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The properties of $(2F_o - F_c)$ and $(F_o - F_c)$ electron-density maps at medium-to-high resolutions

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This paper reports on the efficacy of $(F_o - F_c)$ *versus* $(2F_o - F_c)$ electron-density maps at 3.2 Å resolution. Firstly, a study is reported of a simple truncation at 2.3 and 3.2 Å of the 1.6 Å resolution crystal structure of concanavalin A at room temperature [Emmerich *et al.* (1994), *Acta Cryst.* D**50**, 749–756] with 149 known bound water molecules. Secondly, the concanavalin A 1.6 Å resolution model was re-refined but with the data truncated to 3.2 Å. In a similar evaluation, these procedures were repeated for the apocrustacyanin A1 cryotemperature 1.4 Å resolution model [Cianci *et al.* (2001), *Acta Cryst.* D**57**, 1219–1229]. Maps at 1.4, 2.3 and 3.2 Å resolutions were first generated and the structure was then re-refined at 3.2 Å and additionally at 2.3 Å resolution. The results on concanavalin A show that the number of bound water molecules that are resolved decreases by two thirds from 1.6 to 3.2 Å, but that key structural waters, for example at the transition metal and the calcium ion, are still resolved in the $(F_o - F_c)$ map but not in the $(2F_o - F_c)$ map. For apocrustacyanin A1, the results with these two difference maps were less clear-cut. Two key structural bound waters (w93 and w105) were selected that had been previously identified in β-crustacyanin [Cianci *et al.* (2002), *Proc. Natl Acad. Sci. USA*, **99**, 9795–9800] in protein–carotenoid interactions. The behaviour of w93 is similar to that of concanavalin A key waters, but that of w105 is not. These behaviours were therefore explored in finer resolution increments, namely 2.9, 2.7 and 2.5 Å. Finally, further tests on 'real' data sets for peanut lectin and concanavalin A at medium resolution confirm these map properties, namely that an $(F_o - F_c)$ difference electron-density map is more effective than a $(2F_o - F_c)$ map in showing bound water structure at lower resolutions (∼3.2 Å). This result is important since a growing number of protein crystal structure studies are concerned with multi-macromolecular complexes and are at such resolutions. Details of the bound solvent can still be revealed at 3.2 Å *via* the $(F_o - F_c)$ map calculation. The physical basis of the limitation of the $(2F_o - F_c)$ map presumably lies in the series-termination error effect on such a map involving the first negative ripple from the protein atom to which a bound water oxygen is hydrogen bonded, sufficiently cancelling its peak. In addition, re-refinements at 3.2 Å show distances that can agree with known values but B values that do not agree with known values.

1. Introduction

The recently determined β-crustacyanin structure at 3.2 Å resolution revealed a bound water at each keto oxygen of one end of each astaxanthin (Cianci *et al.*, 2002). The $(F_o - F_c)$ map peaks reported in Cianci *et al.* (2002) were clear at each

Table 1
Details of the test protein crystal sytems.

	β-Crustacyanin	Concanavalin A	Apocrustacyanin A1	Peanut lectin–sugar complex	Concanavalin A–sugar complex
Unit-cell parameters					
a, b, c (Å)	155.5, 155.5, 168.5	88.7, 86.5, 62.5	41.65, 80.7, 110.8	128.95, 126.68, 77.28	119.7, 119.7, 68.9
α, β, γ (°)	90, 90, 120	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90
Space group	$P6_322$	$I222$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Starting resolution (Å)	3.2	1.6	1.4	2.8	2.75
Temperature/source	Cryo/SRS 14.1	Room/EMBL Hamburg + Cu $K\alpha$	Cryo/SRS 9.6	Room/Cu $K\alpha$	Room/Cu $K\alpha$
PDB code	1gka	1scs	1h91	1qf3	1bxh
No. of atoms in asymmetric unit	2996	1960	3373	7405	7391
Bound waters	30	149	439	373	73
$\langle F/\sigma(F)\rangle$	21.6	27.7	35.0	60.3	24.1
$\langle F/\sigma(F)\rangle$ at measured resolution limit	5.7 (3.2 Å)	13.3 (1.60 Å)	12.5 (1.4 Å)	18.0 (2.8 Å)	9.5 (2.75 Å)
$\langle F/\sigma(F)\rangle$ at truncated resolution limit	5.7 (3.2 Å)	12.8 (3.2 Å)	55.0 (3.2 Å)	38.3 (3.2 Å)	19.2 (3.2 Å)

bound water site, but the $(2F_o - F_c)$ map evidence was weak at one site and absent at the other. The evidence for the two water sites was backed up by the clear presence of one water at each corresponding position (within 0.5 Å) in the 1.4 Å resolution apocrustacyanin Al structure. The fact that the $(F_o - F_c)$ electron-density difference map at 3.2 Å resolution showed these two bound waters, which were obviously functionally important, and that the $(2F_o - F_c)$ map did not, was the genesis of the present work in further evaluating these standard and much-used tools of protein crystallography.

