to $1/F_o^2$ for $F_o \geq 4F_o$ (min.) and $\frac{1}{4}F_o \times F_o$(min.) otherwise (Marsh & Schomaker, 1979). Except where indicated, final shifts were less than 0·1 e.s.d. Calculations were carried out on a VAX-750, using the *CRYM* system of crystallographic programs.

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SHORT COMMUNICATION

Contributions intended for publication under this heading should be expressly so marked; they should not exceed about 1000 *words; they should be forwarded in the usual way to the appropriate Co-editor; they will be published as speedily as possible.*

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Least-squares refinement of two protein molecules per asymmetric unit with and without non-crystallographic symmetry restrained. By A. TULINSKY and R. A. BLEVINS,* *Department of Chemistry, Michigan State University, East Lansing, MI* 48824, *USA*

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Abstract

Monoclinic crystals of α-chymotrypsin (α-CHT) possess two molecules per asymmetric unit related by non-crystallographic twofold symmetry. The structure has been refined as such at 1·67 Å resolution [free refinement: Blevins & Tulinsky (1985). *J. Biol. Chem.* **260**, 4264-4275] and, now, with the equivalence imposed. The equivalence was restrained to conform to expected errors in coordinates (moderate refinement) and to a stringent restraint of 0·05 Å (tight refinement). All three refinements led to a highly acceptable geometry and R values (0·179-0·198) along with other key indicators. As anticipated, the tight refinement produced a highly twofold-related structure whereas the moderate refinement produced non-equivalence not unlike that observed in the free refinement: main-chain folding was equivalent but side chains on the surface and in the dimer interface were in general not equivalent. The determination of the solvent structure deteriorated spectacularly in going to the tight equivalence restraint. In cases of high resolution and high quality data, imposition of non-crystallograhic symmetry appears ill-advised since the data will preserve the equivalence. At lower resolution, and/or with inferior data, restraining symmetry could be advantageous and expedient in obtaining a consensus structure.

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The restrained least-squares refinement of α-chymotrypsin (α-CHT) at 1·67 Å resolution as two molecules per asymmetric unit has shown that the side chains are generally unequivalent around the surface and in the dimer interface region (Blevins & Tulinsky, 1985*a*) (referred to hereafter as free refinement), which is consistent with other crystallographic observations (Mavridis, Tulinsky & Liebman, 1974; Tulinsky, Mavridis & Mann, 1978; Tulinsky, 1980). However, the main-chain folding and certain other important regions of the two molecules, such as the catalytic and specificity sites, are practically identical. Since there are many instances of more than one molecule per asymmetric unit in protein crystals, we have investigated the effect of imposing equivalence as a restraint in refinement. Since the restraint is not exact, a 'tight' and a 'moderate' alternative were pursued and these results are compared with those of the free refinement (Blevins & Tulinsky, 1985*a*).

The refinement of α-CHT dimer was performed using Hendrickson's program *PROLSQ* (Hendrickson & Konnert, 1980): (1) by imposing non-crystallographic twofold symmetry restrained to conform to expected errors in coordinates (0·20-0·25 Å)† (moderate) and (2) as in (1) but with a stringent restraint of 0·05 Å (tight). The results of the free refinement have appeared elsewhere (Blevins & Tulinsky, 1985*a*, *b*) along with a description of the experimental procedures employed to obtain and process the

† 0·50 Å proved to be equivalent to no restraint.

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Table 1. *Final R factors of independent refinements* ($R = \sum \|F_o| - |F_c\| / \sum |F_o|$)

Range	(5·0–1·67)Å			(8·0–1·67)Å		
Structure	Free	Moderate	Tight	Free	Moderate	Tight
Final	0·179	0·185	0·198	0·194	0·201	0·214
No solvent	0·218	0·221	0·224	0·227	0·238	0·241
H included*	0·180	0·187	0·199	0·201	0·206	0·217

* All protein H atoms were included at calculated positions; if free rotation was possible, they were included at 'idealized' positions.

observed data used in the refinements. All the refinements began with the same twofold symmetrical structure (Birktoft & Blow, 1972), the refinements were carried out in resolution stages/ranges (3·0, 2·5, 2·0, 1·67 Å) and the same protocol was generally followed. From the results of the free refinement an estimate of 0·20 Å was obtained for coordinate error which formed the basis of the moderate refinement.

