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Anomalous Signal of Solvent Bromides used for Phasing of Lysozyme

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The anomalous signal of bromide ions, present in the crystal structure of tetragonal hen egg-white lysozyme through the substitution of NaCl by NaBr in the crystallization medium, was used for phasing of X-ray data collected to 1.7 Å resolution with a wavelength near the absorption edge of bromine. Phasing of a single wavelength data set, based purely on anomalous $\delta f''$ contribution, led to easily interpretable electron density, equivalent to the complete multiwavelength anonalous dispersion phasing based on four-wavelength data. The classic small-structure direct methods program SHELXS run against all anomalous differences gave a successful solution of six highest peaks corresponding to six bromide ions in the structure with data limited up to a resolution of 3.5 Å. Interpretable maps were obtained at a resolution up to 3.0 Å using programs MLPHARE and DM. Bromide ions occupy well ordered positions at the protein surface. Phasing based on the single wavelength signal of anomalous scatterers introduced into the ordered solvent shell can be proposed as a tool for solving structures of well diffracting crystals.

Keywords: anomalous scattering; single wavelength; solvent bromide ions; phasing; lysozyme

Introduction

Two sources of information are typically used to identify the heavy atoms or anomalous scatterers in crystals: the dispersive differences, based on the $\delta f'$ contribution or anomalous differences resulting from the $\delta f''$ signal. To utilize the dispersive differences two data sets are necessary, either with and without heavy atoms, as in the classic isomorphous replacement approach, or at two different wavelengths, as in the MAD technique. In contrast, the anomalous differences are estimated from a single data set collected at one wavelength. Characteristics of these two kinds of data are somewhat different. If measured from two crystals, dispersive differences can be affected by non-isomorphism. Scaling of two data sets also presents a non-trivial problem. The anomalous data collected from one crystal are only available for acentric reflections. In both types of data only large differences are meaningful; small differences may result either from cancellation of vector contributions of several scatterers or from the anomalous (or heavy atom) phase being close (or orthogonal) to the protein phase for a given reflection. This means that only strong differences are useful for the identification of heavy or anomalous scatterer sites. In the direct methods approach this is fulfilled automatically, since they use the subset of largest normalized structure factors; however, those figures of merit which are based on weak reflections are useless.

Bromine is a very convenient anomalous scatterer with its K absorption edge at 0.920 Å. It is the most popular atom used for MAD phasing of DNA and RNA after incorporation of bromouracil instead of thymine. Similarly, selenium is widely used for MAD phasing of proteins based on selenomethionine, with its absorption edge at 0.9795 Å. These two elements lie next to each other in the periodic table and have 34 (Se) and 35 (Br) electrons. Both of them have anomalous corrections $\delta f''$ of about four electrons and $\delta f'$ about −10 electrons beyond the edge, as estimated by the program CROSSEC (Cromer, 1983); the white lines may be a little higher. The use of Br and Se for MAD is often complicated because many sites are typically occupied by these atoms in macromolecules-methionine accounts for up to 5% of amino acids in proteins and thymine about 25% of bases in nucleic acids. This makes the interpretation of

Abbreviations used: MAD, multiwavelength anoualous dispersion; HEWL, hen egg-white lysozyme; ARP, ; CFOM, combined figure-of-merit; SAD,.

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Patterson functions difficult. However, in recent years considerable progress has been achieved in the application of direct methods for solving the multi-site anomalous scatterer substructures in macromolecules. Classic direct methods programs like MULTAN or SHELXS were shown to be of use (Mukherjee *et al*., 1989) but the algorithms based on the Shake-n-Bake approach applied in SnB (Miller *et al*., 1994) and SHELXM (Sheldrick, 1997) have proven to be most successful (Smith *et al*., 1998; Dauter *et al*., 1999).

Phasing of macromolecular structures based purely on the anomalous differences is not widely used in crystallographic practice, but several examples have been published. In their classic work Hendrickson & Teeter (1981) utilized the anomalous signal to identify six sulfur atoms in the structure of crambin and subsequently to solve its structure. One of the most recent examples is the solution of rusticyanin based on the anomalous signal of the single copper atom in the native protein structure (Harvey *et al*., 1998).

