possible, but provides a very simple and accurate method if it is. The systematic geometry also has the advantage of providing many independent structure factors, unlike the more accurate critical voltage method, which provides only a relationship between structure factors. The intersecting-Kikuchi-line method can also provide useful approximate values of structure factors, and is by far the easiest to apply since it relies on distance measurements taken from film, rather than intensity measurements.

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Note added in proof: The use of eigenvalue perturbation theory for absorption in acentric crystals is an approximation. See Bird, James & King [*Phys. Rev. Lett.* (1989), **63**, 1118]. The sign of the phase triplet may be determined as described by Marthinsen & Høier [*Proc. EMSA*, (1989), p. 484].

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Segmented Anisotropic Refinement of Bovine Ribonuclease A by the Application of the Rigid-Body TLS Model

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Abstract

The anisotropic displacements of selected rigid groups in bovine ribonuclease A have been refined from X-ray diffraction data by the application of the rigid-body **TLS** model. The rigid groups chosen were the side chains of tyrosine, histidine and phenylalanine and the planar side chains of aspartic acid, glutamic acid, glutamine, asparagine and arginine. The method has also been applied to the co-crystallizing active-site sulfate anion. This has enabled the description of the motion of the above-mentioned side-chain atoms by anisotropic displacement ellipsoids from a 1·45 Å refinement. The hydrophobic side

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groups in the protein core show mainly translational motion, with mean-square librations of 20 deg^2 which are similar to those found in some close-packed crystals of small organic molecules. Librational displacements are much more significant in the hydrophilic side groups where their magnitudes can be correlated with solvent accessibility. Large librations of some solvent exposed side chains correspond with the breakdown of a simple **TLS** model and the existence of multiple orientations of the side groups. The **TLS** model has also been applied to the whole protein molecule and shows that the average motion is approximately isotropic with little librational character.

Introduction

An understanding of the flexibility of biological macromolecules is particularly relevant in the study of structure-function relationships. The static and dynamic disorder in protein and nucleic-acid crystals provides information about the low-energy deformations of macromolecules. This disorder limits the X-ray diffraction of most macromolecular crystals to resolutions poorer than 1 Å and causes a weakening of the Bragg reflections together with a complementary increase in X-ray diffuse scattering. This poor data-to-parameter ratio has limited most X-ray studies of macromolecules to models with isotropic mean-square displacement parameters for individual atoms (*e.g.* Artymiuk, Blake, Grace, Oatley, Phillips & Sternberg, 1979).

In order to effectively improve this ratio, Hendrickson & Konnert (1980) assumed a riding model for bond dynamics and applied restraints between isotropic mean-square displacement amplitudes (MSDA) of bonded atoms. This idea was later extended to the refinement of anisotropic displacements (Konnert & Hendrickson, 1980). However, when this strategy was evaluated by molecular dynamics it was shown that the riding model might not be valid (Yu, Karplus & Hendrickson, 1985; Haneef & Moss, 1987).

However, where diffraction data of unusually high resolution ($\sim 1{\cdot}0$ Å) have been available, as in the case of avian pancreatic polypeptide (Glover, Haneef, Pitts, Wood, Moss, Tickle & Blundell, 1983), individual anisotropic atomic displacements have been refined and analysed. Such cases are exceptional and in most protein structure analyses anisotropic models of atomic displacements can only be employed in structure refinement by limiting the number of parameters. The treatment of selected groups of atoms as rigid bodies provides one way of improving the observation-to-parameter ratio, and this technique has already been extensively employed in refining the positional parameters of macromolecules (Sussman, Holbrook, Church & Kim, 1977).

The mean-square displacements of rigid bodies were first studied by Cruickshank (1956) who described rigid-molecule vibrations in terms of a translation (**T**) and a libration tensor (**L**). He calculated the components of these tensors by least squares from the individual anisotropic atomic temperature factors (U) of crystalline naphthalene. Later Schomaker & Trueblood (1968) showed that the description of the general motion of a rigid body in the harmonic approximation required a screw-rotation tensor (**S**) in addition to **T** and **L**. The **S** tensor describes the correlation between the rigid-body translation and libration. Pawley (1972) advocated the direct refinement of the **TLS** tensors in cases where internal modes of vibration could be neglected and demonstrated that in certain molecular crystals no significant improvement resulted when refinements employing individual atomic anisotropic thermal parameters were compared with such refinements.

Several authors have discussed the relation between structure-factor models, rigid-body displacements and the consequences for the calculation of interatomic distances. Good accounts have been given by Schomaker & Trueblood (1968), Johnson (1970), Johnson & Levy (1974) and Willis & Pryor (1975). Different notations have been employed in the literature and not all authors have made the same approximations. The theory on which this analysis is based is set out in the Appendix.

The work described in this paper uses the **TLS** model (Schomaker & Trueblood, 1968) in the context of geometrically restrained least-squares refinement of protein structures. The anisotropic displacements of relatively rigid groups were modelled by translation, libration and screw-rotation tensors whose components were refined thus effecting a saving on the number of parameters which would be required for a free anisotropic refinement. Individual anisotropic MSDAs of the group atoms were calculated from the refined **TLS** tensors. A refinement has also been carried out in which the **TLS** model has been applied to the whole protein molecule.

Holbrook and co-workers have recently applied the **TLS** method to a dodecamer of DNA (Holbrook & Kim, 1984; Holbrook, Dickerson & Kim, 1985) and have extracted information about the mobility of bases, ribose and phosphate moieties. Their determination used the program *CORELS* (Sussman, Holbrook, Church & Kim, 1977), where the **TLS** parameters were refined with the positional parameters held fixed. The method described here uses the program *RESTRAIN* (Haneef, Moss, Stanford & Borkakoti, 1985) which enables positional parameters to be refined at the same time as the **TLS** parameters and demonstrates the applicability of this method to the refinement of protein structures.

