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Hypothesis

Mechanism of catalysis of Fe(II) oxidation by ferritin H chains

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Recombinant H chain ferritins bearing site-directed amino acid substitutions at their ferroxidase centres have been used to study the mechanism of catalysis of Fe(II) oxidation by this protein. UV-difference spectra have been obtained at various times after the aerobic addition of Fe(II) to the recombinants. These indicate that the first product of Fe(II) oxidation by wild type H chain apoferritin is an Fe(III) μ-oxo-bridged dimer. This suggests that fast oxidation is achieved by 2-electron transfer from two Fe(II) to dioxygen. Modelling of Fe(III) dimer binding to human H chain apoferritin shows a solvent-accessible site, which resembles that of ribonucleotide reductase in its ligands. Substitution of these ligands by other amino acids usually prevents dimer formation and leads to greatly reduced Fe(II) oxidation rates.

Ferritin; Ferroxidase centre; Iron (III) oxo-bridged dimer; Iron (III) monomer

1. INTRODUCTION

Ferritin plays an essential role in iron metabolism. Its ability to sequester iron is important both because it husbands an essential element and because free iron is potentially toxic. The ferritin molecule is a hollow shell built from 24 protein subunits in which iron is deposited as ferrihydrite, an Fe(III) hydrolysis product [1]. Apoferritin subunits are of two types, H and L, which cooperate in ferrihydrite production [2]. Each H chain contains a ferroxidase centre at which Fe(II) oxidation is catalysed so that Fe(III) is provided rapidly for ferrihydrite nucleation [3–6]. L chains lack these centres, but their cavity-faces favour nucleation because they are relatively rich in carboxylic amino acids [6]. Here, however, our main concern is to explain how H chains catalyse Fe(II) oxidation.

Mössbauer spectroscopic measurements have shown that when Fe(II) is added in air at pH 7.0 to recombinant human H chain apoferritin (8 or 38 Fe/mol, 0.5 mM Fe), oxidation is complete in under 1 min [7]. The iron (III) at 1 min is in the form of isolated Fe(III) atoms (20%), μ-oxo-bridged Fe(III) dimers (70%) and small Fe(III) clusters (10%). Although isolated Fe(III) species are observed, Fe(III) dimers are not formed when Fe(II) is added to an H chain variant in which two residues at the ferroxidase centre are changed [7]. With this variant, Fe(II) oxidation was even slower than in the protein-free control (in which neither monomer nor dimer Fe(III) was seen). Here we report changes in UV-difference spectra after Fe(II) addition to recombinant human H chain apoferritin variants bearing one or more amino acid substitution at their ferroxidase centres.

Previously it has been found by X-ray crystallography that the ferroxidase centres of human H chains bind Tb^{3+} ions at two alternative positions about 3.1 Å apart [5]. Ligands of these metal sites, Glu-27, Glu-61, Glu-62, His-65 and Glu-107, are conserved in mammalian H chains, as are two residues which hydrogen bond to Glu-107, namely Tyr-34 and Gln-141 [2]. Several of these residues have been replaced for our spectroscopic study. Another conserved residue, Ala-144, near the ferroxidase centre, may also be essential for activity because its replacement by a larger residue (Leu as in L chains) seems to impede access from or to cavity. A computer graphics diagram of the centre is shown in Fig. 1 modelled with a μ-oxo-bridged Fe(III) dimer in place. We propose that these dimers are the product of rapid oxidation from a pair of Fe(II) atoms and that this accounts for the catalytic action of the ferroxidase centre.

