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## Unbiased Three-Dimensional Refinement of Heavy-Atom Parameters by Correlation of Origin-Removed Patterson Functions

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### Abstract

A procedure is presented for three-dimensional refinement of heavy-atom parameters without the use of phase information in the methods of single and multiple isomorphous replacement. This procedure is based on the Patterson-function correlation method of Rossmann [*Acta Cryst.* (1960), **13**, 221–226] except that the origins of the Patterson functions are now removed from this correlation and centric and acentric reflections are treated separately. The resulting procedure is shown theoretically and by application to three test cases to yield accurate and essentially unbiased estimates of the occupancies, thermal parameters, and positions of heavy-atom sites.

### Introduction

Often a difficult step in the determination of the structure of a crystalline protein by the methods of single or multiple isomorphous replacement (SIR or MIR) is the refinement of positions, occupancies, and thermal factors of the heavy atoms bound in the isomorphous derivatives.

Two general methods have been commonly used to carry out this refinement. The method of Dickerson,

Kendrew & Strandberg (1961) requires several isomorphous derivatives, all of which (Dickerson, Weinzierl & Palmer, 1968) or some of which (Blow & Matthews, 1973) are used to determine approximate phases for the protein structure. These phases are in turn used to refine the heavy-atom parameters for one or more derivatives. In the SIR method, and often during early stages of the MIR method, however, it is necessary to refine the heavy-atom parameters for a single derivative. Since this refinement method requires several derivatives, it cannot be effectively used in these cases. Also, as Blow & Matthews (1973) have pointed out, this method does not yield unbiased estimates of the occupancies of heavy-atom sites.

The least-squares method of Rossmann (1960) and related methods due to Hart (1961) and Kartha (1965) require only a single isomorphous derivative and do not involve the calculation of phases. These procedures are based on the concept that the difference between derivative and native structure-factor amplitudes is related to the true heavy-atom structure-factor amplitude. In the method of Rossmann (1960), heavy-atom parameters are refined so as to minimize the sum over all reflections  $hkl$  of the residual  $R$ :

$$R = \sum_{hkl} \omega^{hkl} [(F_{ph,obs}^{hkl} - F_{p,obs}^{hkl})^2 - (f_{calc}^{hkl})^2]^2, \quad (1)$$

where  $F_{ph,obs}^{hkl}$  and  $F_{p,obs}^{hkl}$  are observed derivative and native structure-factor amplitudes for a reflection with indices  $hkl$ ,  $f_{calc}^{hkl}$  is a heavy-atom structure-factor amplitude calculated from the estimated heavy-atom

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parameters, and  $\omega^{hkl}$  is a weighting factor. This procedure is essentially a correlation of the coefficients of the difference Patterson function for this derivative,  $(F_{ph,obs}^{hkl} - F_{p,obs}^{hkl})^2$ , with the coefficients of the hypothetical Patterson function calculated from the estimated heavy-atom parameters,  $(f_{calc}^{hkl})^2$ . The principal disadvantage of this and related methods is that the origins of the Patterson functions are included in this correlation, so that a heavy atom in *any* position will yield a smaller value of  $R$  than no heavy atom at all. This means that if incorrect sites are included in a starting heavy-atom model this refinement method will generally yield a positive occupancy for these sites. This property can also be deduced from (1) by noting that  $(F_{ph,obs}^{hkl} - F_{p,obs}^{hkl})^2$  is always positive and that a heavy atom at any position will yield a positive value of  $(f_{calc}^{hkl})^2$ .

An additional minor disadvantage of the method of Rossmann (1960) is that, for acentric reflections,  $(F_{ph,obs}^{hkl} - F_{p,obs}^{hkl})^2$  does not equal  $(f_{calc}^{hkl})^2$ , the square of the heavy-atom structure factor, even in the absence of error. In fact, as noted by Lipson & Cochran (1966) its expected value is  $(f_{calc}^{hkl})^2/2$ . Consequently, the occupancies of heavy-atom sites are not accurately determined using acentric reflections. The  $F_{HLE}$  method of Kartha (1965) does not suffer from this disadvantage but estimates of the heavy-atom structure-factor amplitudes obtained from isomorphous and anomalous differences tend not to be very accurate.

