

A technique for determining the deuterium/hydrogen contrast map in neutron macromolecular crystallography

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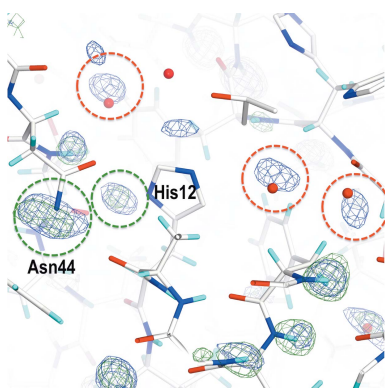
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A difference in the neutron scattering length between hydrogen and deuterium leads to a high density contrast in neutron Fourier maps. In this study, a technique for determining the deuterium/hydrogen (D/H) contrast map in neutron macromolecular crystallography is developed and evaluated using ribonuclease A. The contrast map between the D₂O-solvent and H₂O-solvent crystals is calculated in real space, rather than in reciprocal space as performed in previous neutron D/H contrast crystallography. The present technique can thus utilize all of the amplitudes of the neutron structure factors for both D₂O-solvent and H₂O-solvent crystals. The neutron D/H contrast maps clearly demonstrate the powerful detectability of H/D exchange in proteins. In fact, alternative protonation states and alternative conformations of hydroxyl groups are observed at medium resolution (1.8 Å). Moreover, water molecules can be categorized into three types according to their tendency towards rotational disorder. These results directly indicate improvement in the neutron crystal structure analysis. This technique is suitable for incorporation into the standard structure-determination process used in neutron protein crystallography; consequently, more precise and efficient determination of the D-atom positions is possible using a combination of this D/H contrast technique and standard neutron structure-determination protocols.

1. Introduction

Single-crystal diffraction techniques have been very powerful and popular in structural biology. In particular, X-ray crystallography has shown tremendous power for determining the three-dimensional structures of biological macromolecules owing to the high flux of X-ray beams such as synchrotron radiation. On the other hand, despite the considerably lower flux of neutron sources compared with synchrotron-radiation facilities, neutron crystallography has provided a unique contribution to structural biology: the detection and location of hydrogen (H) or deuterium (D) atoms in biological macromolecules. This is possible because the scattering length of hydrogen to neutrons is comparable to those of other atoms; it is difficult with X-ray crystallography, in which the scattering power is proportional to the electron number of the atom (Schoenborn & Langan, 2004; Tsyba & Bau, 2002; Niimura, 1999; Helliwell, 1997). Combination of the X-ray and neutron diffraction techniques with the advanced crystallographic methods available today has thus been applied to the determination of the complete chemical structures of biological macromolecules (Blum *et al.*, 2009). In fact, some refinement programs for biomacromolecular crystallography have been modified with neutron–X-ray joint refinement



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algorithms (*nCNS*; Adams *et al.*, 2009) or have integrated a module for this type of algorithm (*PHENIX*; Afonine *et al.*, 2010; Adams *et al.*, 2009).

Neutron diffraction experiments are usually carried out using crystals containing D_2O solvent to reduce the background noise owing to the high incoherent neutron scattering of H atoms [$\sigma_{inc(H)} = 80.26$ barn and $\sigma_{inc(D)} = 2.05$ barn; Rauch & Waschkowski, 2003], thereby making efficient data collection possible. More importantly, this large difference in the scattering length between H and D ($b_D = 6.67$ fm and $b_H = -3.74$ fm; Rauch & Waschkowski, 2003), enables the use of crystals containing D_2O solvent to determine H/D exchange as well as the protonation states of macromolecules. Furthermore, the large difference in scattering lengths between D_2O and H_2O ($2b_D - 2b_H = 20.82$ fm) makes it easier to locate D_2O molecules in the solvent region. Neutron crystallography thus provides information important for elucidating various structural aspects of macromolecules, including the stabilities of

their three-dimensional structures, the molecular mechanisms of their functions and their solvent–macromolecule interactions.

The detectability of H atoms can be significantly enhanced by employing the difference Fourier map between a crystal containing D_2O solvent (d-crystal) and one containing H_2O solvent (h-crystal). Assuming that the d-crystal and h-crystal are isomorphous, the difference map between the d-crystal and the h-crystal shows the locations of H atoms replaced by D atoms and water molecules with accentuated intensity while other atoms in the crystals are cancelled out.

The first application of this D/H contrast technique was carried out by Kossiakoff and coworkers (Kossiakoff *et al.*, 1990, 1992; Shpungin & Kossiakoff, 1986; Kossiakoff, 1985). In their pioneering study on trypsin, the D/H contrast map was calculated in ‘reciprocal space’, where the structure factors of the h-crystal were subtracted from those of the d-crystal while taking account of the structure factors arising from the solvent

region (Shpungin & Kossiakoff, 1986). The amplitudes of neutron structure factors ($|F_{D_2O}|$ and $|F_{H_2O}|$ for the d-crystal and h-crystal, respectively) were independently collected at a resolution of 1.8 Å. Because of the large differences in phase between F_{D_2O} and F_{H_2O} , the usual technique of difference map calculation using the difference amplitudes and the phases of F_{H_2O} cannot be employed. They thus introduced components representing the structure factor of H/D-exchangeable atoms (D/H atoms) in the protein (F_{EX}) and the current solvent model (F_{SOL}), and based on the phases of F_{D_2O} determined by an independent refinement of the h-crystal structure, the phases of F_{D_2O} were determined by minimizing the closure error $\varepsilon [= F_{D_2O} - (F_{H_2O} + F_{EX} + F_{SOL})]$ through the refinement of the phases of F_{D_2O} and the solvent model. This sophisticated method provides maps that show densities only in the regions of the replaced D atoms. As a consequence, a good representation of the solvent densities was achieved and water molecules with occupancies of as low as 10% were detected (Kossiakoff *et al.*, 1992). This procedure was, however, designed as an additional refinement after neutron structure determination and was

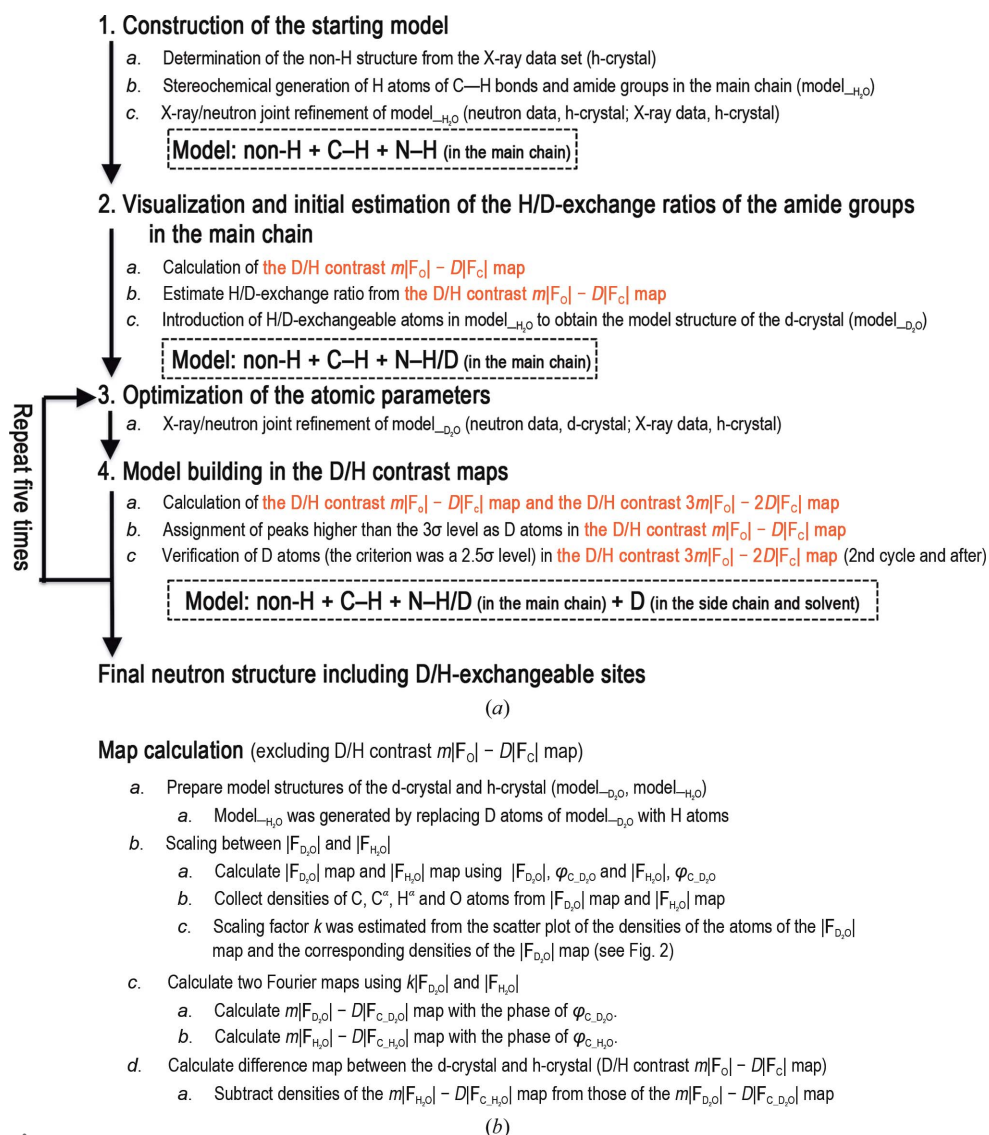


Figure 1

Flowchart of (a) neutron structure determination by the D/H contrast method and (b) map calculation. The definitions of specific terms are summarized in Table 1.

thought to be unsuitable for standard structure determination, which involves iteration of model building and atomic parameter refinement.