The displacements that are found are generally of a larger nature than those found in small-molecule

studies (Cruickshank, 1956) and the purpose of this paper is to assess the use and limitations of the **TLS** model in the refinement of macromolecular structures.

The rigid-body approximation

The mean-square amplitudes of vibration of atoms due to normal modes of molecular vibration have been calculated from vibrational frequencies of small molecules observed in the infrared or Raman spectrum (Cyvin, 1968). Characteristic frequencies of groups of atoms such as phenyl or amide are also observed in macromolecules such as proteins (Yu, Liu & O'Shea, 1972) and provide evidence that the amplitudes of vibration due to internal modes of these groups are similar to those observed in smaller molecules. These mean-square vibrations range from 0·001 to 0·005 $Å^2$ for bonds involving non-hydrogen atoms (Cyvin, 1968). These values are one to three orders of magnitude smaller than the mean-square displacements observed in protein crystal structures as determined by X-ray crystallography which are mostly in the range 0·05 to 0·5 $Å^2$ (Artymiuk *et al.*, 1979; Wlodawer, Borkakoti, Moss & Howlin, 1986). The larger displacements in protein structures are due to the concerted movement of groups or domains of atoms as is confirmed by normal-mode analysis and molecular dynamics (Brooks & Karplus, 1983; Glover, Moss, Tickle, Pitts, Haneef, Wood & Blundell, 1985).

Selected groups of atoms may therefore be treated as rigid bodies for the purpose of determining and refining displacement parameters provided that the errors introduced by this approximation are less than the random errors occurring in the determination of these parameters.

Experimental

The model used for this study was that which resulted from the 1·45 Å isotropic refinement of bovine pancreatic ribonuclease A (Borkakoti, Moss, Stanford & Palmer, 1984).

Groups of atoms in the protein ribonuclease A have been refined anisotropically in terms of **T**, **L** and **S** tensors using a restrained least-squares program described elsewhere (Haneef, Moss, Stanford & Borkakoti, 1985). Earlier isotropic refinement of ribonuclease A had already been undertaken (Borkakoti, Moss & Palmer, 1982). An absorption correction was applied to all X-ray reflections using the method of North, Phillips & Mathews (1968).

The groups selected for **TLS** refinement include: (1) phenyl and imidazole rings (side chains of His, Phe and Tyr); (2) the planar carboxylate, amide and guanidinium moieties (side chains of Asp, Asn, Glu, Gln and Arg); (3) the sulfate group in the active site of ribonuclease; (4) the whole molecule. In the case of the ring systems, **TLS** refinement was applied to side-group atoms beyond and including the CB atom. In this segmented approach to anisotropic refinement, atoms outside the **TLS** groups were refined with isotropic MSDAs and no restraints were applied between the MSDAs of neighbouring atoms.

The geometry in the **TLS** groups was not rigidly constrained because the distances between the mean positions of the atoms of a rigid group are a function of the group libration (see Appendix). Weighted restraints were applied to these distances and the calculated distances in the **TLS** groups were computed using equation (8) (see Table 2). Analysis of rigid-body parameters was performed with programs written in the laboratory (Howlin, 1986) and anisotropic ellipsoids were displayed with the program *ORTEP* (Johnson, 1965). Anisotropic displacement parameters calculated from the rigid-body **TLS** parameters were checked by comparison with those calculated from *XANADU* (Roberts & Sheldrick, 1979). The atomic coordinates and anisotropic displacement parameters will be available from the Brookhaven Protein Data Bank.

Electron density maps using $2|F_o|-|F_c|$ and phases from the refinement as coefficients were inspected on an Evans & Sutherland Picture System 2. Particular attention was paid to examining the density of the anisotropically refined groups for evidence of anharmonicity. The existence of subsites might reveal itself by multimodal or fragmented density or by refined positions which were asymmetrically placed in the available density. On the other hand, subsites which are sufficiently close may give rise to a refined atomic position at the centroid of the true positions. Such situations have been analysed by refinement against simulated structure amplitudes from molecular dynamics by Kuriyan, Petsko, Levy & Karplus (1986).

The accessible surface area was calculated by the method of Lee & Richards (1971) and reflects static accessibility since the calculation takes no account of atomic motion.

Results

Table 1 gives some basic statistics arising from the refinement. The strategy used in applying the **TLS** method to protein refinement has been described by Haneef, Moss, Stanford & Borkakoti (1985).

The planar groups of atoms in the protein side chains were all subjected to **TLS** refinement. To a first approximation the libration tensor **L** can be thought of as describing the component of displacement which is due to the local motion in the side chain whereas the translation tensor **T** describes that part of the displacement which is more global and may be shared with main-chain or other side-chain atoms in the vicinity.

The screw rotation tensor **S** describes the correlation between the translational and librational displacements and except in certain special cases its

Table 1. *Crystal and refinement data for bovine ribonuclease A*

Space group	$P2_1$
Unit cell dimensions (Å,°)	$a = 30{\cdot}45$
	$b = 38{\cdot}37$
	$c = 53{\cdot}22$
	$\beta = 105{\cdot}96$
Resolution (Å)	1·45
Absorption correction	Semi-empirical (North *et al.*, 1968)
Correlation coefficient	0·95
R factor	0·22 (all reflections)
Number of reflections	19 098
Number of protein atoms	951
Number of water atoms	127
Number of geometrical restraints	2458
Number of TLS groups	46
Number of parameters	4220
Reflection:parameter ratio	4:1
Root-mean square deviation from target geometry:	
bond lengths (Å)	0·01
across bond angles (Å)	0·07
planes (Å)	0·01

Table 2. *Mean diagonal elements of the* **T** *and* **L** *tensors associated with the rigid groups in bovine ribonuclease A*

The trace operation is denoted by tr. The mean distance corrections are calculated as indicated in the Appendix.

