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Can Anomalous Signal of Sulfur Become a Tool for Solving Protein Crystal Structures?

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A general method for solving the phase problem from native crystals of macromolecules has long eluded structural biology. For well diffracting crystals this goal can now be achieved, as is shown here, thanks to modern data collection techniques and new statistical phasing algorithms. Using solely a native crystal of tetragonal hen egg-white lysozyme, a protein of 14 kDa molecular mass, it was possible to detect the positions of the ten sulfur and seven chlorine atoms from their anomalous signal, and proceed from there to obtain an electron-density map of very high quality.

Keywords: anomalous scattering of sulfur and chlorine; single wavelength; phasing; lysozyme

Introduction

If one could measure from a crystallography experiment the amplitude and phase of all X-rays diffracted from the crystal, or at least most of them, to a given resolution, a simple Fourier transform would yield an electron-density map at that resolution. From there, the interpretation of this map in terms of an atomic model would proceed. Because only the intensity of the diffracted rays can be measured, various methods were established to obtain an approximate estimation of phases, that would lead, *via* the Fourier transform, to an electron-density map where molecular features can be recognized and interpreted.

For most ''small'' molecules (less than 100 atoms) it is possible to obtain a good approximation of the phases from the experimental knowledge of the diffracted intensities only. Recently direct methods have been applied to macromolecules, but they still require data extending to exceptionally high resolution, at least to 1.2 Å. If the atomic structure of a related molecule is known, the phase problem can sometimes be solved by the method of molecular replacement, by modifying one to fit the diffracted intensities of the other. In all other cases, the phase problem can only be solved by a perturbation method: introducing a heavy atom into the molecule in the crystal (isomorphous replacement), or taking advantage of the resonant diffraction behavior of some atoms at specific X-ray wavelengths (anomalous diffraction). This latter method yields weaker signals, but requires less chemical manipulation of the crystals, and provides more reliable phase information to high resolution.

Atoms differ widely in the strength of their resonant scattering behaviour. In the range of wavelengths used for X-ray diffraction, lanthanides exhibit the largest effects. Standard heavy metals, such as Hg or Pt have $\delta f''$ of about ten electrons at a wavelength just below their L_{III} edges and about four electrons above the edge, which is the worst wavelength selection for anomalous signal. With the copper radiation the anomalous contribution of those metals is about seven electrons. Selenium, often used for multiwavelength anomalous dispersion (MAD) phasing through incorporation of selenomethionine residues instead of normal methionine residues into proteins, has an $\delta f''$ value of

Abbreviations used: MAD, multiwavelength anomalous dispersion; rmsd, root-mean-square deviation; HEWL, hen egg-white lysozyme.

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about four below its K edge, 0.5 above it and about 1.1 anomalous electrons for the copper radiation. In MAD work with selenium precautions are taken to ensure the highest possible accuracy of the measured intensities. Crystals are often aligned to measure the Bijvoet-related reflections on the same exposure or ''inverse beam'' technique utilized to record Friedel mates close in time, to make sure that the measurements of both mates, F^+ and F^-, are done with similar paths of the X-ray beams in the crystal, so as to minimize the systematic errors in the estimation of ΔF_{anom}. Local scaling of intensities during merging of symmetry equivalent reflections serves the same purpose of minimizing the errors, therefore enhancing the weak anomalous signal present in the X-ray data. Three or four data sets at different wavelengths are usually collected for MAD work, allowing us to exploit different combinations of $\delta f'$ and $\delta f''$ contributions. However, with accurate enough measurements it should be possible to obtain phase estimations from a single wavelength data set, using only the anomalous signal based on $\delta f''$ contribution.

The K X-ray absorption edges of the elements of the third period lie in the low energy region, below 3 keV or in terms of wavelength, beyond 4 Å. It is therefore not realistically possible to utilize the maximum anomalous scattering effect of such elements as chlorine, sulfur or phosphorus for phasing of X-ray diffraction data. However, those elements retain some anomalous scattering effect even far from their absorption edges, at wavelengths that are commonly used for collecting X-ray data. At the copper Kα wavelength of 1.54 Å, a sulfur atom has a $\delta f''$ value of 0.56 and chlorine 0.70 anomalous electron as estimated from program CROSSEC (Cromer, 1983). Close to 1 Å, often used at synchrotron beamlines, S and Cl have $\delta f''$ values of 0.24 and 0.31 electron, respectively.

A seminal paper of Hendrickson & Teeter (1981) describes the structure solution of the small protein crambin from the anomalous diffraction of the sulfur atoms naturally present in the molecule. This points to the possibility of a universal phasing method, since sulfur is present in almost all proteins. Phosphorus can be used for the same purpose in nucleic acids. The main obstacle so far has been the quality of the data required to take advantage of the vanishingly small signal of the sulfur atom. We demonstrate that it is now becoming possible by using currently available data collection and statistical phasing methods.