We present here a modified version of the procedure of Rossmann (1960) in which the 'origins' of the Patterson functions are effectively removed from the correlation given in (1) and in which centric and acentric reflections are treated separately. We then show that the new technique yields unbiased estimates of occupancies of heavy-atom sites and compare the technique to those of Rossmann (1960) and Dickerson, Weinzierl & Palmer (1968) in three test cases.

#### Notation and approximations used

We use the following definitions and approximations throughout this paper.  $F_p \exp\{i\varphi_p\}$  and  $F_{ph} \exp\{i\varphi_{ph}\}$  are the true native and derivative structure factors for a particular reflection and are related by

$$F_{ph} \exp\{i\varphi_{ph}\} = F_p \exp\{i\varphi_p\} + F_H \exp\{i\alpha\} + \mu \exp\{i\theta\}, \quad (2)$$

where  $F_H \exp\{i\alpha\}$  is the true structure factor due to heavy atoms in the derivative structure and  $\mu \exp\{i\theta\}$  is the difference between derivative and native structure factors owing to lack of isomorphism (Terwilliger & Eisenberg, 1983). For convenience, we assume that there is only one heavy-atom site in the current model and that only the occupancy of this site is to be determined. We may therefore write

$$F_H \exp\{i\alpha\} \equiv a_0 f_0 \exp\{i\beta\} + \eta \exp\{i\gamma\} \quad (3)$$

or

$$F_{ph} \exp\{i\varphi_{ph}\} = F_p \exp\{i\varphi_p\} + a_0 f_0 \exp\{i\beta\} + \eta \exp\{i\gamma\} + \mu \exp\{i\theta\}, \quad (4)$$

where  $a_0$  is the true occupancy of this site,  $f_0 \exp\{i\beta\}$  is the structure factor due to an atom with unit occupancy and the true temperature factor located at this site, and  $\eta \exp\{i\gamma\}$  is the structure factor due to all other heavy atoms in the derivative structure. All the above structure factors are assumed to be distributed according to Wilson (1949) statistics (Terwilliger & Eisenberg, 1983). Based on the heavy-atom model with estimated occupancy  $a$ , we can calculate an estimate of the heavy-atom structure factor,  $f_{calc} \exp\{i\psi\}$ . Since we are uncertain only about the occupancy of this site, we may write that  $f_{calc} \exp\{i\psi\} = a f_0 \exp\{i\beta\}$ . We now make the assumption that  $a_0 f_0$ ,  $\eta$ , and  $\mu$  are small relative to  $F_p$  so that we may write

$$F_{ph} - F_p \doteq a_0 f_0 \cos\{\beta - \varphi_p\} + \eta \cos\{\gamma - \varphi_p\} + \mu \cos\{\theta - \varphi_p\}. \quad (5)$$

We have measurements  $F_{ph,obs}$  and  $F_{p,obs}$  of the derivative and native structure-factor amplitudes with variances  $\sigma_{ph}^2$  and  $\sigma_p^2$ . Equation (5) may thus be rewritten using  $F_{ph,obs}$  and  $F_{p,obs}$  as

$$F_{ph,obs} - F_{p,obs} \doteq a_0 f_0 \cos\{\beta - \varphi_p\} + \eta \cos\{\gamma - \varphi_p\} + \mu \cos\{\theta - \varphi_p\} + \delta_{ph} - \delta_p \quad (6)$$

where  $\delta_{ph}$  and  $\delta_p$ , assumed to follow Gaussian distributions, are the errors in measurement of  $F_{ph,obs}$  and  $F_{p,obs}$ , respectively. Now define a new variable  $\zeta$ :

$$\zeta \equiv \eta \cos\{\gamma - \varphi_p\} + \mu \cos\{\theta - \varphi_p\} + \delta_{ph} - \delta_p \quad (7)$$

