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THE LOCATION OF REDOX CENTERS IN BIOLOGICAL MEMBRANES DETERMINED BY RESONANCE X-RAY DIFFRACTION

II. ANALYSIS OF THE RESONANCE DIFFRACTION DATA

J.K. BLASIE [a,b], J.M. PACHENCE [a,b], A. TAVORMINA [a,b], M. ERECINSKA [a], P.L. DUTTON [a], J. STAMATOFF [c], P. EISENBERGER [c] and G. BROWN [d]

[a] *Department of Biochemistry and Biophysics,* [b] *Department of Chemistry, University of Pennsylvania, Philadelphia, PA 19104,* [c] *Bell Telephone Laboratories, Murray Hill, NJ 07974, and* [d] *Stanford Synchrotron Radiation Laboratory, Stanford, CA 94305 (U.S.A.)*

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In the preceding paper (Stamatoff, J., Eisenberger, P., Blasie, J.K., Pachence, J.M., Tavormina, A., Erecinska, M., Dutton P.L. and Brown, G. (1982) Biochim. Biophys. Acta 679, 177–187), we described the observation of resonance X-ray scattering effects from intrinsic metal atoms associated with redox centers in membrane proteins on the lamellar X-ray diffraction from oriented multilayers of reconstituted membranes. In this paper, we discuss the possible methods of analysis of such data and present the results of our model refinement analysis concerning (a) the location of the cytochrome *c* heme iron atom in the profile structure of a reconstituted membrane containing a photosynthetic reaction center-cytochrome *c* complex and (b) the location of the heme *a* and a_3 iron atoms in the profile structure of a reconstituted membrane containing cytochrome oxidase. The former results are of special importance because they provide a test of the validity of the resonance diffraction data and the methods of analysis, since the location of cytochrome *c* in the reaction center-cytochrome *c* membrane profile is known independently of the resonance diffraction experiments.

Introduction

In the preceding paper [1] we described the dependence of the ratios of several integrated lamellar X-ray reflections $R_e(h/h')$ from oriented membrane multilayers on the incident X-ray beam energy in the neighborhood of a K absorption edge for an intrinsic metal atom of an integral protein in the membrane. We ascribed the energy dependence of these ratios to resonance X-ray scattering effects from the intrinsic metal atoms because (a) the form of the energy dependence of $R_e(h/h')$ was approximately that for the f' part of the atomic scattering factor for the metal atom as required by the symmetry of the multilayer unit cell profile, (b) the magnitude of the energy dependence of $R_e(h/h')$ was approximately that expected considering the concentration of the metal atom in the multilayer and the estimated X-ray scattering (electron density) contrast for the membrane profile at high protein content and (c) such effects were not observed in several different types of control experiments.

Having clearly demonstrated the measurement of such resonance X-ray scattering effects [1], we felt that in order to establish firmly resonance X-ray diffraction as a direct method for determining the positions of resonance scattering atoms with the membrane structure, a critical control experiment remained to be performed. This control experiment would concern the utilization of the measured resonance X-ray

scattering effects from intrinsic metal atoms within integral membrane proteins on the lamellar X-ray diffraction from oriented membrane multilayers to determine the position of an intrinsic metal in a membrane profile where this position was known independently.

In this paper, we describe the utilization of resonance X-ray scattering effects from cobalt heme-cytochrome *c* on the lamellar X-ray reflections from oriented multilayers of reconstituted membranes containing a photosynthetic reaction center-cytochrome *c* complex in determining the position of the cobalt atom in the membrane profile. The locations of the cytochrome *c*, reaction center, phospholipid and water molecules within this functional membrane profile are known independently from X-ray and neutron diffraction studies (Refs. 2–4 and Pachence, J.M., Dutton, P.L. and Blasie, J.K., unpublished data). In addition, we examine the relationship between the number and accuracy of measurements of the resonance X-ray scattering effects on the lamellar diffraction from membrane multilayers and the resulting accuracy of the determination of the position of the relevant metal atom in the membrane profile. The heme *a* and a_3 iron atoms of cytochrome oxidase and the heme cobalt atom of the reaction center-cobalt- to reaction centers was 100 : 1 and that of cyto- examination.

Methods

a. Reaction center-cytochrome c profile structure

We have previously described the reconstitution of membranes composed of reaction centers from photosynthetic bacteria, mammalian cytochrome *c* and egg lecithin [2]. These membranes were fully functional with regard to the kinetics of the relevant light-induced electron-transfer reactions and the vectorial distributions of reaction centers and cytochrome *c* in the membrane profile were shown to be highly asymmetric (more than 85–90% of the reaction centers were oriented in the membrane profile such that their cytochrome *c*-binding sites occurred on the extravesicular membrane surface). In addition, we have previously determined to approx. 12 Å resolution the separate profile structures of the reaction center molecule, phospholipid and water within the profile structure of these reconstituted membranes utilizing both X-ray and neutron lamellar diffraction from hydrated oriented multilayers of the reconstituted membranes in the absence of cytochrome *c* [3].