Data acquisition and phasing

We have collected X-ray data on the tetragonal crystal of hen egg-white lysozyme (HEWL) using synchrotron radiation of 1.54 Å, thus mimicking the home laboratory copper anode source, and the MAR345 imaging plate scanner. The data collection protocol was standard for single wavelength experiments, except for the high multiplicity of observations, resulting mainly from four measurement passes with different resolution limits and exposure times (Table 1). The high symmetry of the crystal also contributed to data multiplicity. Apart from that no attempts were made to

Table 1. Data collection and model rfinement

A. *Data*							
Beam line	X9B at NSLS						
Wavelength (Å)	1.54						
Pass	High		Medium		Low		Very low
Resolution (Å)	3.5-1.53		10-1.8		25-2.9		60-4.0
Exposure (seconds)	180		120		15		3[a]
ϕ-Rotation (°)	1		1		2		3
Images	501-720[b]		1-180		201-245		301-420[b]
MAR plate diameter (mm)	345		240		240		240
Resolution range (Å)		60-1.53				1.56-1.53	
Completeness (%)		100				100	
$R(I)$ merge[c] (%)		4.6				19	
I/σ		73				10	
Multiplicity		23				16	
B. *Refinement*							
R factor (number of reflections)	0.22 (17,008)						
R free (number of reflecitons)	0.30 (915)						
Protein/solvent atom sites	1001/224 Wat+8 Cl^-+1Na^+						
C. *rmsd from idealized geometry*							
Bond lengths (Å)	0.026						
Angle distances (Å)	0.044						
Chiral volumes ($Å^3$)	0.180						
Planarity (Å)	0.016						
DPI[d] (Å)	0.108						

[a] This is an effective exposure, whose images were exposed 15 seconds with an attenuated beam.
[b] Thirty two images from the high resolution pass and two from the very low resolution pass were discarded as exposed during synchrotron particle injection, without the X-ray beam.
[c] $R(I) = \Sigma\ |I_i - \langle I\rangle| / \Sigma\ \langle I\rangle$.
[d] DPI, diffraction precision indicator according to Cruickshank (1999).

measure Bijvoet-related reflections close in time or on the same images. The crystal orientation was arbitrary. It was realized *a posteriori* that due to rather large crystal size, coupled with relatively long wavelength, the data probably suffer from absorption; no correction for this effect was attempted except that, as usual, part of it was taken into account during inter-image scaling. The images were exposed with constant time, and the decay of synchrotron beam intensity resulted in image scales differing by up to 50 % within the highest resolution data collection pass.

The intensities were integrated with the HKL2000 (DENZO, Otwinowski & Minor, 1997), refining all parameters (including crystal mosaicity) against batches of three images at a time. Scaling and merging was done with the same package, taking care to obtain the accurate estimates of not only intensities, but also their associated uncertainties. The resulting intensities were used directly for SHELXM (Sheldrick, 1997, 1998), but were converted to amplitudes using the program TRUNCATE (French & Wilson, 1978) for subsequent phasing and map calculations. The statistics of anomalous differences as a function of resolution are shown in Figure 1. According to Hendrickson & Teeter (1981) the expected average ratio of $\langle|\Delta F|\rangle/\langle F\rangle = 2^{1/2}(N_A^{1/2}\ \delta f''_A)/(N_P^{1/2}\ Z_{eff})$, which for lysozyme with N_P of about 1100 atoms with the average $Z_{eff} = 6.7$ electrons and assuming $N_A = 15$ sulfur atoms with $\delta''_A = 0.56$ electron at the copper Kα wavelength gives 1.4 %, a similar value as for crambin with its six sulfur atoms per 400 protein atoms. As seen in Figure 1(a), $\langle|\Delta F|\rangle/\langle F\rangle$ is close to that value in the resolution ranges up to 2 Å; at higher resolution it rises, probably due to the higher noise in the estimation of both ΔF and F. This is corroborated by the ratio of $|\Delta F|/\sigma(\Delta F)$ (Figure 1(b)), which drops below 1.0 at a resolution higher than 1.8 Å. At longer wavelengths the expected $\langle|\Delta F|\rangle/\langle F\rangle$ ratio is higher, for example for $\lambda = 1.8$ Å it reaches 1.8 %, although the detrimental absorption effects would also be higher. It remains to be checked experimentally which wavelength is optimal for use of an anomalous signal of sulfur, with data collected at a tunable synchrotron source.

