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A Procedure for Joint Refinement of Macromolecular Structures with X-ray and Neutron Diffraction Data from Single Crystals

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Abstract

A procedure is presented for the stereochemically restrained least-squares refinement of macromolecular structures with neutron and X-ray diffraction data from single crystals. This procedure has been tested by refining a model of ribonuclease A using neutron data to minimal spacings of 2·8 Å and X-ray data from within 2·0 Å spacings. Joint X-ray and neutron refinement is well conditioned and tends to avoid false minima that may occur when a medium-resolution structure is refined solely with the neutron structure factors.

Introduction

Several methods for the refinement of the single-crystal neutron diffraction data collected on proteins have been tried in the last few years, but none of these has been completely satisfactory. The structures of metmyoglobin and carbonmonoxymyoglobin were refined by the real-space techniques at 2 Å (R = 32%) and 1·8 Å (R = 37%) resolution respectively (Schoenborn & Diamond, 1976; Norvell & Schoenborn, 1976). The structure of triclinic lysozyme was refined by Bentley & Mason (1981) using the least-squares technique of Agarwal (1978) and the idealization procedure of

Dodson, Isaacs & Rollett (1976). After 30 cycles of refinement, the crystallographic R factor was 28·2% at 1·5 Å resolution. The above-mentioned neutron investigations started with only moderately well refined X-ray structures and did not lead to completely acceptable neutron models. One of the difficulties in the least-squares refinement was caused by the tendency of the H atoms to be pushed away from the atoms to which they were bonded. This was not merely due to the short, unresolved bond distances involving hydrogens, since deuterium atoms appeared not to have been similarly affected. In contrast, the neutron refinement of trypsin by Kossiakoff & Spencer (1980) gave more encouraging results and proved that histidine 57 in the active site is doubly protonated. This refinement was done at 2·2 Å resolution starting from a very highly refined X-ray model of Chambers & Stroud (1979). Both the X-ray and neutron refinement were accomplished with the use of the curvature-gradient technique and energy minimization (Chambers & Stroud, 1977). The R factor for the initial X-ray model was 15·7% at 1·5 Å resolution, and that for the final neutron model at 2·2 Å was 18·7%. Part of this success was probably due to the high quality of the starting model and to the reasonably high resolution of the neutron data. However, in this case, as in the earlier work, the extent of agreement of the refined neutron model with X-ray data was not reported.

Obviously, an atomic model for a crystal structure should be consistent with both the X-ray and the neutron diffraction data. Hence, more accurate atomic parameters can be expected from a simultaneous refinement against the data from both kinds of radiation than from either separate refinement. Since the degree of overdetermination will be increased in a joint refinement, improved refinement behavior might also be expected. All the data for such a procedure should, of course, be measured from essentially identical crystals. In particular, for macromolecules both the X-ray and the neutron data should be measured from identically deuterated crystals in equilibrium with the same mother liquor. Such a suggestion for the joint analysis of X-ray and neutron data from macromolecules was made by Hoppe (1976) in the discussion following a presentation on real-space refinement techniques for neutron refinement. Recently, a procedure for the refinement of the structural and charge-density parameters against both the X-ray and neutron diffraction data has been described by Coppens, Boehme, Price & Stevens (1981). However, this technique is not directly applicable to macromolecules since very high-resolution data are required.

For several reasons we found joint refinement to be particularly attractive for our work on the neutron structure analysis of ribonuclease A. First, no refined structure of this enzyme was available at the outset of the neutron investigation; thus refinement of the X-ray model was necessary. This was accomplished at 2·5 Å resolution using partially deuterated crystals treated in the same manner as the large crystals used for X-ray data collection (Wlodawer, 1980). Second, neutron data were initially collected only to 2·8 Å resolution, and thus the ratio of observed intensities to the number of atomic parameters was very unfavorable, even for a program utilizing stereochemical restraints (Hendrickson & Konnert, 1980, 1981). In addition, we did not expect the hydrogens in the aliphatic side chains to exchange, and the total scattering length of a CH_2 group is very close to zero. At a resolution as low as 2·8 Å we did not expect such groups to provide any useful contribution to the refinement process. Third, we decided that the joint refinement against the neutron and X-ray data might, in general, be useful, since at any resolution it doubles the number of diffraction data while the number of refinable parameters is not increased above that for a separate neutron refinement. This considerably increases the ratio of observations to parameters.

This paper describes the approach that we have used for the joint refinement of a protein model with X-ray and neutron diffraction data and compares results from the joint refinement of ribonuclease A with those obtained in a separate neutron refinement.

