Combining solution wide-angle X-ray scattering and crystallography: determination of molecular envelope and heavy-atom sites

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Solving the phase problem remains central to crystallographic structure determination. A six-dimensional search method of molecular replacement (*FSEARCH*) can be used to locate a low-resolution molecular envelope determined from small-angle X-ray scattering (SAXS) within the crystallographic unit cell. This method has now been applied using the higher-resolution envelope provided by combining SAXS and WAXS (wide-angle X-ray scattering) data. The method was tested on horse hemoglobin, using the most probable model selected from a set of a dozen bead models constructed from SAXS/WAXS data using the program *GASBOR* at 5 Å resolution ($q_{\max} = 1.25 \text{ \AA}^{-1}$) to phase a set of single-crystal diffraction data. It was found that inclusion of WAXS data is essential for correctly locating the molecular envelope in the crystal unit cell, as well as for locating heavy-atom sites. An anomalous difference map was calculated using phases out to 8 Å resolution from the correctly positioned envelope; four distinct peaks at the 3.2σ level were identified, which agree well with the four iron sites of the known structure (Protein Data Bank code 1ns9). In contrast, no peaks could be found close to the iron sites if the molecular envelope was constructed using the data from SAXS alone ($q_{\max} = 0.25 \text{ \AA}^{-1}$). The initial phases can be used as a starting point for a variety of phase-extension techniques, successful application of which will result in complete phasing of a crystallographic data set and determination of the internal structure of a macromolecule to atomic resolution. It is anticipated that the combination of *FSEARCH* and WAXS techniques will facilitate the initial structure determination of proteins and provide a good foundation for further structure refinement.

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1. Introduction

X-ray crystallography is the foremost technique for determining the structures of biomolecules. Once a crystal is obtained, solving the phase problem is the greatest hurdle for crystallographic structure determination (Shen *et al.*, 2006). Powerful techniques have been developed for recovering phase information in the past few decades. The traditional phasing methods are multiple or single isomorphous replacement (MIR or SIR), usually with anomalous scattering and molecular replacement. With the advances in synchrotron radiation techniques, the multiple- and single-wavelength anomalous dispersion methods (MAD and SAD) have become increasingly popular, but these often require the incorporation of selenium into protein crystals by using selenomethionine during the expression of the protein. If the structure of a homologous protein is available, molecular replacement is commonly used for initial phase determination,

by placing a known model molecule into the crystal unit cell of the target homologous molecule. However, there are numerous proteins of interest that either cannot be produced in bacteria, a process that is often required to incorporate selenomethionine, or have no homologous known structure (Hao, 2006).

Development of phasing methods that do not require either the production of proteins with heavy atoms or knowledge of homologous protein structures would provide an attractive alternative path to structure solution. Considerable effort has been devoted to exploring new low-resolution phasing methods which do not require the presence of heavy atoms (see reviews by Lunin *et al.*, 2002; Urzhumtsev & Podjarny, 1995; Rabinovich & Shakked, 1984; Yu Lunin *et al.*, 1995; Hauptman *et al.*, 2002). Among them, an envelope-based phasing method that utilizes the low-resolution envelope determined by solution small-angle X-ray scattering (SAXS) of macromolecules has shown promising results (Hao *et al.*, 1999; Ockwell *et al.*, 2000; Hao, 2001). The CCP4-supported

computer program named *FSEARCH* (Hao *et al.*, 1999) was developed for obtaining a molecular-replacement solution by performing a simultaneous six-dimensional search on orientation and translation using a pre-determined envelope from SAXS, electron microscopy images or the coordinates of a homologous structure, to find the best match between experimental structure factors, F_{obs} , and calculated structure factors, F_{calc} .

In this method, the molecular envelope determined by SAXS data is described in terms of relatively low coefficients of spherical harmonics (L less than 7). The method was first tested by locating the molecular envelope within the crystallographic unit cell using experimental diffraction data from nitrite reductase and superoxide dismutase by a four-dimensional search with the aid of non-crystallographic symmetry (Hao *et al.*, 1999; Ockwell *et al.*, 2000). An exhaustive six-dimensional search was later reported using an envelope constructed from a Protein Data Bank (PDB) file to phase very low resolution X-ray data (Liu *et al.*, 2003). This envelope phasing is a promising technique that does not require the presence of incorporated atoms; it instead utilizes the low-resolution envelope of the protein.