This variable can be shown to have the properties:

$$\langle \zeta \rangle = 0 \quad (8a)$$

$$\langle \zeta^2 \rangle = k \langle \eta^2 \rangle + k \langle \mu^2 \rangle + \sigma_{ph}^2 + \sigma_p^2 \quad (8b)$$

$$\langle [\zeta^2 - \langle \zeta^2 \rangle]^2 \rangle = 2 \langle \zeta^2 \rangle^2, \quad (8c)$$

where the brackets indicate an average value in a given range of resolution and  $k$  is  $\frac{1}{2}$  for acentric and 1 for centric reflections. The factor  $k$  is different from 1 for acentric reflections because  $F_p \exp\{i\varphi_p\}$ ,  $F_H \exp\{i\alpha\}$ , and  $\mu \exp\{i\theta\}$  are then generally not collinear. It may be verified that this will be the case even if the heavy atoms in the derivative structure are in a pseudo-centric arrangement.

#### Theory

Our method of determining heavy-atom parameters consists of minimizing the residual  $R'$ :

$$\begin{aligned}
 R' = \sum_{hkl} \omega^{hkl} \{ & (F_{ph,obs}^{hkl} - F_{p,obs}^{hkl})^2 \\
 & - \langle (F_{ph,obs} - F_{p,obs})^2 \rangle \\
 & - k[(f_{calc}^{hkl})^2 - \langle (f_{calc})^2 \rangle] \}^2, \quad (9)
 \end{aligned}$$

where the sum is over all reflections  $hkl$ , the angular brackets indicate an average in an appropriate resolution range,  $\omega^{hkl}$  is a weighting factor (see below), and  $k$  is  $\frac{1}{2}$  for acentric and 1 for centric reflections. The usefulness of (9) lies in the fact that, if  $(f_{calc}^{hkl})^2$  is based on an incorrect model site,  $\{(F_{ph,obs}^{hkl} - F_{p,obs}^{hkl})^2 - \langle (F_{ph,obs} - F_{p,obs})^2 \rangle\}$  is uncorrelated with  $\{(f_{calc}^{hkl})^2 - \langle (f_{calc})^2 \rangle\}$ , yet for a model site corresponding to the data, the two bracketed quantities are correlated. The expected occupancy of an arbitrary site will therefore essentially be zero. The effect of subtracting the quantities in angular brackets is to determine the correlation between the coefficients of 'observed' and 'calculated' Patterson functions *after* removal of thermally broadened origins. The factor  $k$  compensates for the fact that, as noted above, the expected value of  $(F_{ph,obs}^{hkl} - F_{p,obs}^{hkl})^2$  for acentric reflections is half that for centric reflections while the expected value of  $(f_{calc}^{hkl})^2$  is the same for each.

We now determine the proper value of the weighting factor  $\omega^{hkl}$  and show that minimization of (9) with respect to the occupancy of a heavy-atom site yields an essentially unbiased estimate of the occupancy. Using (6) and (7) we may write

$$(F_{ph,obs} - F_{p,obs})^2 \doteq [a_0 f_0 \cos(\beta - \varphi_p) + \zeta]^2 \quad (10)$$

and

$$\langle (F_{ph,obs} - F_{p,obs})^2 \rangle \doteq ka_0^2 \langle f_0^2 \rangle + \langle \zeta^2 \rangle, \quad (11)$$

where  $k$  is as above and the brackets indicate average values in a range of resolution, determined separately for centric and acentric reflections. We may further define a new variable  $\Delta$  which has a mean value of zero:

$$\begin{aligned}
 \Delta \equiv & a_0^2 f_0^2 [\cos^2(\beta - \varphi_p) - k] + [\zeta^2 - \langle \zeta^2 \rangle] \\
 & + 2\zeta a_0 f_0 \cos(\beta - \varphi_p) \quad (12)
 \end{aligned}$$

and write that

$$\begin{aligned}
 (F_{ph,obs} - F_{p,obs})^2 - \langle (F_{ph,obs} - F_{p,obs})^2 \rangle \\
 \doteq a_0^2 k [f_0^2 - \langle f_0^2 \rangle] + \Delta. \quad (13)
 \end{aligned}$$