More recently, we have collected lamellar X-ray diffraction from hydrated oriented multilayers of membranes reconstituted from reaction centers, egg lecithin and cytochrome *c*; the mole ratio of lecithin to reaction centers was 100 : 1 and that of cytochrome *c* to reaction centers was systematically varied from 0 to 3.3. The multilayers were formed from unilamellar vesicular dispersions of the reaction center/lecithin vesicles and the cytochrome *c* was added to these dispersions of preformed vesicles [2]. The reaction center/bound cytochrome *c* ratio was determined directly via absorption spectroscopy of the oriented membrane multilayer. Unit cell electron-density profiles were calculated to approx. 12 Å resolution utilizing methods described in detail previously [2] for the membranes at various reaction center/cytochrome *c* ratios. The systematic differences between the lecithin/reaction center membrane profile and the lecithin/reaction center/cytochrome *c* membrane profiles with increasing cytochrome *c* content were analyzed via appropriate difference profiles in order to determine that region of the membrane profile in which the cytochrome *c* molecules were located. These difference profiles were calculated directly from the scaled reaction center and reaction center/cytochrome *c* membrane profiles where the scaling procedure assumed that the average unit cell electron density would be relatively insensitive to the reaction center/cytochrome *c* ratio for small variations in this ratio and that reasonably large portions of the unit cell profile would be relatively unperturbed by the binding of cytochrome *c* to the reaction centers. The latter assumption was readily justified from a simple inspection of the various unscaled profiles.

b. Cobalt-cytochrome c preparation

The heme iron atom in horse heart cytochrome *c* was replaced by cobalt utilizing methods developed by Vanderkooi [5]. We are grateful to Vanderkooi for providing us with the cobalt-cytochrome *c* for these resonance X-ray scattering studies.

c. Cytochrome oxidase membrane profile structure

Reconstituted membranes composed of beef heart

cytochrome oxidase, egg lecithin and bovine cardiolipin (oxidase/lecithin/cardiolipin mole ratio of 1 : 100 : 5) were prepared in the form of unilamellar vesicles as determined by electron microscopy which were homogeneous in density as described by others [6]. The vectorial distribution of cytochrome oxidase within the profile of these reconstituted membranes was determined by published methods [7,8] and was similarly found to be relatively symmetric (about 50 ± 10% of the oxidase molecules are oriented within the membrane profile with their cytochrome *c*-binding sites exposed at the extravesicular surface of the membrane).

The lamellar X-ray diffraction from hydrated oriented multilayers formed from the unilamellar vesicular dispersions of these reconstituted membranes was collected using methods previously described in detail [2]. This diffraction was analyzed using the swelling method solution to the phase problem [9], the algorithm of Stamatoff and Krimm [10] and a minimum of four different multilayer periodicities (160 Å $\leqslant D \leqslant$ 180 Å) having demonstrated that the oriented multilayers were composed of stacks of flattened unilamellar vesicles using methods previously described [2]. This analysis has provided the electron-density profile for these membranes currently to a resolution of approx. 25 Å.

d. Measurement of resonance X-ray scattering effects

The methods utilized to collect and reduce the lamellar X-ray diffraction data as a function of incident X-ray beam energy in the neighborhood of the cobalt K absorption edge for reaction center/cobalt-cytochrome *c* membranes and the iron K absorption edge for cytochrome oxidase membranes were described in detail in the preceding paper [1]. It was shown that the resonance X-ray scattering effects from the cobalt and iron atoms on the lamellar X-ray diffraction from the respective membrane multilayers could most conveniently be expressed in the form of differential resonance scattering effects evident in the energy dependence of the ratios of integrated lamellar reflections. We shall therefore utilize these differential resonance scattering effects in our model refinement analysis described below.

e. Model refinement analysis

There are several approaches for determining the position(s) of the resonance scattering atom(s) in the membrane profile from the resonance scattering effects of these atoms on the lamellar X-ray diffraction from oriented membrane multilayers.

(1) If neither the number of possible positions of the resonance scattering atoms in the membrane profile nor the phases of the lamellar reflections are known independently, an appropriate difference Patterson function can be utilized. This difference Patterson function would utilize as coefficients the expression $[|F_{E2}(h)|-|F_{E1}(h)|]^2$, where $F_{E1}(h)$ is the multilayer unit cell profile structure factor for diffraction order h and an incident X-ray beam energy $E1$ in the near vicinity of an absorption edge for the resonance scattering atom(s) and $F_{E2}(h)$ would be that structure factor at an X-ray beam energy $E2$ far from such an absorption edge (several hundred electronvolts). This coefficient can be used when the generally small resonance scattering effects (see Ref. 1) would not be expected to alter the phase of F_{E1} relative to F_{E2}. As a result, this difference Patterson function contains only the autocorrelation function of the distribution of resonance scattering atoms in the membrane profile. This function can be readily interpreted if the number of possible positions of the resonance scattering atoms in the membrane profile is small to provide these positions to an accuracy limited by the resolution of the Patterson function and the $|F_{E1,2}(h)|$ data. A disadvantage of this approach is the need to measure the resonance scattering effects themselves (as opposed to differential effects) for all $|F(h \leqslant h_{max})|$ including those very small $F_E(h)$ for which $[|F_{E2}(h)| - |F_{E1}(h)|]$ may be large.

(2) If the number of possible positions of the resonance scattering atoms in the membrane profile is not known independently, but the phases of the lamellar reflections are known independently, an appropriate difference Fourier synthesis for the unit cell scattering profile may be utilized. The coefficients for the synthesis would be $F_{E2}(h) - F_{E1}(h)$ and the synthesis would therefore contain only the distribution of the resonance scattering atoms in the unit cell (or membrane) profile to an accuracy limited by the resolution of the synthesis and the $|F_{E1,2}(h)|$ data. A disadvantage of this approach is again that described for the difference Patterson function in Ref. 1 above.