Refinement procedures

The procedure that we have adopted here for the joint refinement of macromolecular structures against both X-ray and neutron diffraction data required relatively minor conceptual modifications to the procedures for stereochemically restrained refinement (Konnert, 1976; Hendrickson & Konnert, 1980, 1981) that we have used previously (Wlodawer, 1980). This procedure introduces stereochemical and other prior knowledge about the structure into the least-squares minimization. These geometrical 'observations' serve as restraints on the atomic parameters.

There may be several qualitatively different kinds of observations. These include the structure-factor data, 'ideal' bond lengths and angles, planarity of certain groups, chirality at asymmetric centers, non-bonded contacts, restricted torsion angles, non-crystallographic symmetry, and limitations on bond and angle fluctuation due to thermal motion (Konnert & Hendrickson, 1980). Thus the function to be minimized is in the form

$$\Phi = \sum \varphi_i, \tag{1}$$

where each of the separate observational functions, φ_i, is usually (but not always) in the form

$$\varphi_i = \sum \frac{1}{\sigma_j^2} [f_j^{\text{obs}} - f_j^{\text{calc}}(\{x\})]^2. \tag{2}$$

Here each term relates to a particular observational quantity f^{obs} for which a corresponding theoretical value f^{calc} can be calculated from the set of refinable parameters $\{x\}$, or possibly some subset of these. Each term is weighted by the inverse of the estimated variance for the particular observation or, in the early stages, possibly by some other variance estimate. The joint refinement simply requires adding another term in (1). Thus we now have

$$\Phi = \varphi_{\text{x-ray}} + \varphi_{\text{neutron}} + \varphi_{\text{bonds}} + \varphi_{\text{planes}} + \cdots. \quad (3)$$

The major tasks involved in implementing the joint-refinement procedure were those needed anyway for neutron refinement, namely the incorporation of H atoms. The changes to the actual refinement program, *PROLSQ*, to permit simultaneous use of both X-ray and neutron data mainly involved setting appropriate switches for reading the respective data sets, calculating structure factors and derivatives based on the appropriate scattering factors, and including a separate scale-factor refinement. Also, a provision for a special class of non-bonded contacts, those involving H atoms that participate in hydrogen bonds, was included. Changes to *PROTIN*, the program that prepares the restraint observations for particular protein structures, were more extensive. A variety of program modifications were needed to allow for H atoms. In addition, new standard groups that include H positions were compiled as described below. All the restraint dictionaries were also appropriately upgraded. Distances involving H positions have been put into special weighting categories.

At present the H positions are simply identified as being either hydrogen or deuterium and are given the appropriate scattering lengths in the neutron calculation. It may prove to be important to permit a variable hydrogen/deuterium occupancy ratio. Currently, we exclude the H atoms from the X-ray structure factor calculation in a joint refinement. However, we can include the H atoms in a separate X-ray structure refinement. In that case the target 'ideal' distances involving hydrogens are shrunk to reflect the expected displacement of the hydrogen electron density along the *X*—H bond.

Standard groups

The success of the restrained least-squares procedure is, to a large extent, dependent on the proper choice of the standard groups used in the construction of the file containing the target models. For the joint-refinement version of the program (and for a version capable of refining H positions in high-resolution X-ray refinement), we have prepared a new version of the standard-group dictionary. This version is similar, but not identical, to the one previously constructed by Sielecki, Hendrickson, Broughton, Delbaere, Brayer & James (1979), with the difference that H positions were included and some of the sources for amino acid structures replaced. The references from which these coordinates were taken are summarized in Table 1.

Some comments are in order. To assure accuracy in the nuclear positions for H atoms, we have used neutron structures whenever available. Most of the coordinates used to construct the dictionary were sent to us by Dr T. F. Koetzle (Brookhaven National Laboratory). These data included the coordinates for L-proline monohydrate and L-tryptophan hydrochloride which are not given explicitly in the respective publications. Neutron coordinates were not available for three amino acids: isoleucine, leucine, and methionine. The first two were included in the dictionary on the basis of the X-ray structure of a neurotensin tetrapeptide Pro-Tyr-Ile-Leu (Cotrait, Geoffre, Hospital & Precigoux, 1979). The H atoms were placed in that