Here, as an alternative to the method developed by Kossiakoff and coworkers, we have developed a technique to calculate the D/H contrast map in which the contrast between the D₂O and H₂O crystals is calculated by subtraction not in ‘reciprocal space’ but in ‘real space’. In this method, the amplitudes of the two neutron data sets of the d-crystal and h-crystal ($|\mathbf{F}_{\text{D}_2\text{O}}|$ and $|\mathbf{F}_{\text{H}_2\text{O}}|$) are scaled, the same type of Fourier transformation (*e.g.* $m|\mathbf{F}_\text{O}| - D|\mathbf{F}_\text{C}|$ and $2m|\mathbf{F}_\text{O}| - D|\mathbf{F}_\text{C}|$ *etc.*) is then carried out independently using the two data sets, and finally the difference map is calculated by subtracting the $|\mathbf{F}_{\text{H}_2\text{O}}|$ map from the $|\mathbf{F}_{\text{D}_2\text{O}}|$ map in real space. This method allows the D/H atoms to be visualized as with Kossiakoff’s method but with a simpler protocol, which does not require any specialized programs except the existing refinement programs (*nCNS*, *PHENIX* and *CCP4*; Winn, 2011) and additional Perl scripts to support the refinement programs. With our method all $|\mathbf{F}_{\text{D}_2\text{O}}|$ and $|\mathbf{F}_{\text{H}_2\text{O}}|$ can be used in the calculation, while with Kossiakoff’s method subtraction was possible only for $|\mathbf{F}_{\text{D}_2\text{O}}|$ and $|\mathbf{F}_{\text{H}_2\text{O}}|$ with the same indices.

This technique has been incorporated into the standard protocol of analysis, and the usefulness of this technique was evaluated by carrying out neutron crystallographic analysis of ribonuclease A (RNase A).

2. Procedure

As shown in Fig. 1, the procedure developed here consists of the following four steps: (1) construction of the starting model, (2) visualization and initial estimation of the H/D-exchange ratios of the amide groups in the main chain using the D/H contrast map, (3) optimization of the atomic parameters and (4) model building in the D/H contrast map. It is similar to the standard procedure for neutron macromolecular crystallography, except that the difference map is used in steps (2) and (4). The detailed protocol for each step is given below. Note that the notation used is summarized in Table 1.

2.1. Step 1: construction of the starting model

Three diffraction experiments are carried out to collect the amplitudes of the neutron structure factors of the d-crystal and h-crystal ($|\mathbf{F}_{\text{D}_2\text{O}}|$ and $|\mathbf{F}_{\text{H}_2\text{O}}|$) and the amplitudes of the X-ray structure factors of the h-crystal ($|\mathbf{F}_{\text{XRAY}}|$). The atomic

Table 1
Specified terms in the manuscript.

Term	Explanation
h-crystal	An H ₂ O-solvent protein crystal
d-crystal	A D ₂ O-solvent protein crystal
$ \mathbf{F}_{\text{H}_2\text{O}} $	The amplitude of the structure factor of the h-crystal
$ \mathbf{F}_{\text{D}_2\text{O}} $	The amplitude of the structure factor of the d-crystal
$k \mathbf{F}_{\text{D}_2\text{O}} $	The scaled amplitude of the structure factor of the d-crystal
model _{-H₂O}	The model structure of the h-crystal
model _{-D₂O}	The model structure of the d-crystal
$ \mathbf{F}_{\text{C-H}_2\text{O}} $	The amplitude of the structure factor calculated from model _{-H₂O}
$ \mathbf{F}_{\text{C-D}_2\text{O}} $	The amplitude of the structure factor calculated from model _{-D₂O}
$\varphi_{\text{C-H}_2\text{O}}$	The phase of the structure factor calculated from model _{-H₂O}
$\varphi_{\text{C-D}_2\text{O}}$	The phase of the structure factor calculated from model _{-D₂O}
$ \mathbf{F}_{\text{H}_2\text{O}} $ map	Fourier map calculated using $ \mathbf{F}_{\text{H}_2\text{O}} $ and $\varphi_{\text{C-H}_2\text{O}}$
$ \mathbf{F}_{\text{D}_2\text{O}} $ map	Fourier map calculated using $ \mathbf{F}_{\text{D}_2\text{O}} $ and $\varphi_{\text{C-D}_2\text{O}}$
$m \mathbf{F}_{\text{H}_2\text{O}} - D \mathbf{F}_{\text{C-H}_2\text{O}} $ map	Fourier map calculated using $(m \mathbf{F}_{\text{H}_2\text{O}} - D \mathbf{F}_{\text{C-H}_2\text{O}})$ and $\varphi_{\text{C-H}_2\text{O}}$
$m \mathbf{F}_{\text{D}_2\text{O}} - D \mathbf{F}_{\text{C-D}_2\text{O}} $ map	Fourier map calculated using $(m \mathbf{F}_{\text{D}_2\text{O}} - D \mathbf{F}_{\text{C-D}_2\text{O}})$ and $\varphi_{\text{C-D}_2\text{O}}$
$2m \mathbf{F}_{\text{H}_2\text{O}} - D \mathbf{F}_{\text{C-H}_2\text{O}} $ map	Fourier map calculated using $(2m \mathbf{F}_{\text{H}_2\text{O}} - D \mathbf{F}_{\text{C-H}_2\text{O}})$ and $\varphi_{\text{C-H}_2\text{O}}$
$2m \mathbf{F}_{\text{D}_2\text{O}} - D \mathbf{F}_{\text{C-D}_2\text{O}} $ map	Fourier map calculated using $(2m \mathbf{F}_{\text{D}_2\text{O}} - D \mathbf{F}_{\text{C-D}_2\text{O}})$ and $\varphi_{\text{C-D}_2\text{O}}$
$3m \mathbf{F}_{\text{H}_2\text{O}} - 2D \mathbf{F}_{\text{C-H}_2\text{O}} $ map	Fourier map calculated using $(3m \mathbf{F}_{\text{H}_2\text{O}} - 2D \mathbf{F}_{\text{C-H}_2\text{O}})$ and $\varphi_{\text{C-H}_2\text{O}}$
$3m \mathbf{F}_{\text{D}_2\text{O}} - 2D \mathbf{F}_{\text{C-D}_2\text{O}} $ map	Fourier map calculated using $(3m \mathbf{F}_{\text{D}_2\text{O}} - 2D \mathbf{F}_{\text{C-D}_2\text{O}})$ and $\varphi_{\text{C-D}_2\text{O}}$
D/H contrast $m \mathbf{F}_\text{O} - D \mathbf{F}_\text{C} $ map	Difference map between $m \mathbf{F}_{\text{D}_2\text{O}} - D \mathbf{F}_{\text{C-D}_2\text{O}} $ map and $m \mathbf{F}_{\text{H}_2\text{O}} - D \mathbf{F}_{\text{C-H}_2\text{O}} $ map
D/H contrast $2m \mathbf{F}_\text{O} - D \mathbf{F}_\text{C} $ map	Difference map between $2m \mathbf{F}_{\text{D}_2\text{O}} - D \mathbf{F}_{\text{C-D}_2\text{O}} $ map and $2m \mathbf{F}_{\text{H}_2\text{O}} - D \mathbf{F}_{\text{C-H}_2\text{O}} $ map
D/H contrast $3m \mathbf{F}_\text{O} - 3D \mathbf{F}_\text{C} $ map	Difference map between $3m \mathbf{F}_{\text{D}_2\text{O}} - 2D \mathbf{F}_{\text{C-D}_2\text{O}} $ map and $3m \mathbf{F}_{\text{H}_2\text{O}} - 2D \mathbf{F}_{\text{C-H}_2\text{O}} $ map
D/H contrast map	General term for the above three difference maps

coordinates and the *B* factors of the non-H atoms of the protein are determined using the X-ray diffraction data set of the h-crystal. To this non-H structure, the unexchangeable H atoms of the C—H bonds and the N—H bonds in the main chain are added stereochemically (model_{-H₂O}). The atomic parameters of this structure are then optimized by X-ray–neutron joint refinement with *PHENIX* (Afonine *et al.*, 2010; Adams *et al.*, 2009), using the amplitudes of the X-ray and neutron data sets of the h-crystal ($|\mathbf{F}_{\text{H}_2\text{O}}|$ and $|\mathbf{F}_{\text{XRAY}}|$).