Lysozyme is one of the most thoroughly studied protein crystal structures, since its first solution in the laboratory of D.C. Phillips (Blake *et al*., 1967). Its most common crystal form, in the space group $P4_32_12$, grows in the presence of NaCl, and in the early studies one of the solvent sites was identified as chloride on the basis of its high electron-density. Several anion binding sites were identified in different crystal forms of HEWL. In the triclinic form nitrate and acetate sites were found (Walsh *et al*., 1998); in the monoclinic form three nitrate ions per molecule of HEWL were identified (Rao & Sundaralingam, 1996). More halide ion sites were found in the HEWL structures refined at higher resolution on the basis of electron density. In monoclinic HEWL 17 iodide atoms are positioned around two enzyme molecules in the asymmetric unit (Steinrauf, 1998). In tetragonal HEWL four bromide sites were recently described (Lim *et al*., 1998). In our recent analysis of anomalous scatterers present in tetragonal HEWL crystallized from NaCl solution eight chloride sites were identified on the basis of anomalous difference Fourier synthesis (Dauter *et al*., 1999).

The presence of halide binding sites in lysozyme has prompted us to study the feasibility of estimating the protein phases employing the anomalous scattering of bromide ions present in the solvent and bound at the surface of the protein. We compared the phasing results obtained by using four wavelengths for data to that using a purely anomalous signal from a single wavelength data set.

Results and Discussion

Incorporation of anomalous scatterers into crystals of lysozyme was straightforward and involved substitution of NaCl by NaBr in the crystallization solution. From this modified solution crystals appeared as readily as from the standard NaCl-containing solution. Some of them tended to adopt the prismatic shape with most prominent {110} faces instead of the usually dominating {111} habit. Nevertheless all of them were in the expected space group $P4_32_12$. The crystal used for data collection had cell dimensions $a = 78.60$ Å, $c = 37.20$ Å. The MAD diffraction data were collected using four wavelengths in the vicinity of the bromine absorption edge and the results are summarized in Table 1.

The crystal stayed in arbitrary orientation during data collection and no attempt was made to use the inverse beam technique. The data were collected by the standard single wavelength approach, changing the wavelength after each data set had been finished. However, high data quality was ensured by high multiplicity of measurements; each Friedel mate was, on average, recorded about four times.

Table 1. Diffraction data and refinement statistics

A. *Data*				
Beam line	X9B at NSLS			
Resolution (Å)	20-1.7			
Data set	Lyso10	Lyso11	Lyso12	Lyso13
Wavelength (Å)	0.9188	0.9195	0.9198	0.9201
$R(I)$ merge[a] (%)	6.3 (23.1)[b]	5.0 (24.3)	3.9 (16.3)	3.5 (18.2)
$I/\sigma(I)$	46 (8.5)[b]	43 (6.5)	65 (14.6)	50 (9.9)
B. *Refinement*				
R factor (number of reflections)	0.21 (11,991)			
R_{free} (number of reflections)	0.29 (1318)			
Protein/solvent atom sites	1010/197 Wat+6 Br^-+1 Na^+			
rmsd from idealized geometry:				
Bond lengths (Å)	0.032			
Angle distances (Å)	0.053			
Chiral volumes (Å^3)	0.263			
Planarity (Å)	0.021			
DPI[c] (Å)	0.153			

[a] $R(I) = \Sigma|I_i - \langle I\rangle|/\Sigma\langle I\rangle$.
[b] Values in parentheses refer to the highest resolution shell, 1.73-1.70 Å.
[c] DPI, diffraction precision indicator according to Cruikshank (1999).

