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Use of Single Isomorphous Replacement Data of Proteins – Resolving the Phase Ambiguity and a New Procedure for Phase Extension

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Abstract

A procedure combining direct methods and solvent flattening to break the phase ambiguity intrinsic to the single isomorphous replacement (SIR) technique has been tested with the experimental SIR data of the known protein RNase Sa at 2.5 Å resolution. The use of direct methods provided better initial phases for the solvent-flattening procedure, while the solvent-flattening procedure greatly improved direct-method phases leading to a traceable Fourier map. A small subset of known phases at low resolution makes direct phasing of SIR data much easier. Accordingly a method for extending low-resolution phases to high-resolution ones is proposed making use of additional SIR information. This reduces the problem of finding a value in the range of 0–2π for each unknown phase to that of just making a choice between two possible values. Tests with the known protein RNase Sa showed that the method is able to extend phases from a resolution of 6 to 2.5 Å leading to an easily traceable Fourier map. The solvent-flattening technique and the combination of which with direct methods were used for the phase extension. Either procedure yielded reasonably good results, but on the whole, the result from the combination of direct methods with solvent flattening is better. Results of the latter procedure were further compared with that from direct phasing of the 2.5 Å SIR data and with that from phase extension by solvent flattening without SIR information. An improvement gained by the use of SIR information is evident.

1. Introduction

Many procedures have been proposed to use single isomorphous replacement (SIR) data from proteins. The main problem encountered is how to resolve the intrinsic phase ambiguity. Among existing methods the solvent-flattening technique (Wang, 1981, 1985) is the most efficient in practice. On the other hand, different kinds of direct methods have been proposed to break the phase ambiguity. Coulter (1965) attempted to use the tangent formula. Fan (1965*a*,*b*) and similarly Karle (1966) suggested the use of 'component relation'. Hendrickson (1971) tried to break the ambiguity by multiplying the bimodal SIR distribution with the distribution of three-phase structure invariants given by Cochran (1955). Hauptman (1982) integrated direct methods with SIR data. Tests with error-free data showed that the method is capable of reliably identifying a large number of three-phase structure invariants. Improved procedures have been reported by Fortier, Moore & Fraser (1985); Klop, Krabbendam & Kroon (1987); Hao & Fan (1988); and Kyriakidis, Peschar & Schenk (1993). However, in the above publications no test results were provided with experimental protein diffraction data. The first successful application of direct methods to experimental SIR data of a known protein was carried out by Fan & Wang (1988) [see also partial results quoted in Woolfson & Fan (1995)] based on the method of Fan & Gu (1985), which is an improvement and generalization of the method of Fan (1965*a*,*b*). Giacovazzo, Siliqi & Zanotti (1995) obtained an interpretable Fourier map from a set of experimental SIR data of a known protein. Their approach was based on that of Giacovazzo, Cascarano & Zheng (1988), which is related to the method of Hauptman (1982). Langs, Guo & Hauptman (1995) applied Hauptman's method separately to three sets of experimental SIR data from a common native protein and then the three resultant Fourier maps were merged yielding a result better than the traditional MIR map. In this paper we present a method combining the direct method of Fan & Gu (1985) and the solvent-flattening technique of Wang (1981, 1985). Tests on experimental SIR data from a known protein of moderate size showed that the combination of direct methods and solvent flattening led to a better result than that obtained by solvent flattening alone.

During our test on breaking the SIR phase ambiguity by direct methods, we found that a small subset of known phases at low resolution could greatly strengthen the power of direct-method phasing. This led to the proposal of a new procedure for extending phases from low-resolution data to high-resolution data. Such a procedure might be important in protein crystallography. In the early stage of solving protein structures, diffraction phases are often obtained at