The molecular basis of the bathochromic shift mechanism was revealed by the β-crustacyanin crystal structure (Cianci *et al.*, 2002). The β-crustacyanin is responsible for 100 nm of the total 150 nm shift effect. The final 50 nm shift arises from α-crustacyanin (comprised of eight β-crustacyanins). A program of research is now starting to determine the crystal structure of α-crustacyanin in order to understand the relative contributions of these molecular factors involved in the bathochromic shift. One such factor is a hydration/dehydration effect, a process which shows the colour change blue to red and is reversible, unlike the cooking of a lobster, which produces the same colour change and is irreversible. We can probably expect that the data from the α-crustacyanin protein complex will again be at medium resolution. Therefore, a better understanding of the relative effectiveness of these two electron-density difference map types is of keen interest to us. Indeed, in general, a growing number of protein crystal structure studies are concerned with multi-macromolecular complexes and are at such resolutions.

One of the evaluations presented involves two different protein structure models in which the bound water structure is known at high resolution. Concanavalin A, is a room-temperature synchrotron-radiation protein structure at 1.6 Å resolution (Emmerich *et al.*, 1994) and apocrustacyanin A1, is a cryo-temperature synchrotron-radiation protein structure at 1.4 Å resolution. Concanavalin A is a lectin- and saccharide-binding protein isolated from the jack bean *Canavalia ensiformis* (Sharon, 1993). Concanavalin A has two metal-binding sites and each ion coordinates two water molecules in its own coordination sphere. Apocrustacyanin A1 is one of the subunits of the protein β-crustacyanin and has been studied at 1.4 Å resolution (Cianci *et al.*, 2001); two of the bound waters are of keen interest, being involved in keto-oxygen water coordination of astaxanthin, as mentioned above. The diffraction data sets were simply truncated to a lower resolution and the efficacy of the two types of difference map was compared. The protein structure was then re-refined at the truncated resolution to examine whether or not the visibility of the key bound waters was improved.

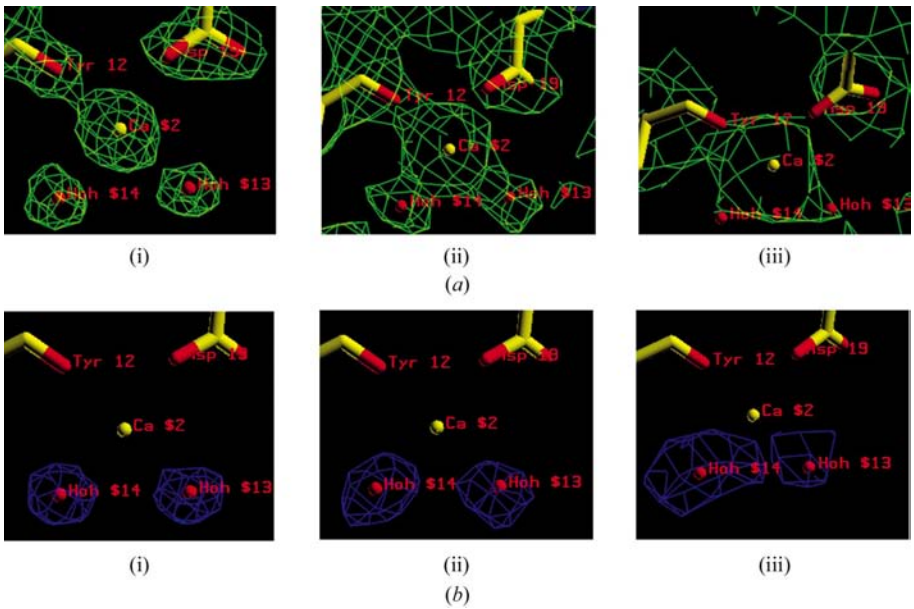


Figure 1
Co,Ca concanavalin A simple truncation. (*a*) The $(2F_o - F_c)$ map at the Ca site. (i) 1.6 Å, 2 r.m.s., (ii) 2.3 Å, 1 r.m.s., (iii) 3.2 Å, 0.8 r.m.s. using the PDB file with waters in the model. (*b*) The $(F_o - F_c)$ map at (i) 1.6 Å, 5σ, (ii) 2.3 Å, 4σ, (iii) 3.2 Å, 3σ using the PDB file without waters in the model.