Prior to attempting the phase determination, the presence of the anomalous signal in the data was checked by calculation of the (ΔF, $\phi_{calc} - 90°$) Fourier map, which was displayed together with the model obtained from straightforward refinement by programs REFMAC (Murshudov *et al.*, 1997) and ARP (Lamzin & Wilson, 1997) to an R factor of 21 %. The anomalous difference map (Figure 2(a) and (b)) showed significant density for all ten sulfur atoms within the protein and in addition eight peaks corresponding to solvent atoms around the protein surface, ascribed to chloride ions present in the crystallization medium in high concentration. Figure 2(c) shows the (F_o, ϕ_{calc}) map for the same region of the structure as Figure 2(b); it is evident that chlorine atoms could not be identified on the basis of this map, since the corresponding peaks are indistinguishable from water.

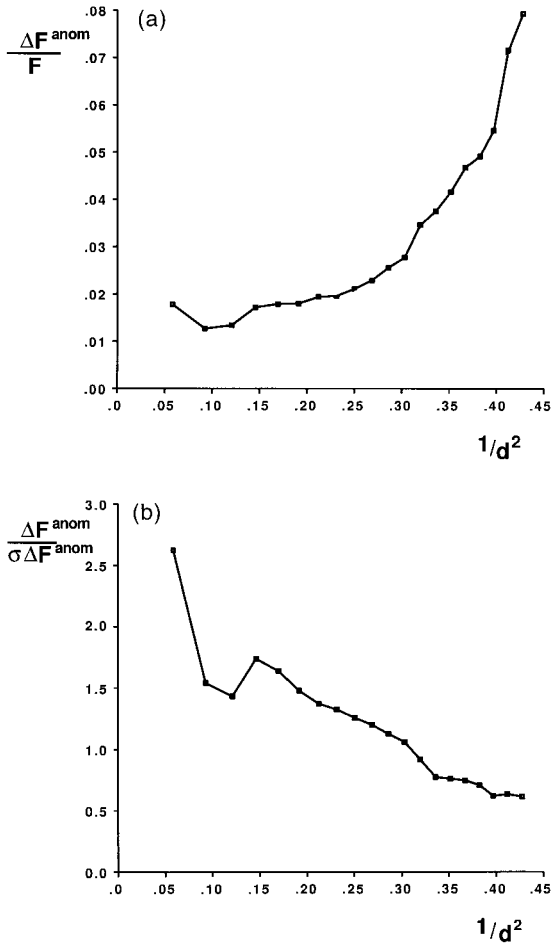

Figure 1. Statistics of the anomalous signal in the data. (a) $|\Delta F|/F$; (b) $|\Delta F|/\sigma(\Delta F)$ as a function of resolution. The expected $|\Delta F|/F$ ratio for lysozyme is about 0.014 (see the text); the experimental value is close to that value up to 2 Å, beyond that errors in the estimation of ΔF and F tend to increase this ratio.

All measured intensities were input to SHELXM (Sheldrick 1997, 1998) which used the Bijvoet differences for the solution of the anomalous scatterers partial structure. SHELXM is based on ''half-baked'' principle, consisting of the refinement of phases in reciprocal space combined with the discrimination of model atoms in direct space, analogously to the Shake-and-Bake algorithm of the SnB program (Miller *et al.*, 1993, 1994) . SHELXM has proven to be very successful in solving structures of macromolecular crystals diffracting to atomic resolution, and has also been used to find positions of anomalous scatterers in MAD experiments (Sheldrick, 1998).

A total of 2324 normalized structure factors ($E > 1.4$) were used and the 100 strongest phase triplets involving each phase were redetermined by the tangent formula. There were 17 correct sol-