relatively low resolution. Phase extension is, therefore, an important part of the whole process. In principle, phase extension can be achieved either by starting with a structural model or by starting with a set of initial phases. The former procedure is more reliable and accurate, if the structural model is essentially correct. However, the Fourier map phased by multiple isomorphous replacement (MIR) may not be at a high enough resolution for building up a reliable structural model. It is, thus, important to extend phases before interpretation is attempted. To do this, phase extension from a set of initial phases is necessary. Early examples of extending protein phases without relying on a structural model involved different kinds of direct methods, such as the least-squares refinement procedure based on the Sayre equation (Sayre, 1974), the use of Karle–Hauptman determinants (de Rango, Mauguen & Tsoucaris, 1975*a*,*b*) and the use of a modified tangent formula (Blundell, Pitts, Tickle, Wood & Wu, 1981). Later the solvent-flattening technique (Wang, 1981, 1985) was successfully used in the determination of the protein troponin C for extending phases from 3.8 to 3.0 Å leading to a complete solution of the structure (Sundaralingam, Bergstrom, Strasburg, Rao, Roychowdhury, Greaser & Wang, 1985). More recently, histogram-matching and maximum-entropy methods have also been used for phase extension of proteins. In practice, both methods were incorporated with the solvent-flattening technique to improve their efficiency (Zhang & Main, 1990*a*,*b*; Xiang, Carter, Bricogne & Gilmore, 1994). Our procedure differs from all the above in that it makes use of additional information from single isomorphous replacement (SIR). Yet our method could easily be combined with others. Hence, it is not intending to replace but rather to strengthen existing methods. The philosophy behind using SIR data in phase extension is as follows. In the early stage of solving unknown proteins by the MIR technique, it is often found that good-quality MIR phases are only available at low resolution, say up to 4 or even 6 Å. It is also often true that while the MIR technique yields only low-resolution phases, crystals of the native protein and one of the heavy-atom derivatives may well diffract to much higher resolution. Even so, such SIR information is not used until at least one more good-quality isomorphous derivative is prepared to complete a new set of MIR data at higher resolution. This is owing to the difficulty in resolving the phase ambiguity intrinsic to SIR data. However, it is desirable to have some way to use SIR information directly so as to avoid the preparation of additional heavy-atom derivatives. One way to do this is to find methods for direct phasing of SIR data. This will be treated in the first part of the present paper. On the other hand, when a set of low-resolution phases are already known, it is preferable to use all the available information, low-resolution known phases and high-resolution SIR doublets. This is why the use of SIR information in phase extension is possible and necessary. Our method of phase extension can be regarded as a procedure for breaking SIR phase ambiguity based on a set of known phases. This makes the phasing procedure much more powerful. The method can also be regarded as a procedure of phase extension using SIR information. The consequence of this is that each unknown phase will take one of the two possible values rather than range from 0 to 2π and, thus, the phasing process will be greatly simplified.

2. Methods

2.1. *Solvent flattening*

The solvent-flattening technique we used in the test is a standard approach and implemented *via* programs in the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). The solvent content was assumed to be 50% in the present test. When a stable mask is obtained during the iteration, this mask will be applied back to the original Fourier map calculated with averaged SIR phases and then the iteration will be repeated until it converges to a new stable mask. By doing this, it is often possible to gain additional improvement on the final phases.

2.2. *Direct-methods phasing*

The phase ambiguity inherent in SIR data is expressed as,

$$\varphi_H = \varphi'_H \pm |\Delta\varphi_H|, \qquad (1)$$

where φ_H denotes the phase of structure factors of the native protein, φ'_H is the phase contributed from the replacing atoms and $\Delta\varphi_H$ is the phase difference between φ_H and φ'_H. (1) reduces the phase problem of finding a value in the range of 0–2π to a sign problem of making a choice between $+1$ and -1. However, it needs the *a priori* knowledge of the replacing-atom substructure. In order to simplify the phasing procedure it is worth doing this to find first the replacing-atom sites, which is just routine work in protein crystallography. The absolute value of $\Delta\varphi_H$ is calculated from,

$$\cos\Delta\varphi_H = (F_{H,D}^2 - F_{H,R}^2 - F_{H,N}^2)/2F_{H,R}F_{H,N}, \qquad (2)$$

where $F_{H,D}$, $F_{H,R}$ and $F_{H,N}$ are structure-factor magnitudes of the heavy-atom derivative, the partial structure of replacing atoms and the native protein, respectively. Before using (2) we need to know the temperature and scaling factor of the replacing-atom substructure. This can be achieved by normalizing the isomorphous differences $|F_{H,D} - F_{H,N}|$ against the known replacing-atom substructure (see Fan, Hao,