Table 2
Example temperature factors and hydrogen-bonding distances of concanavalin A solved at 1.6 Å and re-refined at 3.2 Å.

Structure resolution (Å)	1.6	3.2
B_{H_2O}† (Å^2)		
w11	9.6	6.9
w12	8.3	3.3
w13	9.9	2.1
w14	7.3	2.2
B_{Bp}‡ (Å^2)		
Cobalt	9.5	3.5
Calcium	7.7	2.9
Asp10	7.4	3.5
His24	9.7	3.3
Tyr12	9.1	6.7
Asp19	10.2	3.4
DB_L§ (Å)		
Co (w11, w12)	2.19, 2.24	1.84, 1.88
Co (Asp10, His24)	2.12, 2.20	2.19, 2.03
Ca (w13, w14,)	2.40, 2.42	2.31, 2.42
Ca (Tyr12, Asp19)	2.32, 2.32	1.97, 2.35

† B_{H_2O} is the temperature factor for waters w11 and 12 bound to the cobalt ion and waters w13 and 14 bound to the calcium ion. ‡ B_{Bp} is the temperature factor for the cobalt and calcium ions, Asp10 and His24 coordinated to the cobalt ion and Tyr12 and Asp19 coordinated to the calcium ion. § DB_L is the hydrogen-bonding distance for waters and residues that are coordinated to the cobalt and calcium ions.

The advantage of these two model systems was that the answer was known, *i.e.* a much higher resolution structure was available. The disadvantage was that the truncated data set is 'unreal'. Therefore, two further examples were examined in which the data were investigated at a resolution very close to 3.2 Å. These were peanut lectin (Ravishankar *et al.*, 1999) and concanavalin A sugar complexes (Moothoo *et al.*, 1999) measured to 2.8 and 2.75 Å resolution, respectively.

2. 'Simple' data truncation of concanavalin A at 3.2, 2.3 and 1.6 Å resolution

Concanavalin A data (PDB code 1scs) were used to produce electron-density maps with simple data-resolution cutoffs of 2.3 and 3.2 Å compared with 1.6 Å. The 1.6 Å room-temperature reference structure contains 149 bound waters (Table 1). $(F_o - F_c)$ and $(2F_o - F_c)$ electron-density maps, using σ_A coefficients, were generated *via SFALL*, *RSTATS*, *FFT*, *MAPMASK* and *MAPMAN* (Collaborative Computational Project, Number 4, 1994) at the three resolution levels 1.6, 2.3 and 3.2 Å. The water structure was examined in $(2F_o - F_c)$ maps using the *O* graphics software (Jones *et al.*, 1991) for the three resolutions (Supplementary Table 1[1]). In particular, the waters that coordinate to the ions Co and Ca in the protein (named 11, 12, 13 and 14 in the PDB file) were inspected. In Fig. 1(*a*) the $(2F_o - F_c)$ maps of Co,Ca concanavalin A at 1.6, 2.3 and 3.2 Å at the Ca site are shown; both of the Ca-liganded waters retain their peaks at 1.6 and at 2.3 Å resolution but not at 3.2 Å.

The 'simple' data-truncation procedure was repeated when water molecules were removed from the model and their positions checked in the $(F_o - F_c)$ maps (Supplementary Table 1). The waters now retain their peaks in this map up to and including 3.2 Å resolution (Fig. 1*b*).

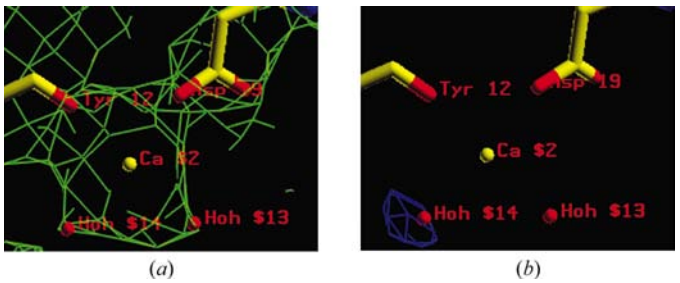
3. Concanavalin A refined against the 1.6 Å data truncated at 3.2 Å resolution

The model 1scs (with 149 bound waters; see Table 1) was re-refined with the restrained option in *REFMAC*5 (Collaborative Computational Project, Number 4, 1994; Murshudov *et al.*, 1997) with isotropic temperature factors ($R = 0.12$; $R_{free} = 0.20$; FoM = 0.87). Maps were generated *via FFT*, *MAPMASK* and *MAPMAN* (Collaborative Computational Project, Number 4, 1994) against the diffraction data to 3.2 Å resolution.