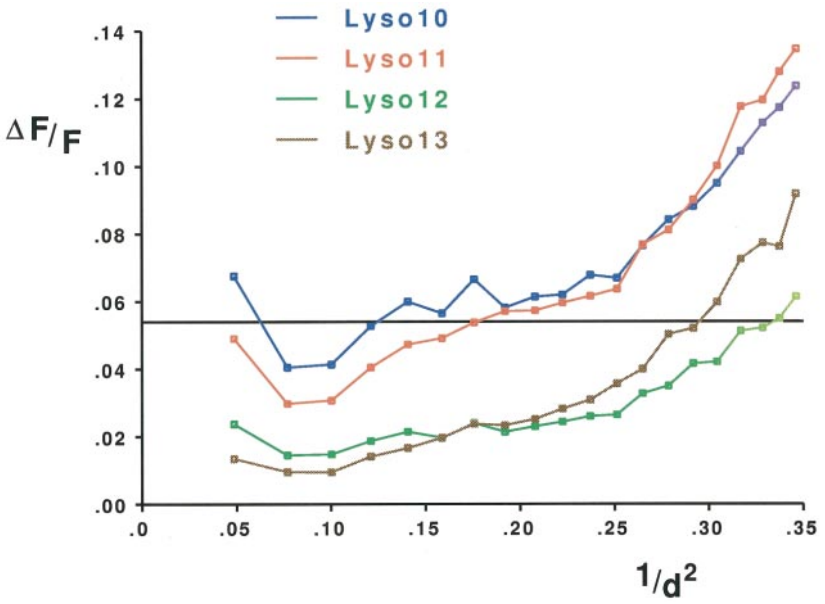


Figure 1. The $|\Delta F_{anom}|/F$ ratio for four data sets as a function of resolution.

Figure 1 shows the $\Delta F_{anom}/F$ ratio as a function of resolution for four data sets. The expected strength of an anomalous signal is given by the formula (Hendrickson & Teeter, 1981):

$$\Delta F/F = 2^{1/2}(N_A^{1/2}\delta f'')/(N_P^{1/2}Z_{eff})$$

where N_A is the number of anomalous scatterers (here six bromine ions with an $\delta f''$ value of 3.8 at the short wavelength side of the absorption edge) and N_P is the number of protein atoms (1001 in lysozyme) with the average scattering of Z_{eff} of 6.7 electrons. According to this formula the expected $\Delta F/F$ ratio for Lyso10 data is 0.054. Figure 1 shows that the anomalous signal in Lyso10 is close to that value in resolution ranges lower that 2 Å. At higher resolution the noise in both ΔF and F increases their ratio. As expected, the anomalous signal is smaller in Lyso11 (corresponding to the inflection point of the EXAFS spectrum) and negligible in Lyso12 and Lyso13 data.

The Lyso13 data set having a minimal anomalous signal was used to refine the lysozyme model. As a starting model the 8LYS coordinate set (Blake *et al.*, 1967) was used. All solvent water molecules were removed and the solvent structure was built-up automatically by ARP on the basis of electron-density maps calculated using weights from the maximum likelihood refinement program REFMAC.

At the early stage of refinement the phases and anomalous differences in the Lyso10 data were used to calculate the anomalous difference map (ΔF_{anom}, $\varphi_{calc} - 90°$), which clearly showed six peaks at the surface of the protein (Figure 2). The positions of those peaks corresponded to six of the eight chloride ions identified in the structure of the tetragonal lysozyme (Dauter *et al.*, 1999) and contained the four bromide sites found earlier (Lim *et al.*, 1998). The presence of bromide ions in only six out of eight halide sites occupied by chloride atoms in the structure of HEWL crystallized from NaCl (Dauter *et al.*, 1999) is a consequence of the different concentration of halide ions in the crystallization solution and differences in the ionic radii and coordination properties between Br^- and Cl^- ions.

The six bromide ions were included in the refinement of the Lyso13 model. *B* factors of some bromide ions refined to rather high values; this may reflect partial occupancies in the structure. Those bromide ions showed a lower signal in the anomalous difference map. The refinement converged with an *R* factor of 21 % and R_{free} of 29 %.

All anomalous differences within the data set Lyso10 were used in SHELXS without any discrimination, and the program was run with default parameters for 1000 phase sets five times with different resolution cut-offs: 1.7, 2.0, 2.5, 3.0 and 3.5 Å. Table 2A shows the frequency of resulting phase sets in ranges of combined figure-of-merit (CFOM). Up to a resolution of 3.0 Å there is a clear contrast between successfully phased sets with CFOM < 0.14 and the majority of phase sets leading to wrong solutions. At 3.5 Å there is no contrast, but still the phase set with the lowest CFOM gave a correct solution in terms of six bromide peaks, albeit less accurately positioned. Table 2B lists peaks in the best solution at each resolution. There is a clear correlation between the peak height and the *B* factor of the corresponding bromine atom resulting from the refinement of the lysozyme model, except for the pair Br204 and Br205, which consistently showed higher *E*-map density for the atom with the higher *B* factor.