Gu, Qian, Zheng & Ke, 1990). In practice, values of $\cos(\Delta\varphi_H)$ calculated from (2) may be outside the range of -1 to $+1$ because of the error in data measurement and normalization. In our experience if there are only a few reflections, say less than 1%, with $\cos(\Delta\varphi_H)$ outside the range of -1 to $+1$, we can simply reduce values greater than $+1$ to $+1$ and increase values smaller than -1 to -1. However, if there are too many reflections with $\cos(\Delta\varphi_H)$ outside the range of -1 to $+1$, the whole set of $\cos(\Delta\varphi_H)$ will not be used directly in subsequent calculations. Instead, they are first sorted in descending order and then modified to fit into a uniform distribution between $+1$ and -1. New values of $\cos(\Delta\varphi_H)$ obtained in this way are then used in the following calculations. The probability for $\Delta\varphi_H$ being positive is (Fan & Gu, 1985),

$$P_+(\Delta\varphi_H) = \tfrac{1}{2} + \tfrac{1}{2}\tanh\left\{\sin(|\Delta\varphi_H|)\left[\sum_{H'} m_{H'} m_{H-H'} \kappa_{H,H'} \times \sin(\Phi'_3 + \Delta\varphi_{H',\text{best}} + \Delta\varphi_{H-H',\text{best}})\right]\right\}, \quad (3)$$

where,

$$m_H = \exp(-\sigma_H^2/2)\{[2(P_+ - \tfrac{1}{2})^2 + \tfrac{1}{2}][1 - \cos(2\Delta\varphi_H)] + \cos(2\Delta\varphi_H)\}^{1/2}, \quad (4)$$

$$\tan(\Delta\varphi_{H,\text{best}}) = 2(P_+ - \tfrac{1}{2})\sin|\Delta\varphi_H|/\cos\Delta\varphi_H, \quad (5)$$

$$\kappa_{H,H'} = 2\sigma_3/\sigma_2^{3/2}|E_{-H}E_{H'}E_{H-H'}|, \sigma_n = \sum_j Z_j^n, \quad (6)$$

and,

$$\Phi'_3 = \varphi'_{-H} + \varphi'_{H'} + \varphi'_{H-H'}. \quad (7)$$

The factor $\exp(-\sigma_H^2/2)$ in (4) is related to the 'lack-of-closure error' (Blow & Crick, 1959) and is calculated according to Hendrickson & Lattman (1970). (3) can be used together with (4) and (5) to break the phase ambiguity. At the beginning P_+ is set to $\frac{1}{2}$ for all reflections. (4) and (5) are then used to calculate values of m_H and $\Delta\varphi_{H,\text{best}}$ for each reflection. The results are in turn substituted into (3) to obtain a new set of P_+. The minimum value of $\kappa_{H,H'}$ for accepting $\sum_2$ relationships was set to 0.03 in our test. With the values of P_+ so obtained, (4) and (5) produce new values of m_H and $\Delta\varphi_{H,\text{best}}$. The values of $\Delta\varphi_{H,\text{best}}$ are further converted to $\varphi_{H,\text{best}}$ using (8),

$$\varphi_{H,\text{best}} = \varphi'_H + \Delta\varphi_{H,\text{best}}, \quad (8)$$

Finally a Fourier map weighted by m_H and phased by $\varphi_{H,\text{best}}$ is calculated, which is the output of direct-method phasing and will be the input for solvent flattening.

In principle, there are two different ways to assign initial values to P_+. One is to assign all reflections to have an initial P_+ of $\frac{1}{2}$. This implies a single-solution phasing procedure. The other is to assign random values to P_+ and a multi-solution procedure will be implemented. The latter is the only choice if the replacing atoms are in centrosymmetric arrangement (see Yao & Fan, 1985). Otherwise, the single-solution procedure is simpler and faster and has been proved to be very efficient (Fan & Wang, 1988).

2.3. *Combining direct methods with solvent flattening*

The Fourier map resulting from the preceding section will be used as the input of solvent flattening. When a stable mask is obtained during the iteration, this mask will be applied back to the Fourier map calculated with averaged SIR phases and then the iteration will be repeated until it converges to a new stable mask.