2.2. Step 2: visualization and initial estimation of the H/D-exchange ratios of the N—H bonds in the main chain using the D/H contrast map

The initial estimation of the H/D-exchange ratios of the N—H bonds in the main chain are carried out and incorporated into the model structure before atomic parameter refinement, as was performed previously (Chatake *et al.*, 2004; Ishikawa *et al.*, 2008). This D/H refined model facilitates convergence of the model and is thus suitable as a starting model for the next step (optimization of the atomic parameters). The H/D-exchange ratios of the N—H bonds in the main chain are estimated from the difference map between the $m|\mathbf{F}_\text{O}| - D|\mathbf{F}_\text{C}|$ -type maps of the d-crystal and h-crystal (the D/H contrast $m|\mathbf{F}_\text{O}| - D|\mathbf{F}_\text{C}|$ map), which is obtained by subtracting the $m|\mathbf{F}_{\text{H}_2\text{O}}| - D|\mathbf{F}_{\text{C-H}_2\text{O}}|$ map from the

$m|\mathbf{F}_{\text{D}_2\text{O}}| - D|\mathbf{F}_{\text{C-D}_2\text{O}}|$ map. This type of map contains the densities of atoms which are not included in the model structure. As shown in Figs. 2(a) and 2(b), positive and negative densities are observed at the positions of the exchangeable H atoms in the standard $m|\mathbf{F}_{\text{O}}| - D|\mathbf{F}_{\text{C}}|$ maps of the d-crystal and h-crystal, respectively. Thus, in the D/H contrast $m|\mathbf{F}_{\text{O}}| - D|\mathbf{F}_{\text{C}}|$ map, the densities at the sites of the H/D exchanges are observed with an enhanced height ($b_{\text{D}} - b_{\text{H}} = 10.41$ fm; Fig. 2c). Note that this map is used only for the purpose of the search for H/D-exchangeable atoms because the densities in this map disappear after the corresponding H/D-exchangeable atoms are included in the model structure (Fig. 2d). Calculation of the D/H contrast maps is the key technique in this protocol, and the details are described below in §2.5.

In both model- H_2O and model- D_2O , H atoms are assigned at all N–H atoms in the main chain. In the D/H contrast $m|\mathbf{F}_{\text{O}}| - D|\mathbf{F}_{\text{C}}|$ map, positive densities should appear at the positions where H/D exchanges occur, corresponding to the difference between the scattering-length densities of D and H atoms. The ratio of H/D exchange at each H/D-exchangeable

site in the main chain is estimated from the density at the site in the D/H contrast $m|\mathbf{F}_{\text{O}}| - D|\mathbf{F}_{\text{C}}|$ map, using the density of the N atom in the $k|\mathbf{F}_{\text{D}_2\text{O}}|$ map as a standard (the averaged density of the N atom corresponds to the neutron scattering factor of N; 9.36 fm).

2.3. Step 3: optimization of the atomic parameters

In this step, X-ray–neutron joint refinement is carried out using the neutron and the X-ray data sets ($|\mathbf{F}_{\text{D}_2\text{O}}|$ and $|\mathbf{F}_{\text{XRAY}}|$) to optimize the atomic parameters of model- D_2O . *PHENIX* is used for this refinement with default settings. The atomic coordinates, the individual *B* factors and the main-chain H/D-exchange ratios are sequentially optimized. In the final cycle, atomic positional optimization is excluded from the refinement. The atomic parameters of model- H_2O are obtained by converting the D atoms of model- D_2O to H atoms.

2.4. Step 4: model building on the D/H contrast maps

The D atoms in the side chain and the water molecules near the protein surface are added to the optimized model, by

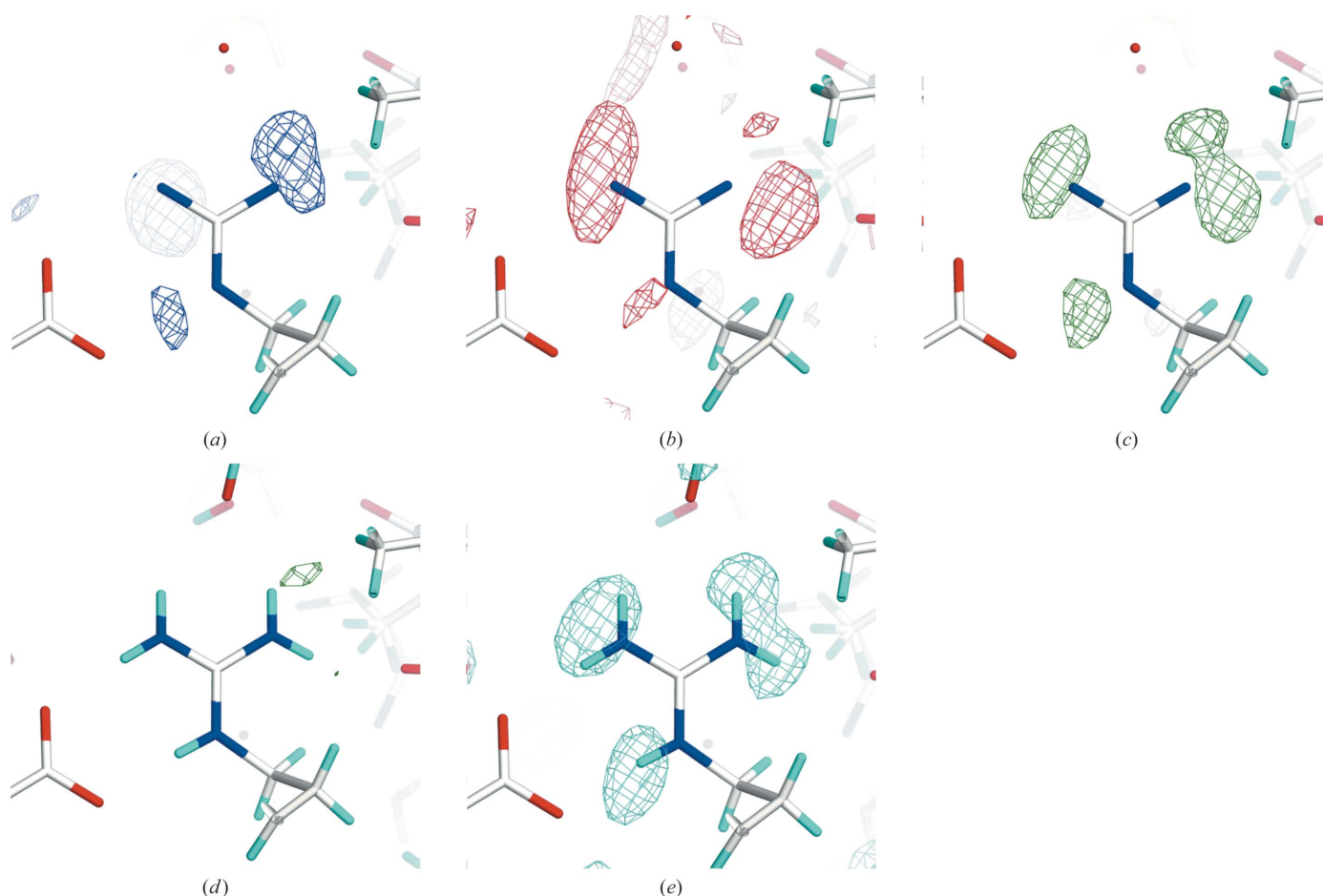


Figure 2

Neutron Fourier maps near Arg10 before and after the introduction of D/H atoms to the guanidinium group of Arg10 in the model structure. (a) $+2.5\sigma$ $m|\mathbf{F}_{\text{D}_2\text{O}}| - D|\mathbf{F}_{\text{C-D}_2\text{O}}|$ map of the d-crystal before introduction, (b) -2.5σ $m|\mathbf{F}_{\text{H}_2\text{O}}| - D|\mathbf{F}_{\text{C-H}_2\text{O}}|$ map of the h-crystal before introduction, (c) $+3.0\sigma$ D/H contrast $m|\mathbf{F}_{\text{O}}| - D|\mathbf{F}_{\text{C}}|$ map before introduction, (d) $+3.0\sigma$ D/H contrast $m|\mathbf{F}_{\text{O}}| - D|\mathbf{F}_{\text{C}}|$ map after introduction and (e) $+3.0\sigma$ D/H contrast $2m|\mathbf{F}_{\text{O}}| - D|\mathbf{F}_{\text{C}}|$ map after introduction. The model structures before and after introduction were from the first and the last cycle of steps 3 and 4 in Fig. 1(a), respectively. Fourier maps and model structures were drawn using *Cuemol2* (<http://www.cuemol.org/>). The definitions of specific terms are summarized in Table 1.

reference to the D/H contrast $m|\mathbf{F}_O| - D|\mathbf{F}_C|$ maps, in which the positive peaks correspond to the H/D-exchange sites. Peaks higher than the 3σ level are assigned as D atoms in the D/H contrast $m|\mathbf{F}_O| - D|\mathbf{F}_C|$ map. In order to verify the D/H atoms included in the model structure, the difference map between the $3m|\mathbf{F}_O| - 2D|\mathbf{F}_C|$ -type maps of the d-crystal and the h-crystal is calculated by subtracting the $3m|\mathbf{F}_{\text{H}_2\text{O}}| - 2D|\mathbf{F}_{\text{C-H}_2\text{O}}|$ map from the $3m|\mathbf{F}_{\text{D}_2\text{O}}| - 2D|\mathbf{F}_{\text{C-D}_2\text{O}}|$ map. This kind of map is more sensitive to misassignment during modelling than the $2m|\mathbf{F}_O| - D|\mathbf{F}_C|$ map usually employed in structure analysis (Lamzin & Wilson, 1997). The D/H atoms, which are included in the model structure, are visualized in the D/H contrast $3m|\mathbf{F}_O| - 2D|\mathbf{F}_C|$ map. Atoms with peaks lower than the 2.5σ level in the D/H contrast $3m|\mathbf{F}_O| - 2D|\mathbf{F}_C|$ map are excluded from the model structure. In the solvent region, peaks that are visually confirmed in the D/H contrast $m|\mathbf{F}_O| - D|\mathbf{F}_C|$ map are assigned as D atoms in solvent water molecules.