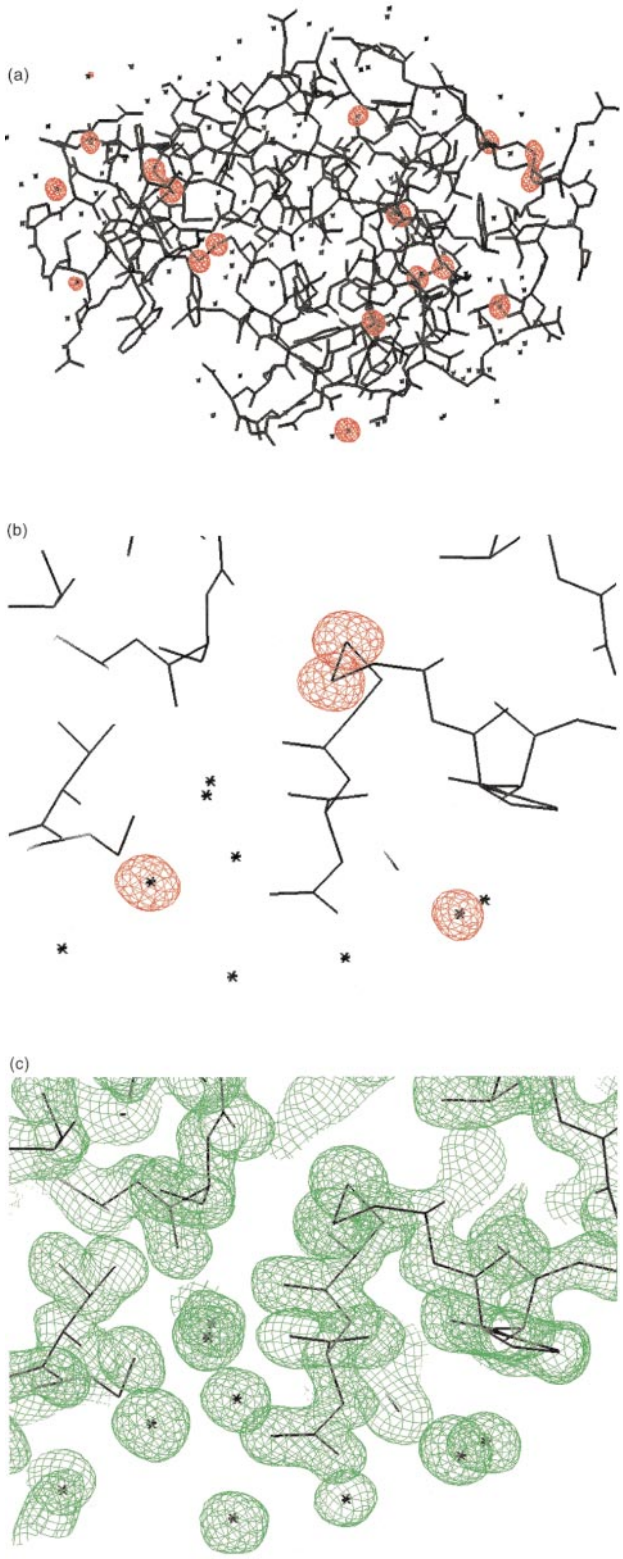

Figure 2. Electron density maps illustrating the anomalous scatterers in the structure of lysozyme. (a) and (b) The (ΔF_{anom}, $\phi_{calc} - 90^\circ$) anomalous difference Fourier synthesis at 5σ for the whole molecule and for the region near the surface of the protein. (c) The (F_o, ϕ_{calc}) synthesis for the same region at the surface, as in (b); the ''anomalous'' solvent atoms are indistinguishable from ordinary water molecules.

utions, which required an average of 75 minutes per solution on a 200 MHz Pentium Pro computer; the success rate per trial was about 0.2 %. The correlation coefficient between E_o^2 and E_c^2 was about 45 % for the correct solutions, and for false solutions had a highest value of 22 %. Table 2 lists the 17 highest peaks obtained from SHELXM with the corresponding atoms of the structure, their peak heights in the (ΔF_{anom}, $\phi_{calc} - 90^\circ$) map and their temperature factors resulting from the model refinement. The rank of SHELXM peaks is correlated to the height of ''anomalous'' peaks and the

Table 2. Anomalous scatterers in the structure

Peaks from SHELXM				Atoms in lysozyme model			
No.	1.53 Å *E*-map density	No.	2.2 Å *E*-map density	Atom	No.	ΔF_{anom} – map density	*B* factor
1	99.9	1	99.9	CL 201	1	148	15.2
2	98.8	5	87.2	SG 115	7	111	11.7
3	98.0	13	53.4	SG 30	6	112	12.2
4	97.6	4	88.8	SG 80	3	128	10.7
5	97.1	2	96.7	SD 12	5	116	11.3
6	97.1	11	55.3	SG 64	2	129	11.2
7	97.1	6	85.0	SD 105	4	126	10.0
8	92.2	7	84.4	CL 202	8	105	19.0
9	90.9	3	89.0	SG 127	12	89	16.9
10	85.7	12	54.3	CL 203	11	92	19.6
11	57.6	15	46.3	SG 6	13	83	18.7
12	54.7	9	81.0	SG 94	10	102	13.7
13	54.1	10	79.4	SG 76	9	102	13.6
14	52.5			CL 205	15	76	25.8
15	50.9			CL 204	14	74	25.2
16	48.7	14	50.9	CL 206	16	67	27.7
17	44.1	16	46.1	CL 207	17	41	29.6
18		8	84.4	CL 208	18	28	33.4

The peak heights are relative. Atomic temperature factors are from the refinement of the model. The electron density was interpolated at the atomic positions in the (ΔF, $\phi_{calc} - 90°$) map, which had an rms of 6.

refined atomic temperature factors, *B*. The use of normalized structure factors, *E*, by the program tended to emphasize the atoms (here sulfur) with lower *B*-values. Chlorine atoms are ''heavier'' with $\delta f''$ of 0.70 than sulfur with 0.56 electron, but they have higher *B*-factors as they are bound loosely at the protein surface and they are generally not the highest peaks in the *E*-map. SHELXM found the 17 best anomalous scatterers among 17 highest peaks, only the last chlorine with the highest *B*-factor and lowest signal was not found.