2.4. *Phase extension*

The phase extension was achieved by using (3) to break the phase ambiguity for reflections with unknown phases while keeping the known phases at low resolution unchanged throughout the phasing process. Before using (3), values of m_H and $\Delta\varphi_{H,\text{best}}$ should be calculated for each reflection. For reflections with known phases, the figure of merit m_H is set to unity while $\Delta\varphi_{H,\text{best}}$ is replaced by $\Delta\varphi_H$ calculated from the difference between the known phase of the native protein and that of replacing atoms. For all reflections with unknown phases, (4) and (5) are used to calculate values of m_H and $\Delta\varphi_{H,\text{best}}$ assuming P_+ equal to $\frac{1}{2}$. With the initial values of m_H and $\Delta\varphi_{H,\text{best}}$ so obtained, (3) is used to calculate a new set of P_+ for all unknown-phase reflections. The minimum value of $\kappa_{H,H'}$ for accepting $\sum_2$ relationships was set to 0.03 in our test. New values of P_+ are then substituted into (4) and (5) yielding a new set of m_H and $\Delta\varphi_{H,\text{best}}$ for reflections with unknown phases. These new values together with those corresponding to the known phases are converted into m_H and $\varphi_{H,\text{best}}$ and input to the solvent-flattening procedure. The above procedure will be denoted as procedure I hereafter. During our test, a programming bug led accidentally to a different procedure, which differs from the above by only the calculation of m_H and $\Delta\varphi_{H,\text{best}}$ for known-phase reflections, *i.e.*,

$$m_H = \cos(\Delta\varphi_H), \quad (9)$$

and,

$$\Delta\varphi_{H,\text{best}} = \begin{cases} 0, \text{ if } \text{sign}(\cos\Delta\varphi_H) = 1 \\ \pi, \text{ if } \text{sign}(\cos\Delta\varphi_H) = -1, \end{cases} \quad (10)$$

where $\Delta\varphi_H$ is calculated from the known phases of the native protein and the replacing-atom substructure. It was surprising that the second procedure, referred to as procedure II hereafter, gave results better than those from procedure I.

3. Test data

Data used in the present test were from the native and the platinum derivative of ribonuclease Sa (RNase Sa) at 2.5 Å resolution (Dodson, Sevcik, Dodson & Zelinka, 1987; Sevcik, Dodson & Dodson, 1991). The crystals belong to space group $P2_12_12_1$ with unit-cell parameters $a = 64.90$, $b = 78.32$ and $c = 38.79$ Å. There are two molecules in the asymmetric unit, each with 96 amino-acid residues. Five platinum positions were found in the asymmetric unit but they are only partially occupied and the sum of the partial occupancies gives six Pt atoms in the whole unit cell.

4. Results and discussion

4.1. *Direct phasing of 2.5 Å SIR data*

All the observed reflections up to 2.5 Å, 7264 in total, were used in the test. SIR phase doublets were obtained from the intensity data and the known replacing-atom substructure. Table 1 shows the cumulative mean phase errors of averaged SIR phases (column 2) in comparison