(3) If the number of possible positions of the reso-

nance scattering atoms in the membrane profile is small and known independently (as is the case for an intrinsic metal atom of a membrane protein of which the vectorial distribution in the membrane profile was known independently), and the phases of the lamellar reflections are known independently, a model refinement procedure can be used to determine the position(s) of the resonance scattering atoms in the unit cell (or membrane) profile to an accuracy limited only by the errors in the $|F_{E1,2}(h)|$ data. Advantages of this approach are that only a small number of $|F_{E1,2}(h)|$ data need be utilized, depending on the number of independent possible positions of resonance scattering atoms in the unit cell profile, differential resonance scattering effects can be utilized and that very small $|F_{E1,2}(h)|$ data need not necessarily be measured. We note that the usual limitation of accuracy imposed by the resolution of the $|F_E(h)|$ data utilized is removed in this case due to the additional constraints placed on the data by the information known independently of the resonance scattering experiment.

As described in sections a and b above, the independently known information required for the model refinement analysis is available for the reconstituted reaction center/cytochrome *c* and cytochrome oxidase membranes. We have therefore used a model refinement analysis to predict the differential resonance scattering effects for the various ratios of integrated lamellar reflections observable from these oriented membrane multilayers as a function of positions of the known number of resonance scattering metal atoms in the multilayer unit cell profile. For the reaction center/cobalt-cytochrome *c* membranes, the single membrane profile contains only one unique cobalt atom position, since the cytochrome *c* occurs only in the extravesicular surface of the membrane profile (Refs. 2 and 4 and this paper) and its heme optical absorption is highly dichroic (see Ref. 11 for methodology). Therefore, the multilayer unit cell profile contains only two cobalt atom positions in the unit cell profile which are related by the mirror plane of symmetry between the two apposed single membrane profiles contained in the unit cell profile. For the cytochrome oxidase membranes, the single membrane profile contains only two unique iron atom positions and a total of four iron atom positions, since each cytochrome oxidase contains the heme *a* and a_3 groups and the vectorial distribution of oxidase molecules is approximately symmetric in the membrane profile, presumably about the center of the single membrane profile, and since the heme groups' optical and EPR absorption is highly dichroic (see Ref. 11 for methodology). Therefore, the multilayer unit cell profile can contain a total of eight iron atoms with a mirror plane between the two groups of four iron atoms in each of the two apposed single membrane profiles in the unit cell profile. The unit cell profiles were calculated using the $|F_{E2}(h)|$ data collected for X-ray beam energies far from the appropriate K absorption edge and the phases derived via the swelling method [1,2,9,10]. The scattering contrast of these profiles was estimated from a comparison of the integrated lower order reflections from an oriented membrane multilayer of reasonably known unit cell profile contrast (e.g., dipalmitoylphosphatidylcholine) with those from the protein-containing membrane multilayer under identical conditions of incident X-ray beam flux, multilayer/X-ray beam intersection geometry, data collection and multilayer lamellar background scattering [1]. These scaled unit cell profiles were then used to predict a calculated set of $|F_{E1}(h)|_c$ data at the appropriate K absorption edge as a function of the position(s) of the unique resonance scattering metal atom(s) in the unit cell profile; the calculated set of $|F_{E1}(h)|_c$ data utilized the known concentration of the resonance scattering metal atom in the multilayer unit cell and a resonance scattering f' effect of 6–8 electrons for the iron and cobalt atoms at their respective K absorption edges [1]. A comparison of the experimental differential resonance scattering effects expressed as:

$$\left(\frac{|F_{E1}(h)|^2}{|F_{E1}(h')|^2} - \frac{|F_{E2}(h)|^2}{|F_{E2}(h')|^2}\right)\Bigg|\left(\frac{|F_{E2}(h)|^2}{|F_{E2}(h')|^2}\right) \equiv R_e(h/h')$$

with the calculated differential resonance scattering effects expressed as:

$$\left(\frac{|F_{E1}(h)|_c^2}{|F_{E1}(h')|_c^2} - \frac{|F_{E2}(h)|^2}{|F_{E2}(h')|^2}\right)\Bigg|\left(\frac{|F_{E2}(h)|^2}{|F_{E2}(h')|^2}\right) \equiv R_c(h/h')$$

for a small set of h as a function of the positions of the known number of unique resonance scattering atoms in the unit cell profile can be used to firmly establish these positions. (We note that for our multi-

layer specimens and data collection system, the value of a particular integrated lamellar reflection $I_{E1,2}(h)$ is simply proportional to $h^{-1}|F_{E1,2}(h)|^2$, taking into account the specimen-beam intersection as a function of Bragg angle θ, the Lorentz correction, the mosaic spread of the multilayer and X-ray absorption in the multilayer.) It is readily apparent from Eqn. 8 in the preceding paper [1] and the expression for $R_c(h/h')$ that the number of comparisons required between R_e and R_c at different values of h depends simply on the number of possible unique positions of the resonance scattering metal atoms in the unit cell profile. The accuracy of the determination of these positions depends primarily on the experimental errors in the $|F_{E1,2}(h)|$ data; this accuracy can be improved by a redundancy in the comparisons of $R_e(h/h')$ and $R_c(h/h')$ by simply measuring more reflections at a fixed multilayer periodicity or fewer reflections at a number of periodicities. The effects of errors in the estimated contrast of the scaled unit cell profiles on the accuracy of the determination of the positions of the resonance scattering atoms in the unit cell profile have been investigated by examination of the effects of computational variation of the estimated contrast (over a reasonably wide range of possible values) on the comparisons of $R_e(h/h')$ and $R_c(h/h')$.