Steps 3 and 4 are repeated until the assignment of H/D atoms for the protein and water molecules converges. This D/H contrast method makes model building of the D/H atoms easier and makes convergence of the refinement faster than the standard method. It reduces the time required for building D/H atoms in the model structure in each cycle and the total number of cycles (five cycles, as shown in §3). Consequently, faster and more precise structure determination of D/H atoms can be accomplished. The D/H contrast $2m|\mathbf{F}_O| - D|\mathbf{F}_C|$ map is calculated using the final model structure for the visualization of the D/H atoms in the model structure (Fig. 2e) and the

residual components of D/H atoms because this map shows the sum of the densities for the model and the actual structures at normal height (Drenth, 1999).

2.5. Calculation of the D/H contrast maps

In this procedure, various kinds of D/H contrast maps are incorporated into the standard protocol of neutron crystallographic refinement. For calculation of these maps, the $|\mathbf{F}_{\text{D}_2\text{O}}|$ and $|\mathbf{F}_{\text{H}_2\text{O}}|$ data sets need to be scaled. However, because $|\mathbf{F}_{\text{D}_2\text{O}}(0, 0, 0)| > |\mathbf{F}_{\text{H}_2\text{O}}(0, 0, 0)|$, the general scaling algorithm, which uses the least-squares method, between $|\mathbf{F}_{\text{D}_2\text{O}}|$ and $|\mathbf{F}_{\text{H}_2\text{O}}|$ leads to an incorrect scale. We have thus developed a scaling protocol in real space. Assuming that the d-crystal and h-crystal are isomorphous, the Fourier maps calculated using $|\mathbf{F}_{\text{D}_2\text{O}}|$ and $\varphi_{\text{C-D}_2\text{O}}$ have the same density as those calculated using $|\mathbf{F}_{\text{H}_2\text{O}}|$ and $\varphi_{\text{C-H}_2\text{O}}$, except in the H/D-exchangeable regions. The densities of C, C $^\alpha$, O, N and H $^\alpha$ atoms are thus extracted from the $|\mathbf{F}_{\text{D}_2\text{O}}|$ and $|\mathbf{F}_{\text{H}_2\text{O}}|$ maps, respectively, and used for calculation of the scaling factor k . The $|\mathbf{F}_{\text{D}_2\text{O}}|$ and $|\mathbf{F}_{\text{H}_2\text{O}}|$ maps are calculated using the amplitudes $|\mathbf{F}_{\text{D}_2\text{O}}|$ and the phases $\varphi_{\text{C-D}_2\text{O}}$ obtained from the model structure of the d-crystal (model $_{\text{-D}_2\text{O}}$) and using $|\mathbf{F}_{\text{H}_2\text{O}}|$ and $\varphi_{\text{C-H}_2\text{O}}$ obtained from the h-crystal (model $_{\text{-H}_2\text{O}}$), respectively, using *nCNS* with a grid spacing of 0.36 Å. Densities at the position of the C, C $^\alpha$, O, N and H $^\alpha$ atoms were collected individually from the $|\mathbf{F}_{\text{D}_2\text{O}}|$ and $|\mathbf{F}_{\text{H}_2\text{O}}|$ density maps, in which the density for each atom is estimated by the summation of the densities within a radius of

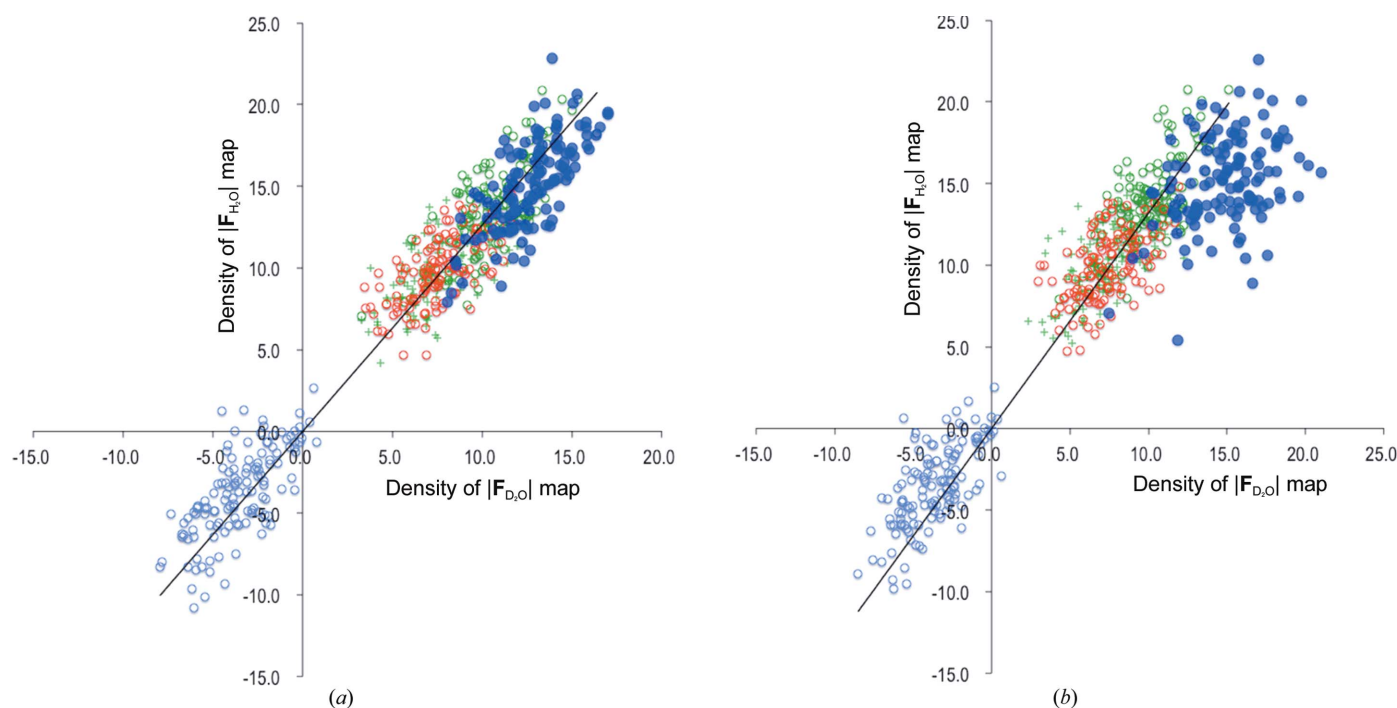


Figure 3

Scatter plots showing the relationship between the scattering-length densities of the atoms in the d-crystal and those in the h-crystal. The densities of the d-crystal and h-crystal were obtained from the $|\mathbf{F}_{\text{D}_2\text{O}}|$ and $|\mathbf{F}_{\text{H}_2\text{O}}|$ maps, respectively. The densities of the atoms in the d-crystal are shown on the horizontal axis and the densities of the corresponding atoms in the h-crystal are shown on the vertical axis. The densities of C (green open circles), C $^\alpha$ (green crosses), H $^\alpha$ (blue open circles), O (red open circles) and N (blue closed circles) atoms in the main chain were plotted. A black line is the linear approximation with an intercept of 0 on this plot. (a) and (b) are the scatter plots in the first and the final scaling processes, respectively. The definitions of specific terms are summarized in Table 1.

Table 2

Parameters for the diffraction experiments and structure determination.

Values in parentheses are for the outer shell.

	d-crystal (neutron)	h-crystal (neutron)	h-crystal (X-ray)
Crystal data			
Space group	$P2_1$		
Unit-cell parameters ($\text{\AA}$, $^\circ$)	$a = 30.34$, $b = 38.37$, $c = 53.12$, $\beta = 105.75$		
Data collection			
$d_{\min}$ ($\text{\AA}$)	1.80	1.80	1.52
$R_{\text{merge}}^\dagger$ (%)	11.4 (34.4)	15.4 (28.1)	5.8 (13.6)
Completeness (%)	72.1 (51.3)	72.5 (48.4)	91.7 (95.2)
$\langle I/\sigma(I) \rangle$	5.5 (2.1)	5.6 (2.9)	43.3 (20.8)
Structure determination			
Resolution ($\text{\AA}$)	51.2–1.8	30.7–1.8	16.8–1.52
R factor (%)	19.7	22.0	13.1
R_{free} (%)	22.1	22.2	15.6
R.m.s.d., bond lengths ($\text{\AA}$)	0.010	0.010	0.006
R.m.s.d., angles ($^\circ$)	0.980	0.980	1.008
PDB code	4zz4	5d97	5d6u

† The atomic coordinates of the h-crystal (model_{H₂O}) were the same as those of the d-crystal (model_{D₂O}) except that the D atoms in the d-crystal were replaced with H atoms.