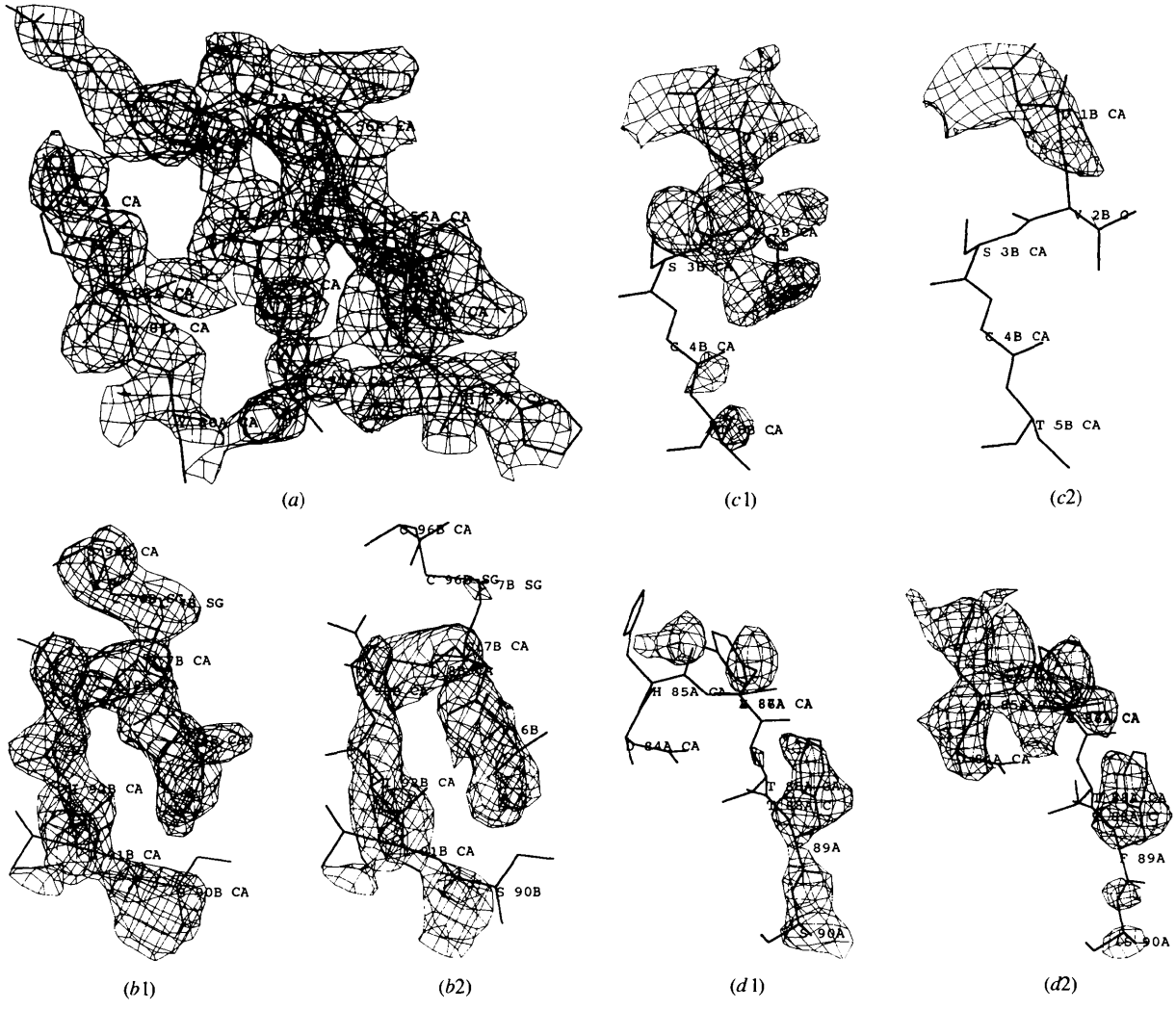


Fig. 1. Portions of the electron-density map resulting from two procedures of breaking the SIR phase ambiguity. Maps in (*a*), (*b*1), (*c*1), (*d*1) are phased by 'direct method + solvent flattening' and those in (*b*2), (*c*2) and (*d*2) are phased by solvent flattening alone. Amino-acid residues of portions on (*a*) and (*b*1) are within the region having an averaged correlation coefficient equal to 0.66. Portions on (*c*1), (*c*2), (*d*1) and (*d*2) belong to the worst 20% of the whole Fourier map, in which electron densities match poorly with the final structure model. The maps are contoured at 1σ except that on (*a*), which is contoured at 1.5σ. Some contours far from but projected near by the skeleton on the plot have been erased manually. The superimposed model is plotted according to the final atomic parameters from the original authors.

Table 1. *Comparison of mean phase errors for different procedures of breaking the SIR phase ambiguity*

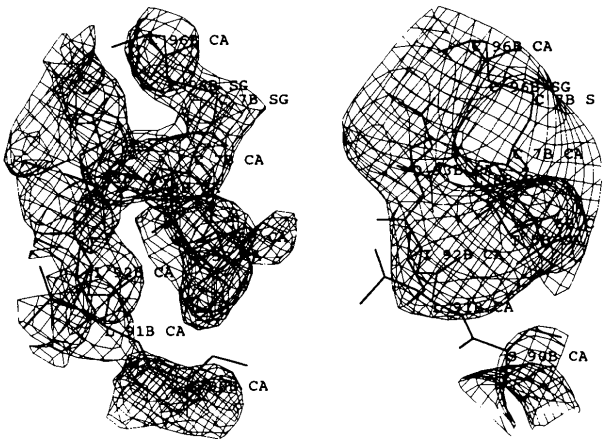
Reflections were sorted in descending order of the observed structure-factor amplitude and then cumulated into eight groups. The number of reflections in each group is listed in the first column. Values of average phase error were calculated against the final structure model.