It is obvious that, once an envelope is properly located in the unit cell, phase extension to higher resolution is essential to make use of all the diffraction data. This process is challenging and would clearly benefit from the highest possible resolution of the initial envelope model. Measurement of X-ray scattering from solution, including small-angle and wide-angle X-ray scattering (SAXS/WAXS), has proven to be a fundamental tool in the study of the low-resolution structure, and conformational changes in response to external conditions, for proteins and other biological macromolecules in near physiological environments (for reviews see Trehwella, 1997; Koch *et al.*, 2003). In addition to a molecular envelope described in terms of spherical harmonics (Svergun & Stuhmann, 1991; Svergun *et al.*, 1996), a more detailed three-dimensional bead model can now be reliably obtained, and domain structure can be determined, from a one-dimensional scattering profile (Barone *et al.*, 1999; Svergun *et al.*, 2001). Detailed modeling of macromolecular complexes can also be carried out, using rigid body refinement (Petoukhov & Svergun, 2005). WAXS has the potential to provide higher-resolution structural information for disordered and partially ordered systems. Significant progress in solution scattering has substantially improved the resolution and reliability of the structural models.

In this paper, we report an investigation of using the *FSEARCH* program with WAXS data to correctly position a molecular envelope represented by a dummy bead model in the crystallographic unit cell. It is found that inclusion of wide-angle X-ray scattering data is essential for correctly locating the low-resolution bead model in the crystallographic unit cell, as well as for locating heavy-atom sites. The initial phases obtained can serve as a starting model for a variety of phase-extension techniques, which can ultimately result in determination of the internal structure of a macromolecule to crystallographic data resolution.

2. Experiment and methods

SAXS data were collected from horse hemoglobin solution at 277 K at the Cornell High Energy Synchrotron Source (CHESS) G1 Station, Ithaca, NY, USA. WAXS data were collected at the F1 beamline at CHESS using a setup designed and tested to ameliorate radiation damage for several test proteins including bovine hemoglobin up to $q = 2.5 \text{ \AA}^{-1}$. Full experimental details have been published elsewhere (Hong & Hao, 2009); the method is briefly described here.

In a solution scattering experiment, protein aggregation results from radiation damage induced by synchrotron radiation, which can easily break down the monodisperse assumption. The accumulation of aggregates mostly interferes with the small-angle region, where the size and shape information of the target protein resides. Obviously, accurate molecular size and shape are essential for the success of envelope-based phasing. We have tackled the problem of radiation damage by measuring solution scattering using a two-dimensional scan data collection mode with a small stationary cell at low temperature (277 K) (Hong & Hao, 2009). Equine hemoglobin was dissolved to 30 mg ml^{-1} , and the protein samples were centrifuged through a centrifugal device at $10\,000 \text{ r min}^{-1}$ for 10 min; the upper clear liquids were used for the scattering experiment. The low-angle data were extrapolated to infinite dilution and merged with the higher-angle data measured at high protein concentrations to yield final SAXS scattering curves. The obtained SAXS and WAXS data were merged into a full scattering curve for molecular-shape generation.

Dummy residue modeling was performed from the experimental scattering pattern using the *GASBOR* program (Svergun *et al.*, 2001), which is a versatile *ab initio* approach and can be used with high scattering vectors. The molecular shape was determined using scattering vectors up to $q_{\text{max}} = 1.25$ and $0.25 \text{ \AA}^{-1}$ for SAXS/WAXS and SAXS-only data, respectively, within a spherical search diameter of $D_{\text{max}} = 65 \text{ \AA}$, with no symmetry constraints. *GASBOR* searches for the best model configuration, minimizing the discrepancy function, $f(X) = \chi^2 + \alpha P(X)$, between the calculated and experimental curves using the simulated annealing method. $\alpha P(X)$ is a looseness penalty with positive weight $\alpha > 0$, and χ is the discrepancy between the experimental $I_{\text{exp}}(q_j)$ and the calculated $I_{\text{calc}}(q_j)$ curves:

$$\chi^2 = \frac{1}{N-1} \sum_j \left[\frac{I_{\text{exp}}(q_j) - c I_{\text{calc}}(q_j)}{\sigma(q_j)} \right]^2, \quad (1)$$

where N is the number of experimental points, c is a scaling factor and $\sigma(q_j)$ is the experimental error at the momentum transfer q_j . A shape model of dummy residues was generated by a random-walk CR chain and was folded in such a way as to minimize the discrepancy between the calculated scattering curve and the experimental data. *GASBOR* uses a simulated annealing approach to find a chain-compatible spatial distribution of dummy residues that corresponds to the distribution of C α atoms of amino acid residues.