To estimate the resolution necessary to obtain the solution of anomalous partial structure, analogous SHELXM jobs were run against data extending to lower resolution limits. To minimize the effect of ''truncating'' weak high resolution data and leaving only artificially strong intensities, the data from only three exposure passes extending to 1.8 Å, without the strongest set, were used. The successful solutions were obtained up to a limit of 2.2 Å, with 16 correct peaks, although their *E*-map rank did not correlate any more with the density of the atoms (Table 2). This resolution may be required to resolve sulfur atoms within the S-S bridges of about 2.03 Å length.

Seventeen atoms from the SHELXM solution were input to SHARP, where all parameters, including those modeling the residual error, were refined using a full-fledged maximum likelihood approach (La Fortelle & Bricogne, 1997). Solvent flattening was then performed by the program SOLOMON (Abrahams, 1997), driven by a script specific to the SHARP interface. The difference between experimental phases and final refined ones was, on average, 28.5°. The phase differences are shown in Figure 3, together with the correlation between the corresponding electron density maps as a function of resolution. The correlation coefficient was calculated in resolution bins in the reciprocal space but it is strictly equivalent to the correlation between electron density maps computed at the same resolution (G.B., unpublished results). The SHARP/SOLOMON electron density map is shown in Figure 4(a) and its correlation coefficient with the (F_{obs} ϕ_{calc}) map is 0.80. The analogous procedure at 2.2 Å gave an interpretable map, although of somewhat poorer quality (Figure 4(b)).

The protein phases were also calculated with MLPHARE (Otwinowski, 1991) and then submitted to density modification with DM (Cowtan, 1994). The resulting electron density map is illustrated in Figure 4(c). It has a correlation coefficient of 0.76 with the final refined map.

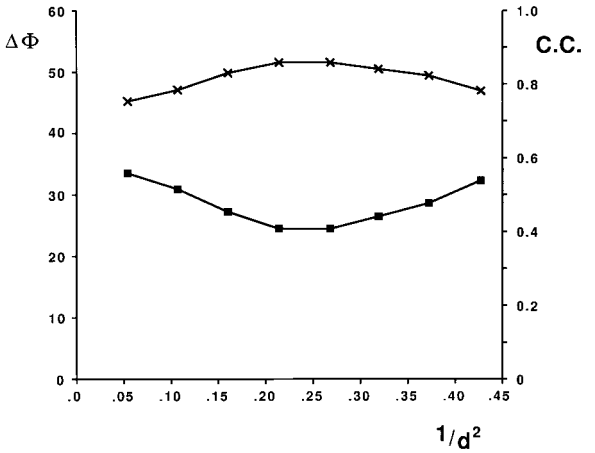

Figure 3. The phase difference between the SHARP/SOLOMON and refined phases as a function of resolution (squares) and the correlation coefficient (see the text) between electron density maps calculated with those phases (crosses).