Table 1. *Standard-groups dictionary*

Group	Reference	Agreement factor R^*
L-Alanine	(1)	0·022
L-Arginine	(2)	0·034
L-Asparagine	(3)	0·026
L-Aspartic acid	(4)	?
L-Cysteine	(5)	0·045
L-Glutamic acid	(6)	0·026
L-Glutamine	(7)	0·032
L-Glycine	(8)	0·032
L-Histidine	(9)	0·058
L-Isoleucine	(10)	0·062
L-Leucine	(10)	0·062
L-Lysine	(11)	0·030
L-Methionine	(12)	0·053
L-Phenylalanine	(13)	0·084
L-Proline	(14)	0·077
L-Serine	(15)	0·039
L-Threonine	(16)	0·068
L-Tryptophan	(17)	0·052
L-Tyrosine	(18)	0·040
L-Valine	(19)	0·031

References: (1) Lehmann, Koetzle & Hamilton (1972*a*). (2) Lehmann, Verbist, Hamilton & Koetzle (1973). (3) Verbist, Lehmann, Koetzle & Hamilton (1972*a*). (4) Ramanadham, Sequeira, Rajagopal & Momin (1981). (5) Kerr, Ashmore & Koetzle (1975). (6) Lehmann, Koetzle & Hamilton (1972*b*). (7) Koetzle, Frey, Lehmann & Hamilton (1973). (8) Jönsson & Kvick (1972). (9) Lehmann, Koetzle & Hamilton (1972*c*). (10) Cotrait *et al.* (1979). (11) Koetzle, Lehmann, Verbist & Hamilton (1972). (12) Chen & Parthasarathy (1977). (13) Al-Karaghouli & Koetzle (1975). (14) Verbist, Lehmann, Koetzle & Hamilton (1972*b*). (15) Frey, Lehmann, Koetzle & Hamilton (1973). (16) Ramanadham, Sikka & Chidambaram (1973). (17) Andrews, Farkas, Frey, Lehmann & Koetzle (1974). (18) Frey, Koetzle, Lehmann & Hamilton (1973). (19) Koetzle, Golič, Lehmann, Verbist & Hamilton (1974).

* R is defined as $\sum||F_o| - |F_c||/\sum|F_o|$ except for L-threonine, where it is defined on F^2.

structure in the final stages of refinement and in theoretical positions corrected for the distance underestimation inherent in the X-ray structures. The standard group for methionine was derived from the X-ray structure of *N*-formyl-L-methionine (Chen & Parthasarathy, 1977), since this structure was better refined than the available X-ray structure of methionine (Torii & Iitaka, 1973). We have idealized the H positions given for this structure to expected nuclear positions. The neutron structure of histidine was determined from a crystal containing a single proton (HE2) on the imidazole ring nitrogens, with the ND1 nitrogen hydrogen-bonded to the amino nitrogen N through hydrogen H2. Since we expected that some of the histidine side chains would be found doubly protonated in the protein structures, hydrogen HD1 was added at its calculated position.

We are aware of the fact that some of the standard groups constructed in this fashion will have to be modified in the future, since, for example, the structures of protonated aspartic or glutamic acids are not expected to be identical to their unprotonated counterparts. An increase in the number of standard groups is indicated, but the present dictionary should suffice for the purpose of studies such as this one at comparatively low resolution.

Refinement of ribonuclease A

We earlier refined the X-ray structure of the monoclinic ribonuclease A ($P2_1$, $a = 30{\cdot}18$, $b = 38{\cdot}4$, $c = 53{\cdot}32$ Å, $\beta = 105{\cdot}85°$) at 2·5 Å resolution using a previous version of the restrained-refinement program, and the refinement was subsequently continued at 2·0 Å resolution (Wlodawer, Bott & Sjölin, unpublished). Details of data collection and refinement procedures were presented elsewhere (Wlodawer, 1980). Both the X-ray and neutron data were collected on partially deuterated crystals treated identically during soaking in hydrogen-free mother liquor. X-ray intensities out to 2·0 Å spacings were measured on a Picker FACS-1 diffractometer using an ω step-scan procedure with individual backgrounds for each reflection. Essentially all reflections were observed (96% have $I > 3\sigma$). Neutron data to 2·8 Å were collected on a flat-cone diffractometer at the National Bureau of Standards reactor (Prince, Wlodawer & Santoro, 1978), but the diffractometer was operated in the ω-scan mode, neglecting the data falling outside the equatorial plane of the instrument. The number of observed reflections ($F > 2\sigma$) was 2773, 87% of all reflections at 2·8 Å resolution.

The X-ray refinement was terminated when the R factor reached 15·9% for the data between 10 and 2·0 Å and the root-mean-square (r.m.s.) deviation of bond lengths from ideality was 0·022 Å. Other stereochemical features were restrained to a comparable level of ideality (Table 3). Estimated deviations of the final atomic positions from true values (Luzzati, 1952) were 0·175 Å. The model included a phosphate molecule in the active site and 176 partially occupied water sites. This model has been used as the starting point for the joint refinement.