The uniqueness and stability of the generated shapes were checked by repeating modeling. A dozen independent models produced consistent results, as all output models yielded nearly identical scattering patterns, with a discrepancy of $\chi = 1.6$ (1) (Fig. 1, black line) and $\chi = 1.2$ (1) (Fig. 1, green line) from the respective corrected experimental data obtained by subtracting a constant in the scattering range up to 1.25 and 0.25 Å⁻¹, respectively. These final models were aligned and averaged using *SUPCOMB* (Kozin & Svergun, 2001), and *DAMSEL*, in the *DAMAVAR* package (Volkov & Svergun, 2003), was employed to align all of the possible pairs of models and to identify the most probable model, which should yield the smallest average discrepancy.

Crystals of horse hemoglobin were obtained using the vapor-diffusion method. The best crystals, up to 1.0 mm in their maximum dimension, were found at the Index F3 of the crystallization screen (Hampton Research) [5%(v/v) Tacsimate pH 7.0; 0.1 M HEPES pH 7.0 buffer; precipitant: polyethylene glycol monomethyl ether 5000]. SAD data on horse hemoglobin were collected at the Fe *K*-edge (peak) on MacCHESS F2 station to 2.1 Å resolution. Data reduction and scaling were performed with *HKL-2000* (Otwinowski *et al.*, 1997). One crystalline form of horse hemoglobin has space group *C2*, with unit-cell parameters $a = 106.99$, $b = 62.64$, $c = 53.73$ Å, $\alpha = 90$, $\beta = 111.99$, $\gamma = 90^\circ$. The low-resolution limit was 53 Å. The data have a completeness of 93% to 2.1 Å and 79% to 9 Å. No solution for heavy-atom sites could be found with *SAPI*, a program to locate heavy-atom sites by direct methods (Hao *et al.*, 2003), possibly as a result of the low average redundancy (3.6) of the SAD data.

To utilize the molecular-envelope information, one must determine the orientation and position of the envelope in a crystallographic unit cell. Unlike a Patterson-function-based

Table 1

The positions with lowest *R* values found in the real-space search.

The most probable model was constructed using the program *GASBOR* at 25 Å resolution ($q_{\max} = 0.25$ Å⁻¹).

(a) Initial five-dimensional (α , β , γ , x and z) search within the whole unit cell with 5° steps in α , β and γ and 1 Å steps in x , y and z .

α	β	γ	x	y	z	<i>R</i>
125	300	245	6	0	45	0.476
125	300	245	16	0	20	0.477
145	285	245	43	0	45	0.486
35	230	340	101	0	13	0.486
35	230	340	48	0	13	0.486

(b) Finer search around the solution found from the initial search with 0.5° steps in α , β and γ and 0.5 Å steps in x , y and z .

α	β	γ	x	y	z	<i>R</i>
124.5	300.5	245.5	6	0	45.0	0.470
125.0	300.5	245.5	16	0	20	0.471
124.5	300.5	245.5	16	0	20	0.471
125.0	300.5	245.5	6	0	44.5	0.473
125.0	300.5	245.5	6	0	45	0.475

molecular-replacement method (Rossmann & Blow, 1962), *FSEARCH* (Hao, 2001) can perform a direct real-space search on orientation and translation to find the best match between experimental structure factors and those calculated from a presumed location of the molecular envelope in the unit cell. A modified version of the *FSEARCH* program for running multiple jobs on a computer cluster, or on a multiple-thread machine, was employed to perform simultaneous rotational and translational searches within the unit cell.

3. Results and discussion

To investigate the difference between the SAXS model and the SAXS/WAXS model, a theoretical scattering profile (Fig. 1, blue dotted line) of the most probable model (MPM) based on the SAXS data alone at $q \leq 0.25$ Å⁻¹ was calculated using *CRY SOL* (Svergun *et al.*, 1995). It can be seen that, except in the fitted SAXS range, the theoretical scattering profile is quite different from the experimental WAXS data, *i.e.* unrepresentative of the actual intramolecular structure.

The SAXS model and the SAXS/WAXS model were further examined by superimposing them on the high-resolution crystallographic structure (PDB code 1ns9; Robinson *et al.*, 2003) using the option 'Align PDB' of the *MASSHA* program (Konarev *et al.*, 2001), which invokes the program *SUPCOMB* (Kozin & Svergun, 2001). The SAXS/WAXS model resulted in a smaller spatial discrepancy (1.289) than the SAXS model (1.386) against the crystallographic structure.

