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SHORT COMMUNICATIONS

The Structure of a Centrosymmetric Protein Crystal

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ABSTRACT **Crystals of racemic rubredoxin, prepared by independent chemical synthesis of the two enantiomers, have been grown and characterized. The unit cell contains two molecules, one of each enantiomer. Examination of the intensity distribution in the diffraction pattern revealed that the crystals are centrosymmetric. This was confirmed by solution of the structure to 2 Å resolution via molecular replacement methods. The electron density maps are of very high quality due to the fact that the phase of each reflection must be exactly 0° or exactly 180°. These results demonstrate the feasibility of using synthetic racemic proteins to yield centrosymmetric protein crystals with electron density maps that have very low phase error and model bias.**

Key words: molecular replacement, rubredoxin, phase error, intensity distribution

INTRODUCTION

X-Ray crystallographic methods are extremely powerful for determining molecular structures. Such methods are based on calculating the electron density function for a crystal via a Fourier expansion.[1] A major complication of these methods is that such calculations require both the amplitudes and the phases of the terms in the Fourier series, and only the former can be directly measured. This leads to the "phase problem" which has at least two levels. First, initial phases of sufficient accuracy must be obtained to produce an electron density map that can be interpreted to yield a preliminary molecular model. Second, the phases must, in most cases, be refined by some means to improve the quality of the electron density map to reveal more subtle features of the structure. For crystals of many small molecules, extremely high quality electron density maps can be obtained. This is especially true for crystals that are centrosymmetric, a feature which requires that the phase of each term in the expansion be exactly 0° or exactly 180° rather than potentially taking on any value from 0 to 360°, as is the case for noncentrosymmetric crystals. For naturally occurring macromolecules such as proteins, the formation of centrosymmetric crystals is impossible since they are chiral and only one of the two enantiomers is present.

To generate centrosymmetric protein crystals, we prepared a racemic protein solution by mixing independently synthesized proteins constructed from either all L- or all D-amino acids. The protein we selected for initial studies was rubredoxin from *Desulfovibrio desulfuricans,* a 45 amino acid iron-binding protein. Previous characterization of the enantiomeric proteins via circular dichroism spectroscopy, metal binding titrations, and sensitivity to proteolysis revealed the expected properties.[2] Racemic protein solutions can be used to try to produce centrosymmetric crystals. However, assuming that crystals can be obtained from such solutions, it is not assured that these crystals will be centrosymmetric. In this initial report, we demonstrate that we have been able to produce crystals of racemic rubredoxin that are centrosymmetric. This has been confirmed by solving the structure to 2 Å resolution via molecular replacement. Examination of the electron density maps illustrates some of the advantages of such crystals.

MATERIALS AND METHODS

Rubredoxin enantiomers were synthesized and purified as previously described.[2] Crystals were grown by the hanging drop method[3] prepared by mixing 2 μl of a racemic D,L-rubredoxin–Fe^{3+} solution at 13 mg/ml with 2 μl of reservoir buffer: 1.7 M ammonium sulfate, 0.1 M sodium citrate, pH 5.25. The original conditions to crystallize L-rubredoxin[5] resulted in overnight growth of large red needle clusters or clumps of small crystals. To slow crystal growth, hanging drops were prepared with a pH gra-

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dient, pH 5.0 to 5.6, and incubated at 4°C. A diffraction quality crystal grew after 4 weeks. Using identical conditions, except for increased drop size (6–8 μl), good quality crystals grow from the racemic mix at 4°C after 2–3 weeks, except that the plates have a tendancy to grow in spiral fans.

X-ray diffraction studies were performed on a single crystal and data was collected on a Siemens area detector with CuK_α radiation. Data reduction was performed using the XENGEN software, and reflections were examined in a range from infinity to 2.0 Å resolution. This data set consisted of 4239 unique, nonzero reflections. This data set is 89% complete from 12 to 2.0 Å, with an R-merge of 4.8%.