The agreement of the final model from the 2·0 Å X-ray refinement with the 2·8 Å set of neutron structure amplitudes was $R = 32{\cdot}0\%$. This model neglects almost a third of the neutron scattering power since H atoms were not included; hence, this comparatively high value is not surprising. Kossiakoff & Spencer (1980) found a similar value for trypsin at 2·2 Å resolution at an equivalent stage in the analysis.

H atoms were appended to the model using a program provided by R. Feldmann at the National Institutes of Health. In the initial approximation, all H atoms attached to O or N were assigned as deuterium and those attached to C as hydrogen. The addition of H and D atoms lowered R to 30·6%, even though some were added wrongly (for example, methyl, amino, and hydroxyl groups were oriented arbitrarily), and the exchangeability of the others was not predicted properly. At this stage the D atoms were not added to the solvent molecules.

This model was refined jointly with the neutron data to 2·8 Å spacings and X-ray data to 2·0 Å. Data from spacings greater than 10 Å were excluded from refinement. A total of 2575 neutron observations and 7708 X-ray observations were included. The progress

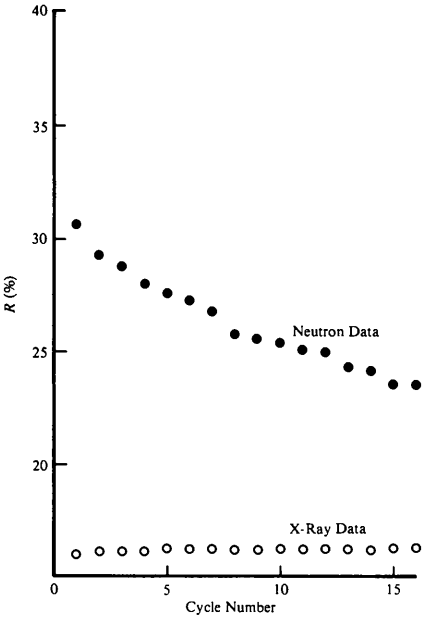

Fig. 1. Progress of joint refinement of the X-ray and neutron data for ribonuclease A at 2·0 and 2·8 Å resolution respectively, starting with the coordinates from 2·0 Å X-ray refinement.

of refinement is shown in Fig. 1. The relative weights for the contribution of the neutron and X-ray structure amplitudes were chosen in such a way that the resulting model was much more influenced by the X-ray data. Thus the X-ray R factor changed little, from 15·9% to 16·3%, in fifteen cycles of refinement. At the same time, the neutron R factor was lowered from 30·6 to 23·6%. The r.m.s. shift in atomic positions from the

Table 2. *Ribonuclease A refinement results*

Model	Number of atoms	Number of diffraction data X-ray	Neutron	R_X*	R_N†	R.m.s. positional shift from starting X-ray model All non-solvent	Non-hydrogen
X-ray alone (2·0 Å)	1132	7708	(2575)‡	15·9%	(32·0%)‡	–	–
Joint	1859	7708	2575	16·3	23·6	0·31 Å	0·12 Å
Neutron alone (2·8 Å)	1859	(2799)	2575	(36·0)	15·6	0·65	0·37
(2·0 Å)		(7708)		(38·6)			
Joint continuation from neutron alone	1859	7708	2575	16·8	22·2	0·51	0·22
X-ray alone (2·8 Å)	1132	2799	–	11·8	–	–	0·13
X-ray alone (3·4 Å)	1132	1605	–	7·1	–	–	0·22

* R factor for X-ray data based only on non-hydrogen atoms.
† R factor for neutron data.
‡ Parentheses indicate R values and data that were not included in the refinement of a particular model.

Table 3. *Final weighting parameters and breadths of realized distributions*

Sigmas are as defined by Hendrickson & Konnert (1980) and enter into the least-squares weighting as inverses of the variance [see equation (2)]. The same weighting was used for each refinement test.