0.5 $\text{\AA}$ from their positions in these maps. A set of densities estimated from the corresponding atoms in the $|\mathbf{F}_{\text{D}_2\text{O}}|$ and $|\mathbf{F}_{\text{H}_2\text{O}}|$ maps are obtained for each atom. These sets of densities are used to make a scatter plot as shown in Fig. 3(a). The scale factor (k) is determined as the slope of the linear fit through the origin to these data points. Note that the N atoms in the main chain are excluded from this measurement because their densities are strongly affected by the variable densities of the neighbouring H/D-exchangeable atoms after assignment of their H/D-exchange ratios (see Fig. 3b), while this effect is small in the first map calculation (see Fig. 3a). Note also that although in general two fitting parameters, the scale factor and the temperature factor, are applied to scale two data sets, only the scale factor is applied here. This is because the overall B factors of the two crystals are nearly the same (8.41 and 8.40 $\text{\AA}^2$ for the h-crystal and d-crystal, respectively) in our particular case of RNase A.

Various kinds of $|\mathbf{F}_\text{O}| - |\mathbf{F}_\text{C}|$ maps ($m|\mathbf{F}_\text{O}| - D|\mathbf{F}_\text{C}|$ maps, $2m|\mathbf{F}_\text{O}| - D|\mathbf{F}_\text{C}|$ maps and $3m|\mathbf{F}_\text{O}| - 2D|\mathbf{F}_\text{C}|$ maps) are then calculated using the scaled data sets ($k|\mathbf{F}_{\text{D}_2\text{O}}|$, $|\mathbf{F}_{\text{C-D}_2\text{O}}|$ and $\varphi_{\text{C-D}_2\text{O}}$) and ($|\mathbf{F}_{\text{H}_2\text{O}}|$, $|\mathbf{F}_{\text{C-H}_2\text{O}}|$ and $\varphi_{\text{C-H}_2\text{O}}$), depending on the situations in the procedure.

Finally, the difference map is generated by subtracting the densities of the map for the h-crystal from those for the d-crystal. Three types of D/H contrast map, which are listed in Table 1, are calculated in order to visualize the D/H atoms in this study.

2.6. Advantages and disadvantages of the present method

The key technique in the present analysis, the calculation of the D/H contrast maps, is carried out in 'real space', while the previous method developed by Kossiakoff and coworkers performed this in 'reciprocal space'. The D/H contrast maps have the following characteristics.

(i) The density at each H/D-exchangeable site is 1.56 times that at the corresponding site in the standard neutron Fourier map of the d-crystal.

(ii) Only discrete densities corresponding to the D/H atoms can be observed, while continuous densities for all atoms except H atoms (H atoms have negative densities) are observed in the standard neutron Fourier maps.

(iii) All amplitudes of the structure factors for the d-crystal and h-crystal data sets ($|\mathbf{F}_{\text{D}_2\text{O}}|$ and $|\mathbf{F}_{\text{H}_2\text{O}}|$) can be included in the calculation, while in the previous method only the $|\mathbf{F}_{\text{D}_2\text{O}}|$ and $|\mathbf{F}_{\text{H}_2\text{O}}|$ values appearing in both data sets could be used.

(iv) It is easy to calculate the D/H contrast maps compared with the previous method, because the existing programs for neutron crystallography can be used for the present map calculations. On the other hand, in the previous method the specific program(s) developed by the authors were necessary for the map calculations. Moreover, the D/H contrast maps are applicable to model building for the D/H atoms in a similar manner to the $m|\mathbf{F}_{\text{D}_2\text{O}}| - D|\mathbf{F}_{\text{C-D}_2\text{O}}|$, $2m|\mathbf{F}_{\text{D}_2\text{O}}| - D|\mathbf{F}_{\text{C-D}_2\text{O}}|$ and $3m|\mathbf{F}_{\text{D}_2\text{O}}| - 2D|\mathbf{F}_{\text{C-D}_2\text{O}}|$ maps in the standard neutron structure determination.

Regarding point (iii), in neutron crystallography the completeness of the neutron data set can often be below 90% (the completeness of monochromatic neutron macromolecular crystallography is 84% on average, as estimated from data available in August 2012). Moreover, the effective resolution of the h-crystal data set is usually lower than that of the d-crystal data set. The present technique thus provides a significant advantage for the calculation of the neutron D/H contrast maps. Regarding point (iv), incorporation of the D/H contrast maps into the standard protocol for neutron analysis is easy, thereby making accurate and rapid analysis of the D/H atoms possible. We employed the *nCNS*1.0 and *PHENIX* programs for map calculations and X-ray–neutron joint refinement, respectively, supplemented with additional Perl scripts written by ourselves and *COORDCONV* in the *CCP4* suite (Winn *et al.*, 2011).

Note that this method requires two isomorphous crystals containing the same solution components in D_2O and H_2O , respectively, and thus two neutron data sets need to be collected using these two crystals at the same temperature.

3. Materials and methods

3.1. Sample preparation

We prepared crystals of RNase A in D_2O solvent and H_2O solvent for analysis. Crystallization of RNase A from bovine pancreas was carried out using the interfacial liquid diffusion technique reported by Berisio *et al.* (1999). A protein solution (400 μl) consisting of 40 mg ml^{-1} RNase A with 0.1 *M* sodium acetate buffer pH 5.8 in H_2O was placed in an NMR-sample capillary (diameter 6 mm) and cooled for 30 min in a freezer at 233 K. 2-Propanol (400 μl) cooled to 277 K was then gently added onto the top of the cooled protein solution, and this crystallization solution was incubated at 293 K. Crystals appeared at the bottom of the capillary after several months of

incubation, and the growth of the crystals was terminated after approximately one year. The maximum volume of the h-crystals thus obtained was approximately 24 mm³. This h-crystal was used for collection of the neutron and X-ray data sets.

The d-crystal was prepared by replacing the H₂O in the h-crystal with D₂O using the vapour-diffusion technique. A capillary of 40 mm in length containing an h-crystal at the bottom was turned upside down, and the end of the capillary was soaked in D₂O solution containing 20 mg ml⁻¹ RNase A, 0.05 M sodium acetate buffer pD 5.8 and 50% 2-d₈-propanol. This crystal was used for the neutron experiment after five months of incubation at 293 K.

3.2. Diffraction experiments

The neutron diffraction experiments were performed using the BIX-3 diffractometer (Tanaka *et al.*, 2002) at the JRR-3 reactor of the Japan Atomic Energy Agency, Tokai, Ibaraki, Japan. The incident neutrons were monochromated to 2.9 Å. Data collection for the h-crystal and d-crystal was carried out at room temperature using the oscillation method with an oscillation angle of 0.5° and no overlap angle between the frames. 375 and 360 diffraction images were collected from the h-crystal and the d-crystal, respectively. It took 9 d to collect each of the data sets. Note that the effective resolution for the h-crystal neutron data set was comparable to that of the d-crystal (1.8 Å for both) and that the overall *B* factors for both the h-crystal and the d-crystal were nearly the same despite the fact that the h-crystal was unfavourable for neutron analysis because of the high background noise owing to incoherent scattering from the H atoms.

The diffraction images were processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997), which were modified for the neutron diffractometer employed. The h-crystal used for neutron data collection was also used for X-ray data collection because of the negligible radiation damage by neutrons. The X-ray diffraction images were collected at room temperature at the BL38B1 station of SPring-8, integrated and merged using *HKL-2000* (Otwinowski & Minor, 1997). The parameters for the neutron and X-ray analyses are summarized in Table 2.

3.3. X-ray structure determination

The atomic coordinates and the *B* factors of the non-H atoms were determined using the X-ray data set for the h-crystal. The initial phases were determined by the molecular-replacement method using the *AMoRe* program (Navaza, 1994), employing the coordinates of the RNase A structure at pH 7.1 (PDB entry 1kf5; Berisio *et al.*, 2002). *PHENIX* (Adams *et al.*, 2010) and *Coot* (Emsley *et al.*, 2010) were used for optimization of the atomic parameters and model building, respectively. After iterations of atomic parameter refinement and model building, the non-H structure was determined at a resolution of 1.52 Å. This structure was employed for the analysis of the neutron data sets as described above.

4. Results and discussion

4.1. D/H contrast map

Fig. 4 shows the D/H contrast $m|\mathbf{F}_O| - D|\mathbf{F}_C|$ map (coloured blue) of RNase A just after introducing the H atoms of the C—H bonds and the main-chain amide groups. The standard $m|\mathbf{F}_{D_2O}| - D|\mathbf{F}_{C-D_2O}|$ map of the d-crystal (coloured green) is superimposed on this map for comparison. Even in this initial map, significant improvement from the standard $m|\mathbf{F}_{D_2O}| - D|\mathbf{F}_{C-D_2O}|$ map is observed: the density at each H/D-exchangeable site of the main-chain amide groups is clearly observed, densities are also found near the positions of a considerable number of D₂O molecules (shown in red broken circles in Fig. 4) and the D atoms of the functional groups in the protein are also observed with higher densities than those seen in the standard map (shown in green broken circles in Fig. 4). It should be noted that the densities of three D₂O molecules are clearly observed in the first D/H contrast map, while the D₂O molecules have ambiguous and/or low densities in the first step of structure determination in standard neutron crystallography. Moreover, these densities in the D/H contrast map indicate the positions of the D atoms in the D₂O molecules, while the positions of the D₂O molecules determined by X-ray crystallography correspond to the positions of their O atoms. This result suggests that the O atoms and the D/H atoms of the water molecules can be determined individually in the X-ray map and the D/H contrast map,