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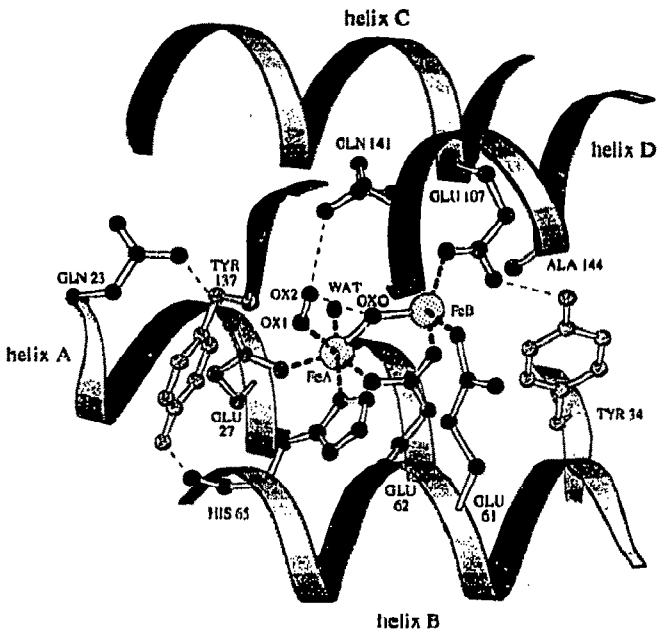


Fig. 1. Proposed site of the μ-oxo-bridged iron dimer with bound dioxygen. The figure is based on the coordinates of human H ferritin [5]. The iron labelled FeA shows octahedral coordination, with a water molecule (WAT) in the sixth position, whilst FeB shows tetrahedral coordination. Metal-to-ligand bonds are in the range 1.84 Å (OXO) to 2.35 Å (H_2O), with an Fe-Fe separation of 3.25 Å and an Fe-O-Fe angle of 125°. Hydrogen bonds are indicated by thin dashed lines. The metal site is viewed from the internal cavity. Rotation about the Cα–Cβ bond allows Glu-61 to swing into the cavity. The model was produced using the molecular modelling program FRODO [19], implemented on an Evans and Sutherland series 'V' stereo workstation and the drawing was produced using the package MOLSCRIPT [20].

2. MATERIALS AND METHODS

2.1. *Protein purification*

Site directed mutagenesis, overexpression in *Escherichia coli* and purification of recombinant human H chain ferritins were performed as in [8]. Iron was removed from the ferritins with sodium dithionite according to [7]. After reduction, the protein was purified by ion exchange chromatography with Q Sepharose Fast Flow (Pharmacia, Milton Keynes, UK). The protein in BBS was absorbed on a column (1.6 × 14 cm) equilibrated with 0.05 M MES, pH 6.2 (buffer A), and washed with three column volumes of buffer A before elution at 1 ml/min with a salt gradient. The latter was formed using an LKB (Pharmacia) HPLC Gradient Controller from buffer A and 0.5 M NaCl in buffer A as solvent B. The gradient corresponded to 0.4% B/min. Ferritin containing fractions with a 280:260 nm absorbance ratio >1.7 were pooled, concentrated, dialysed against 10 mM NaCl in an 8MC ultrafiltration cell (Amicon, High Wycombe, UK) and stored at 4°C. Bovine serum albumin (BSA) was from Sigma (Poole, Dorset, UK).

2.2. *UV difference spectroscopy*

Spectra were recorded using a Cary 1 spectrophotometer. The apoferritin solution (1 mg/ml) in MES buffer was placed in both sample and blank compartments and a base line recorded. 0.5 μl H_2O was added to the blank and 0.5 μl $(NH_4)_2Fe(SO_4)_2$ (Aldrich, UK, 99.997% pure) solution added to the sample to give a final concentration of 8.4 μM. The spectrophotometer was set to record repeated spectra at either 1 or 5 min intervals.