Feature	Weighting sigma	R.m.s. discrepancy from ideal values X-ray alone	Neutron alone	Joint	Joint after neutron
Bonding distances (Å)					
Bond length (1–2 neighbor)					
Non-hydrogen	0·02	0·022	0·019	0·022	0·023
Hydrogen involving	0·03	–	0·025	0·024	0·023
Angle-related distance (1–3 neighbor)					
Non-hydrogen	0·04	0·060	0·049	0·063	0·065
Hydrogen involving	0·05	–	0·044	0·064	0·067
Intraplanar distance (1–4 neighbor)	0·05	0·064	0·042	0·042	0·048
Planar groups					
Deviation from plane (Å)	0·02	0·017	0·012	0·017	0·019
Chiral centers					
Chiral volume (Å^3)	0·15	0·228	0·185	0·205	0·214
Non-bonded contacts (Å)					
Single torsion	0·5	0·230	0·206	0·215	0·209
Multiple torsion	0·5	0·262	0·338	0·360	0·345
Possible hydrogen bond	0·5	0·343	0·399	0·327	0·315
Torsion angles (°)					
Planar (*e.g.* peptide ω)	5·0	3·4	3·5	3·7	4·8
Staggered (*e.g.* aliphatic χ)	15·0	23·2	23·1	23·1	21·9
Transverse (*e.g.* aromatic χ_2)	15·0	19·0	19·5	19·1	17·5
Thermal factors (Å^2)					
Main-chain bond	1·0	1·005	0·843	0·664	0·696
Main-chain angle	1·5	1·638	1·259	1·288	1·319
Side-chain bond	1·0	1·469	1·212	1·414	1·489
Side-chain angle	1·5	2·457	2·051	2·393	2·500
Restraints against excessive shifts					
Positional parameters (Å)	0·25				
Thermal parameter (Å^2)	3·0				
Occupancy parameter	0·05				
Diffraction data		Weighting sigma (fraction of $\|F_o - F_c\|$)			
X-ray structure amplitudes	–	0·55	0·55	0·48	
Neutron structure amplitudes	–	0·44	0·36	0·41	

X-ray starting point was 0·31 Å overall and only 0·12 Å for non-hydrogen protein atoms (solvent excluded). The stereochemical weights used in this refinement (as well as in all refinements discussed below) were the same as in the original X-ray refinement and the resulting models had similar stereochemistry (Table 3).

The results from this joint refinement of ribonuclease show that the procedure has fulfilled our expectations. The refinement was stable and converged within an acceptable number of cycles. The inclusion of X-ray data prevented large shifts in the positions of non-hydrogen atoms while geometrical restraints removed the tendency reported for lysozyme (Bentley & Mason, 1981) for bonds involving hydrogen to become too long. However, some of these properties could be expected from stereochemically restrained refinement with neutron data alone. Thus in the interest of comparison, we have carried out a separate neutron refinement and, as a control, we have also made separate X-ray refinements at lower resolution. In addition, a joint refinement was continued from the model after separate neutron refinement. The results from these various tests are summarized in Tables 2 and 3 and in Fig. 3.

The separate refinement with 2·8 Å neutron data was also well behaved. The R factor was lowered to 15·6% in 21 cycles starting from the same initial model as that used in the joint refinement. However, this was done at the cost of increasing the X-ray R factor to 38·6% (see Fig. 2). This refinement was also accompanied by much larger positional shifts than in the joint refinement. In this case the r.m.s. shift from the starting X-ray model was 0·65 Å (0·37 Å for non-hydrogen, non-solvent atoms). The largest atomic shifts were on the order of 1·5 Å. Not unexpectedly, these occurred in the side chains of lysine, glutamine, asparagine, arginine, and valine.

The occurrence of excessive shifts in the separate neutron refinement is due more to the nature of neutron scattering than to the limited extent of the neutron data. We tried X-ray refinement with the data of the same resolution or having the same observation-to-parameter ratio as the neutron refinement. These refinements against limited X-ray data, first at 2·8 Å (2799 reflections) and later at 3·4 Å (1605 reflections), were much better behaved than the neutron refinement. The R factor was lowered from 14·4 to 11·8% in six cycles at 2·8 Å and from 14·1 to 7·1% in nine cycles at 3·4 Å. The r.m.s. shift from initial coordinates for the non-solvent atoms was 0·13 Å in the 2·8 Å test and 0·21 Å in the 3·4 Å test. The r.m.s. difference between the 2·8 and 3·4 Å models was 0·16 Å. Side-chain atoms from only one residue, lysine 37, had shifts as large as 0·75 Å at 2·8 Å resolution and 1·1 Å at 3·4 Å, but since there is no density for this residue in the difference Fourier maps at 2·0 Å, its position was always considered uncertain. Other maximum shifts at 2·8 Å did not exceed 0·35 Å, only a third of the size noticed for over a dozen side chains in the separate neutron refinement. Only a few 0·5 Å shifts were found in 3·4 Å refinement.