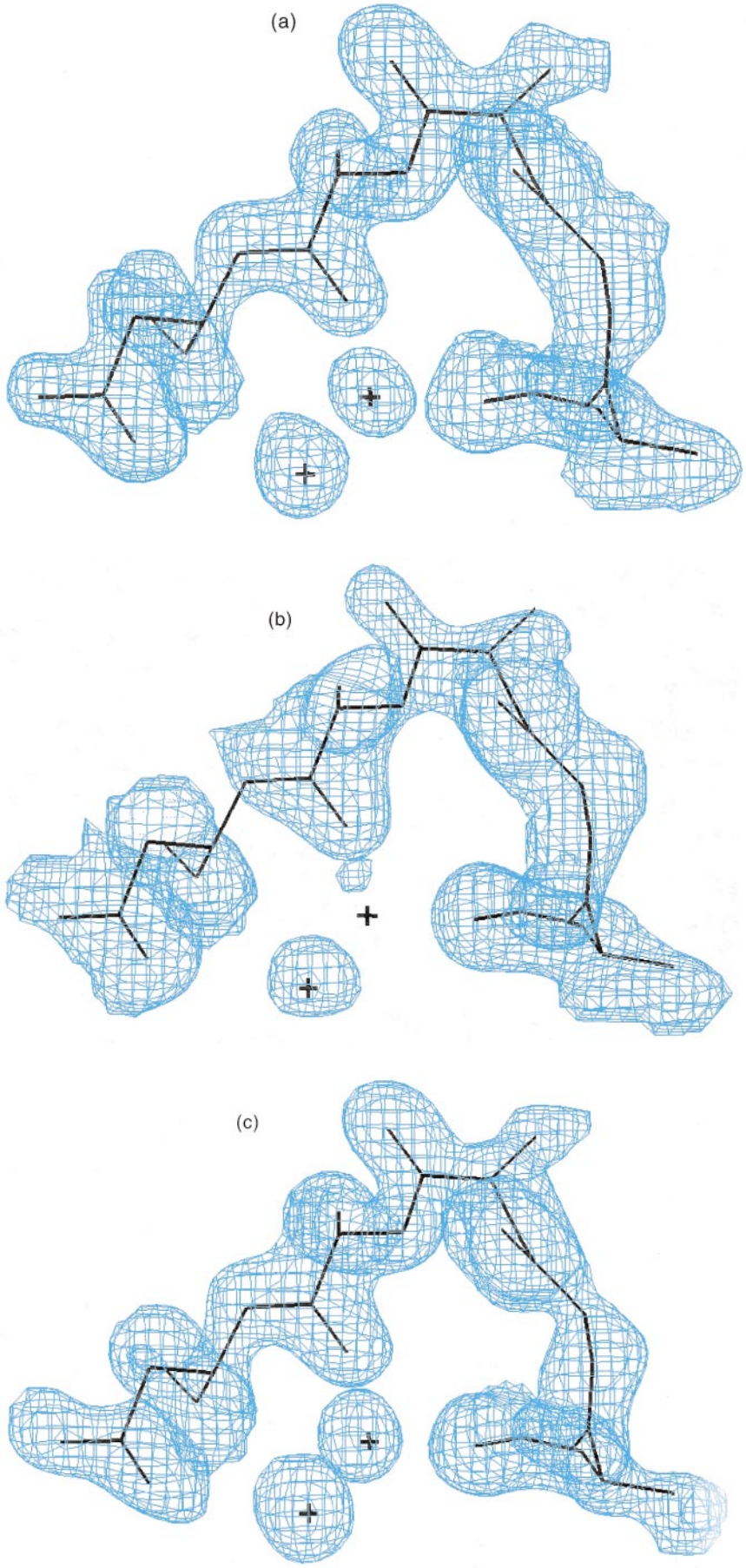

Figure 4. The electron density maps (at 1σ level) for the region around the sodium cation. (a) Calculated with phases estimated by SHARP and SOLOMON at 1.53 Å; (b) analogous map at 2.2 Å; (c) calculated with phases from MLPHARE and DM at 1.53 Å.

Anion sites

One chloride site was found in the early structure determination of tetragonal HEWL (Blake *et al*., 1967) on the basis of its low *B* factor. In a recent paper (Lim *et al*., 1998) four bromide ions in tetragonal HEWL were identified and a probable fifth site postulated on the basis of the difference in electron density between bromine and chlorine atoms. In other crystal forms HEWL is known to bind nitrates and acetates (Walsh *et al*., 1998) or iodides (Steinrauf, 1998). The sites of eight chloride atoms found in the present work (Figure 5(a) to (h)) on the basis of their anomalous signal agree with the previously identified halide sites.

In general all chloride atoms are bound rather loosely by hydrogen bonds from protein hydroxyls (Cl201-Tyr23, Cl202-Ser24) or amides (Cl201-Asn113, Cl202-Gly26, Cl203-Thr69, Cl204-Ile88, Cl206-Asn65, Cl207-Asn74) and from water molecules (all chloride atoms) and by hydrophobic interactions with parts of protein. Only a few chloride atoms lie in the vicinity of positively charged groups (Cl205-Lys33, Cl207-Arg73, Cl208-Arg68). It is interesting that one chloride (Cl203) shares two coordinating water molecules with the sodium cation (Figure 5(i)). Only two chloride atoms (Cl201 and Cl208) are positioned between two molecules of lysozyme, the remaining six sites are in contact with only one molecule of protein.

It is possible that some chloride atoms which refined to high temperature factors are not fully occupied, sharing their site with water. However, all eight of them showed significant density in the anomalous difference synthesis (Table 2).

Conclusions

The small but significant anomalous dispersion signal of 17 sulfur and chlorine atoms is enough to estimate the protein phases of lysozyme, a protein with 129 amino acid residues, by the single wavelength anomalous dispersion (SAD) approach. The data have been collected by standard procedures, except for high multiplicity of intensity measurements. The positions of anomalous scatterers were found by ''half-baked'' direct methods approach and the protein phases were obtained by a maximum likelihood estimation coupled with the density modification. At 1.53 Å the resulting electron density maps were of very high quality, clearly showing the complete protein chain with almost all its side-chains. The procedure was successful up to 2.2 Å resolution. The success of phasing on the basis of weak anomalous scatterers is mostly due to the accurate measurements of intensities on the MAR image plate scanner and to powerful algorithms implemented in the programs used for data reduction (HKL2000), anomalous atoms finding (SHELXM), phasing (SHARP and MLPHARE) and density modification (DM and SOLOMON).