The six bromide sites identified from the *E*-map were input to MLPHARE for refinement of their parameters and evaluation of protein phases. At each resolution (1.7, 2.0, 2.5 3.0 and 3.5 Å) two trials were performed, one using all four wavelength data sets and the other using only Lyso10

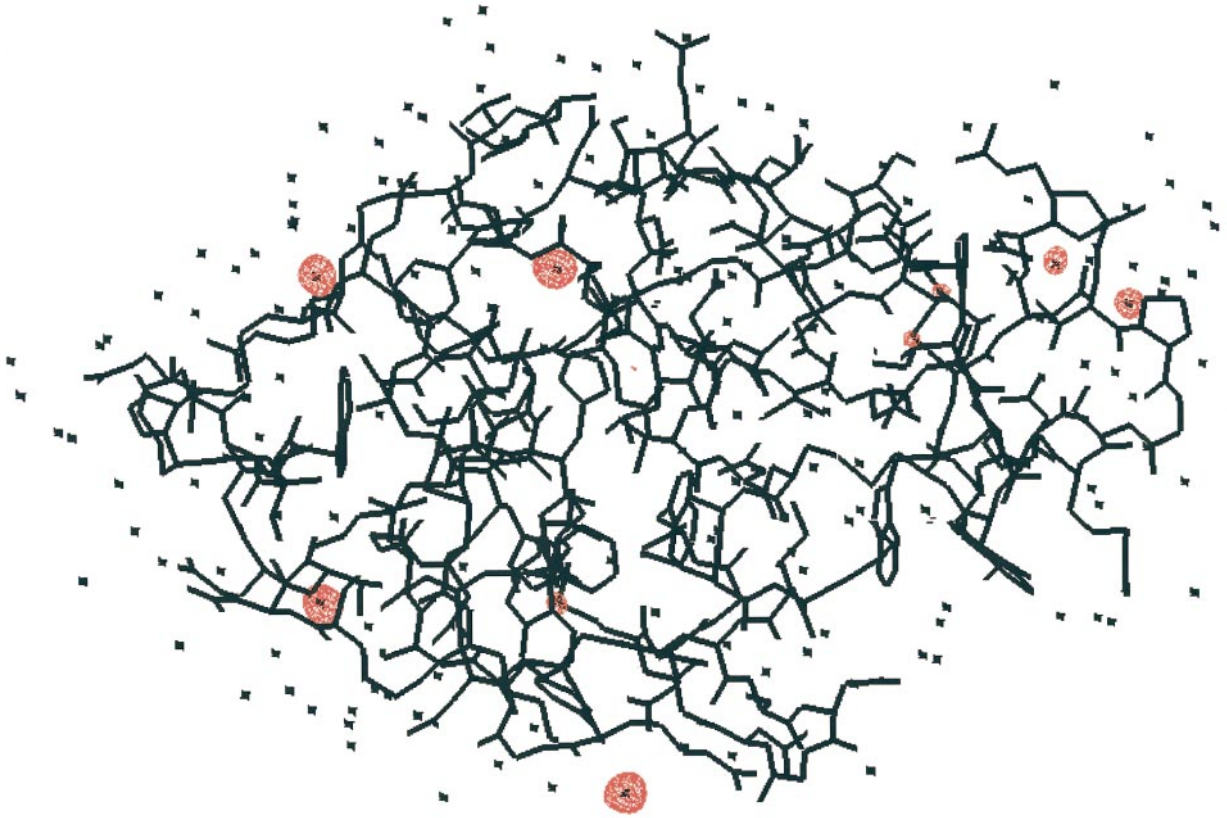


Figure 2. The anomalous difference map contoured at 5σ showing six bromide sites around the molecule of lysozyme. In addition, three sulfur atoms also show an anomalous signal at this level of the map.

data. In 4-λ calculations the atomic parameters were refined against an anomalous signal in Lyso10 and Lyso11 data and against a dispersive signal in Lyso12 and 13 data. In 1-λ calculations the parameters were refined only against an anomalous signal of Lyso10 data. Each MLPHARE phase set was subsequently input to the density modification by DM run in ''combine and omit''

Table 2. Results of SHELXS at different resolution

A. *Frequency of successful phase sets*

	1.7 Å	2.0 Å	2.5 Å	3.0 Å	3.5 Å
0.06-0.10	39	38	27	0	0
0.10-0.14	1	1	7	20	4
0.14-0.18	0	0	2	4	54
0.18-0.22	0	1	2	156	281
0.22-0.26	4	14	72	147	177
0.26-0.30	3	22	89	112	137
0.30-0.34	19	22	48	57	85
0.34-0.38	7	14	59	79	54
0.38-0.42	7	14	56	37	50
0.42-0.46	6	13	35	18	33
0.46-0.52	8	10	25	27	23
0.50-0.54	8	17	34	22	29
0.54-0.58	4	15	23	14	21
0.58-9.99	894	819	531	207	52