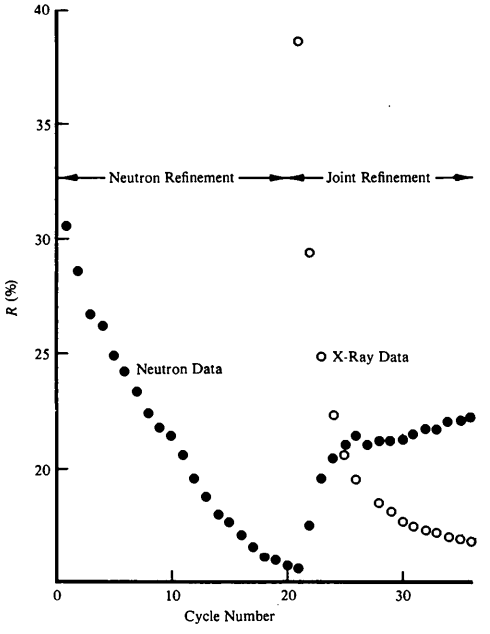

Fig. 2. Progress of the neutron refinement of ribonuclease A at 2·8 Å resolution, followed by joint X-ray and neutron refinement.

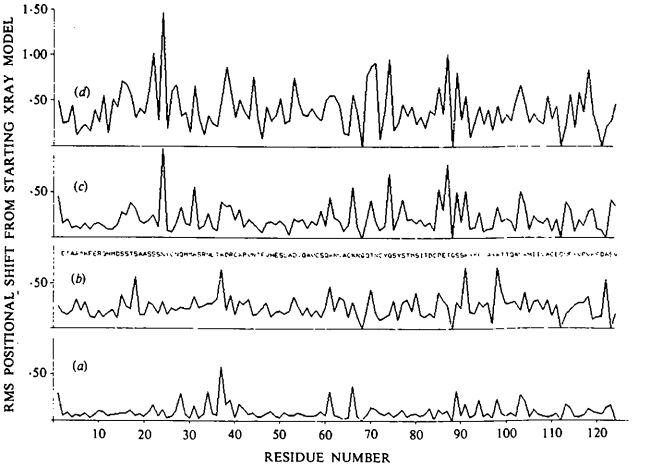

Fig. 3. Root-mean-square positional shift of non-hydrogen atoms belonging to the side chains, plotted as a function of residue number. All shifts are relative to the original X-ray model resulting from the refinement at 2·0 Å resolution. One-character residue names are provided. (*a*) Results of the joint refinement starting from the X-ray coordinates. (*b*) Results of the X-ray refinement at 3·4 Å resolution. (*c*) Results of the joint refinement starting from the coordinates obtained in the separate neutron refinement at 2·8 Å. (*d*) Results of the separate neutron refinement at 2·8 Å resolution.

To investigate the capability of the joint-refinement procedure for restoring the agreement with the X-ray data after separate neutron refinement, we have continued the joint refinement starting from the model based on neutron refinement alone. The progress is shown in Fig. 2. The final R factors (16·8 and 22·2% for X-ray and neutron data respectively) are not very different from those obtained in the joint refinement starting from the X-ray model, and the r.m.s. discrepancy between the final atomic coordinates is 0·47 Å for the two joint refinements (0·18 Å for the non-hydrogen atoms). The r.m.s. deviation from the starting model is 0·22 Å for non-hydrogen atoms. Although the apparent agreement with the X-ray data was essentially completely restored, the two models are clearly not identical. Nevertheless, most of the large deviations from the initial X-ray model that occurred in the separate neutron refinement are no longer present. A majority of the remaining discrepancies are similar in size to the uncertainty of the determination of the atomic positions, but some side chains were clearly not restored to their proper orientation.

Fourier maps often give a far better impression of the quality of a structural model than do refinement statistics. We have examined the agreement of models obtained in the joint refinement and in the separate neutron refinement with density maps prepared by Fourier synthesis. Fragment ΔF maps were computed from coefficients of $(F_o - F_c')\alpha_c'$ where a fragment of interest (usually comprising about 10% of the structure) had been deleted in calculating the partial structure factors, $F_c' \exp(i\alpha_c')$. We have used molecular-graphics displays to study such maps systematically, based both on the X-ray data and on the neutron data. Particular attention was paid to side chains for which results from the two types of refinement disagreed.

