). *J. Appl. Cryst.* **53**, 837–840.
- Kossiakoff, A. A. (1985). *Annu. Rev. Biochem.* **54**, 1195–1227.
- Kossiakoff, A. A., Shpungin, J. A. & Sintchak, M. D. (1990). *Proc. Natl Acad. Sci. USA*, **87**, 4468–4472.
- Kossiakoff, A. A., Sintchak, M. D., Shpungin, J. & Presta, L. G. (1992). *Proteins*, **12**, 223–236.
- Kusaka, K., Hosoya, T., Yamada, T., Tomoyori, K., Ohhara, T., Katagiri, M., Kurihara, K., Tanaka, I. & Niimura, N. (2013). *J. Synchrotron Rad.* **20**, 994–998.
- Langan, P. & Greene, G. (2004). *J. Appl. Cryst.* **37**, 253–257.
- Liebschner, D., Afonine, P. V., Baker, M. L., Bunkóczi, G., Chen, V. B., Croll, T. I., Hintze, B., Hung, L.-W., Jain, S., McCoy, A. J., Moriarty, N. W., Oeffner, R. D., Poon, B. K., Prisant, M. G., Read, R. J., Richardson, J. S., Richardson, D. C., Sammito, M. D., Sobolev, O. V., Stockwell, D. H., Terwilliger, T. C., Urzhumtsev, A. G., Videau, L. L., Williams, C. J. & Adams, P. D. (2019). *Acta Cryst.* **D75**, 861–877.
- Niimura, N. (1999). *Curr. Opin. Struct. Biol.* **9**, 602–608.
- Niimura, N., Minezaki, Y., Nonaka, T., Castagna, J. C., Cipriani, F., Høghøj, P., Lehmann, M. S. & Wilkinson, C. (1997). *Nat. Struct. Biol.* **4**, 909–914.
- Niimura, N. & Podjarny, A. (2011). *Neutron Protein Crystallography*, pp. 142–152. New York: Oxford University Press.
- Ostermann, A. & Schrader, T. (2015). *J. Large-Scale Res. Facil.* **1**, A2.
- Phillips, D. C. (1967). *Proc. Natl Acad. Sci. USA*, **57**, 483–495.
- Plaza-Garrido, M., Salinas-Garcia, M. C., Alba-Elena, D., Martínez, J. C. & Camara-Artigas, A. (2020). *Acta Cryst.* **D76**, 845–856.
- Plaza-Garrido, M., Salinas-Garcia, M. C. & Camara-Artigas, A. (2018). *Acta Cryst.* **D74**, 480–489.
- Ramos, J., Laux, V., Haertlein, M., Boeri Erba, E., McAuley, K. E., Forsyth, V. T., Mossou, E., Larsen, S. & Langkilde, A. E. (2021). *IUCrJ*, **8**, 372–386.
- Ramos, J., Laux, V., Haertlein, M., Forsyth, V. T., Mossou, E., Larsen, S. & Langkilde, A. E. (2021). *Acta Cryst.* **D77**, 1579–1590.
- Sauter, C., Otálora, F., Gavira, J.-A., Vidal, O., Giegé, R. & García-Ruiz, J. M. (2001). *Acta Cryst.* **D57**, 1119–1126.
- Schoenborn, B. P. & Nunes, A. C. (1972). *Annu. Rev. Biophys. Bioeng.* **1**, 529–552.
- Shpungin, J. & Kossiakoff, A. A. (1986). *Methods Enzymol.* **127**, 329–342.
- Tanaka, I., Chatake, T., Fujiwara, S., Hosoya, T., Kusaka, K., Niimura, N., Yamada, T. & Yano, N. (2020). *Methods Enzymol.* **634**, 101–123.
- Tanaka, I., Kurihara, K., Chatake, T. & Niimura, N. (2002). *J. Appl. Cryst.* **35**, 34–40.
- Tanaka, I., Kusaka, K., Hosoya, T., Niimura, N., Ohhara, T., Kurihara, K., Yamada, T., Ohnishi, Y., Tomoyori, K. & Yokoyama, T. (2010). *Acta Cryst.* **D66**, 1194–1197.
- Vocadlo, D. J., Davies, G. J., Laine, R. & Withers, S. G. (2001). *Nature*, **412**, 835–838.
- Wang, J., Dauter, M., Alkire, R., Joachimiak, A. & Dauter, Z. (2007). *Acta Cryst.* **D63**, 1254–1268.
- Yano, N., Yamada, T., Hosoya, T., Ohhara, T., Tanaka, I., Niimura, N. & Kusaka, K. (2018). *Acta Cryst.* **D74**, 1041–1052.