B. *Peak heights in* E*-maps (in arbitrary units) at different resolution.* B *factors are from refinement of Lyso13*

Atom	B(Å^2)	1.7 Å	2.0 Å	2.5 Å	3.0 Å	3.5 Å
Br201	23.0	230	224	223	215	176
Br202	24.5	176	186	188	190	195
Br203	26.4	166	177	166	159	155
Br204	30.7	109	123	125	120	110
Br205	34.9	117	126	147	165	157
Br206	46.8	69	75	81	88	103
First wrong		47	57	76	83	68

Table 3. Results of phasing at different resolution

Phased on resolution (Å)	Four wavelengths				One wavelength			
	FML	FDM	Δφ (°)	C.C.	FML	FDM	Δφ (°)	C.C.
1.7	0.48	0.79	42.9	0.87	0.34	0.79	43.4	0.89
2.0	0.57	0.79	45.9	0.82	0.42	0.79	45.9	0.82
2.5	0.62	0.78	48.8	0.72	0.48	0.77	47.4	0.76
3.0	0.65	0.77	50.1	0.69	0.48	0.75	50.5	0.71

FML is the figure-of-merit after MLPHARE and FDM figure-of-merit after DM. Δφ is the average difference of phases from DM and from the refined set. C.C. is the map correlation for the protein main-chain between the DM map and final F_{obs} map, both calculated at the appropriate resolution.

mode for 20 cycles. Results of phasing are summarized in Table 3.

Judging by figure-of-merit the results of 4-λ MLPHARE are considerably better than 1-λ. At this stage the additional dispersive signal improves the phasing process considerably. However, density modification is so powerful that it levels off the quality of output phase sets. There is very little difference in the quality criteria of the DM phases. Indeed, map correlation and average ($\varphi_{DM} - \varphi j_{CALC}$) phase difference is no worse for 1-λ phasing than for 4-λ (as seen in Figure 3(a) and (b). In general, up to a resolution of 3.0 Å the resulting electron-density maps were interpretable and at 1.7 and 2.0 Å their quality was outstanding, clearly showing almost all amino acid side-chains. Figure 3 illustrates the electron density for a region of the protein resulting from phasing at different resolution.

These results show that the density modification procedure becomes more powerful with increasing resolution. Beyond 2 Å it is capable of producing phase sets differing, on average, from the ''true'' phases by about 40°, leading to high quality electron-density maps even if the starting phases are relatively inaccurate.

Conclusions

Anomalous scatterers diffused in the solvent regions of macromolecular crystals have been used for evaluation of phases at low resolution, e.g. for the estimation of the protein envelope (Fourme *et al.*, 1995). If such atoms or ions adopt definite places at the protein surface, like bromide ions in the crystal structure of lysozyme, they can be successfully utilized for phasing of reflections at high resolution, leading to the solution of crystal structure *via* MAD or SAD methods. In the crystallization of macromolecules the frequently used reagents, such as chlorides, sulfates, and salts of calcium or sodium, can be replaced by heavier analogues, like bromides, selenates, salts of strontium or rubidium. Those elements have easily accessible X-ray absorption edges with about four anomalous electrons per atom; with several such scatterers present in the crystal structure they may provide sufficient phasing power to solve the unknown structures.

The solvent atoms, even in the first hydration shell, usually have somewhat higher temperature factors than most of protein atoms, hampering the phasing power of such anomalous scatterers. On the other hand, selenomethionine residues also often display high mobility or disorder.

It can be expected that methods of phasing based on incorporation of anomalous scatterers within the solvent will gain popularity thanks to the minimal technical complications involved. Solvent anomalous scatterers should only negligibly perturb the structure of the protein, much less than the incorporation of heavy atoms or even substitution of sulfur by selenium in methionine residues.

The phasing and phase modification algorithms implemented in several available programs have become so powerful that it may often be sufficient to collect only one wavelength data set containing maximized anomalous signal instead of several wavelength data sets, as in the MAD approach. If a crystal diffracts strongly enough, it may be more beneficial to enhance the quality of the data increasing the multiplicity of measurements by collecting more images rather than collecting less redundant data at several wavelengths. In addition, such an approach alleviates the need to use precisely selected wavelength points on the fluorescence spectrum, it is enough to maximize the $\delta f''$ signal at the low-wavelength side of the absorption edge.