In the $(2F_o - F_c)$ map only 42 waters appeared in density, *i.e.* 107 of the known structure waters were without significant density (Supplementary Table 2). Fig. 2(*a*) shows the $(2F_o - F_c)$ map of the calcium atom and its waters, which only show as a bulge of density. Comparing Fig. 1(*a*) with Fig. 2(*a*), there is no benefit from the re-refinement against the truncated 3.2 Å data.

The model refinement was then repeated with the PDB file without waters ($R = 0.13$; $R_{free} = 0.19$; FoM = 0.87) and the procedure was repeated (Supplementary Table 2). Inspecting the $(F_o - F_c)$ electron-density map, 77 waters appeared in 2σ or 1σ density. 72 of the 149 starting waters were without density. The cobalt and calcium again preserved their waters, but as seen from Fig. 1(*b*)(iii) compared with Fig. 2(*b*) water 13 is now seen only at the 2σ level. The $(2F_o - F_c)$ map is again not as effective in showing the known bound water positions, yielding 42 waters. The number of common waters between these two populations is 36 (Supplementary Table 2).

Table 2 reports the *B*-factor values and bond distances for the calcium and cobalt ions and for various ligands. The *B*-factor values are significantly smaller at 3.2 Å resolution. The distances to the metal ions also tend to show smaller distances when refined at 3.2 Å.



(*a*) (*b*)

Figure 2
Co,Ca concanavalin A data truncated at 3.2 Å resolution after re-refinement using the PDB file with waters in the model. $(2F_o - F_c)$ density map contoured at 0.8 r.m.s. for the calcium ion. (*b*) The $(F_o - F_c)$ density map contoured at the 3σ level for the calcium ion (water 13 is visible at the 2σ level).

[1] Supplementary data have been deposited in the IUCr electronic archive (Reference: EN0077). Services for accessing these data are described at the back of the journal.

4. 'Simple' data truncation of apocrustacyanin A1 at 3.2, 2.3 and 1.4 Å resolution

Apocrustacyanin A1 data (Table 1; PDB code 1h91) was used to study the behaviour of the bound waters in $(2F_o - F_c)$ and $(F_o - F_c)$ electron-density maps. The model of the protein in the asymmetric unit consists of a homodimer, with 215 waters in monomer *A* and 224 waters in monomer *B*. The apocrustacyanin A1 structure data at 1.4 Å resolution, measured at cryotemperature, were initially cut at 2.3 and 3.2 Å. The structure factors and the electron-density maps were generated *via SFALL*, *RSTATS*, *FFT*, *MAPMASK* and *MAPMAN* (Collaborative Computational Project, Number 4, 1994) at 2.3, 3.2 and 1.4 Å resolution. Firstly, maps were generated using the PDB file with waters and the $(2F_o - F_c)$ maps were inspected using the *O* graphics software (Jones *et al.*, 1991). The waters named w93 in the PDB file for the monomer *A* and water w105 for the monomer *B* were selected for scrutiny as they are involved in the astaxanthin keto–oxygen hydrogen bonding at one end of the carotenoid in the β-crustacyanin. Specifically, these two waters are located at corresponding positions to the water called w1 in the PDB file of the β-crustacyanin structure solved at 3.2 Å resolution (Cianci *et al.*, 2002).

Here, the simple truncation map comparison did not give as clear-cut a result as that above with concanavalin A; the $(F_o - F_c)$ map did not show waters w95 and w105 at 3.2 Å resolution (see Fig. 3). Therefore, a third evaluation was made at finer intervals of resolution between 2.3 and 3.2 Å to determine the resolution at which the waters w93 and w105 disappeared in the $(F_o - F_c)$ map. Maps at 2.9, 2.7 and 2.5 Å were generated and checked again. Supplementary Table 3 details the different behaviour of the two waters in the $(2F_o - F_c)$ *versus* the $(F_o - F_c)$ omit maps. Basically, waters w93 and w105 were not in density in the $(2F_o - F_c)$ map at

Table 3
Temperature factor and hydrogen-bond distances of apocrustacyanin A1 solved at 1.4 Å and re-refined at different resolutions.