	F_{obs}-weighted mean phase error (°)			
No. of reflections	Averaged SIR phases	Direct-method resolved phases	Solvent flattening based on averaged SIR phases	Solvent flattening based on direct-method resolved phases
1000	64.26	58.36	47.27	45.34
2000	63.86	59.22	48.61	47.39
3000	64.38	60.15	50.64	49.90
4000	65.07	61.22	52.51	51.74
5000	65.96	62.32	54.06	53.59
6000	66.69	63.27	55.56	55.14
7000	67.30	64.09	56.52	56.22
7264	67.39	64.19	56.66	56.36

with that after the phase ambiguity has been resolved by the direct method (column 3). It is evident that the SIR phases were improved by the direct method. Two separate solvent-flattening procedures were then carried out. One of which used averaged SIR phases as input, while the other started from direct-method phases. Cumulative mean phase errors after solvent flattening are listed in the last two columns of Table 1. As is seen the result derived from direct-method phases is slightly better than that from averaged SIR phases. Overall correlation coefficients of the two resultant Fourier maps are 0.498 and 0.486, respectively, for that from 'direct method + solvent flattening' and that from solvent flattening alone. On the Fourier map obtained by 'direct method + solvent flattening' there are regions containing more than 63% of amino-acid residues, the average correlation coefficient of which for the main chain equals 0.66. Part of the electron-density map belonging to this portion is shown in Fig. 1(*a*). Within this portion the quality of the map from 'direct method + solvent flattening' is better than that of the map from solvent flattening alone. This is seen from Figs. 1(*b*1) and 1(*b*2), in which the two resultant Fourier maps are compared with respect to the same portion of electron density containing the disulfur bond on molecule *B*. On the other hand there are about 20% of the whole Fourier map obtained from 'direct method + solvent flattening' in which electron densities match poorly with the final structure model. Some parts of this portion are given in Figs. 1(*c*1), 1(*c*2), 1(*d*1) and 1(*d*2). Within this portion the quality of electron density resulted from 'direct method + solvent flattening' is rather the same as that from solvent flattening alone.

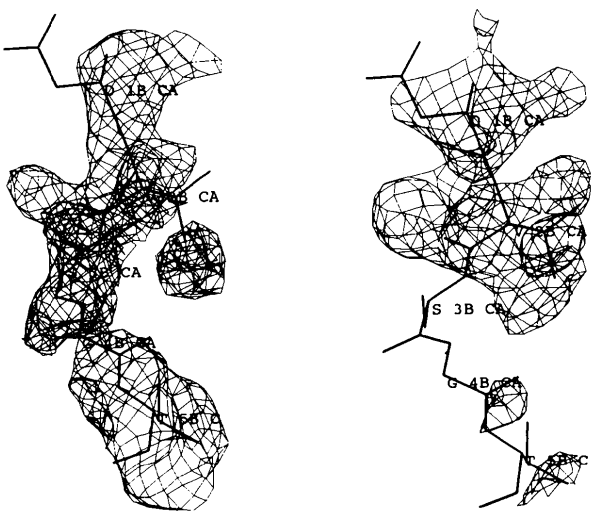
4.2. *Phase extension from 6 to 2.5 Å resolution*

All the observed reflections within 2.5 Å, 7264 in total, were used in the test. Among them 586 reflections at 6 Å resolution were assumed to have known phases, which were calculated from the final structure model. SIR phase doublets were obtained from the intensity data and the known replacing-atom substructure. Phases of the remaining 6678 reflections were then derived based on the SIR phase doublets and the known phases within 6 Å resolution. Mean phase errors of different phasing procedures are listed in Table 2. A prominent



(*a*) (*b*)

Fig. 2. Comparison of electron-density maps calculated before and after the phase extension. A portion of the electron-density map containing the disulfur bond in molecule *B* is plotted with contours at 1σ. (*a*) Electron-density map after phase extension to 2.5 Å resolution. (*b*) Electron-density map at 6 Å resolution before phase extension.