In March 1999 among 7805 X-ray structures deposited in the Protein Data Bank, 4654 data sets (60 %) extended to at least 2.2 Å resolution, 3543 (45 %) to 2.0 Å and 1853 (24 %) to 1.8 Å. A substantial number of macromolecular crystals seems to diffract well enough to meet the resolution and data quality criteria for application of the phasing methods analogous to described here. The applicability of this approach depends also on the number of anomalous scatterers (inherent, like sulfur here, or introduced, like solvent chloride atoms) per macromolecule of a given size.

Since the anomalous signal alone of sulfur and chlorine atoms suffices to obtain a good estimation of protein phases (if the resolution and data quality permit) it can be expected that in cases where the protein contains atoms with a larger anomalous scattering effect the structure may be solved using a single data set collected at a wavelength maximizing the $\delta f''$ contribution, even with somewhat more modest data quality and resolution. The use of the anomalous effect of sulfur (and possibly phosphorus or chlorine) for phasing data collected at synchrotron or home laboratory sources can be envisioned for relatively strongly diffracting crystals. Such an approach significantly reduces the burden of introducing the extra anomalous scatterers to the protein and of collecting data at precisely adjusted wavelengths.

Methods

The tetragonal crystals of HEW lysozyme (from Sigma) were grown in 2 ml batches of 20 mg ml^{-1} protein solution in sodium acetate buffer (pH 4.6) containing 10 % (w/v) NaCl. Before using for data collection, the crystal was transferred for five seconds to the solution escribed above, but containing 30 % (v/v) glycerol.

A single specimen of size 0.3 mm × 0.3 mm × 0.4 mm was quickly transferred in the fiber loop to a stream of nitrogen gas and frozen at 100 K at the goniostat of the MAR345 imaging plate scanner at the beam line X9B of the National Synchrotron Light Source, Brookhaven National Laboratory. X-ray data were collected in four passes with different exposure times, resolution limits and oscillation range. The crystal sat in the loop so that it was rotated about the axis lying in the *ab*-plane, 8° from the (1 1 0) direction. Images were exposed in the ''time'' mode and those which were recorded during synchrotron injection were discarded; 537 images were used in total. The intensities were integrated and merged using the HKL2000 data processing system through its graphics interface. The results are shown in Table 1. Among 403,234 individual measurements are 17,923 Friedel-merged reflections and 15,283 unique Friedel-pairs (of non-centrosymmetric reflections); this constitutes all possible data in the resolution range 60-1.53 Å.

Programs REFMAC, ARP, MLPHARE and DM were from the CCP4 (Collaborative Computational Project Number 4, 1994) suite. Program QUANTA (Oldfield, 1996; from Molecular Simulations Inc., San Diego) was used for graphics display of the model and for the preparation of Figures.