Owing to the *C2* symmetry, the y value could be set to zero. For the SAXS model using $q_{\max} = 0.25$ Å⁻¹, the five-dimensional search within the unit cell using crystallographic data (53–9 Å) produced a rather poorly defined solution: $\alpha = 124.5$, $\beta = 300.5$, $\gamma = 245.5^\circ$, $x = 6$, $y = 0$, $z = 45$ Å. Details are shown in Table 1. For the SAXS/WAXS model using $q_{\max} = 1.25$ Å⁻¹, the five-dimensional search within the unit cell, using the same

Figure 1

X-ray-scattering patterns of horse hemoglobin in solution (filled circles), and calculated scattering patterns from the reconstructed models: *GASBOR* shape model using scattering vectors, q , up to 1.25 Å⁻¹ (5 Å resolution, red line), and up to 0.25 Å⁻¹ (25 Å resolution, green line). A theoretical scattering profile (blue dotted line) was calculated using *CRY SOL* from the most probable model (MPM) based on the SAXS data alone at $q \leq 0.25$ Å⁻¹.

Table 2

The positions with lowest R values found in the real-space search.
The most probable model was constructed using the program *GASBOR* at 5 Å resolution ($q_{\text{max}} = 1.25 \text{ \AA}^{-1}$).

(a) Initial five-dimensional (α, β, γ, x and z) search within the whole unit cell with 5° steps in α, β and γ and 1 Å steps in x, y and z .

α	β	γ	x	y	z	R
115	220	295	75	0	2	0.462
115	220	295	65	0	27	0.463
115	220	295	11	0	27	0.464
115	220	295	21	0	2	0.465

(b) Finer search around the solution found from the initial search with 1° steps in α, β and γ and 1 Å steps in x, y and z .

α	β	γ	x	y	z	R
112	221	292	75	0	2	0.446
112	221	292	75	0	1	0.454
112	221	292	76	0	2	0.456
113	221	292	75	0	2	0.456

Table 3

Average phase errors of the *FSEARCH* results against the crystal structure (Robinson *et al.*, 2003) as a function of resolution.

Resolution range (Å)	Phase error of SAXS model (°)	Phase error of SAXS/WAXS model (°)
14	111.8	59.8
12	99.2	62.3
10	96.0	70.0
8	94.0	77.5

crystallographic data (53–9 Å), produced a clear solution: $\alpha = 112, \beta = 221, \gamma = 292^\circ, x = 75, y = 0, z = 2 \text{ \AA}$. Details are shown in Table 2. *FSEARCH* also calculates the amount of overlap between symmetry-related copies of the search model. In this solution, there is no overlap. It should be noted that the search result for the SAXS/WAXS model exhibits a lower R factor than that for the SAXS model in the same resolution range, which indicates that the model constructed with the WAXS data possesses better internal mass distribution than that based on the data of the SAXS portion only.

The crystal structure of horse hemoglobin was originally solved by Perutz and co-workers many years ago (Boyes-Watson *et al.*, 1947; Perutz, 1954). A recent structure model at pH 7.1 was used as the standard model (PDB code 1ns9; Robinson *et al.*, 2003) for map comparisons. The SAXS and SAXS/WAXS models were transformed to their respective new positions and orientations using *PDBSET* from the *CCP4* package (Potterton *et al.*, 2003). Fig. 2(a) shows the SAXS model in the unit cell, together with the symmetry-related molecules of the crystal structure generated using *COOT* (Emsley & Cowtan, 2004). A considerable portion of the SAXS model is found to overlap with adjacent symmetry-related molecules, indicating that this solution of *FSEARCH* is incorrect. Indeed, the phase error of this solution against the crystal structure is random (see column 2 in Table 3). Figs. 2(b) and 2(c) show the SAXS/WAXS model. This molecular envelope provides excellent agreement with the crystal structure (Robinson *et al.*, 2003), with a respectable phase error up to 8 Å resolution (see column 3 in Table 3). Note that all the commonly used molecular-replacement methods based on the Patterson function require discrimination between intermolecular vectors and intramolecular vectors to match the diffraction data and have difficulty handling models derived from solution

Figure 2
Location of the generated molecular envelope in the crystal unit cell of horse hemoglobin. The molecular-replacement solution was found with *FSEARCH* for a model using scattering vectors (a) out to $0.25 \text{ \AA}^{-1}$ and (b) out to $1.25 \text{ \AA}^{-1}$. The latter solution is viewed at three orthogonal orientations in (c). In (a) and (b), the SAXS and SAXS/WAXS models are represented by cyan balls, with adjacent symmetry-related molecules of the crystal structure (Robinson *et al.*, 2003), generated using the *COOT* software (Emsley & Cowtan, 2004), shown as chains. In (c), the bead model for the molecular envelope is shown as spheres of various colors, and the crystal structure is shown as a yellow ribbon.

scattering or electron microscopy because of the low resolution of the models (Hao, 2006). Even for a search model derived from a known PDB structure, conventional molecular replacement may not be able to yield correct solutions when the diffraction data resolution is lower than 10 Å (Liu *et al.*, 2003).