Rigid group*	tr(**T**)/3 (Å²)	tr(**L**)/3 (deg²)	Mean interatomic distance correction (Å)
Phenyl	0·034	20	0·01
Phe	0·034	20	—
Tyr	0·034	20	—
His	0·034	15	0·01
Glu/Gln	0·180	64	0·03
Glu	0·164	57	—
Gln	0·196	71	—
Asp/Asn	0·585	144	0·04
Asp	0·625	103	—
Asn	0·546	185	—
Arginine	0·105	23	0·01
Whole molecule	0·117	1·5	0·001

* Excluding Tyr 76, His 119, Asp 83, Gln 28 and Arg 39 for the reasons discussed in the text.

Figures for translation and libration tensors for Glu, Gln, Asp, Asn and Arg are given as mean translation parallel to the plane ($\frac{1}{2}$[tr(**T**) − **n**′**Tn**]) and mean libration perpendicular to the plane (**n**′**Ln**) where the unit vector perpendicular to the four-atom plane is **n**, tr denotes the trace operation and ′ denotes a matrix transpose.

interpretation is less obvious. For this reason and because its trace cannot be determined from Bragg reflections, it is not further considered in this paper. Table 2 gives results on the groups subjected to **TLS** refinement. The statistics used to quantify the mean values of the **T** and **L** tensors and their anisotropies are defined in the legends to the table. The **TLS** model does not allow a complete separation of librational and translational displacements for planar four-atom groups. The model does, however, still allow the distinction between librations about an axis perpendicular to the plane and translations in the plane. It is these quantities which have been analysed for planar four-atom groups and the mean values are displayed in Table 2. The principal axes of libration and associated mean-square displacements are displayed in Table 3.

Table 3. *Mean-square librations and associated axes for rigid groups in bovine ribonuclease A*

Rigid group	Mean-square libration (deg²)	Libration axes		
Whole molecule	0·6	0·8125	−0·4308	0·3928
	1·4	0·2159	−0·4036	−0·8891
	2·6	−0·5416	−0·8072	0·2349
Histidine 12	12·8	0·7723	0·6214	0·1316
	18·5	0·0164	−0·2267	0·9738
	24·4	−0·6350	0·7500	0·1853
Tyrosine 25	6·1	0·5826	−0·7034	−0·4072
	16·0	0·8063	0·3986	
	19·0	0·1024	0·5606	−0·8218
Tyrosine 73	21·1	0·3584	−0·6357	0·6836
	22·6	0·5338	−0·4612	−0·7088
	23·8	0·7659	0·6189	0·1740
Tyrosine 76	19·7	0·6577	0·5238	−0·5413
	30·0	0·6863	−0·7130	0·1439
	45·8	−0·3105	−0·4661	−0·8284
Tyrosine 92	18·2	0·7079	0·4684	0·5287
	18·9	0·6663	−0·1944	−0·7199
	21·1	0·2344	−0·8619	0·4497
Tyrosine 97	2·6	−0·4941	0·3611	0·7909
	12·3	0·7090	0·6938	0·1261
	23·7	−0·5032	0·6230	−0·5989
Tyrosine 115	11·8	0·8115	0·2381	0·5336
	14·2	0·1374	0·8099	−0·5702
	25·3	0·5679	−0·5361	−0·6246
Histidine 119	21·3	0·6844	−0·6430	−0·3438
	45·8	0·6911	0·4219	0·5868
	49·0	0·2323	0·6392	−0·7331
Histidine 119B	29·5	−0·7289	−0·2980	−0·6164
	44·2	0·3557	−0·9341	0·0310
	84·9	−0·5850	−0·1967	0·7869

Rigid groups on the outside of the ribonuclease molecule show larger displacements with a greater librational character. In several of the externally placed rigid groups very large librations are found, *e.g.* Asp 83 and Gln 28. These groups have mean-square librations of 233 and 257 deg² respectively compared with the average librations found for Asp and Gln side chains which are 103 and 71 deg² respectively. Such large values as are found for Asp 83 and Gln 28 are likely to be associated with marked anharmonic behaviour. These two side groups have been refined as double-site occupancy models in a higher-resolution refinement of ribonuclease A (Svensson, Sjölin, Gilliland, Finzel & Wlodawer, 1986).

Those residues where electron density maps suggested that a one-site **TLS** model was too simplistic were not included in the averages presented. In these cases the density was generally fragmented and suggested a multimodal distribution. In only one such case (His 119) have we tried to apply the **TLS** model to the individual sites.

Results are presented for each type of amino-acid residue refined but interest also centres on the relation between anisotropy and hydrogen bonding. Many of

the groups refined form H-bonded clusters which may be important for the structural integrity of the molecule as a whole (Harris, Borkakoti, Moss, Palmer & Howlin, 1987).

(1) *Phenylalanines*

The three phenylalanine groups in ribonuclease A, Phe 8, Phe 46 and Phe 120, were refined as rigid bodies including the CB atom and the phenyl ring. Results are shown in Table 2.

The mean-square libration of 20 deg^2 found in the phenylalanine rings is comparable to that found in crystal structures of small molecules (Cruickshank, 1957). As such structures do not usually possess solvent channels, the environment of their rigid groups is similar to that of the core of a protein molecule where the phenylalanine rings in the present study occur. Most of their rigid-body motion can be described in terms of translation. This suggests that these groups share much of the motion of their local environment in the protein core.