Fig. 4(*a*) shows four amino acids (asparagine 24, tyrosine 25, cysteine 26, and asparagine 27) in an X-ray fragment ΔF map. It is clear that the coordinates obtained in the joint refinement agree with the map while the coordinates of both asparagines from the separate neutron refinement do not agree. The non-hydrogen atoms of the tyrosine are almost in the same positions, while the cysteine sulfur is slightly displaced after separate neutron refinement. The same coordinates are shown in the neutron difference Fourier map in Fig. 4(*b*). It is clear that the density for both asparagines is not well defined, owing to phasing errors and the interaction between hydrogen and carbon scattering lengths, which are of opposite signs.

Fig. 5 shows another region in which the results of both refinements were in substantial disagreement. It is clear that while the electron density maps for glutamine 69 and asparagine 71 are of very high quality, the nuclear density map was much poorer, and this may explain the discrepancy. The lack of density near CG 69 is a quite common feature, since the scattering of a C atom is almost completely balanced by the contribution of the attached two H atoms, and the resolution of the data is 2·8 Å. (The scattering length for C is $0{\cdot}66 \times 10^{-12}$ cm; for H, $-0{\cdot}38 \times 10^{-12}$ cm; and for the CH_2 group at low resolution, $-0{\cdot}1 \times 10^{-12}$ cm.)

Not all of the regions in the nuclear density maps are of low quality. Fig. 6 shows, in more detail, the comparison of the X-ray and neutron maps for the unambiguous tyrosine 25. The density for the deuterium DZ in the neutron map is obvious, and the sharp drop of positive density of the phenyl ring compared to the X-ray map is apparent. This map was contoured at the lower level ($\pm 4\sigma$), and the negative density corresponding to some of the ring hydrogens is visible. Thus we can conclude that the neutron map contains useful information in some areas, even though it is ambiguous in the others. It is in these ambiguous areas where the coordinates from separate refinement are most likely to be wrong.

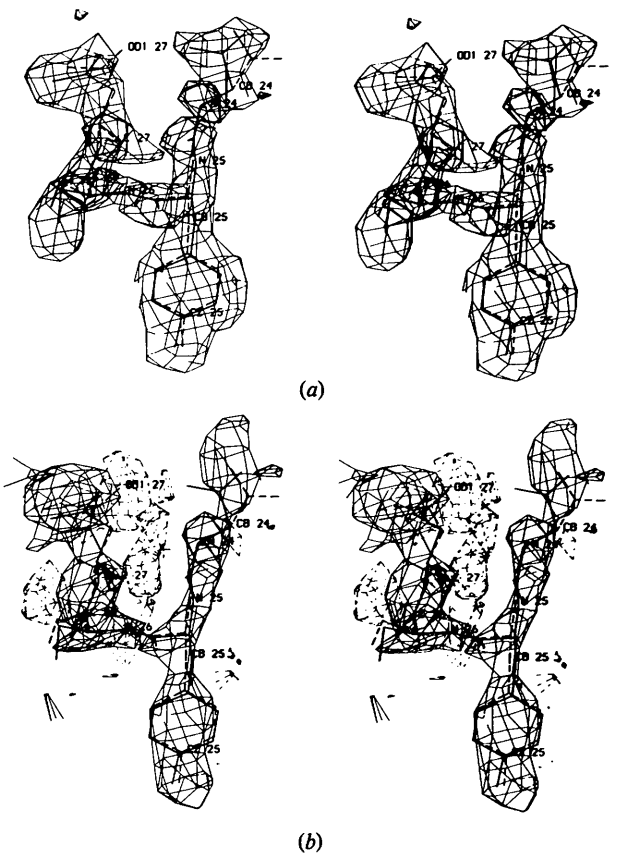

Fig. 4. 'Fragment ΔF' Fourier maps calculated using the phases from the joint X-ray and neutron refinement, subtracting the contribution of the residues 24–27. The maps were displayed using the program *BILDER*, written by R. Diamond. Coordinates after the joint refinement are marked in solid lines, those after the separate neutron refinement are dashed. (*a*) Electron density contoured at 5σ level. (*b*) Nuclear density contoured at $\pm 5\sigma$ level. Positive contours are solid, negative contours dashed.