Now, using (13) and substituting  $a^2 f_0^2$  for  $(f_{calc})^2$  in (9), we have a new expression for the residual which is to be minimized:

$$\begin{aligned}
 R' \doteq \sum_{hkl} \omega^{hkl} \{ & [a_0^2 k (f_0^2 - \langle f_0^2 \rangle) + \Delta] \\
 & - a^2 k (f_0^2 - \langle f_0^2 \rangle) \}^2. \quad (14)
 \end{aligned}$$

The quantity of interest is  $a^2$  and it is clear that this equation is in a linear least-squares form with an error of  $\Delta$  in the dependent variable. The value of  $a^2$  expected upon minimization of  $R'$  with respect to  $a^2$  is  $a_0^2$ , hence

the estimate of (the square of) the occupancy is unbiased.

The best value for the weighting factor  $\omega^{hkl}$  in a linear least-squares residual such as (14) is the reciprocal of the expected value of the square of the error term  $\Delta$ :

$$\omega^{hkl} = 1/\langle \Delta^2 \rangle. \quad (15)$$

Using (8a), (8b), (8c) and (12), and substituting  $\langle (f_{calc})^2 \rangle$  for  $a_0^2 \langle f_0^2 \rangle$ , we find

$$\langle \Delta^2 \rangle = \begin{cases} 2E_{\text{CENT}}^2 (E_{\text{CENT}}^2 + 2\langle (f_{calc})^2 \rangle) & \text{centric reflections, (16a)} \\ 2E_{\text{ACENT}}^2 (E_{\text{ACENT}}^2 + \langle (f_{calc})^2 \rangle) + \frac{1}{4}\langle (f_{calc})^2 \rangle^2 & \text{acentric reflections, (16b)} \end{cases}$$

where, referring to (8a), (8b), (8c),

$$E_{\text{CENT}}^2 \equiv \langle \zeta^2 \rangle_{\text{centric reflections}} \quad (17a)$$

$$E_{\text{ACENT}}^2 \equiv \langle \zeta^2 \rangle_{\text{acentric reflections}}. \quad (17b)$$

It may be noted from (8b) that

$$E_{\text{CENT}}^2 \doteq 2E_{\text{ACENT}}^2. \quad (18)$$

We will show in a future paper (Terwilliger & Eisenberg, 1983) that  $E_{\text{CENT}}^2$  and  $E_{\text{ACENT}}^2$  may be estimated from standard lack-of-closure residuals averaged over all possible phases, weighted by the phase probability. Since  $\langle (f_{calc})^2 \rangle$  is simply the mean-square calculated heavy-atom structure-factor amplitude, all the quantities in (16a) and (16b) are readily available for any given model for heavy-atom sites.

Although we have shown here only that an essentially unbiased estimate of the occupancy of a single heavy-atom site is obtained using this technique, the same approach may be used to show that unbiased estimates of the occupancies of heavy-atom sites will also be obtained when several sites are present in the heavy-atom model. In either case, estimates of thermal factors will also be nearly unbiased, as they simply reflect 'occupancy' as a function of resolution. Similarly, coordinates of heavy atoms may be refined, since shifting the location of a heavy-atom site is essentially equivalent to changing coordinately the occupancies of heavy atoms at the current and at an adjacent site.

## Discussion

In both the method of Rossmann (1960) and that presented here, the determination of heavy-atom parameters is based on maximizing the correlation between  $(F_{ph,obs}^{hkl} - F_{p,obs}^{hkl})^2$  and  $(f_{calc}^{hkl})^2$  or, equivalently, between Patterson functions based on these quantities. The principal difference between the

Table 1. *Estimated heavy-atom parameters in a model case after refinement to convergence by various methods*

The true occupancy of the two correct sites is 0.30. At the beginning of each refinement the positional error in each of these sites was 0.69 Å and all four sites had an occupancy of 0.30. 'Origin-removed Patterson' refers to the present method. Rossmann refers to the method of Rossmann (1960). Dickerson refers to the method of Dickerson, Weinzierl & Palmer (1968).