The program X-PLOR[4] was used for the molecular replacement solution. The rotation function resulted in a major peak 70% greater than any unrelated peak. This solution was optimized using the Patterson correlation method and used in a translation

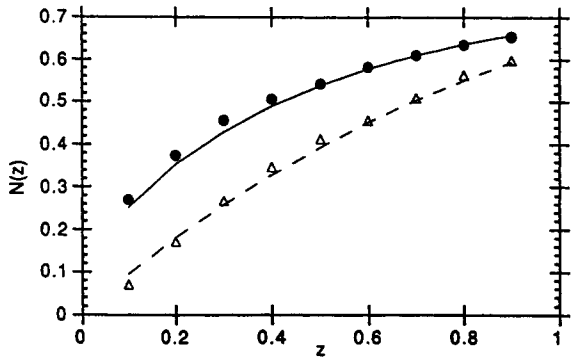

Fig. 1. Graphical comparison of intensity distributions. Experimental points are shown for the D,L-rubredoxin–Fe^{3+} (●), collected in this work. Data for L-rubredoxin–Fe^{3+} (△) were calculated from the published coordinates.[5] Theoretical curves are shown for a centrosymmetric distribution (——) and the non-centrosymmetric distribution (– – –). See ref. 7 for details.

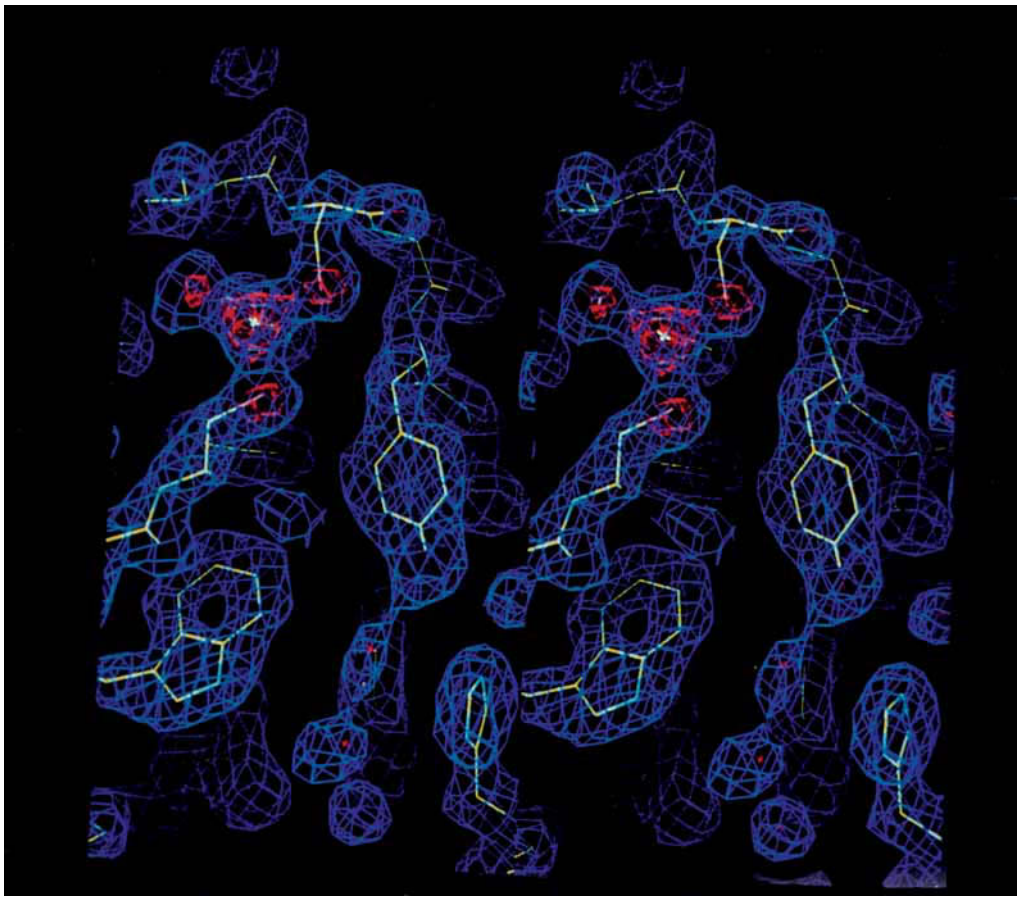

Fig. 2. Stereo electron density maps of the centrosymmetric crystals.[12] This electron density map was calculated using ($2|F_o| - |F_c|$) coefficents with the model-derived phases. The blue contours represent 1.0 σ density, whereas the red depicts 4.0 σ density. The protein model is shown and waters are indicated by red crosses. Notably, one can see several hydrophobic residues, the Fe–S_4 cluster, and well-defined density around the backbone carbonyls. The red 4.0 σ density not only clearly demonstrates the location of the Fe–S_4 cluster, but also this region of the map presented no problems during refinement.