Both the magnitude and the anisotropy of the MSDAs of the atoms of the phenylalanine rings (calculated as the ratio of the largest to the smallest eigenvalues of the U tensor) tend to increase as one moves out along the side chain from the CB atom. Hence for Phe 46 (which occurs in a β-sheet region) the anisotropy of CB = 3·0, CG = 3·8, CD1 = 4·1, CE1 = 5·1, CZ = 5·7. The increasing anisotropy shows that the larger displacements are constrained by the local packing requirements.

(2) *Tyrosines*

The six tyrosine groups in ribonuclease A were refined as rigid bodies including the CB atoms, yielding rigid groups containing eight atoms.

Mean-square librations were of the same order as those of the phenylalanine residues (20 deg^2) with the exception of Tyr 76. This tyrosine is externally situated on the molecule and has an accessible surface area (summed over all atoms in the side chain) of 40 Å^2 compared with 1·0 Å^2 for a buried residue. The mean libration of Tyr 76 is 67 deg^2 which is three times larger than the mean tyrosine value and is consistent with the external nature of this side chain, where it is less constrained by other protein atoms.

The tyrosine groups (with the exception of Tyr 76) occur in buried positions and their displacements are largely translational in character. The magnitude of the mean libration of these groups, like the phenylalanines, is similar to that observed in crystals of small molecules (Cruickshank, 1956) and is consistent with the idea that the interior of the protein is close packed. The motion of buried groups may be strongly influenced by this close-packed environment.

Anisotropic refinement has also modified the appearance of electron density maps. The hole in the centre of the phenyl rings of Phe 46 and Tyr 73 has opened up and some differentiation of the side chain density into individual atoms is noted. Fig. 1 shows how anisotropic refinement can lead to better definition of the ring atoms of a phenylalanine group where isotropic refinement has caused partial infill of the aromatic ring. The example shown here is phenylalanine 46.

(3) *Histidines*

The librational behaviour of the histidines in ribonuclease A is generally similar to that of the phenylalanines and tyrosines, with the exception of

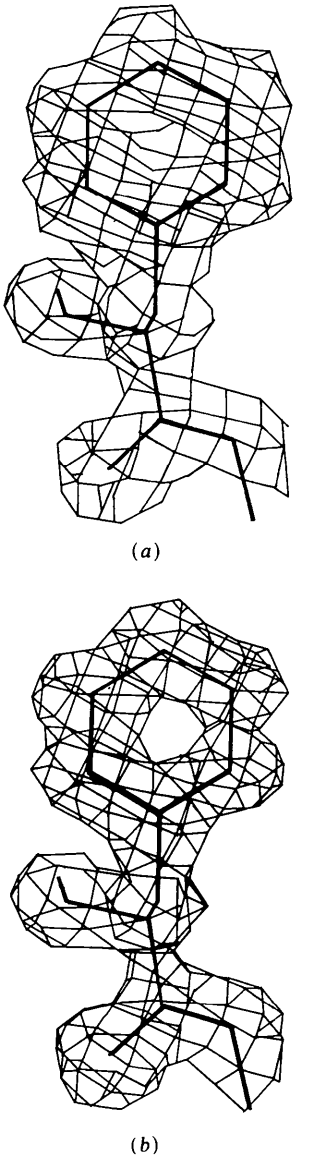

Fig. 1. Electron density at the 0·5 e Å^{-3} level for phenylalanine 46 of bovine ribonuclease A, (*a*) before **TLS** refinement, (*b*) after **TLS** refinement.

catalytically important His 119. In the major (*A*) site and minor (*B*) site of histidine 119 librations of the order of 50 deg^2 are found. A study of the electron density maps after anisotropic refinement shows even greater disorder than that found in the earlier isotropic maps (Borkakoti *et al.*, 1984) and is consistent with molecular dynamics studies of RNase A (Haneef, 1985; Brünger, Brooks & Karplus, 1985). It appears that the high conformational flexibility observed with this residue cannot be properly accounted for by a two-site anisotropic model but a more realistic representation may be difficult to parameterize at a resolution of 1·4 Å. Thus the ellipsoidal representations of the side chain atoms shown in Fig. 2 may only partially account for the multimodal density. This view shows the large nature of the displacements occurring in His 119 with respect to the ordered His 12 and Phe 120. Histidine 12 has H-bonding contacts to the sulfate anion and to Asn 44. The latter contact will be discussed later.

Fig. 2 also shows the co-crystallizing sulfate ion in the proximity of His 119. It should be noted that other studies have shown that the position of the side group of His 119 and the degree of order of this residue are sensitive to the nature of any moiety bound in the active site (Borkakoti, 1983; Wlodawer, Svensson, Sjölin & Gilliland, 1988).

The difference in the disorder of His 12 and His 119 is marked and it is interesting to note the buried nature of His 12 (accessible surface area = 1·0 Å^2) and its multiple H-bonded contacts as against the exposed His 119 (accessible surface area = 17·7 Å^2) with its fewer H-bonding contacts.

(4) *Aspartates and asparagines*

In the case of aspartates, asparagines, glutamates, glutamines and arginines the planar four-atom groups at the end of the respective side chains were subjected to **TLS** refinement. Results are shown in Table 2. In general the librations and translations observed for these groups are much larger than those observed for the ring groups discussed previously. This reflects their more exposed positions closer to the surface of the enzyme molecule.

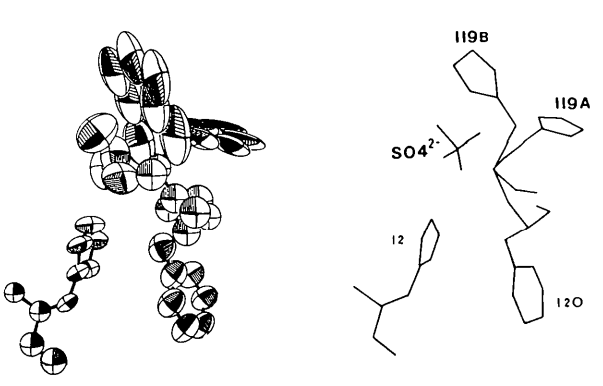

Fig. 2. *ORTEP* plot of the active site of bovine ribonuclease A showing His 12, His 119 A and B, Phe 120 and the sulfate anion at the 50% probability level.