Results

a. Reaction center-cytochrome c membranes

The differential resonance scattering effect $R_e(h/h')$ measured for the reaction center/cobalt-cytochrome *c*

TABLE I

EXPERIMENTAL DIFFERENTIAL RESONANCE X-RAY DIFFRACTION EFFECTS

Reaction center/cobalt cytochrome *c* multilayers.

$R_e(h/h')$		
$R_e(3/2)$	−32.0% ±	5.3 [a]
$R_e(4/2)$	+7.9% ±	1.3
$R_e(6/2)$	+23.0% ±	6.1

[a] Errors were propagated assuming that all non-edge ratios of integrated lamellar reflections are equivalent and independent. This requires that the energy dependence of the transfer function for the data collection system is removed by utilizing these ratios (see Ref. 1).

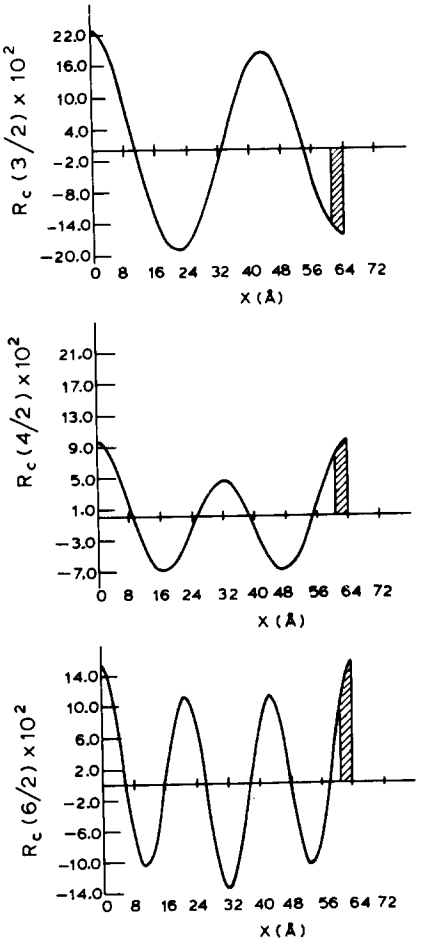

Fig. 1. The calculated differential resonance X-ray diffraction effects $R_c(h/h')$ for $h' = 2$ and $h = 3, 4$ and 6 as a function of the position of the one unique cobalt atom in the half-unit cell profile ($0 \text{ Å} \leq X \leq D/2 = 64$ Å) which contains the single membrane profile of the reconstituted reaction center/ cobalt cytochrome *c* membrane. The estimated unit cell contrast used was 0.008 e/Å^3. A comparison of the signs and conservative estimates of the relative magnitudes of the measured $R_e(h/h')$ values (Table I) with their respective $R_c(h/h')$ functions restricts the allowed positions of the cobalt atom to the one stippled region indicated within $61 \text{ Å} \leq X \leq 64$ Å. See text for further details (A) $h = 3$, $h' = 2$; (B) $h = 4$, $h' = 2$; (C) $h = 6, h' = 2$.

complex for $h' = 2$ and $h = 3, 4$ and 6 in the neighborhood (±200 eV) of the measured cytochrome *c* cobalt K absorption edge (at 7735 eV) are shown in Fig. 1 of the preceding paper [1]. The sources of the experimental errors in these measurements are discussed in that paper [1]; no such differential reso-

nance scattering effects for these reflections are observed for the reaction center/iron-cytochrome *c* complex over the same energy range about the cobalt K edge as expected.

For the model refinement analysis of these experimental resonance scattering effects, we utilize the $R_e(h/h')$ obtained from the average of $|F_{E2}|$ at ±200 and ±100 eV with respect to the cobalt K absorption edge; these values of $R_e(h/h')$ and their errors are shown in Table I.

The calculated differential resonance scattering effect $R_c(h/h')$ for $h' = 2$ and $h = 3, 4$ and 6 as a function of the position of the one possible unique cobalt atom in the half-unit cell profile ($0\ \text{Å} \leqslant X \leqslant D/2 = 64\ \text{Å}$) for an estimated unit cell contrast of 0.008 e/Å^3 are shown in Fig. 1. Inspection of Fig. 1 indicates that a precise measurement (no errors) of one particular $R_e(h/h')$ value generally would result in h possible solutions ($h > h'$) for the position of the cobalt atom in the half-unit cell upon comparison of that $R_e(h/h')$ value with its respective calculated $R_c(h/h')$ function. This number of solutions resulting from the comparison of only one $R_e(h/h')$ value with its respective $R_c(h/h')$ function results as a direct mathematical consequence of the expression of the measured differential resonance scattering effects. Further inspection of Fig. 1 indicates that the introduction of errors into the measurement of a particular $R_e(h/h')$ value results in the restriction of the possible solutions for the position of the cobalt atom in the half-unit cell to allowed regions upon comparison of the $R_e(h/h')$ value with its respective $R_c(h/h')$ function. The widths of these regions derive directly from the errors in the measured $R_e(h/h')$ value.