Resolution (Å)	3.2	2.9	2.7	2.5	2.3	1.4
B_{H_2O} †(Å^2)						
w93	2.3	11.5	14.6	9.1	8.2	13.8
w105	2.1	19	17.8	28.1	23.7	22.1
B_{Hp}‡(Å^2)						
Tyr*A*56	3.3	7.4	7.8	11.6	11.1	14.6
Tyr*A*97	2.0	7.5	9.6	10.9	12.0	13.5
Tyr*B*56	2.0	13.2	15.9	19.2	21.2	20.7
Tyr*B*97	5.2	7.1	5.5	6.4	7.8	12.0
HD_{H_2O}§ (Å)						
w93 (*A*56)	2.73	2.72	2.62	2.68	2.70	2.74
(*A*97)	3.68	3.21	3.11	3.00	2.94	2.94
w105 (*B*56)	2.73	3.05	2.92	2.93	2.94	2.69
(*B*97)	2.91	2.67	2.86	2.94	2.98	3.04

† B_{H_2O} is the temperature factor for the waters w93 and w105. ‡ B_{Hp} is the temperature factor for the hydrogen partner atoms bound to the waters w93 and w105. § HD_{H_2O} is the hydrogen-bond distance for the waters w93 and w105 from their hydrogen partner atoms.

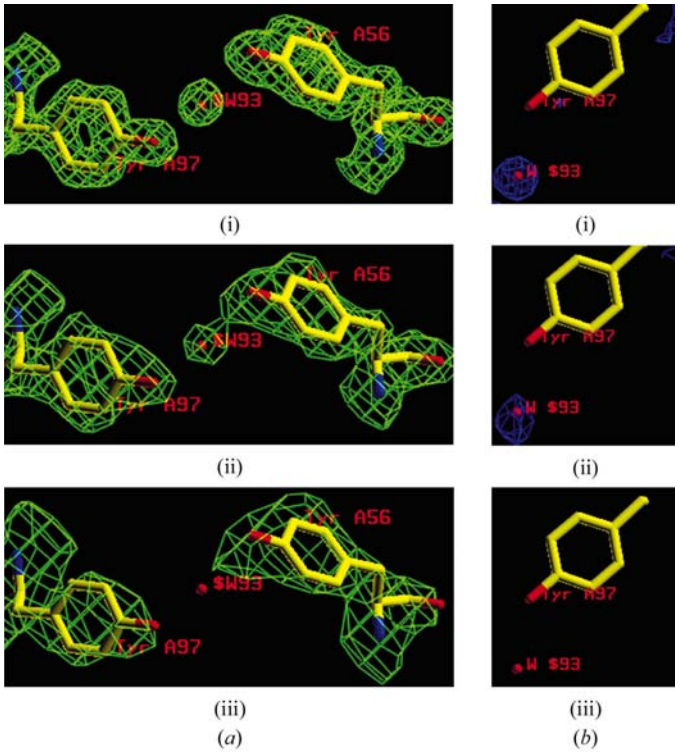


Figure 3
Simple truncation of apocrustacyanin A1 at different resolution ranges. (*a*) The $(2F_o - F_c)$ maps at (i) 1.4 Å, (ii) 2.3 Å and (iii) 3.2 Å at 1 r.m.s. using the PDB file with waters in the model. (*b*) The $(F_o - F_c)$ maps at (i) 1.4 Å, (ii) 2.3 Å and (iii) 3.2 Å contoured at 2σ using the PDB file without waters in the model.