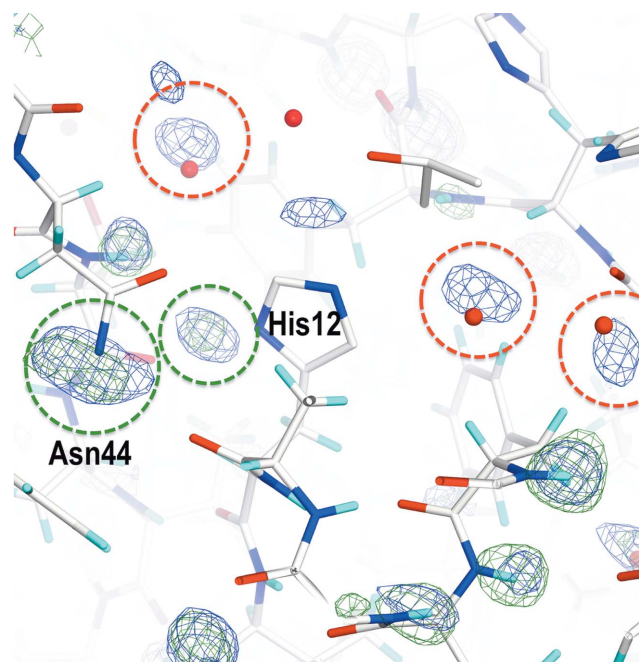


Figure 4
The D/H contrast $m|\mathbf{F}_O| - D|\mathbf{F}_C|$ map and the $m|\mathbf{F}_{D_2O}| - D|\mathbf{F}_{C-D_2O}|$ map superimposed on stick models of RNase A. These maps were calculated in the first cycle of the present structure-determination process (step 2 in Fig. 1). No structure factors for D atoms were included in the model structure. The blue and green contours indicate the +3 σ level contours on the D/H contrast $m|\mathbf{F}_O| - D|\mathbf{F}_C|$ map and the $m|\mathbf{F}_{D_2O}| - D|\mathbf{F}_{C-D_2O}|$ map, respectively. View of the protein surface near Lys7 and its accessible solvent region. The definitions of specific terms are summarized in Table 1.

respectively. If the d-crystal and h-crystal are not isomorphous, the density owing to the structural differences between the two crystals should be observed in the D/H contrast map. However, such densities were not observed, suggesting that these crystals were sufficiently isomorphous for the present D/H contrast method. Consequently, this high detectability of the water molecules allowed the precise and rapid determination of the hydration structures using the D/H contrast map technique developed here.

Fig. 5 shows three types of Fourier maps calculated using the final model structure including all detectable D and H atoms. The X-ray $2m|F_{\text{XRAY}}| - D|F_{\text{C-H}_2\text{O}}|$ and neutron $2m|F_{\text{D}_2\text{O}}| - D|F_{\text{C-D}_2\text{O}}|$ maps (Figs. 5a and 5b, respectively) represent the continuous densities of all of the atoms except the D atoms and those including the D atoms, respectively. In these maps, the boundaries between the D atoms and neighbouring atoms are ambiguous. On the other hand, the D/H contrast $2m|F_{\text{O}}| - D|F_{\text{C}}|$ map (Fig. 5c) represents the discrete densities corresponding to the sites of the individual H/D-exchangeable atoms. Fig. 5(d) shows a superposition of the X-ray (Fig. 5a) and D/H contrast (Fig. 5c) maps. This combination of the maps provides a good visualization of the atom densities, in which the D/H atoms and other atoms can be clearly distinguished.

4.2. Protonation and orientation of the O—H and N—H bonds in amino-acid residues

Table 3 shows a list of the functional groups containing H/D-exchangeable sites in RNase A, categorized according to their chemical structures. Assuming that all of the carboxyl groups are unprotonated, they are excluded from the list. No tryptophan is contained in this protein. Previous X-ray crystallographic analyses reported that RNase A had alternative conformations at several residues when the crystals were grown in high concentration of 2-propanol (Berisio *et al.*, 1999, 2002), while these conformational variations were not observed in previous neutron crystallographic analyses of RNase A (Yagi *et al.*, 2009; Wlodawer *et al.*, 1983). The present X-ray structure also contains seven alternative conformers of these functional groups, and low electron densities are observed at the ends of all of the Lys side chains. The D/H atoms in such functional groups are disordered, resulting in no significant densities greater than the threshold for the assignment of the D/H atoms during model building. Of the remaining 49

functional groups, 24 have detectable D/H atoms in the D/H contrast map. For the remaining 25 functional groups, the D/H atoms are not located in the model structure because the densities corresponding to these D/H atoms are too low in the D/H contrast maps. More than half of them are on the surface of RNase A, and the structural restraints owing to hydrogen bonding and other interactions appear to be weaker than those for the D/H atoms for which the positions are determined. It is plausible that the D atoms adopt multiple positions owing to the rotational flexibility of these functional groups. Such multiple conformations have been observed in previous D/H contrast neutron crystallography analyses (McDowell & Kossiakoff, 1995; Kossiakoff *et al.*, 1990). The determination of the hydroxyl conformations using the D/H contrast map created by Kossiakoff and coworkers would provide further information about the multiple positions of the H/D-exchangeable atoms in these hydroxyl groups.

Some typical examples demonstrating the improvements achieved using the technique developed here are shown in Fig. 6. As shown in Fig. 6(a), whereas the orientation of the O_H—D_H bond of Tyr115 is ambiguous in the standard

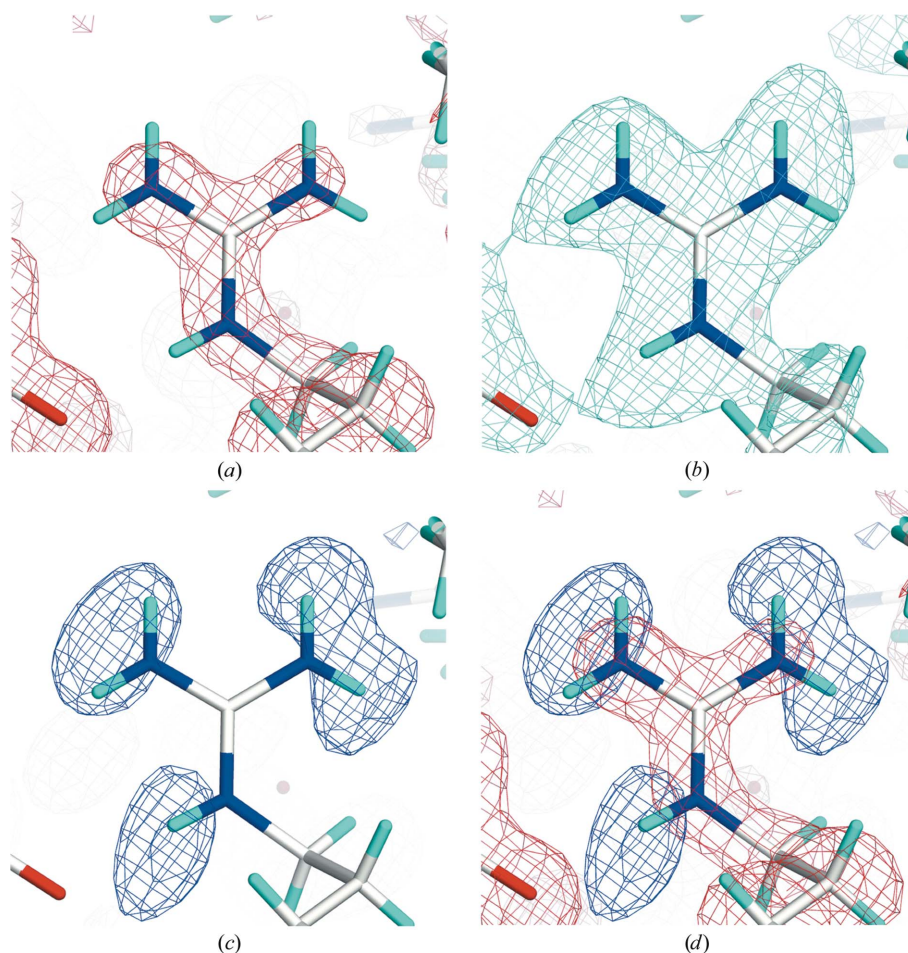


Figure 5 Various Fourier maps near Arg10 in RNase calculated using the final model structure. (a) Red, (b) cyan and (c) blue contours represent the $+1.5\sigma$ X-ray $2m|F_{\text{XRAY}}| - D|F_{\text{C-H}_2\text{O}}|$, $+1.5\sigma$ neutron $2m|F_{\text{D}_2\text{O}}| - D|F_{\text{C-D}_2\text{O}}|$ and $+2.5\sigma$ D/H contrast $2m|F_{\text{O}}| - D|F_{\text{C}}|$ densities, respectively. (d) Superimposed X-ray and neutron D/H contrast $2m|F_{\text{O}}| - D|F_{\text{C}}|$ maps. The definitions of specific terms are summarized in Table 1.