		Origin-removed Patterson	Rossmann	Dickerson
Correct sites				
Site 1	Occupancy	0.24	0.28	0.44
	Positional error (Å)	0.08	0.22	0.67
Site 2	Occupancy	0.28	0.47	0.40
	Positional error (Å)	0.10	0.44	0.58
Incorrect sites				
Site 3	Occupancy	0.03	0.14	0.25
Site 4	Occupancy	0.04	0.20	0.38

methods is that in the present technique the origins are effectively removed from the Patterson functions in this correlation by the subtraction of the terms within angular brackets in (9). Consequently, random sites will have a calculated occupancy of nearly zero and the estimated occupancy of a true site will be essentially unbiased.

In order to test this prediction and to compare the present method with others, a model set of data was created using 3449 native structure-factor amplitudes measured to 2.0 Å resolution from the form II crystals of melittin (Terwilliger & Eisenberg, 1982*a,b*) and phases calculated from the partially refined model for these crystals.

Structure-factor amplitudes for one 'derivative' were calculated using five heavy-atom sites with equal occupancies and temperature factors such that the normalized value of  $\langle |F_{ph}| - |F_p| \rangle = 0.26$ . During the test of each refinement method, heavy atoms with the correct occupancies and temperature factors were initially placed 0.69 Å from each of two of the correct sites. Additionally, heavy atoms with the same occupancy and temperature factor were placed at two arbitrary sites. Table 1 shows the estimates of heavy-atom parameters obtained after simultaneous refinement of occupancies and coordinates to convergence with the present method and with that of Rossmann (1960). Although the method of Dickerson, Weinzierl & Palmer (1968) was not intended for use with only one derivative, we include the results of a similar refinement using this method as well. When this method was used, one of the two 'most probable' SIR phases was used in the heavy-atom refinement. It is evident from Table 1 that in this SIR case the origin-removed Patterson method of refinement yields estimates of positions and occupancies which are substantially more accurate than either the method of Rossmann (1960) or that of Dickerson, Weinzierl & Palmer (1968). In

particular, the estimated occupancies of the two incorrect sites are near zero only for the present method.

We have also compared the accuracy of the phases yielded by the three methods of refinement when applied to measured data from two types of protein crystals. One set of data consisted of structure-factor amplitudes from B-phycoerythrin crystals and from three isomorphous derivatives (Fisher, Woods, Fuchs & Sweet, 1980). The other consisted of structure-factor amplitudes from native form II melittin crystals and from two isomorphous derivatives of these crystals to 2.8 Å resolution (Terwilliger & Eisenberg, 1982*a,b*). In each of these cases, an objective measure of the accuracy of the resultant phases was available. Since the crystals of B-phycoerythrin contain a non-crystallographic dyad (Fisher, Woods, Fuchs & Sweet, 1980), the correlation of the electron density with two-fold-related electron density is an objective measure of the accuracy of the phases used to calculate the electron density. For the form II crystals of melittin, the correlation of multiple isomorphous replacement (MIR) phases with the phases calculated based on the partially refined structure of melittin (Terwilliger & Eisenberg, 1982*a*) is a measure of the accuracy of the MIR phases.

In the refinement of heavy-atom parameters for B-phycoerythrin, the method of Dickerson, Weinzierl & Palmer (1968) was first used until the parameters had converged. An electron density map at 5 Å resolution was calculated which had a dyad symmetry-overlap integral (Fisher, Woods, Fuchs & Sweet, 1980) of 0.44. Heavy-atom parameters were then refined using the present method, minor sites were deleted, and a new electron density map was calculated which had a symmetry-overlap integral of 0.53, substantially higher than the previous value.