If we utilize only the signs of the measured $R_e(h/h')$ values from Table I, it is readily apparent from Fig. 1 that all three of the measured R_e values are sufficient to restrict the possible positions of the cobalt atom to only one moderately narrow region in the half-unit cell profile within $58\ \text{Å} \leqslant X \leqslant 64\ \text{Å}$. However, if we utilize conservative estimates of the relative magnitudes of the measured R_e values (e.g., from Table I, $R_e(3/2) \approx R_e(6/2) > R_e(4/2)$), it is apparent from Fig. 1 that the comparisons of only $R_e(4/2)$ with $R_c(4/2)$ and $R_e(6/2)$ with $R_c(6/2)$ are sufficient to restrict further the possible cobalt atom positions to only one allowed region within $61\ \text{Å} \leqslant X \leqslant 64\ \text{Å}$. Utilization of the conservative estimate of the relative magnitude of $R_e(3/2)$ in addition provides no further restriction to the allowed positions.

The computational variation of the unit cell scattering contrast from 0.005 to 0.015 e/Å^3 in steps of 0.001 e/Å^3 does not seriously affect the restriction of

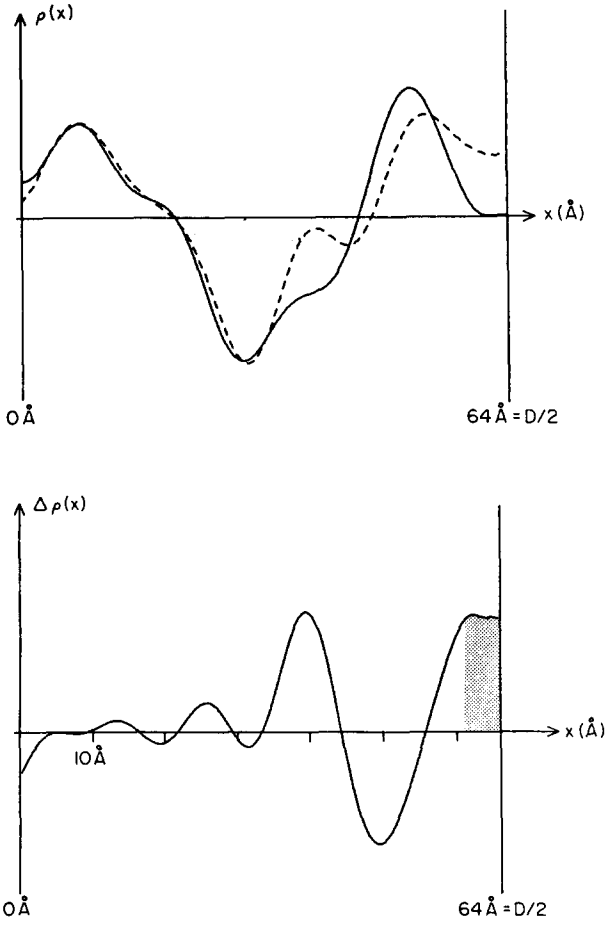

Fig. 2. (A) Scaled electron-density profile for the lecithin/reaction center single membrane profile at approx. 12 Å resolution contained within the half-unit cell profile (solid line). (B) Scaled electron-density profile for the lecithin/reaction center/cytochrome *c* single membrane profile at approx. 12 Å resolution for a cytochrome *c*/reaction center mole ratio of 0.8 contained within the half-unit cell profile (dashed line). (C) The difference electron-density profile calculated as the difference between the scaled reaction center/cytochrome *c* and reaction center single membrane profiles. The relative scaling procedure is described in the text. The allowed positions of the cytochrome *c* heme cobalt (or iron) atom in the half-unit cell profiles $0\ \text{Å} \leqslant |X| \leqslant D/2 = 64\ \text{Å}$ provided by the analysis of the resonance X-ray diffraction data are indicated by the stippled region.

the possible positions of the unique cobalt atom in the half-unit cell profile to only one allowed region 61 Å $\leqslant X \leqslant$ 64 Å. This insensitivity of the comparisons of the relative R_e values with their R_c functions to scattering contrast arises because the zeros of the $R_c(h/h'; X)$ function and the ratios of $R_c(h1/h'; X)/$ $R_c(h2/h'; X)$ are invariant to this contrast variation ($h1$ and $h2$ are simply two different h values different from h').

Therefore, it can be seen that for the case of one unique resonance scattering metal atom in the unit cell profile, the measurement of differential resonance scattering effects for only two to three values of (h/h') can highly restrict the allowed position of the unique metal atom to a single moderately narrow region in the unit cell profile even at this modest level of experimental accuracy. Further measurement at additional values of (h/h') or at different multilayer periodicities for the same (h/h') values is redundant, thereby permitting some further restriction in the width of the allowed region or some relaxation of the experimental accuracy otherwise required of fewer measurements.