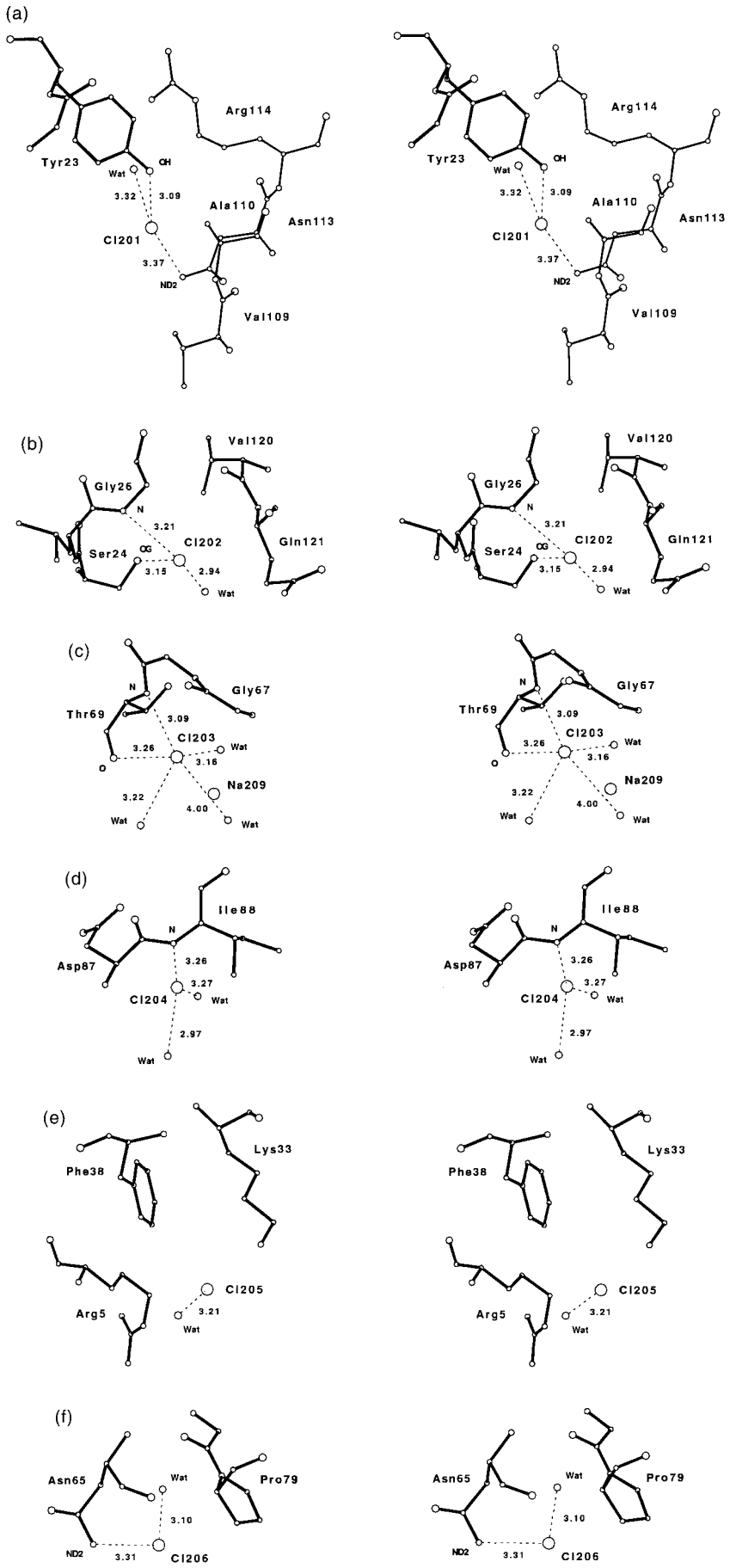

Figure 5. (*legend opposite*)

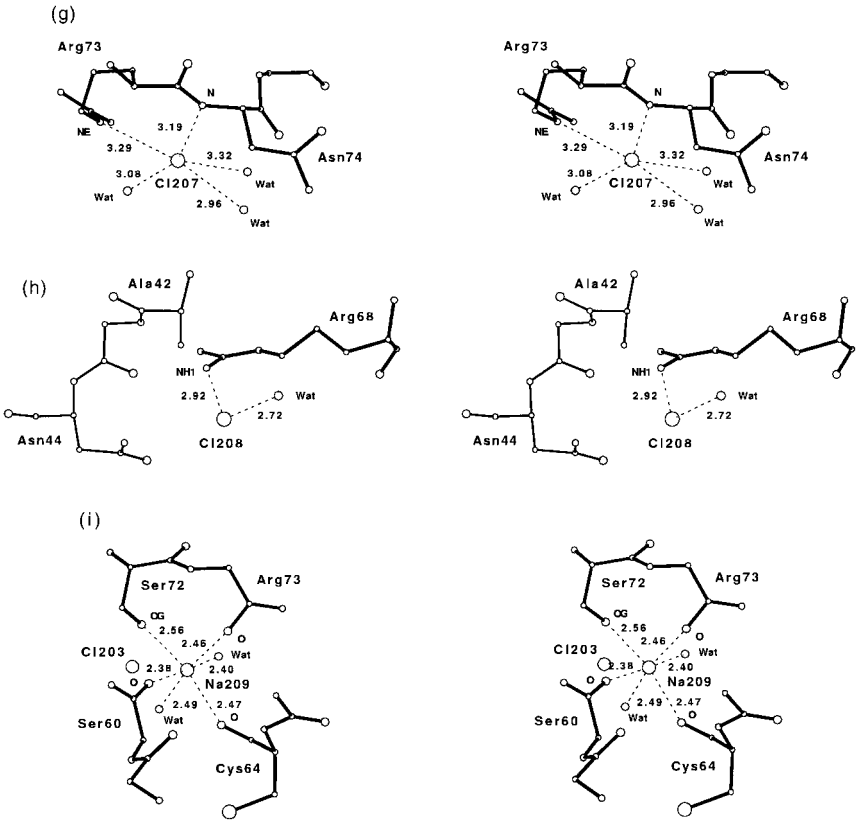

Figure 5. (a) to (h) Coordination of eight chlorine anions present in the solvent structure of lysozyme, Cl201-208; (i) coordination of the sodium cation. The close contacts to ligands are shown as broken lines and their distances are given.

Brookhaven Protein Data Bank accession numbers

The coordinates and structure factors have been deposited in the Brookhaven Protein Data Bank with the entry codes 1LZ8 and R1LZ8SF, respectively.

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