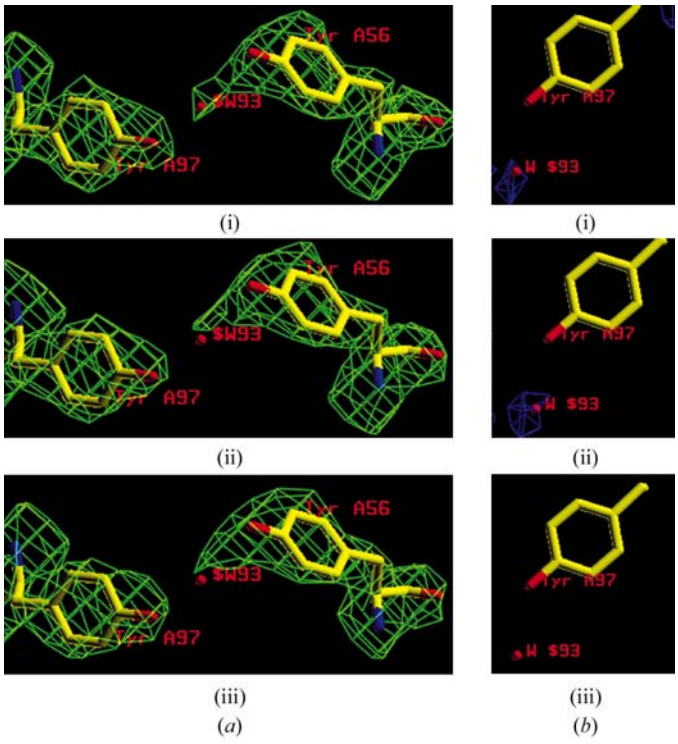


Figure 4
Simple truncation of apocrustacyanin A1 at finer intervals of resolution than in Fig. 3. The $(2F_o - F_c)$ maps at (i) 2.5 Å, (ii) 2.7 Å and (iii) 2.9 Å contoured at 1 r.m.s. using the PDB file with the waters in the model. (*b*) The $(F_o - F_c)$ maps at (i) 2.5 Å, (ii) 2.7 Å and (iii) 2.9 Å contoured at 2σ using the PDB file without waters in the model.

2.7 Å. In the $(F_o - F_c)$ maps, both water w93 and water w105 appeared in density at 2.7 Å at 2σ; only w105 appeared in the $(F_o - F_c)$ map at 2.9 Å, albeit with poor density. At 2.5 Å both waters appeared in both maps (see Figs. 4*a* and 4*b*). As Fig. 4(*a*) shows, the $(2F_o - F_c)$ map does not give a clearly resolved peak at any of these resolutions, whereas the $(F_o - F_c)$ map is clear at 2.7 Å or better (Fig. 4*b*).

5. Apocrustacyanin A1 refined against the 1.4 Å data truncated at various resolutions

Supplementary Table 4 details the re-refinement of apocrustacyanin A1 against the truncated data sets at various resolutions. In this case, the $(F_o - F_c)$ map clarity of waters w93 and w105 is not improved by the refinement. However, the clarity of these waters in the $(2F_o - F_c)$ map is improved. Table 3 gives the *B*-factor values for waters w93 and w105 (B_{H_2O}) and their hydrogen-bonding partners (B_{Hp}). Table 3 also reports the hydrogen-bonding distances of water w93 to Tyr*A*56 and Tyr*A*97 and w105 to Tyr*B*56 and Tyr*B*97 (HD_{H_2O}).

Comparing the *B* factors with the true values (*i.e.* at 1.4 Å), the B_{H_2O} values increase or decrease in a non-systematic way, but become very small in the 3.2 Å refinement (2.35 and 2.07 Å^2).

Comparing the distances to hydrogen-bonding partners for w93 and w105, the values remain reasonable for residues *A*56, *B*56 and *B*97 and gradually become less and less reasonable for residue *A*97.

6. Tests on real medium-high resolution data sets

The advantage of the two previous model systems was that the answer was known, *i.e.* a much higher resolution structure was available. The disadvantage was that a truncated data set is 'unreal'. Therefore, two further examples were looked at where the data were measured at a resolution very close to 3.2 Å. These are peanut lectin (Ravishankar *et al.*, 1999) and concanavalin A (Moothoo *et al.*, 1999) sugar complexes, measured to 2.8 and 2.75 Å resolution, respectively. Table 1 again gives the details of these test structures. The $(2F_o - F_c)$ and $(F_o - F_c)$ maps at 3.2 Å, as well as at their true resolution limits, were calculated. These are compared in Figs. 6(*a*), 6(*b*), 7(*a*) and 7(*b*). These confirm that an $(F_o - F_c)$ difference electron-density map is effective in showing bound water structure at medium-high resolution (~3.2 Å resolution) and a $(2F_o - F_c)$ map is not.

7. Discussion and concluding remarks

For concanavalin A, the investigation of the behaviour of the bound solvent water at 3.2 Å, when the bound water details are known at much higher resolution, clearly shows that the four key waters bound to the Co, Ca sites are preserved at 3.2 Å in the $(F_o - F_c)$ map but not in the $(2F_o - F_c)$ map. The results from the 'real' data sets, peanut lectin and concanavalin A sugar complexes, measured to ~3 Å confirm the properties of these difference map types. A similar trend was obtained for apocrustacyanin A1, but was less marked, in which the