Materials and Methods

Lysozyme from Sigma was crystallized in hanging drops. The crystallization solution consisted of 15 mg/ml of protein, 0.5 M NaBr in 0.1 M sodium acetate buffer (pH 4.6) and the well solution was 1 M NaBr in 0.1 M sodium acetate buffer. For X-ray data collection a crystal was transferred for a few seconds to the mother liquor containing in addition 30% (v/v) glycerol and quickly frozen in the stream of cold nitrogen gas at 100 K in the fiber loop.

A single crystal specimen was used for collecting data at four wavelengths near the bromine Kα absorption edge at the beamline X9B at NSLS, Brookhaven National Laboratory. The Mar345 imaging plate scanner was used in its 180 mm and 150 μm pixel mode. The crystal was rotated approximately about its (101) axis. Two passes were performed for each wavelength, first at 1.7 Å with 1° rotation and two minutes exposure and second at

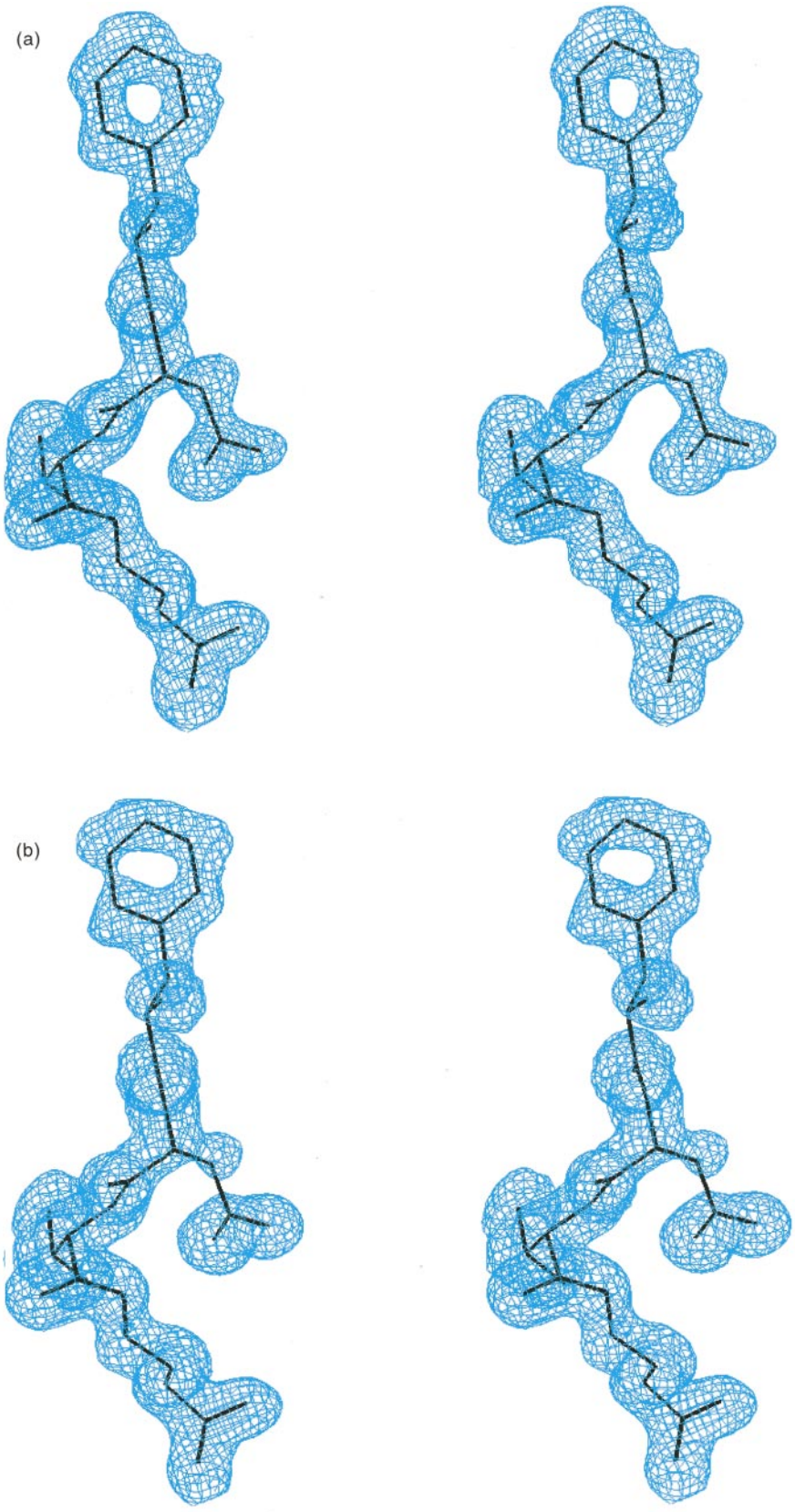
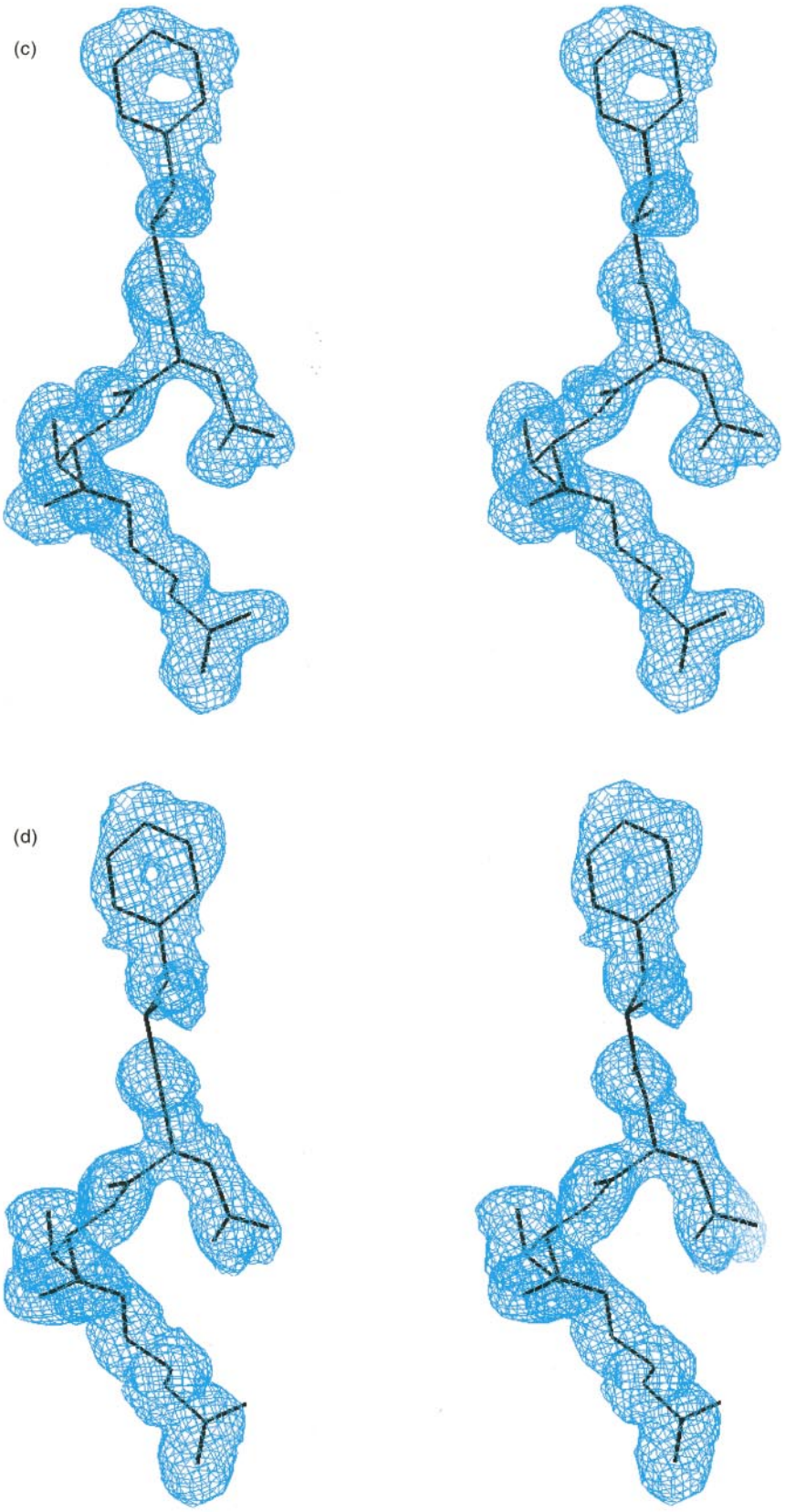