(*a*) (*b*)

Fig. 3. Comparison of electron-density maps obtained from phase extension and from *ab initio* phasing of the SIR data. Electron densities were plotted in the region of residues 1–5 of molecule *B* with contours at 1σ. (*a*) Electron-density map obtained by phase extension from 6 to 2.5 Å resolution. (*b*) Electron-density map from *ab initio* phasing of the 2.5 Å SIR data.

Table 2. *Comparison of mean phase errors for different phasing procedures*

Reflections within 6–2.5 Å were sorted in descending order of the observed structure-factor amplitude and then cumulated into seven groups. The number of reflections in each group is listed in the first column. Values of mean phase error were calculated against the final structure model.

		F_{obs}-weighted mean phase error (°) resulting from different phasing procedures			
	Without using SIR information	Using SIR information			
	Solvent-flattening phase extension		Phase extension from 6 to 2.5 Å		
Number of reflections within the range 6–2.5 Å	from 6 to 2.5 Å	Direct phasing of the 2.5 Å SIR data	Solvent flattening alone	Direct method + solvent flattening Procedure I	Procedure II
1000	74.52	45.83	39.36	38.90	37.48
2000	79.20	49.12	43.50	43.05	42.02
3000	81.57	51.73	46.73	46.11	45.52
4000	82.68	53.75	49.15	48.50	48.10
5000	83.36	55.34	51.32	50.73	50.27
6000	83.88	56.77	53.16	52.61	52.09
6678	83.95	57.35	53.80	53.17	52.69

Table 3. *Comparison of map correlation coefficients for different phasing procedure*

Correlation coefficients were calculated with respect to individual amino-acid residues for the resultant electron-density maps against the 'final' map. The coefficients were then sorted in descending order and cumulated into five groups as indicated in the first column. Number of residues in each cumulated group was counted for the main chain and the side chain separately.

	Number of amino-acid residues					
	Main chain			Side chain		
	Direct phasing	Phase extension		Direct phasing	Phase extension	
Correlation coefficient	of the 2.5 Å SIR data	Solvent flattening alone	Procedure II	of the 2.5 Å SIR data	Solvent flattening alone	Procedure II
≥0.8	14	26	27	2	3	4
≥0.7	42	86	87	22	24	30
≥0.6	91	150	157	51	69	75
≥0.5	139	175	182	85	119	113
≥0.4	170	185	190	118	145	142

feature of which is that the conventional phase extension by solvent flattening without using the SIR information resulted in an averaged phase error at least 20° worse than that from any of the other phasing procedures using the SIR information. It turns out also that results from all the phase-extension procedures using SIR information are reasonably accurate and are evidently better than that from direct phasing of the 2.5 Å SIR data. In addition results from 'direct method + solvent flattening' are better than those from solvent flattening alone, while procedure II is the best. Table 3 lists results of correlation coefficients, which are calculated for the resultant Fourier maps from different phasing procedures. As can be seen again, results from different phase-extension procedures are far better than that from direct phasing of the 2.5 Å SIR data, while procedure II is still better than solvent flattening alone. It is seen in Table 3 that the maps from phase extension using SIR information contain more than 150 (~80% of the total) residues on the main chain correspond to good electron densities, indicated by a correlation coefficient greater than 0.6. This means that the Fourier map resulting from the phase extension by either 'direct methods + solvent flattening' or by 'solvent flattening alone' is easily traceable and the phase extension is considered to be very successful. Fig. 2 shows a portion of the resultant electron-density map after the phase extension by procedure II in comparison with the same portion of the map at 6 Å resolution before phase extension. Fig. 3 shows another portion of the Fourier map resulting from phase extension by procedure II in comparison to that from direct phasing of the 2.5 Å SIR data by the combination of direct method and solvent flattening. All the Fourier maps shown in this paper were calculated using the program *FRODO* (Jones, 1978, 1985).

5. Concluding remarks

The incorporation of direct methods in the solvent-flattening technique strengthened the latter in dealing with SIR data. The improvement gained by using SIR information in phase extension from low-resolution data to high-resolution data is remarkable. It has been succeeded in extending phases from 6 to 2.5 Å resolution for a protein of moderate size. This is rare, if not impossible, by conventional phase-extension techniques without making use of either SIR information or a structural model.

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