Table 3

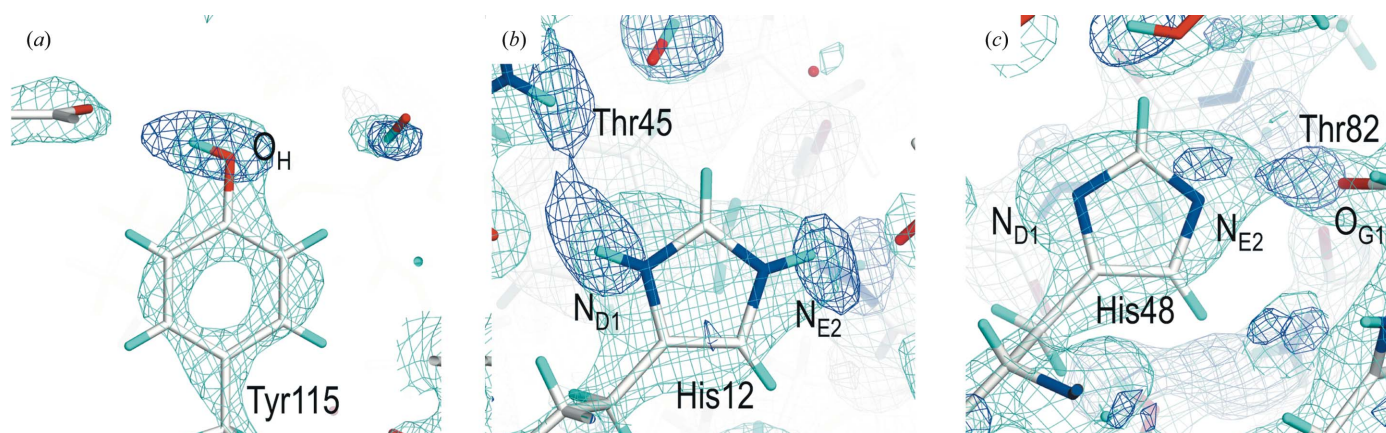
List of functional groups in RNase A having H/D-exchangeable atoms.

The exchangeable atoms which were identified and unidentified in this method are listed in the upper and lower halves of this table, respectively. Underlined functional groups did not have the possibility of making any hydrogen bonds to proton acceptors of the protein. Functional groups marked with an asterisk have alternative conformations.

		Accessible surface area ($\text{\AA}^2$)			
		0–50	50–100	100–150	150+
Assigned atoms					
ND ₃ ⁺ ND ₂			Arg10, Arg33		
			Asn62		
OD	Asn27, Asn44, Asn71		Gln55, Gln69, Gln101		
	Gln60				
	Ser75, Ser80, Ser90				
	Thr36, Thr82		Thr3, Thr87		
Imidazole	Tyr25, Tyr97		Tyr115	Tyr92	Tyr76
	His12, His48				
Unassigned atoms					
ND ₃ ⁺ ND ₂	Lys41		Lys7, Lys104	Lys31, Lys37, Lys61, Lys91, Lys98	Lys1, Lys66
				Arg39, Arg85	
OD			Asn24	Asn34*, Asn67, Asn94, Asn103	Asn113
	Gln11		Gln74	Gln28	
	Ser123		Ser15*, Ser22, Ser23, Ser32*, Ser50, Ser77	Ser16*, Ser18*, Ser21, Ser59*, Ser89	
	Thr17, Thr45, Thr99		Thr78, Thr100	Thr70	
Imidazole	Tyr73				
	His105			His119*	

$2m|F_{D_2O}| - D|F_{C,D_2O}|$ map, the D/H contrast $2m|F_O| - D|F_C|$ map clearly indicates its orientation. In addition, the density for the O_H atom suggests another minor conformation in the opposite direction. Figs. 6(b) and 6(c) show a striking contrast between the two His residues for which the protonation states can be observed. His12 (Fig. 6b) is doubly protonated, as two distinct densities higher than $+2.5\sigma$ corresponding to the D_{N1} and D_{E2} atoms are clearly observed in the D/H contrast $2m|F_O| - D|F_C|$ map. On the other hand, His48 (Fig. 6c) shows only one density higher than $+2.5\sigma$ in the D/H contrast $2m|F_O| - D|F_C|$ map, while it appears to be doubly protonated in the $2m|F_{D_2O}| - D|F_{C,D_2O}|$ map. Moreover, this density is spread out between the N_{E2} atom of His48 and the O_{G1} atom of Thr82 despite the fact that the D_{E2} atom and the D_{G1} atom cannot

show the densities of D atoms. Accordingly, water molecules can be modelled easily as follows. The O atoms of water molecules are determined by the X-ray analysis, the D atoms are then generated stereochemically and finally the orientations of the water molecules are determined by rotating these molecules to fit the densities for their D atoms with the positions of their O atoms fixed. Fig. 7(a) shows a typical example of the water molecules observed in this analysis. The X-ray $2m|F_{XRAY}| - D|F_{C,H_2O}|$ (red) and neutron D/H contrast $2m|F_O| - D|F_C|$ (blue) maps show the densities corresponding to one O atom and that corresponding to two D atoms, respectively (D–O–D type). Fig. 7(b) shows an example of the densities of the partially disordered water molecules that are observed. This map shows the density corresponding to

**Figure 6**

D/H contrast $2m|F_O| - D|F_C|$ maps (blue) and $2m|F_{D_2O}| - D|F_{C,D_2O}|$ maps (cyan) near (a) Tyr115, (b) His12 and (c) His48, for which the contour levels are $+2.5\sigma$ and $+1.5\sigma$, respectively. The definitions of specific terms are summarized in Table 1.

only one D atom, suggesting that the second D atom is rotationally disordered around the observable O—D bond (O—D type) (Chatake *et al.*, 2003; Habash *et al.*, 2000). The remaining water molecules, for which the O atoms were observed in the X-ray map but the D atoms were not observed in the D/H contrast map, are considered to be rotationally disordered around the O atom (O type). In the present analysis, three

types of water molecules (19 D—O—D type, 44 O—D type and 44 O type) are clearly observed. They indicate an obvious improvement from standard neutron crystallography, because, for example, our previous analysis at the same resolution using the same neutron diffractometer (Z-DNA, 1.8 Å) could determine only two types of water molecules (D—O—D type and O type; Chatake *et al.*, 2005). Fig. 7(c) shows a comparison

Table 4
R factor, *R*_{free} and the number of D/H atoms in the model during structure determination.
mc, main chain; sc, side chain.

	Scaling factor†	<i>R</i> factor (<i>R</i> _{free}) (%)		H/D			D
		Neutron (d-crystal)	X-ray (h-crystal)	H	mc	sc	
X-ray structure			13.1 (15.6)	0	0	0	0
Add H atoms of C—H and N—H in mc‡	1.264	21.1 (22.2)	13.9 (16.6)	802	0	0	0
Estimate H/D-exchange ratio of N—H in mc	1.265	20.9 (23.2)	13.8 (16.6)	680	122	0	0
First cycle of model building	1.300	19.8 (22.6)	13.8 (16.8)	680	122	27	60
Second cycle of model building	1.307	19.6 (22.5)	13.8 (16.8)	680	122	33	70
Third cycle of model building	1.316	19.5 (22.5)	13.7 (16.6)	680	122	36	82
Fourth cycle of model building	1.316	19.5 (21.9)	13.7 (16.6)	684	122	40	84
Fifth cycle of model building	1.318	19.7 (22.5)	13.7 (16.6)	684	122	43	81

† Scaling factor. ‡ *R* factor and *R*_{free} were calculated using the h-crystal diffraction data sets only at this step.

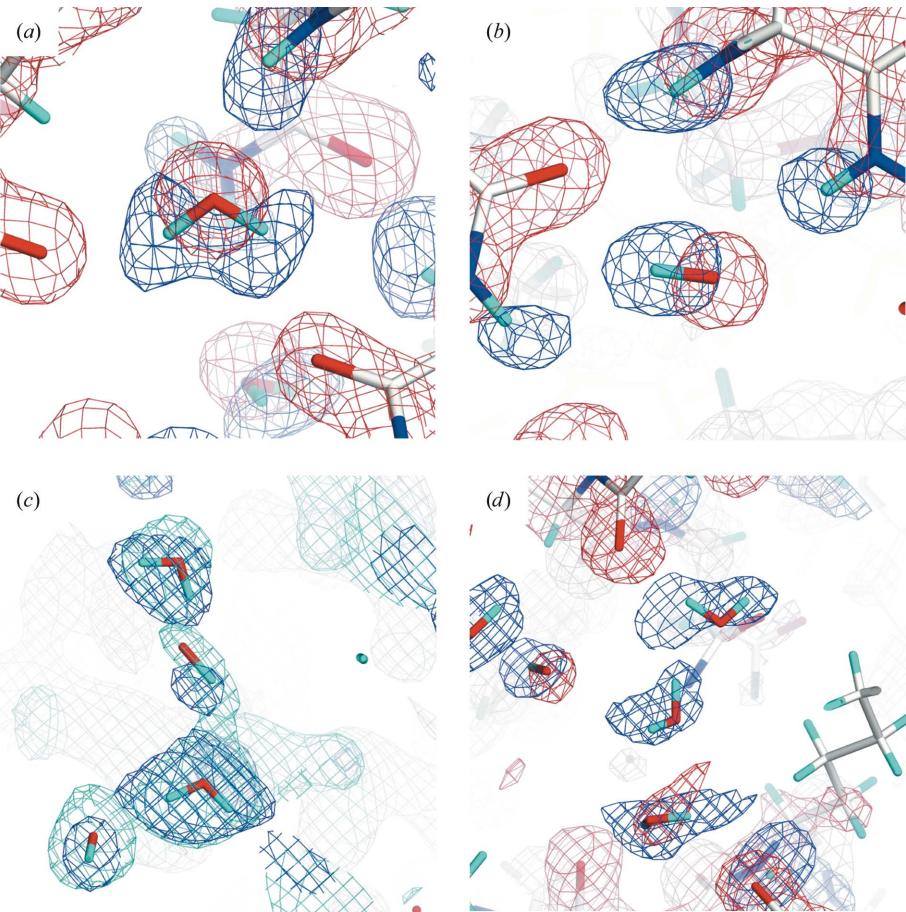


Figure 7
Fourier maps in the solvent region of RNase A. Densities for the D/H contrast $2m|F_O| - D|F_C|$ and X-ray $2m|F_{XRAY}| - D|F_{C-H_2O}|$ maps are contoured in blue and red, respectively. The cyan contours in (c) indicate the density of the neutron $2m|F_{D_2O}| - D|F_{C-D_2O}|$ map. The definitions of specific terms are summarized in Table 1.

between the D/H contrast $2m|F_O| - D|F_C|$ (blue) and $2m|F_{D_2O}| - D|F_{C-D_2O}|$ (cyan) maps of RNase A. In the $2m|F_{D_2O}| - D|F_{C-D_2O}|$ maps only envelopes of densities for the O and D atoms of four water molecules are observed, making the assignment of the D atoms ambiguous. On the other hand, the D/H contrast $2m|F_O| - D|F_C|$ map provides distinct densities corresponding to each of the D atoms, thereby making it possible to assign the D/H atoms unambiguously.