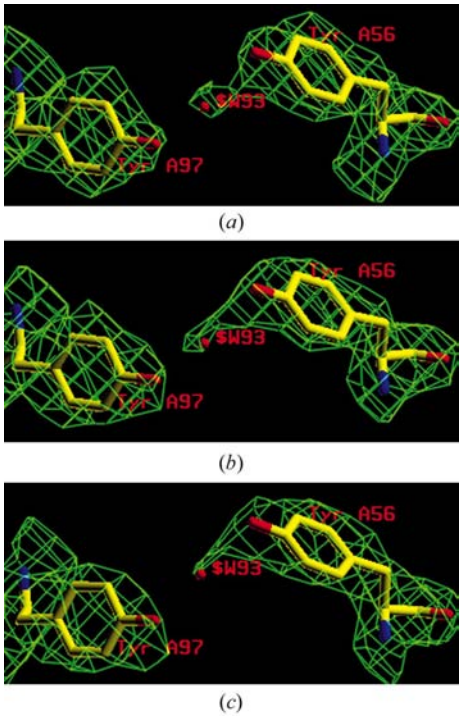


Figure 5
Re-refinements of apocrustacyanin A1 data truncated at different resolution ranges. The $(2F_o - F_c)$ density map contoured at 1 r.m.s. at (*a*) 2.7 Å, (*b*) 2.9 Å and (*c*) 3.2 Å resolution data using the PDB file with waters in the model.

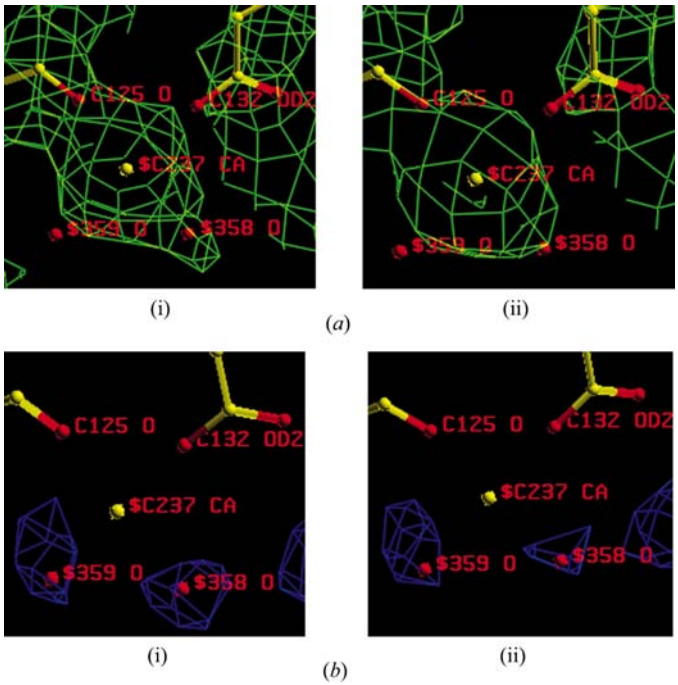


Figure 6
Test involving a data set measured close to 3.2 Å: the case of peanut lectin. (*a*) $(2F_o - F_c)$ electron-density map contoured at 1 r.m.s. (i) at 2.8 Å and (ii) truncated to 3.2 Å. (*b*) $(F_o - F_c)$ electron-density map contoured at 3σ (i) at 2.8 Å and (ii) truncated to 3.2 Å.

fading-out resolution was around 2.7 Å for the $(F_o - F_c)$ map [compared with around 2.3 Å resolution for the $(2F_o - F_c)$ map].

What is the physical basis of the differences in the visibility of bound waters in the $(F_o - F_c)$ *versus* $(2F_o - F_c)$ maps? It is known in general terms that there is a negative ripple effect arising from the series termination of the Fourier summation (James, 1948; Stenkamp & Jensen, 1984). In essence, in an F_o (or F_c) map an electron-density peak is accompanied by a negative ripple at a position 0.92 $d_{\min}$ of the diffraction data resolution and at about 10% of the primary (positive) peak height. Thus, a bound water oxygen that is hydrogen bonding to its partner protein atom is on average 2.8 Å away and usually has a *B* factor higher than that protein partner atom (see, for example, Table 4 for β-crustacyanin). The relatively weaker positive electron density of the bound water oxygen sits neatly right at the negative ripple of the protein atom. At 2 Å $d_{\min}$ this problem has 'died away' at the place of the bound water oxygen, thus explaining the common observation of the benefits of working nearer to 2 Å resolution. However, when one is forced to work at 3 Å resolution one can see that the negative ripple is perfectly positioned to cause (partial) cancellation of the bound water oxygen. In contrast to F_o or F_c or $(2F_o - F_c)$ maps, the $(F_o - F_c)$ map does not have such a series-termination ripple effect and thus there is no cancelling out of the bound water oxygen peak. An additional benefit of an $(F_o - F_c)$ map is the clarity of the isolated spherical electron-density features of bound waters.