The result shown in Fig. 2 illustrates that wide-angle X-ray scattering data, in this case, are essential for correctly locating the low-resolution shape model of bead ensembles in the crystallographic unit cell, mainly because the internal mass distribution inside the molecular shape can be optimized by the WAXS data. This is also true for locating heavy-atom sites. The phases of the correctly located envelope were calculated in the resolution range from 53 to 8 Å using *SFALL* of the *CCP4* package (Potterton *et al.*, 2003). A phased anomalous difference map was generated using *FFT* of *CCP4* (Potterton *et al.*, 2003). At a map level of 3.2 σ , four distinct peaks (yellow

balls) can be identified, which agree well (r.m.s. deviation 5.3 Å) with the four iron sites of the known crystal structure (PDB code 1ns9; Robinson *et al.*, 2003), as shown in Fig. 3. In contrast, the peaks (magenta balls) found using the SAXS model at 25 Å resolution ($q_{\max} = 0.25 \text{ \AA}^{-1}$) are far from the known iron sites (r.m.s. deviation 15.9 Å). To test the reproducibility, an averaged model, which was obtained by averaging all 12 independent models with *DAMAVAR*, was employed as the search model. A solution of $\alpha = 200$, $\beta = 140$, $\gamma = 115^\circ$, $x = 103$, $y = 0$, $z = -1 \text{ \AA}$ was obtained by using *FSEARCH*. At a map level of 3.4 σ , four distinct peaks (Fig. 3, green balls) appear and they also agree well with the iron sites of the known crystal structure.

To further test the reproducibility, another set of 16 independent reconstructions was generated with SAXS/WAXS data. This provided another independent MPM and average models. With the same search strategy, the four distinct peaks of the MPM model are somewhat distant from the known iron sites. However, for the averaged model, an *FSEARCH* solution of $\alpha = 111$, $\beta = 243$, $\gamma = 328^\circ$, $x = 106$, $y = 0$, $z = 17 \text{ \AA}$ was obtained. At a map level of 3.2 σ , the distinct peaks inside the molecule (Fig. 3, white balls) appear in the vicinity of the known iron sites of the crystal structure. It was found that the *FSEARCH* result of the new MPM model was trapped in a local minimum. It is therefore suggested that exhaustive six-dimensional searches with different bead ensembles or at different resolutions would be helpful for the identification of a correct *FSEARCH* solution.

4. Conclusion

We have demonstrated that the molecular envelope determined from SAXS/WAXS solution scattering can be used to locate the heavy-atom sites in the crystallographic unit cell. The bead model constructed with the WAXS data possesses better internal mass distribution than that based on the data of the SAXS portion only. It is found that the WAXS data are essential for correctly locating the low-resolution bead model in the crystallographic unit cell, as well as for locating heavy-atom sites. The initial phase information calculated from the correctly positioned molecular shape can be used for further SAD phasing or serve as a starting model for a variety of phase-extension techniques, such as maximum-entropy and density-modification methods (Hao, 2006). The method has worked for hemoglobin but it will need to be further tested with larger or less well behaved proteins before it can be used as a general phasing technique. It is anticipated that the combination of *FSEARCH* and WAXS techniques will facilitate the initial structure determination of proteins and provide a good foundation for further structure refinement.

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Figure 3

Phased anomalous difference maps calculated from the *FSEARCH*-positioned molecular envelope. Yellow balls represent the peaks identified at the 3.2 σ level of the map calculated from the SAXS/WAXS model using scattering vectors out to 1.25 Å⁻¹. Green balls are the peaks of the average model identified at the 3.4 σ level. White balls are the peaks of a new average model obtained from another set of 16 SAXS/WAXS models at the 3.2 σ level; they are close to the four iron sites of the crystal structure located at the center of the pyrrolic rings of the porphyrin (red balls). Magenta balls are peaks at the 3.0 σ level found on the map calculated from the SAXS-only model using scattering vectors to 0.25 Å⁻¹.

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