The single membrane electron-density profiles for the lecithin/reaction center membrane and the lecithin/reaction center/cytochrome *c* membrane (at the cytochrome *c*/reaction center ratio of 0.8 relevant to the resonance scattering experiments) are shown in Fig. 2 at about 12 Å resolution. The difference profile between the scaled reaction center and reaction center/cytochrome *c* membrane profiles is also shown in Fig. 2. It is readily apparent from the difference profile shown that the binding of cytochrome *c* to the reaction center induces significant changes in the lecithin/reaction center membrane profile only within the region 40 Å $\leqslant X \leqslant$ 64 Å. The magnitude of the changes vary systematically with variation of the cytochrome *c*/reaction center ratio and remain confined to only this region of the membrane profile (data not shown). The cytochrome *c* molecule must therefore be located within the half-unit cell profile within the region 40 Å $\leqslant X \leqslant$ 64 Å; the one region within the half-unit cell profile containing the possible positions of the cytochrome *c* cobalt atom allowed by $R_e(3/2)$, $R_e(4/2)$ and $R_e(6/2)$ of 61 Å $\leqslant$ $X \leqslant$ 64 Å is also shown in Fig. 2 for comparison. We note that the allowed positions of the cytochrome *c* cobalt atom in the unit cell profile as determined from the resonance X-ray scattering data from cobalt do indeed occur within that region of the profile occupied by cytochrome *c* as determined independently from the resonance scattering experiments.

b. Cytochrome oxidase membranes

The differential resonance scattering effect $R_e(h/h')$ for cytochrome oxidase for $h' = 4$ and $h = 2, 3, 5$ and 6 in the neighborhood (±200 eV) of the measured iron K absorption edge at (7125 eV) are shown in Fig. 1 of the preceding paper [1]. The sources of the experimental errors are described in that paper [1]. No such differential resonance scattering effects are observed over a ±200 eV range centered about 7735 eV as expected, since cytochrome oxidase contains no metal atoms with K absorption edges in that energy range (e.g., cobalt).

For the model refinement analysis of these experimental resonance scattering effects, we again utilize the $R_e(h/h')$ obtained from the average of $|F_{E2}|$ at ±200 and ±100 eV with respect to the iron K absorp-

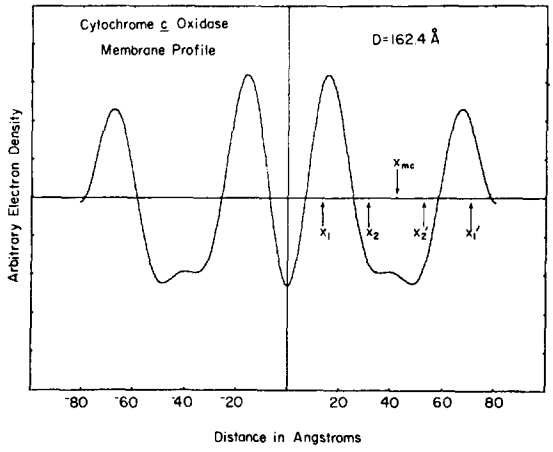

Fig. 3. Electron-density profile for the unit cell containing two apposed cytochrome oxidase membrane profiles at approx. 25 Å resolution. A single asymmetric membrane profile is contained within the half-unit cell 0 Å $\leqslant |X| \leqslant D/2 =$ 81.2 Å. The possible positions of the two unique iron atoms associated with the oxidase heme *a* and a_3 groups in the half-unit cell profile containing a single membrane profile are indicated by the variables $X1$ and $X2$. The nearly symmetrical vectorial distribution of cytochrome oxidase molecules about the membrane center X_{mc} in these single membrane profiles as measured independently suggests that the positions $X1$, $X2$ and $X1' = 2X_{mc} - X1$, $X2' = 2X_{mx} - X2$ are equivalent only to a first approximation (see text for details).