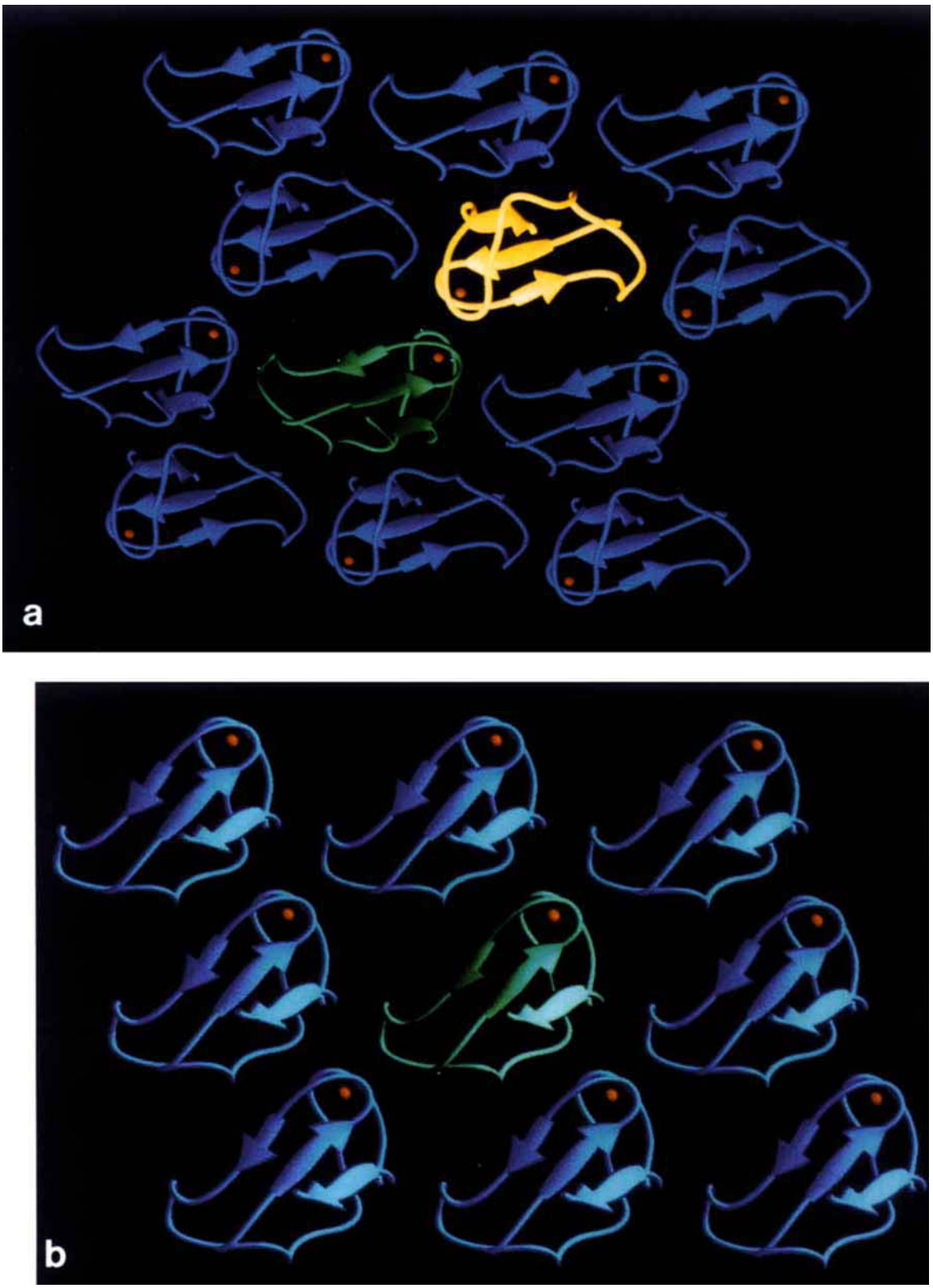

Fig. 3. Crystal packing diagrams for the racemic and natural rubredoxin crystals. (**a**) Ribbons[13] drawing illustrating the packing for the racemic crystals for six pairs of molecules in one plane. The Fe^{3+} atoms are shown in red with one L-isomer and one D-isomer protein molecule depicted in green and yellow, respectively. (**b**) The packing for the natural (L-amino acid only) crystals.[5] A total of nine molecules are shown with one molecule in green.

search. The translation function peak value eclipsed the next best solution by 4.2 standard deviations. The structure was refined by X-PLOR using conventional positional refinement and individual isotropic temperature factors. During molecular replacement and refinement, only reflections with observed structure factors greater than 2 σ were used. The range of temperature factors is 4.6 to 46.0 for waters and, 2.5 to 44.0 for the protein atoms, with a mean of 11.9 overall. The rms deviations from ideality for bond lengths and bond angles are 0.016 Å and 2.8°, respectively.