The Asp/Asn side chains exhibit a mean libration perpendicular to the plane of 144 deg^2 and a mean translation in the plane of 0·58 Å^2. The librations appear to be correlated with accessible surface area. Those with accessible surface areas less than 20 Å^2 show librations of the order of 50 deg^2, whilst those with accessible surface areas greater than 20 Å^2 show librations of the order of 150 deg^2.

One exceptional residue in this group is Asp 83 which has an accessible surface area of 7·7 Å^2 and an anomalously large libration of 233 deg^2, which is reflected in the poor electron density observed for this residue. Our model may be too simplistic and it may be significant that a two-site model has been proposed in sulfate-free ribonuclease A (Wlodawer, Svensson, Sjölin & Gilliland, 1988).

There are several notable H-bonding contacts involving Asp or Asn groups and other protein atoms, *i.e.* Asp 121···His 119, Asn 44···His 12, Ser 16···Asp 14···Tyr 25 and Asn 67···Gln 69···Asn 71. In most cases there is marked anisotropy of the ellipsoids with the major axis of the ellipsoid pointing approximately in the direction of the H-bonding contact. This suggests the possibility of the concerted displacements of the H-bonding partners. An exception is provided by the ND2(Asn 71)···OE1(Gln 69) contact where the ND2 major ellipsoid axis is perpendicular to the hydrogen bond.

(5) *Glutamates and glutamines*

The lower average mean-square libration perpendicular to the plane of 64 deg^2 for these groups reflects the fact that they are less accessible than the Asp/Asn groups. The largest accessible surface area is 20 Å^2, except for Gln 28 which has a librational component of 257 deg^2 and an accessible surface area of 34 Å^2. As in the case of Asp 83, this side chain has poor electron density and may be poorly modelled by this technique. Owing to the smaller variation in accessible surface area, there is a linear increase in libration with accessible surface area for the glutamate residues. Gln 69 provides a further example of anisotropy associated with an H-bonding contact and this is shown in Fig. 3 where both NE2 and OE1 ellipsoids point in the direction of their respective H-bonding partners. The particular H-bonding cluster Asn 67···Gln 69···Asn 71 forms part of the B2 binding site of ribonuclease. This site may be associated with the stabilization of the transition state in the cyclization step of RNA hydrolysis.

(6) *Arginines*

The electron density found for arginine side chains is much better resolved than that for lysine side

chains in ribonuclease. The **TLS** model has been applied to the guanidinium group NE-CZ-NH1-NH2 at the terminus of the arginine side chain.

The arginines show the widest variation in both librations and accessibility. These range from a mean libration (defined in the same way as Asp/Asn and Glu/Gln) of 13 deg^2 for Arg 33 (buried) to 150 deg^2 for Arg 39 which at 46 Å^2 has the highest accessible surface area for a side chain. Arg 10 forms a salt bridge with Glu 2 and this interaction is shown in Fig. 4. This figure also shows the small magnitude of the displacements of the groups involved in this ion pair.

(7) *Sulfate*

The sulfate anion which co-crystallizes with bovine ribonuclease A shows the largest libration of all the rigid groups. The mean libration of this ion is over 200 deg^2 and the relevant ellipsoids are shown in Fig. 2.

This extremely high libration may be explained by the absence of covalent bonds to the sulfate moiety and may be the reason for the absence of any suggestion of tetrahedral geometry in electron density maps. The libration correction of the S–O interatomic distances is 0·06 Å and this correction has significantly improved agreement between target and calculated values in the restrained X-ray refinement. The sulfate is hydrogen bonded to His 119, His 12 and through a water molecule to Lys 41. Fig. 2 shows ellipsoids on the sulfate and His 119B pointing towards each other which again may indicate concerted displacements involving a hydrogen bond.

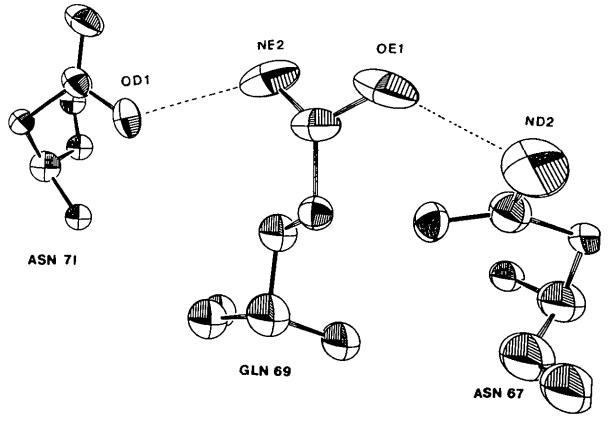

Fig. 3. *ORTEP* plot of Asn 67, Gln 69, Asn 71 at the 22½% probability level.

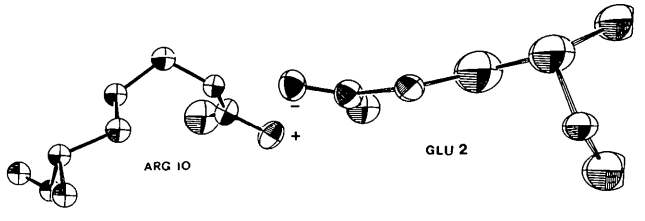

Fig. 4. *ORTEP* plot of Glu 2 and Arg 10 at the 22½% probability level.