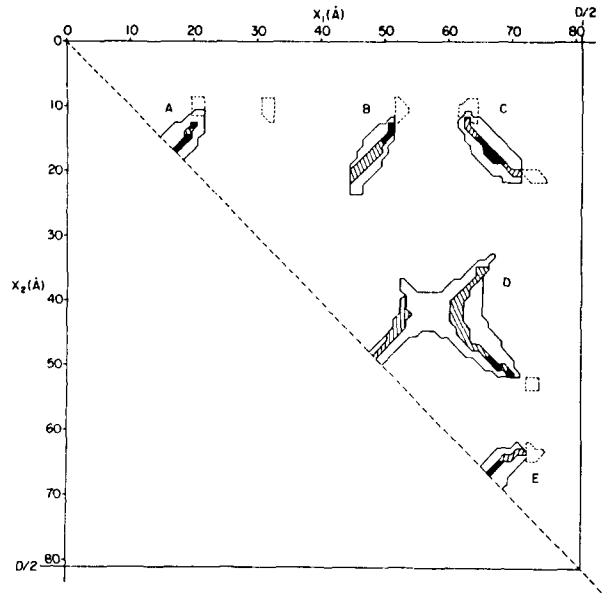

Fig. 4. A graphical representation of the comparison between the measured differential resonance diffraction effects $R_e(h/h')$ with the calculated $R_c(h/h')$ values as a function of the positions $X1$ and $X2$ of the two unique heme a and a_3 iron atoms in the half-unit cell profile. The variables $X1$ and $X2$ each vary independently over $0\ \text{Å} \leqslant X1$, $X2 \leqslant D/2 = 81.2$ Å. A line of symmetry from $X1 = X2 = 0$ Å to $X1 = X2 = D/2$ occurs in this representation because the heme a and a_3 contents of oxidase are identical and the a and a_3 iron atoms are therefore indistinguishable by resonance X-ray scattering. The estimated unit cell scattering contrast used was 0.012 e/Å^3. A comparison of only the signs of the $R_e(h/h')$ values for $h = 2$, 3 and 6 and $h' = 4$ with their respective $R_c(h/h')$ values calculated as a function of $X1$ and $X2$ severely restrict the allowed values of $X1$ and $X2$ to only five regions of moderate size labelled A–E. Utilization of the relative magnitude of $R_e(2/4)$, $R_e(3/4)$ and $R_e(6/4)$ and only the sign of $R_e(5/4)$ (Table II) further restricts the allowed values of $X1$ and $X2$ to the small solid regions within only the three regions A, B and D. Utilization of the $R_e(2/4)$, $R_e(3/4)$ and $R_e(6/4)$ values obtained at a larger multilayer periodicity restricts the allowed values of $X1$ and $X2$ to only the regions outlined with dashed lines. Since the actual values of $X1$ and $X2$ at the two multilayer periodicities must differ by less than 1 Å, only the small solid regions within the two regions A and B can be allowed by the differential resonance diffraction data at the two different multilayer periodicities (162.4 and 168.3 Å). See text for further details.

tion edge; these values of $R_e(h/h')$ and their errors are shown in Table II.

The differential resonance scattering effects $R_c(h/h')$ for $h' = 4$ and $h = 2$, 3, 5 and 6 were calculated as a function of the positions $X1$ and $X2$ of the

TABLE II

EXPERIMENTAL DIFFERENTIAL RESONANCE X-RAY DIFFRACTION EFFECTS

Cytochrome oxidase multilayers.

$R_e(h/h')$		
$R_e(2/4)$	+1.44% ±	0.49 [a]
$R_e(3/4)$	+1.61% ±	1.27
$R_e(5/4)$	−5.71% ±	5.40
$R_e(6/4)$	−1.37% ±	0.86

[a] Errors were propagated assuming that all non-edge ratios of integrated lamellar reflections are equivalent and independent. This requires that the energy dependence of the transfer function for the data collection system is removed by utilizing these ratios (see Ref. 1).

two possible unique iron atoms in the half-unit cell profile ($0\ \text{Å} \leqslant X \leqslant D/2 = 81$ Å) for an estimated unit cell profile contrast of 0.012 e/Å^3. These positions $X1$ and $X2$ are indicated in the low resolution unit cell electron-density profile, derived as described in Methods, shown in Fig. 3. Since the vectorial distribution of cytochrome oxidase molecules in the membrane profile is approximately symmetric, presumably about the center of the single membrane profile denoted by X_{mc}, the four possible positions of the oxidase heme iron atoms in the half-unit cell are $X1$, $X2$ and $X1' = 2X_{mc}-X1$, $X2' = 2X_{mc}-X2$. Our most reliable measurement * of the vectorial distribution of oxidase molecules in the membrane profile indicates a 60 : 40 distributional asymmetry and the derived single membrane electron-density profile is indeed clearly asymmetric ** (Fig. 3) due to the occurrence of several significant odd-order lamellar reflections from the membrane multilayer at both lower and higher scattering angles. Therefore, the po-

* We feel that the fractional reduction in heme a by reduced cytochrome c added to preformed cytochrome oxidase membrane vesicles where the subsequent reduction of heme a_3 is blocked by azide [7] is more reliable than the comparison of the oxygen reduction activity of cytochrome oxidase in membrane vesicles as compared to that in detergent using reduced cytochrome c as the electron donor [7]. Our preference arises from the fact that the oxygen reduction activity of cytochrome oxidase in detergents is highly variable depending on the detergent [8].

** A 50 : 50 symmetric vectorial distribution of cytochrome oxidase molecules in the membrane profile is inconsistent with the asymmetry of the derived single membrane electron-density profile.

sitions $X1$, $X2$ and $X1'$, $X2'$ were taken to be inequivalent, having a weighting of approx. 60 and 40, respectively, in our calculation of $R_c(h/h')$ as a function of $0\ \text{Å} \leqslant X1, X2 \leqslant D/2 = 81\ \text{Å}$.