RESULTS AND DISCUSSION

The crystal was found to be triclinic with unit cell dimensions a = 26.46 Å, b = 19.91 Å, c = 36.85 Å, α = 109.60°, β = 85.54°, γ = 95.16°, V = 18,159 Å^3. Crystals of the natural protein (L-amino acid only) have a unit cell that is approximately one-half of this volume and contains a single molecule.[5] This comparison suggested that our unit cell probably contains two molecules. To determine whether the crystals were, indeed, centrosymmetric, the distributions of intensities were examined, as it has long been known that centrosymmetric and noncentrosymmetric crystals differ in this regard.[6,7] The results are shown in Figure 1. The striking similarity between the theoretical and experimental curves strongly indicated that the crystals are centrosymmetric and, hence, belong to the space group P1 bar with one molecule in the asymmetric unit and, hence, one molecule of each chirality in the unit cell.

In order to solve the structure by the molecular replacement method, a 2 Å data set was examined with the X-PLOR package and the previously determined structure of the natural protein. Both the rotation and translation functions gave unique solutions that were significantly greater than the alternatives. This initial structure yielded an R factor of 49% for data from 15 to 4 Å resolution and 57% for data from 6 to 2 Å. The expected value of R for a totally random structure has been derived[6] and shown to be 83% for a centric distribution. We concluded that the translation function solution was correct. Examination of the electron density at this point revealed clear density for the backbone including carbonyl groups, as well as for most side chains. The structure was refined by conventional positional refinement to convergence to yield an R factor of 34% for data from 6 to 2 Å resolution.

Examination of a difference electron density map showed no unaccounted density within the protein and clear density for a number of water molecules. At this stage, 35 well-defined waters were added to the asymmetric unit and the structure was refined with individual isotropic temperature factors and unit occupancies. The R factor at this stage of refinement was 24%. Luzzati analysis suggests that this corresponds to an R factor of 16% for a noncentrosymmetric crystal with similar coordinate error.[8] A portion of the final electron density map is shown in Figure 2.

After refinement to 24%, the Fe–(S-Cys)$_4$ cluster was examined in X-PLOR by setting the bond lengths and angles to equilibrium values for a tetrahedal geometry, and then lowering the penalties for changes in Fe–S bond distances during positional refinement. The Fe–S bond lengths range from 2.269 to 2.322 Å, with an average of 2.296 Å. The S–Fe–S angles vary from 102.6° to 115.3°, with an average angle of 109.5°. This sort of distortion of the Fe–S$_4$ tetrahedron has been seen in all rubredoxin crystal structures solved to date, and several model compounds.[5]

The electron density is very well defined due, in part, to the nature of the phases for the centrosymmetric crystals. The robustness of the phases can be illustrated by examining the phases at various stages of refinement. Specifically, we compared the phases for the initial, intermediate, and final models (R-factors of 57, 34, and 24%, respectively). If one considers the top 4/5 of the reflections with respect to intensity, then the phases of the initial model agree with those of the final model for 80% of these reflections. The phases from the intermediate model and the final model agree for 94% of these reflections. The small differences between the phases for the early, unrefined models and those for the final model can be ascribed to the phase restrictions for the centrosymmetric crystal as well as to the quality of the search molecule used in the molecular replacement procedure.

The packing in the centrosymmetric crystal as well as that for the natural protein is shown in Figure 3. The packing in one plane is very similar between the two crystals. For the centrosymmetric crystal, this "sheet" of protein molecules of one chirality has a sheet of molecules of the opposite chirality inverted above it.

These results demonstrate that the growth of centrosymmetric crystals of racemic synthetic proteins is, indeed, possible. It should be noted that other outcomes are possible.[9] Racemic proteins could spontaneously resolve into crystals of enantiomerically pure materials or could crystallize with the two enantiomers related by symmetry operations other than an inversion center. However, if conditions can be found that yield centrosymmetric crystals, the present results indicate that electron density maps with very precise and robust phases can be obtained. Such crystals should be useful for examining subtle structural features without limitations of phase bias due to the model used. Finally, it may well be possible to solve centrosymmetric protein structures by direct methods.[2,10,11] We are presently collecting data to higher resolution to realize this possibility for racemic rubredoxin.

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