A total of 86 cycles were carried out in the moderate and 67 in the tight refinement. The r.m.s. deviations of these final structures from ideal geometry are comparable to those of the free refinement. Various R factors are summarized in Table 1 which shows that all three refinements lead to highly acceptable R values. Somewhat of a surprise are the similar R values of the free and tight refinements since the dimer structure is fairly unequivalent (Blevins & Tulinsky, 1985*a*).

Since we were strictly testing the effect of imposing molecular equivalence, graphics was not used to refit individual portions of the molecules. However, since the equivalence restraint was not applied to solvent, solvent peaks were examined with graphics.

Coordinate differences occurred early but the first notable appearance of other significant differences between the refinements occurred when solvent was included in the twofold equivalence calculations. This can be seen from Fig. 1 which shows the number of water molecules included in the calculations and the distribution of the occupancies of water molecules after refinement. The moderate and free refinements display expected behavior. This is not the case with the tight refinement where, after the first wave of 110 water molecules, little other significant solvent structure could be uncovered and/or maintained (Fig. 1, Table 1); from Fig. 1, it can be seen that about half of the water structure of the tight refinement hovers at occupancies near background level.

The solvent structures of the twofold equivalence refinements were compared with that of the free refinement and with themselves; disappointingly, only half of the solvent structure of the free and moderate refinements is the same (within 1·0Å) and this decreases to about $\frac{1}{3}$ for the free–tight refinement comparison. However, the latter is not surprising since water did not contribute significantly in the tight refinement. The lack of agreement in the solvent structure between the different refinements is thus a consequence of the imposition of the equivalence restraint.

The number of water molecules of the non-crystallographic refinements which are within 1·0 Å of those of γ-CHT (Cohen, Silverton & Davies, 1981) is gratifiably large: 68 for the free refinement, 64 for moderate and 48 for tight. In addition, about half of the latter two occur as twofold-related pairs [$\frac{2}{3}$ of the γ-free refinement waters are twofold symmetrical (Blevins & Tulinsky, 1985*b*)]. Most of this class of water molecules was the very first to be uncovered. The extent of such significant structure decreases as the twofold non-crystallographic equivalence restraint of the protein structure is increased so that an undue restraint appears to obscure detail which might be otherwise obtainable.

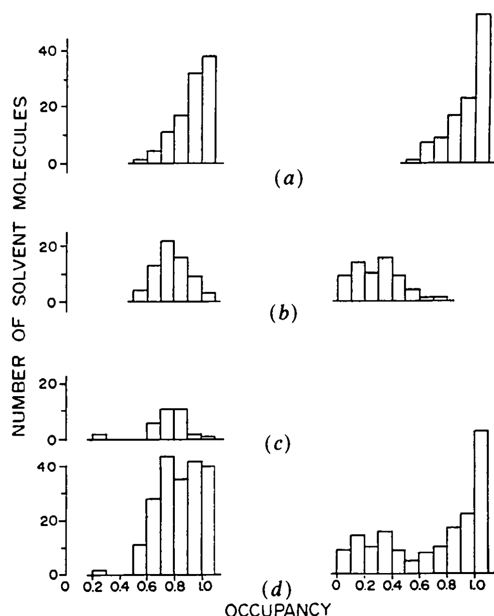

Fig. 1. Manner of inclusion and retention of water molecules in refinement. Moderate: left, tight: right; water included in three stages for moderate [(*a*)–(*c*)] and two stages for tight [(*a*) and (*b*)]; final occupancy distribution shown in (*d*).