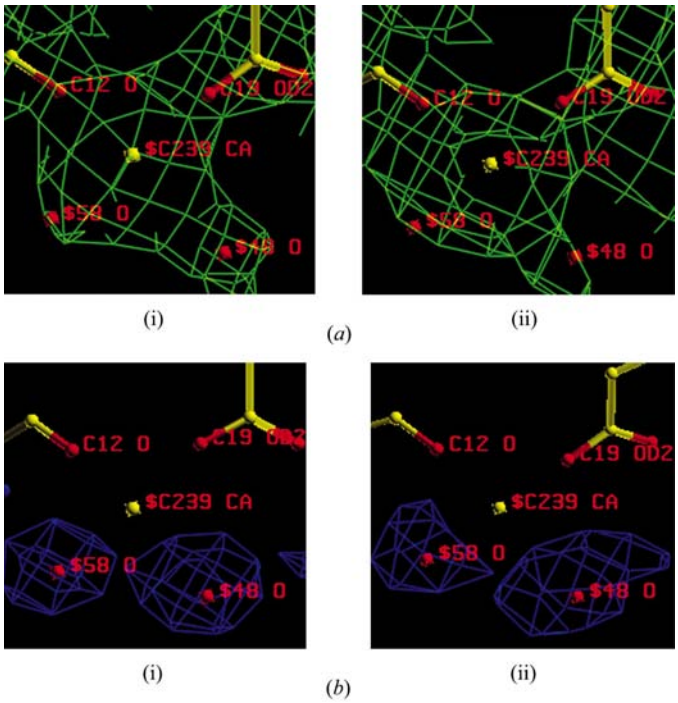


Figure 7
Test involving a data set measured close to 3.2 Å: the case of a concanavalin A–saccharide complex. (*a*) $(2F_o - F_c)$ electron-density map contoured at 1 r.m.s. (i) at 2.8 Å and (ii) truncated to 3.2 Å. (*b*) $(F_o - F_c)$ electron-density map contoured at 3σ (i) at 2.8 Å and (ii) truncated to 3.2 Å.

Table 4
Example temperature factors and hydrogen-bonding distances of β-crustacyanin solved at 3.2 Å resolution.

B_{H_2O}† (Å^2)	
w1	52.3
w2	35.1
B_{Bp}‡ (Å^2)	
Tyr*A*56 (w1)	40.3
Tyr*A*97 (w1)	45.4
O(4) astaxanthin (w1)	39.9
Ser*B*49 (w2)	24.0
Tyr*B*51 (w2)	14.1
O(4′) astaxanthin (w2)	24.5
HD_{H_2O}§ (Å)	
w1 (Tyr*A*56, Tyr*A*97)	2.48, 2.98
w1 O(4) astaxanthin	2.60
w2 (Ser*B*49, Tyr*B*51)	3.17, 2.85
w2 O (4′) astaxanthin	2.73

† B_{H_2O} is the temperature factor for waters w1 and w2 of β-crustacyanin. ‡ B_{Bp} is the temperature factor for Tyr*A*56 and Tyr*A*97 bound to water w1 and Ser*B*49 and Tyr*B*51 bound to water w2; also given are the *B* factors for the two O atoms (O_4) and ($O_{4'}$) of the two carotenoid astaxanthins coordinated to the waters w1 and w2. § HD_{H_2O} is the hydrogen-bonding distance of the waters w1 and w2 with their hydrogen-bonding partners and the distances of the two waters coordinated to the O atoms of the two astaxanthin rings.

On a cautionary note, it is important to recall the obvious fact that higher resolution is better. At medium–high resolutions, the clarity of the $(F_o - F_c)$ density peaks do vary. As pointed out in the β-crustacyanin 3.2 Å study (Cianci *et al.*, 2002), the evidence for each key structural water bound to the keto oxygen of one end of each astaxanthin was the $(F_o - F_c)$ density corroborated by waters in the corresponding positions in apocrustacyanin A1 determined at much higher resolution (1.4 Å). An interesting feature of the water oxygen to keto oxygen bond distances (Table 4) was that they were unusually short (2.60 and 2.73 Å). As we have seen in this paper, the refined distances at 3.2 Å, where the distances are known at higher resolution, show some that agree with the known values and some that do not (Tables 2 and 3). The *B* factors do not agree.

Overall, in conclusion, these observations regarding bound water visibility are important as part of the field of protein crystallography is increasingly moving to the study of large multi-macromolecular complexes at resolutions between 3 and 3.5 Å. Details of the bound solvent can still be revealed at 3.2 Å *via* the $(F_o - F_c)$ map calculation.

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