For the test refinements using the melittin form II structure-factor amplitudes, only the five principal heavy-atom sites in the KI derivative and the single major site in the Hg derivative were included in the starting heavy-atom model. Additionally, one false site at an arbitrary position was added to each derivative with an occupancy and temperature factor essentially equal to that of the site with the highest occupancy in that derivative. Anomalous differences were not used in refinement or in calculating phases. Beginning with the parameters obtained previously (Terwilliger & Eisenberg, 1982*a*), occupancies, thermal parameters, and coordinates were separately refined until convergence using each of the three methods described above. Table 2 shows the agreement [ $\langle \cos(\varphi_{\text{MIR}} - \varphi_{\text{REF}}) \rangle$ ] of the MIR phases ( $\varphi_{\text{MIR}}$ ) obtained using each method with the phases calculated from the partially refined model of the melittin form II structure ( $\varphi_{\text{REF}}$ ). We find that in this test the method of Dickerson, Weinzierl & Palmer (1968) and the present method yield essentially

Table 2. Correlation of MIR phases after various refinement procedures with phases calculated from partially refined model

Values of the correlation  $\langle \cos \{ \varphi_{\text{MIR}} - \varphi_{\text{REF}} \} \rangle$  are tabulated, where  $\varphi_{\text{MIR}}$  is the phase calculated by the Terwilliger & Eisenberg (1983) modification of the method of Blow & Crick (1959), using heavy-atom parameters obtained from the appropriate refinement method (see legend to Table 1).  $\varphi_{\text{REF}}$  are phases calculated from the partially refined model for the form II crystals of melittin (Terwilliger & Eisenberg, 1982a).

	Origin- removed Patterson	Rossmann	Dickerson
All reflections ( $n = 1274$ )	0.31	0.26	0.31
Centric reflections ( $n = 288$ )	0.29	0.31	0.26
Acentric reflections ( $n = 986$ )	0.31	0.25	0.32

equivalent values of this agreement factor, both of which are somewhat higher than that yielded by the method of Rossmann (1960). We note, however, that for centric reflections the agreement between MIR phases and phases calculated from the form II model was higher using the method of Rossmann (1960) than with either of the other two methods.

Based on the three test cases presented here as well as on many other tests which we have carried out, we conclude that the present method of heavy-atom refinement is substantially more useful than either the method of Rossmann (1960) or that of Dickerson, Weinzierl & Palmer (1968) when only one heavy-atom derivative is available (Table 1). For intermediate cases where the heavy-atom parameters for several derivatives are known approximately, the present method still yields substantially more accurate heavy-atom parameters than either of the other methods tested. This was the case in the refinement of heavy-atom parameters for B-phycoerythrin derivatives. When heavy-atom parameters are well known to begin with, as in the case of the melittin form II crystals (Table 2), or when many heavy-atom derivatives are available, any of the three methods yields reasonably accurate results. One of the most difficult procedures in determining protein phases by the method of multiple isomorphous replacement, however, is that of proceeding from a very crude heavy-atom model containing incorrect as well as correct sites to a model consisting only of correct sites with reasonably accurate heavy-atom parameters. We suggest that in this procedure the present method may be a substantial improvement over the other methods tested.

It is perhaps surprising that the present method, which does not require native phase information, yields heavy-atom parameters which are just as accurate as those obtained using the 'phase refinement' method of Dickerson, Weinzierl & Palmer (1968) even when native phases are well known. We suspect that the reason for

this is that when the native phases are precisely known, there are several factors which limit the accuracy of both methods in about the same way. These additional uncertainties include errors in measurement of native and derivative structure-factor amplitudes, lack of isomorphism between native and derivative structures, and the presence of heavy-atom sites in the derivative structures which are not included in the heavy-atom model.

One minor disadvantage of the method described here is that, as heavy-atom parameters for each derivative are refined separately, the procedure may not be used to refine the relative positions of heavy atoms in separate derivatives. In cases where this refinement is necessary, another method, such as that of Blow & Matthews (1973) or that suggested by Rossmann (1960) should be used to refine the coordinates of one of the heavy-atom sites in each derivative relative to each other. Then all heavy-atom positions in all derivatives may be refined independently, maintaining the appropriate coordinate(s) of the principal heavy-atom site in each derivative fixed.

A computer program which carries out the present method of heavy-atom refinement as well as those of Rossmann (1960) and Dickerson, Weinzierl & Palmer (1968) has been deposited with the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, New York 11973, from which copies are available.

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