3. RESULTS AND DISCUSSION

Examples of UV difference spectra are shown in Fig. 2 a–f. Each figure shows successive spectra obtained at the times indicated after the addition of $(NH_4)_2Fe(SO_4)_2$ to the recombinant human H chain apoferritin (4 Fe(II)/molecule) or to BSA. The spectra shown in Fig. 2b,c, d and e are of recombinant human H ferritin with one or more amino acid substitution at the ferroxidase centre. In the sample of Fig. 2a this centre is intact. Samples in Fig. 2a–d are at pH 6.5 and 2e at pH 7.5. Fig. 2f shows spectra obtained during the oxidation of Fe(II) at pH 7.0 in the presence of BSA used as control protein (behaving as a protective colloid). The rate of oxidation at pH 6.5 was negligible. Except for the sample in Fig. 2d and e, the ferritins bear a substitution Lys-86/Gln, remote from the ferroxidase centre, introduced to allow crystallization through metal bridge formation and hence X-ray analysis [5]. In all the samples, spectral changes with time are seen, giving essentially four types of pattern: (1) shown in Fig. 2a and d, (2) in Fig. 2b and c, (3) in Fig. 2e and (4) in Fig. 2f. The spectra of Fig. 2f are featureless. Similar spectra, without any absorbance maxima, have also been obtained with rat or human L ferritin under similar conditions. Patterns like those of Fig. 2b and c, in which a single absorbance maximum develops in the region of 290 nm, have been obtained when any of the seven ferroxidase centre residues are substituted, except for Glu-61 (and Gln-141 which has not yet been examined). In the sample of Fig. 2d and e the substitution Glu-61/Ala has been made along with Glu-64/Ala and Glu-67/Ala. Similar spectra were obtained in a sample containing only the Glu-61/Ala substitution. Glu-64 and -67 lie on the inside surface of the apoferritin shell, but Glu-61 can occupy one of two different positions in the crystal electron density, one participating in the ferroxidase centre (Fig. 1) and the other pointing into the cavity. It has been postulated that movement of this residue may assist the migration of Fe^{3+} from the oxidation site to the nucleation site [5]. In all the examples of Fig. 2 absorbance changes occur over a period of several minutes after the recording of the first spectrum. However, in Fig. 2a and d the first absorbing species is gradually replaced by another and the spectra intersect at an isosbestic point. The first spectrum in Fig. 2d, obtained at pH 6.5, shows two shoulders at approximately 310 and 350 nm and similar shoulders are even more evident in Fig. 2e. However, at the higher pH value, 7.5, this absorbing species is relatively stable. Its spectrum bears a strong resemblance to the 300–400 nm region of the spectra of oxyhaemerythrin and ribonucleotide reductase, the two shoulders being attributed in these proteins to ligand-to-metal charge transfer bands of their μ-oxo-bridged Fe(III) dimers [9,10]. We propose that the 310 and 350 nm absorbances of Fig. 2d and e represent LMT C bands of such dimers attached to ferritin H chains. Fig.