The whole molecule

The refinement of a protein with a single overall isotropic displacement parameter to account for disorder represents the crudest model of atomic mobility in a crystal. A better approximation would be the use of an anisotropic overall parameter. The application of the **TLS** model represents an even better approximation of the overall motion. Fig. 5 shows the ribonuclease molecule and its principal axes of libration. It does, however, require a cautious interpretation because the protein molecule is not a pseudo-rigid body, and thus the tensors do not have a simple interpretation in terms of translation and libration. For example, in addition to accounting for the rigid-body component of molecular libration, the **L** tensor will receive a positive contribution from the larger side chain fluctuations on the outside of the molecule.

Refinement of whole-molecule **TLS** parameters was undertaken using the molecular centre of mass as origin for the **TLS** calculation. Convergence was tested by repeating the refinement with different initial estimates of the **TLS** components. The resulting **T** tensor was almost isotropic with diagonal components of 0·12 Å^2. The **L** tensor components were more anisotropic and small at only 2 deg^2 (Table 3). An isotropic overall **U** tensor calculated from the **TLS**

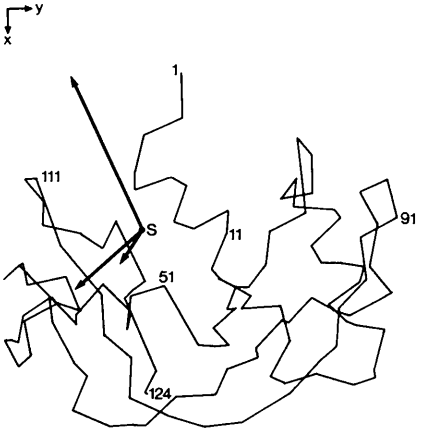

Fig. 5. The *z* projection of the backbone structure of bovine pancreatic ribonuclease showing residue numbers. The thick arrows show the principal libration axes drawn to the scale of mean-square libration amplitudes. The origin of the axes is at the position of a sulfate ion which is in the active site of the enzyme.

parameters is 0·15 Å^2. The value of the overall isotropic displacement obtained by a conventional refinement was identical.

The direction of the principal axes of libration of the molecule is shown in Fig. 5. It is of interest to note that the axis of largest libration lies parallel to the active-site cleft of the molecule. This may reflect an overall molecular motion about this direction. However, the effect of surface side-chain disorder in an approximately kidney-shaped molecule may be to produce an apparent principal axis of libration along an approximate symmetry axis of the molecule.

We conclude that overall molecular motion is almost isotropic and is mainly translational in character. This may be compared with the results of Sternberg, Grace & Phillips (1979), where small librations were found for the whole-molecule refinement of lysozyme.

Estimation of errors

The paucity of data available for macromolecular refinements relative to that available for the refinement of small molecules inevitably means that the MSDA values will be of lower precision.

The estimation of errors in macromolecular refinements is notoriously difficult, owing partly to the lack of full-matrix techniques which make possible the estimates of standard deviations. The **T** and **L** tensors must be positive definite in order to represent physically meaningful displacements and this was the case in the work presented in this paper where the tensor components were directly refined. This suggests that the smaller librations of the hydrophobic groups are meaningful.

There are several causes of systematic errors in the displacement parameters. Any uncorrected absorption will cause shifts in these parameters which are difficult to predict. Another error arises from the broad collimation used on sealed-tube X-ray sources which does not allow background corrections to be made for the scattering of acoustic modes in the crystal. This causes a systematic underestimation of mean-square displacements and might be overcome by the use of highly collimated radiation on a synchrotron source. Neglect of hydrogen atoms may cause some bias in the atomic anisotropies, particularly in the resolution range of the refinement of ribonuclease.

Errors also arise from the model itself. This may easily arise as, for example, when a single-site anisotropic model is used where significant anharmonicity should be recognized. Multiple sites and significant anharmonicity may be the rule rather than the exception for those side groups on the outside of molecules which display large librational components. Such is suggested by the dynamics studies of Kuriyan *et al.* (1986).

Discussion

Following Cruickshank (1957) most studies of rigid-body vibrations in crystals of small molecules have proceeded by a free atomic anisotropic refinement followed by the least-squares fitting of the **TLS** parameters of the quasi-rigid groups to the resulting **U** tensors. Direct refinement of **TLS** parameters in small-molecule work has, however, been advocated and used by Pawley (1972) and such a refinement has recently been carried out on the Z form of a DNA hexamer duplex (Holbrook, Wang, Rich & Kim, 1986). This approach is attractive in macromolecular refinement for several reasons.

Firstly, the poor data-to-parameter ratio in free anisotropic refinement of macromolecules (2:1 in ribonuclease) produces large correlated random errors in displacement parameters. Direct **TLS** refinement allows some control of these errors and enables the **TLS** model to be monitored using electron density maps during the course of refinement so that errors in the atomic model or the need for multiple subsites can be detected as early as possible.

Secondly, direct **TLS** refinement allows the calculated distances employed in restrained refinement to be corrected for the effects of libration. Our work shows that these corrections (up to 0·06 Å for the sulfate ion in ribonuclease) may be considerably larger than the uncertainties in the lengths of some of the target distances. It should be remembered that the target distances themselves have often been derived from small-molecule work where no libration corrections have been applied but the resulting error in such a case will not usually exceed 0·01 Å.

The present work has ignored the contribution of third-order cumulants to the structure factors. The true description of atomic librations in terms of equiprobability surfaces requires these cumulants in order to generate the banana-shaped contours. Rae (1981) has suggested that the need for correction of crystallographic interatomic distances might arise from neglect of third-order cumulants rather than from libration. The neglect of these cumulants does cause distances to be systematically overestimated from equation (8) (see Appendix). However, this effect will usually be insignificant. A calculation with data of 1·5 Å resolution shows that neglect of third-order cumulants in equation (1) leads to an overestimation of less than 0·005 Å in a rigid body undergoing harmonic librations of r.m.s. amplitude 20°.