A comparison of the measured $R_e(2/4)$, $R_e(3/4)$ and $R_e(6/4)$ with their respective $R_c(h/h')$ calculated as a function of $X1$ and $X2$ on the basis of only the signs of the $R_e(h/h')$ values severely restricts the allowed values of $X1$ and $X2$ to five regions of moderate size labelled A–E in Fig. 4. Utilization of the information contained in Table II that the magnitudes of $R_e(2/4)$ and $R_e(6/4)$ are comparable results in the allowed values of $X1$ and $X2$ becoming further restricted to the smaller solid and hatched regions within the regions A–E. Utilization of the additional information that the magnitudes of $R_e(2/4)$, $R_e(3/4$ and $R_e(6/4)$ are all comparable restricts even further the allowed values of $X1$ and $X2$ to only the small solid regions within A–E. The measurement of $R_e(5/4)$ contains the largest experimental error. The sign of the measured $R_e(5/4)$ further restricts the allowed values of $X1$ and $X2$ to only the solid regions within the three regions A, B and D. At this point, if the errors in the measurement of $R_e(5/4)$ were smaller such that the value of $R_e(5/4)$ were indeed that indicated in Table II relative to the other measured $R_e(h/h')$ values, only the one small solid region within region B would contain the allowed values of $X1$, $X2$. However, in the present absence of more accurate data on $R_e(5/4)$, the utilization of $R_e(2/4)$, $R_e(3/4)$ and $R_e(6/4)$ data from the cytochrome oxidase membrane multilayer at a higher periodicity (168.3 vs. 163.4 Å) restricts the allowed values of $X1$ and $X2$ to the small solid regions within only the two regions A and B. (At the higher multilayer periodicity, $R_e(2/4)$ and $R_e(6/4)$ become vanishingly small while $R_e(3/4)$ remains substantial (approx. 1.5%) and positive in sign; these data limit the allowed values of $X1$ and $X2$ to the dashed-box regions in Fig. 4. For the purpose of easily comparing the allowed regions of $X1$ and $X2$ at the two different multilayer periodicities in one figure, the plots of the allowed regions at the two periodicities were superimposed such that the $X1 = X2$ value corresponding to the center of the single membrane profile at X_{mc} superimposed. We would expect that the positions of the iron atoms in the single membrane profile would be relatively invariant to changes in the multilayer periodicity, since the membrane profile is invariant to less than 1 Å, i.e., $X1$ and $X2$ should be the same to within 1 Å for the two periodicities. Inspection of Fig. 4 indicates that the solid regions only within the two regions A and B allowed at the lower periodicity are within less than 1 Å of the allowed regions at the higher periodicity).

In view of the errors in our measured $R_e(h/h')$ values at the two multilayer periodicities available at the present time, we conclude that the allowed values of $X1$ and $X2$ can currently be restricted to the small solid regions within only the two regions A and B. We note that the actual unit cell scattering contrast utilized in the above comparisons of the relative R_e values and the R_c functions is of little consequence to the above results, since the zeros of the $R_c(h/h'; X1, X2)$ function and the ratios $R_c(h1/h'; X1, X2)/R_c(h2/h'; X1, X2)$ are invariant to the computational variation of this scattering contrast.

Discussion

The model refinement analysis of the differential resonance X-ray scattering effects from the cobalt atom in the reaction center/cobalt cytochrome *c* complex contained in oriented membrane multilayers has restricted the position of the one unique cobalt atom in the single membrane profile to a single allowed region only 3 Å in width. This allowed region is fully consistent with the location of the cytochrome *c* molecule in this single membrane profile which was derived independently of the resonance scattering experiments. Therefore, the necessary conol experiment has been performed concerning the utilization of resonance X-ray scattering to determine the position of a dilute intrinsic metal atom in a membrane protein within the membrane profile structure to moderate accuracy.

It is clear that the model refinement analysis of differential resonance scattering effects as presented is capable of determining the positions of the resonance scattering metal atoms in the unit cell profile to a moderately high accuracy if the number of unique metal atoms in the unit cell profile is small and known independently and the phases of the lamellar reflections are known independently. The accuracy of the determined positions simply depends on the accuracy of the measured $|F_{E1,2}(h)|$ data if a

minimal number of measurements of $|F_{E1}(h)|$ and $|F_{E2}(h)|$ are utilized, that number simply depending on the number of possible unique positions of the resonance scattering atoms in the membrane profile. Increasing the number of measurements (more values of h or the same set of h at different multilayer periodicities) makes the determination of the metal atom positions redundant, thereby permitting an improvement in the accuracy of the determination or some relaxation of the accuracy otherwise required of fewer measured $|F_{E1,2}(h)|$ data.

Concerning the use of differential resonance X-ray scattering effects to determine the locations of the heme a and a_3 iron atoms within the cytochrome oxidase membrane profile structure, our results to date indicate that the two unique iron atoms may be located either at $X_1 \simeq 20$ Å and $X_2 \simeq 12$ Å or at $X_1 \simeq 50$ Å and $X_2 \simeq 12$ Å in Fig. 4. We note that it is clear from these results that additional measurements of $R_e(h/h')$ for the same values of h (e.g. 2, 3, 4, 5, 6) at higher accuracy (using a two-dimensional position-sensitive X-ray detector [1] and finer scans of incident X-ray beam energy) or at a greater number of h values or multilayer periodicities will be sufficient to establish firmly the values of X_1 and X_2. As a result, the positions and separations of the heme a and a_3 iron atoms and also the two copper atoms within the cytochrome oxidase membrane profile structure can be determined accurately without ambiguity utilizing resonance X-ray scattering. The positions of the two copper atoms in the membrane profile would of course utilize resonance X-ray scattering data collected about the measured copper K absorption edge.

We note that a determination of the positions of resonance scattering atoms within a membrane profile via the model refinement analysis is strongly dependent on reliable measurements of the vectorial distribution of the membrane protein containing these atoms in the membrane profile.

Finally, these resonance X-ray scattering methods can in principle also be applied to the equatorial scattering from such oriented membrane multilayers. The related analyses of such resonance scattering data can provide the relative positions of the resonance scattering atoms associated with the redox centers projected onto the membrane plane. This information coupled with that concerning their relative positions in the membrane profile projection (as described in this paper) can be used to provide absolute distances between the resonance scattering atoms of these redox centers.

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