Moreover, 12 additional significant densities are observed in the D/H contrast maps, as shown in Fig. 7(d). These densities correspond to the water molecules observed in the previous X-ray structure analysis (PDB entry 1kf4; Berisio *et al.*, 2002) at a higher resolution (1.1 Å) than that of the present X-ray analysis. As known from the above descriptions, detectability of water molecules by the D/H contrast technique at 1.8 Å resolution is superior to the present X-ray crystallography at 1.5 Å resolution. In addition, a number of distinct densities were found in the solvent region. Although these densities were not assigned to the water molecules because their peak heights were lower than the threshold, they are likely to be low-occupancy water molecules, as described previously (Kossiakoff *et al.*, 1992). It is thus concluded that the D/H contrast technique developed here has a considerable ability to determine both the positions and the orientations of water molecules.

4.4. Validity of the present D/H contrast method for neutron macromolecular crystallography

The D/H contrast technique developed here showed a powerful performance in the detection of the D/H atoms in the interior and on the surface of the protein as well as the water molecules around the protein. It should

be noted that this technique is much faster at determining structure and is more precise than the standard analysis. As shown in Table 4, only five cycles of refinement were required to reach the final structure. In the initial estimation of the H/D-exchange ratios of the amide groups in the main chain (step 2), 122 D/H atoms were assigned. For the side-chain functional groups, 43 D/H atoms could be included in the final structure. In the first cycle, 63% (27 of 43) of the D/H atoms of the final model were assigned, while only 37% (16 of 43) D/H atoms were assigned using the standard $m|\mathbf{F}_O| - D|\mathbf{F}_C|$ map of the d-crystal with the same criteria (greater than 3σ level). Regarding the solvent molecules, 60 H/D atoms of water molecules were assigned in the first cycle using the D/H contrast $m|\mathbf{F}_O| - D|\mathbf{F}_C|$ map, while only 12 D atoms were assigned using the standard $m|\mathbf{F}_{D_2O}| - D|\mathbf{F}_{C-D_2O}|$ map of the d-crystal. Note that in the first cycle the R factor of the neutron data set of the d-crystal had a value comparable to that obtained for the final structure.

The technique developed here thus provides an efficient tool to analyze the H/D-exchange sites and the solvent water

molecules. The effectiveness of this technique may, however, be dependent on the resolution of the measurements. To illustrate the performance of this method as a function of the resolution of the data, we truncated the data sets at 2.27, 2.86 and 3.6 Å resolution (which correspond to omitting 1/8, 1/4 and 1/2 of the data, respectively) and repeated the calculations. Typical differences were observed in the solvent region (as seen in the broken squares in Fig. 8). Notably, the D/H contrast map did not deteriorate significantly even when half of the reflections were omitted. However, the map drawn using a quarter of the reflections was blurry, and when only 1/8 of the reflections were used the degradation of the map was extensive and prevented structure determination. The resolution limit of the D/H contrast technique should thus be around 2.5 Å in the present data sets, in which the completeness of the d-crystal and h-crystal data sets are 72.1 and 72.5%, respectively.

The results obtained here indicate that the D/H contrast technique has wide applications in neutron macromolecular crystallography, including (i) improvement of the accuracy of determination of the protonation states and orientations of the functional groups, (ii) investigation of the hydration structures at atomic resolution, including the dynamic behaviour, and (iii) facilitation of the process of model building to place the D/H atoms at positions with rotational degrees of freedom and solvent. The present D/H contrast technique should be effective in better visualizing the maps of the D/H atoms and the hydration of the macromolecules, and should provide an effective tool for the structure analysis even when the standard neutron experiments fail to provide the desired quality.

5. Conclusion

The D/H contrast technique for neutron macromolecular crystallography is developed here. Although this method required two neutron diffraction data sets from two crystals (the d-crystal and the h-crystal), the D/H contrast maps provided a detailed picture of the H/D-exchangeable atoms in RNase A and the hydration of this protein. The present technique using calculations in real space has the following advantages: (i) all of the observed amplitudes ($|\mathbf{F}_{D_2O}|$

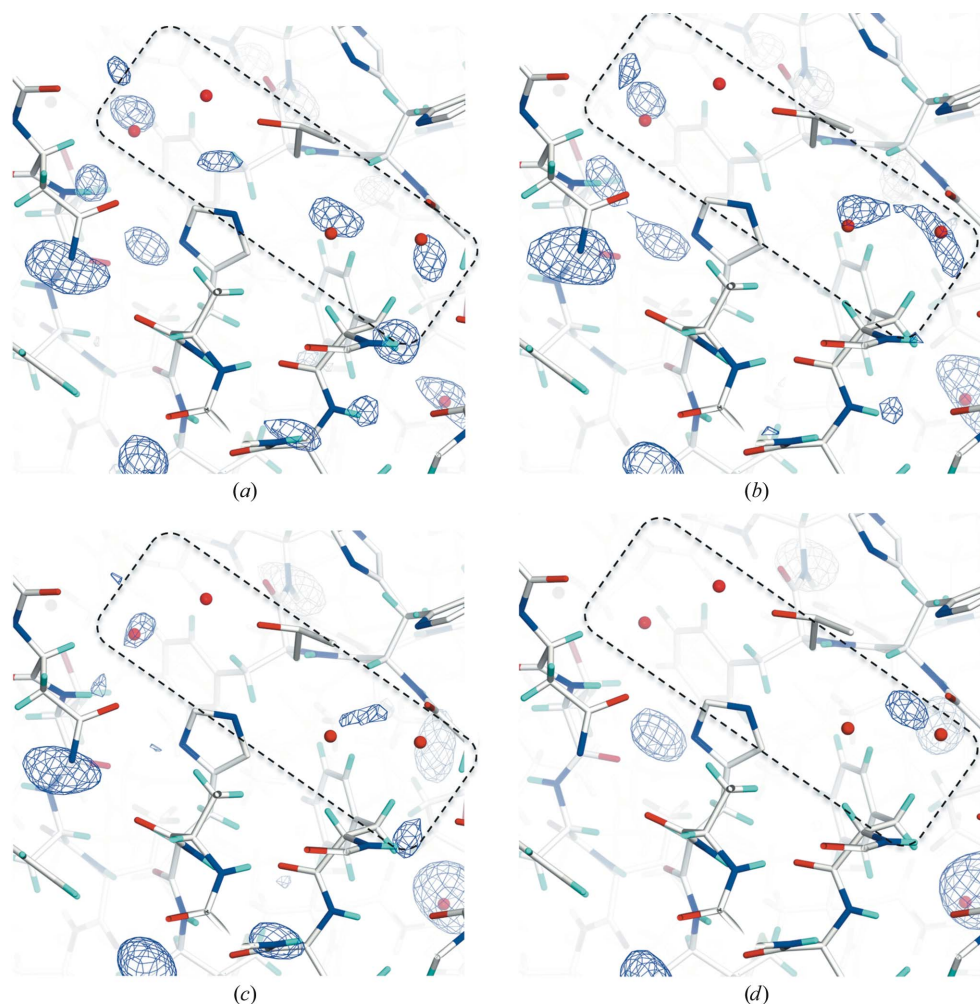


Figure 8
The D/H contrast $m|\mathbf{F}_O| - D|\mathbf{F}_C|$ map at different resolutions (contoured at the $+3\sigma$ level). (a) 1.80 Å, (b) 2.27 Å, (c) 2.86 Å and (d) 3.60 Å resolution maps were calculated using 1, 1/2, 1/4 and 1/8 of all observed reflections, respectively. These maps were calculated in the first cycle of the present structure-determination process (step 2 in Fig. 1a). The definitions of specific terms are summarized in Table 1.

and $|F_{H_2O}|$) of the d-crystal and h-crystal can be used to visualize the D/H contrast, while only the amplitude commonly observed in both $|F_{D_2O}|$ and $|F_{H_2O}|$ can be used in the 'reciprocal' D/H contrast method, and (ii) the technique can be easily incorporated into standard neutron crystallography. This technique should thus contribute not only to the highest quality structure determination but also to improvement of the existing macromolecular structures determined by standard neutron crystallography.

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