The protein used in the current study diffracted to a resolution better than 1·5 Å. Most proteins do not diffract to such high resolution owing to higher thermal and static disorder. In such cases it may be necessary to consider a rigid-body treatment of larger structural units such as α-helices or β-sheets. The larger displacements observed in these proteins are almost certain to involve significant anharmonicity,

as observed in some of the solvent-exposed groups in ribonuclease. More disorder implies a greater proportion of information in the disorder diffuse scattering and less in the Bragg reflections. The diffuse scattering also contains information on the correlation of atomic motions which could enable the complete description of rigid-body motion together with the correlation between the displacements of different rigid groups.

Concluding remarks

The buried hydrophobic residues in the protein have displacements which are largely translational in character. The translational components are of the same order (0·04 Å^2) as the MSDA's of the Cα atoms to which they are attached. These displacements could be accounted for by a riding motion where the side-chain atoms ride on the main chain and have in addition their own small librational components.

The exposed hydrophilic groups which have been subjected to **TLS** refinement display much larger translational and librational components. These larger magnitudes are likely to be associated with poorer approximations to the harmonic model. It is therefore not surprising that in some cases we have not been able to fit this model satisfactorily. It will be interesting to see whether future anisotropic studies can provide further evidence of concerted motion of hydrogen-bonded surface groups.

It should be noted that the anisotropic displacements reported in this work are the combined effect of thermal vibrations and static disorder in the crystals. X-ray diffraction work at different temperatures might be able to distinguish these effects at least in cases where the harmonic model is reasonable.

The **TLS** method can of course be used to investigate the pseudo-rigid body displacements of larger units such as helices, sheets, subunits or even entire protein molecules as in this study and this will be the subject of further work, where it is hoped to use the diffuse as well as the Bragg scattering.

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APPENDIX

The structure-factor model and rigid-body kinematics

The contribution of an atom to a structure factor is the Fourier transform of the convolution of its electron density with the probability density function of the atomic position. By the convolution theorem this transform is the product of the atomic form factor and the transform of the probability density function. For an approximately Gaussian distribution this latter transform may be written in terms of the lower-order cumulants of the density function (Johnson, 1970). In matrix notation this transform may be written

$$\exp(2\pi i\mathbf{h}'\mathbf{x}+2\pi^2\mathbf{h}'U\mathbf{h}+\text{higher-order cumulants}) \quad (1)$$

where a prime denotes a transpose. The first-order cumulant $\mathbf{x}$ is the atomic coordinates and gives the mean position of the atom and the second-order cumulant U represents mean-square displacements from the mean (the conventional 'temperature factor'). The third-order cumulants would represent the skewness of the probability density (zero for a Gaussian distribution). The possible consequences of their neglect in the present work are referred to below.

Rigid-body displacements imply the existence of constraints between the cumulant tensors of the different atoms in the rigid group. Such displacements may be represented by a translation $\mathbf{t}$ followed by a rotation θ about an axis along a unit vector $\mathbf{n}$ (Johnson, 1970). If $\boldsymbol{\lambda}=\theta\mathbf{n}$ then the instantaneous displacement $\mathbf{u}$ of an atom in the rigid body from its position $\mathbf{r}$ at $\mathbf{t}=\boldsymbol{\lambda}=0$ may be represented by a series expansion of the rotation position in terms of vector products.

$$\mathbf{u}=\mathbf{t}+\boldsymbol{\lambda}\times\mathbf{r}+\tfrac{1}{2}\boldsymbol{\lambda}\times(\boldsymbol{\lambda}\times\mathbf{r})+\ldots. \quad (2)$$

In matrix notation this may be written

$$\mathbf{u}=\mathbf{t}+\mathbf{A}\boldsymbol{\lambda}+\tfrac{1}{2}[\boldsymbol{\lambda}\boldsymbol{\lambda}'\mathbf{r}-\mathrm{tr}(\boldsymbol{\lambda}\boldsymbol{\lambda}')\mathbf{r}]+\ldots \quad (3)$$

where tr denotes the trace operator and

$$A=\begin{bmatrix}0 & r_3 & -r_2\\ -r_3 & 0 & r_1\\ r_2 & -r_1 & 0\end{bmatrix}.$$

The cumulants $\mathbf{x}$ and U are related to the first and second moments of the displacements $\mathbf{u}$ by the equations

$$\mathbf{x}=\mathbf{r}+\langle\mathbf{u}\rangle \quad (4)$$

$$U=\langle\mathbf{u}\mathbf{u}'\rangle-\langle\mathbf{u}\rangle\langle\mathbf{u}\rangle'. \quad (5)$$

The first moment of $\mathbf{u}$ yields the distance corrections due to rigid-body libration. Averaging (3) and choosing the origin of rigid-body configuration 6-space so that $\langle\mathbf{t}\rangle=\langle\boldsymbol{\lambda}\rangle=0$ we obtain

$$\langle\mathbf{u}\rangle\simeq\tfrac{1}{2}[L-\mathrm{tr}(L)I]\mathbf{r} \quad (6)$$

where L is a matrix with elements $\langle\lambda_i\lambda_j\rangle$ and represents the rigid-body mean-square libration tensor. I is a unit matrix. Substituting (6) into (4) we obtain

$$\mathbf{x}\simeq\{[1-\tfrac{1}{2}\mathrm{tr}(L)]I+\tfrac{1}{2}L\}\mathbf{r}. \quad (7)$$