Figure 3. (*Legend on page 100*)



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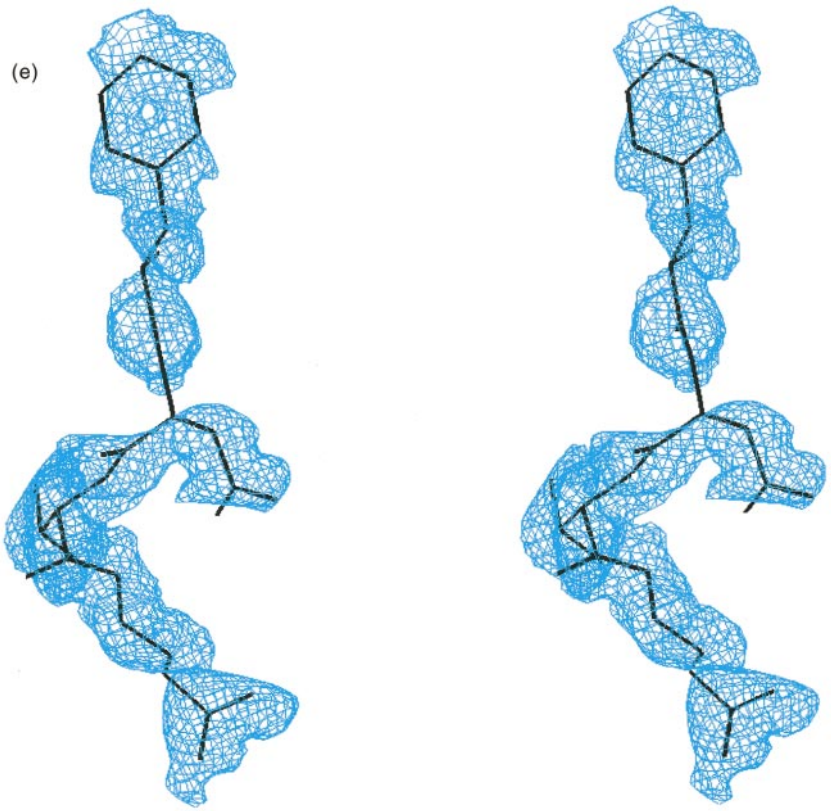

Figure 3. The electron-density synthesis, at the 1σ level, resulting from different phasing trials: (a) four-wavelength data at 1.7 Å resolution; (b), (c), (d) and (e) one wavelength data and a resolution limit of 1.7, 2.0, 2.5 and 3.0 Å, respectively.

2.7 Å with 1.5° rotation and 20 second exposure per image. Each pass consisted of about 90° of total rotation. All four data sets were more than 99.5% complete. The intensities were integrated, scaled and merged with the HKL2000 system (Otwinowski & Minor, 1997) equipped with the graphics interface. Intensities were transformed into amplitudes using TRUNCATE (French & Wilson, 1978). The data collection is summarized in Table 1.

All measured anomalous differences from the Lyso10 data set having a maximal $\delta f''$ contribution were used for solving the partial structure of anomalous scatterers by the classic direct methods program SHELXS (Sheldrick, 1986) at different resolution cut-off limits.

All subsequent calculations were done using programs from the CCP4 suite (CCP4, 1994). The four data sets were put on a common scale by SCALEIT and all phasing trials were performed by MLPHARE (Otwinowski, 1991), and the density modification program DM (Cowtan, 1994) used in the ''combine & omit'' mode. The solvent content was set to 40%, employing the solvent flattening and histogram matching options, with all reflections included in all phase refinement cycles. The data set Lyso13, with a minimal $\delta f''$ contribution was used to refine the lysozyme model to obtain final phases for comparison with those calculated by different phasing options. The refinement was performed with REFMAC (Murshudov *et al.*, 1997) combined with a selection of solvent water molecules by ARP (Lamzin & Wilson, 1997). For inspection of electron-density maps the program QUANTA (Molecular Simulations Inc., San Diego) was used as well as for producing electron-density Figures.

Brookhaven Protein Data Bank accession codes

The final atomic coordinates and all four sets of amplitudes collected at different wavelengths have been deposited at Brookhaven Protein Data Bank, entry codes 1LZ9 and R1LZ9SF.

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