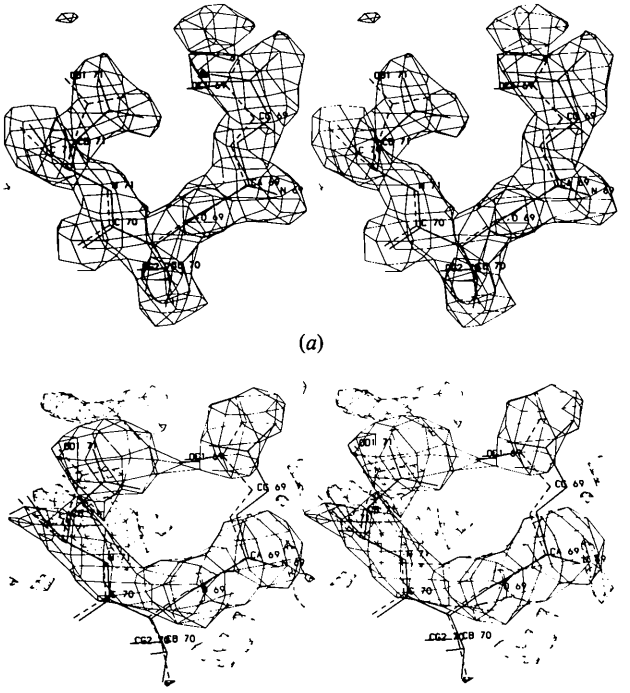

Fig. 5. 'Fragment ΔF' Fourier maps for residues 69–71, displayed in the same way as in Fig. 4.

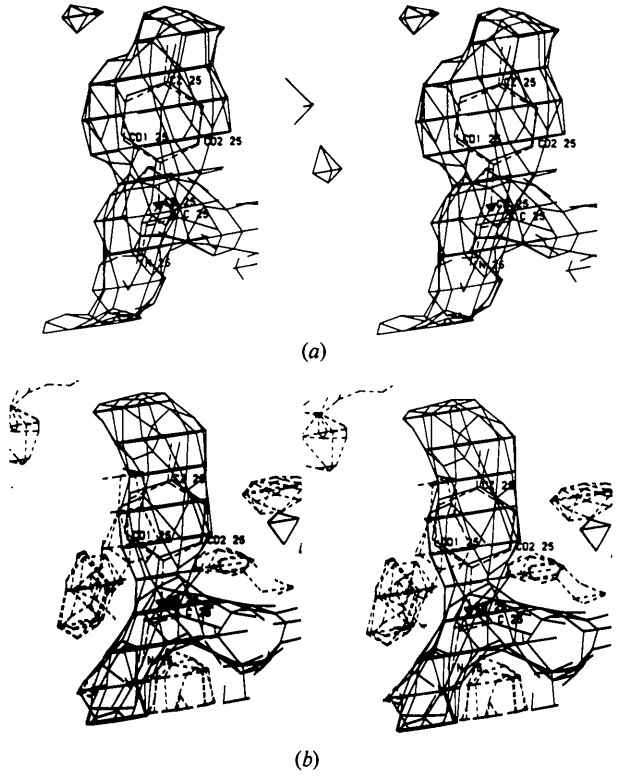

Fig. 6. A detail of the map calculated as in Fig. 4(*b*) but contoured at $\pm 4\sigma$ levels.

Neutron Fourier maps calculated after joint refinement were used in practice to determine the orientation of all four histidine side chains in ribonuclease (Wlodawer & Sjölin, 1981).

Discussion

The results derived from our application of the joint-refinement procedure to a protein structure at medium resolution show that such a procedure is less likely to lead to serious errors than the separate refinement with neutron data alone. Even with an initial model in which many hydrogens were not properly placed, the refinement converged rapidly while the idealized geometry was preserved. The joint refinement, starting from a well refined X-ray model, achieved a substantial improvement in agreement with the neutron data without appreciable change in agreement with the X-ray data. In contrast, the dramatic decrease in the neutron R value during refinement against the neutron data alone was accompanied by great deterioration in the match with the X-ray data and large, meaningless shifts in a number of side chains. This ill behavior of neutron refinement at 2·8 Å resolution is not simply due to the poor observation-to-parameter ratio of the problem since an X-ray refinement with a comparable ratio of diffraction data to variables was well behaved.

The joint-refinement procedure may be advantageous even for protein structures for which the location of hydrogens is of secondary importance. This is because the inclusion of neutron data increases the ratio of observations to parameters. The development of neutron diffractometers equipped with position-sensitive detectors makes the task of data collection easier, and the gain in overdetermination may justify the increased effort involved in the data collection.

Preliminary results from our investigation of the structure of ribonuclease A by a combination of X-ray and neutron diffraction methods have been published separately (Wlodawer & Sjölin, 1981). At this stage in the ribonuclease refinement, careful examination of difference Fourier maps is needed to correct hydrogen/deuterium assignments. We are presently applying the joint-refinement scheme using a new set of neutron data that extends to 2·0 Å spacings. We expect that the convergence of refinement and the final results will be substantially improved by the data extending to a higher resolution. A full report of the structure will be presented in due course.

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