This equation shows that libration causes a generally anisotropic shrinkage of the constellation of points which represent the mean dispositions of the rigid-body atoms. It implies that the distances d_0 between the mean positions of the atoms should be corrected for rigid-body libration. The corrected interatomic distance d may be derived from (7) by taking an inner

product followed by a binomial approximation of the square root. The result is

$$d \simeq d_0\{1+\tfrac{1}{2}[\mathrm{tr}\,(L)-\mathbf{n}'L\mathbf{n}]\}. \quad (8)$$

The second moments of **u** may be related to the mean-square rigid-body displacements by use of (3). Ignoring the third and higher curvilinear moments we have

$$\langle \mathbf{u}\mathbf{u}' \rangle \simeq T + ALA' + AS + S'A' \quad (9)$$

where $T = \langle \mathbf{t}\mathbf{t}' \rangle$ and $S = \langle \boldsymbol{\lambda}\mathbf{t}' \rangle$. The matrices T, L and S represent tensors which describe the mean-square displacements of a rigid body. Equation (9) can be used to relate approximately the mean position **x** and the mean-square displacement U of a rigid-body atom at **r**. We can therefore write

$$U \simeq T + ALA' + AS + S'A'. \quad (10)$$

An equation such as (10) can be written for each atom in the rigid body. By expanding these equations into component form it can be shown that the **TLS** components are completely determined by the individual atomic U's with the exception of the diagonal element of S. This is because these elements are dependent on the correlation between the displacements of different atoms and information on such correlations is not contained in the Bragg reflections. However, the differences between the diagonal elements such as $(S_{11}-S_{22})$ may be determined and hence there are a total of 20 independent parameters which may be determined from Bragg reflections for a general rigid body.

When the atoms of a rigid body lie on a conic section a further indeterminacy in the **TLS** components occurs. Such cases always arise when the rigid group is planar and consists of fewer than six atoms. In the present work this occurs in groups such as carboxylate, amide and guanidinium which consist of four planar atoms. In these cases translational displacements perpendicular to the plane can be described in terms of librations about two axes in the plane of the group and only 19 **TLS** components and linear combinations of components are uniquely determined from Bragg diffraction. An unambiguous determination of the motion of such groups in terms of rigid-body displacements is not possible without the inclusion of higher-order cumulants in the structure-factor expansion.

The elements of the **T** and **S** tensors are dependent on the rigid-body origin. For quoting results in this work, the centre of reaction (Johnson & Levy, 1974) has been chosen as origin for each rigid group. This choice minimizes the trace of T and simultaneously makes S symmetric. With this origin we have from (10)

$$\mathrm{tr}\,(U) \simeq \mathrm{tr}\,(T) + \mathrm{tr}\,(ALA') \quad (11)$$

and $\mathrm{tr}\,(U)/3$ is an estimate of the isotropic U.

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On the Calculation of Small-Angle Diffraction Patterns from Distorted Lattices

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Abstract

A method is presented for the calculation of the diffraction properties of distorted lattices. An isotropic correlation field is used and the influence of finite crystal size is taken into account. An approximate model is described which is based on a fit of the correlation function to a sum of Gaussians. This method, which is applicable to more complex structures than simple lattices, simplifies the calculation of complicated convolution integrals. The method is illustrated with the calculation of the diffraction properties of 2D hexagonal lattices and exponential correlation. Various aspects, such as variations in peak intensity, line width and line shifts, are discussed.

Introduction

Many models have been developed which deal with the statistical properties of disordered lattices. However, calculation of the diffraction properties of such lattices remains a difficult task (Welberry, 1985). One type of disorder which is particularly important in macromolecular crystals is deformation of the regular structure. The earliest concept which has been widely used to describe the diffraction properties of such lattices is that of the paracrystal, developed by Hosemann and co-workers (Hosemann & Bagchi, 1962). In the paracrystal model one considers the distance between neighbours as the basic stochastic variable. Fluctuations in the length and orientation of unit-cell vectors lead to displacement disorder. The advantage of this model, in its simplest form, is that expressions for diffraction properties can be obtained relatively easily. The main disadvantage is that, owing to the random-walk nature of the disturbances, the variance of the length of vectors between successively distant neighbours increases without bound. This implies that the crystal dimensions depend on the statistical properties. In later developments corrections have been made for this behaviour (Hosemann, 1975) but these do not allow a simple calculation of diffraction properties.

An alternative view regards the lattice points as the basic stochastic variables and treats the distorted lattice by allowing the lattice points to deviate from a perfectly regular arrangement. If one chooses a large single-site standard deviation as well as a sufficiently large neighbour correlation the ideal paracrystal and the distorted regular lattice can be shown to be equivalent in one dimension (Welberry, Miller & Carroll, 1980). Generalizing this approach to higher dimensions, Welberry and co-workers developed so-called Gaussian 'growth disorder models' (Welberry, Miller & Carroll, 1980; Welberry & Carroll, 1982). These models do not have the drawback of the ideal paracrystal and lead to manageable formulae for diffraction patterns. Also, examples of lattices, which can be used in optical analogue diffraction experiments, can be easily constructed. The starting point in these models is that the perturbations of the lattice points can be represented by a Gaussian probability distribution, with the additional assumption that, on a square lattice, the diagonal correlation coefficient can be expressed as a product of the primary axial coefficients. This assumption introduces an unavoidable anisotropy in the correlation field and the corresponding diffraction pattern. This feature is also present in the ideal paracrystal model.

Dropping this assumption leads to a general Gaussian model (Welberry & Carroll, 1983) which is much more realistic. However, samples of lattices can only be generated by Monte Carlo methods and expressions for derived diffraction patterns are complicated and hardly amenable to calculation, so that one still has to use optical diffraction analogues to study practical situations.

A third type of approach has been proposed by Thakur *et al.*, who explicitly introduced the interaction potential, felt by the scattering units, into the line-shape expression (Thakur, Tripathy & Lando, 1985). Anharmonic terms in this potential are responsible for line-broadening effects. Although this idea

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