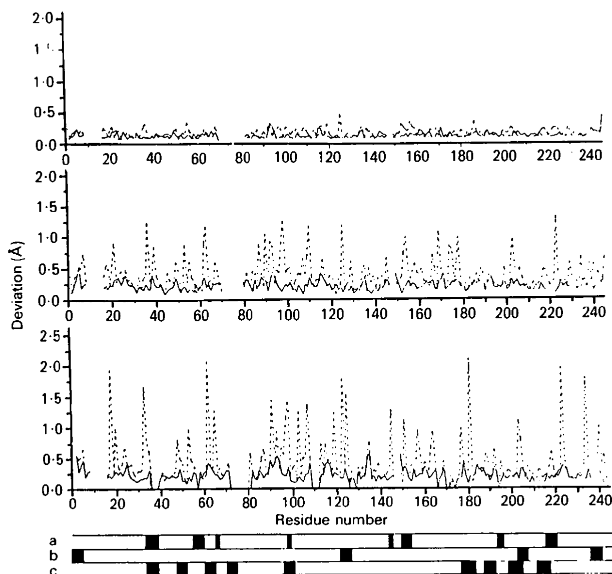

Fig. 2. Root-mean-square asymmetry between individual molecules of α-CHT dimer. Tight refinement: top, moderate: center, free: bottom; main chain: solid, side chain: broken; (*a*) dimer interface regions, (*b*) dyad *B* regions near non-crystallographic twofold axis between dimers, (*c*) external turns; gaps at 14–15 and 147–148 are terminals of *A*, *B*, *C* chains; gaps at 9–13 and 72–80 disordered structure.

The three independent refinements show that non-equivalence has developed in the moderate and the free refinements (Fig. 2). Like the free refinement, the moderate has non-equivalence in the side chains of the exterior of the molecule and the dimer interface residues but shows excellent equivalence for the main-chain folding and shows the same general trends observed for the free refinement (Fig. 2). Comparison of the r.m.s. deviations of Fig. 2 shows that most of the large deviations of the free refinement also occur in that of the moderate whereas the unrealistically severe equivalence restraint of the tight refinement (4 × less than expected error) was sufficient to suppress all indications of non-equivalence. Moreover, the decrease in non-equivalence was accomplished at the apparent expense of only increasing the R factor slightly (about 2%) and with the loss of some solvent structure.

In cases of non-crystallographic symmetry involving high quality diffraction data, restraining the equivalence drastically could be counter-productive and curtail indications of non-equivalence. Since it is clear that equivalence can be retained with accurate data without an external restraint (Blevins & Tulinsky, 1985*a*, *b*), a more relaxed approach would seem prudent. However, there may be certain advantages to restraining non-crystallographic symmetry with lower-order data or in the low-order refinement of more extensive data because non-equivalence develops sluggishly under such circumstances (Cohen, Matthews & Davies, 1970). In the present case it developed decisively at 2·8 Å resolution.

Finally, the routine application of restrained least squares without examining electron density maps is obviously artificial and will necessarily produce limited results in non-equivalence and an appropriate mix of the two is the correct way to proceed. Non-equivalent changes introduced from maps can be easily accommodated in *PROLSQ* along with a decrease in equivalence restraints since the program calculates using all the atoms in the asymmetric unit. Thus, refinement utilizing non-crystallographic symmetry is no faster (computer-time per cycle) than refining the complete asymmetric unit without a symmetry restraint and the resulting phase angles are not those of a symmetrical molecule; however, such a refinement does give the transformation between related molecules and deviations thereof from average coordinates. Even with an averaged structure, if the phases are assigned to observed amplitudes of a non-equivalent structure, the resulting electron density will show non-equivalence. Such was the case for the tight refinement.

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Book Review

Works intended for notice in this column should be sent direct to the Book-Review Editor (J. H. Robertson, School of Chemistry, University of Leeds, Leeds LS2 9JT, England). As far as practicable books will be reviewed in a country different from that of publication.

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Crystal structure analysis: A primer. 2nd ed. By J. P. GLUSKER and K. N. TRUEBLOOD. Pp. xviii + 269. Oxford University Press, 1985. Price hardback £29.00, US $37.50; softback £17.00, US $18.95.

The first edition of this text came out 13 years ago in 1972, and was reviewed then by J. L. Lawrence [*Acta Cryst.* (1972), **A28**, 680], who concluded '... this book can be highly recommended as an undergraduate text ... and ... to any scientist who desires an introduction to structure determination'. Now, in producing their second edition, the authors have made the book still better by updating and judiciously enlarging it. Almost every part has been affected, with modified or expanded text, new (extra) diagrams and photographs, such as the protein-crystal synchrotron-radiation diffraction photograph shown in the section on experimental methods. Direct methods and anomalous dispersion now have a chapter each; four-circle diffractometry is explained in detail, the glossary (a most valuable feature) has been doubled in size, and the index nearly doubled too. Of course, the price has more than doubled: the factor is about seven; but it is to be hoped that at least the paper cover version will nevertheless be within the reach of the students – to whom it is addressed.

One regret – which the authors will surely share. In the year of the award of a Nobel prize in the central core of this subject area, it is sad that this book, despite its 20-page 30-section annotated bibliography, just happens not to contain any reference to the papers, or the names, of Jerry Karle and Herbert Hauptman.

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