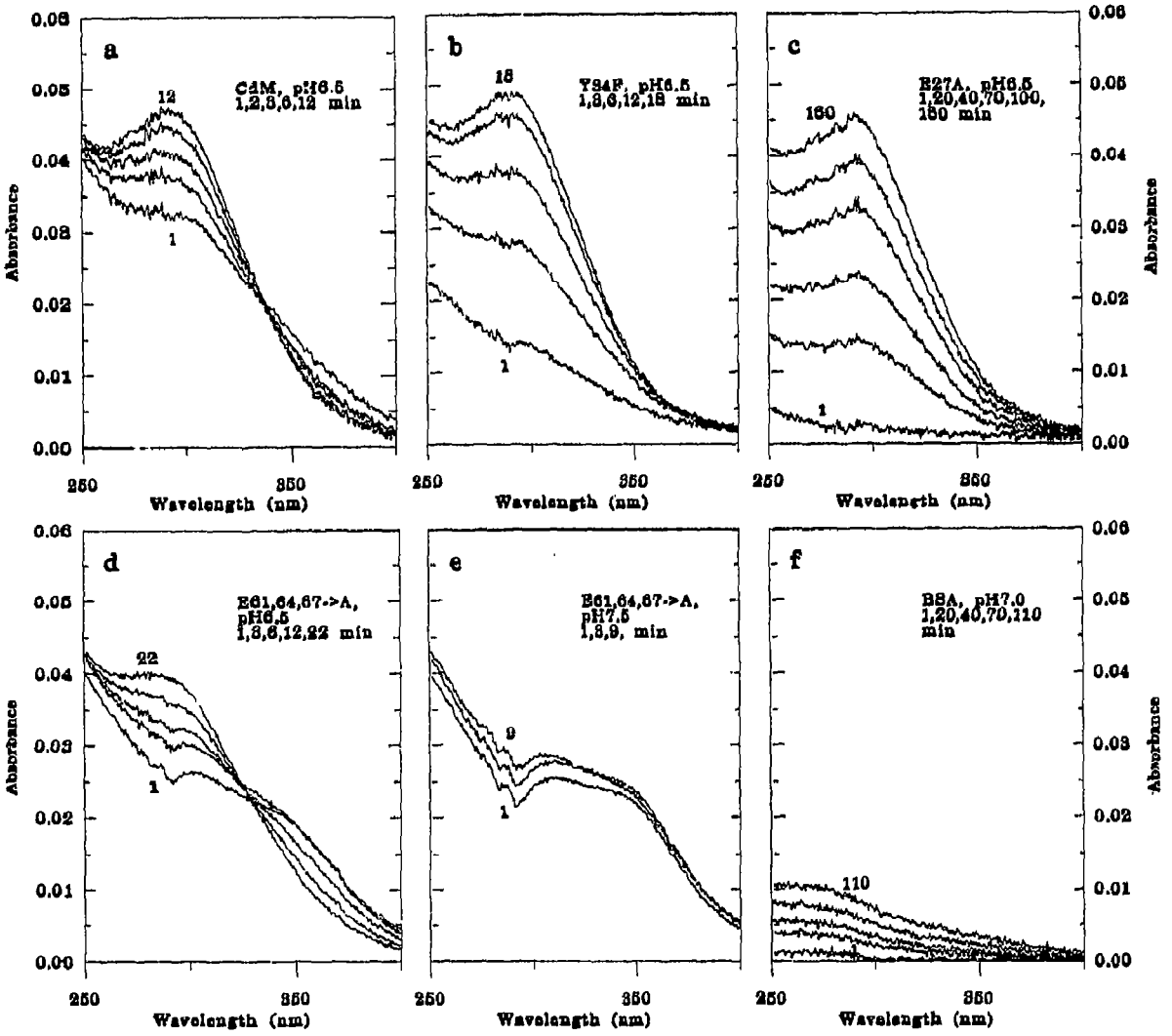
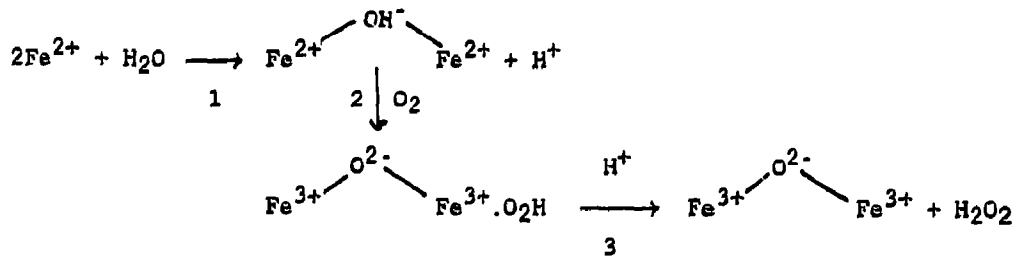


Fig. 2. UV difference spectra of recombinant human H chain apoferritins after the addition of 4 Fe(II)/apoferritin as $(NH_4)_2Fe(SO_4)_2$. The apoferritin in a (CdM) bears a single amino acid substitution Lys-86/Gln on the outside surface of the protein shell, b and c carry this change in addition to those at the ferroxidase centre shown. In d and e, Glu-61 is found alternatively at the ferroxidase centre or on the cavity surface, and Glu-64 and Glu-67 are on the latter.

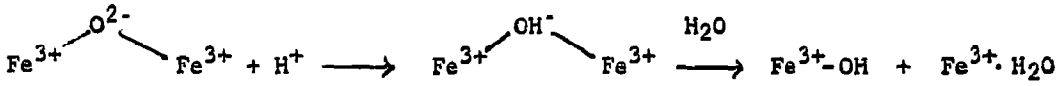
2d shows the disappearance over several minutes at pH 6.5 of the dimer spectrum and its replacement by a species absorbing in the region of 290 nm, essentially like those of Fig. 2b and c. Similar behaviour at this pH is suggested in Fig. 2a, although the dimer bands are now barely visible. We suggest that in these recombinants the 290 nm absorbance results from dimer cleavage. It could represent a composite of two single Fe(III) species, and could also have a contribution arising from the movement of an aromatic residue or residues.

In variants showing only the 290 nm absorbance such as those of Figs. 2b and c, maximum absorbance is reached much more slowly than that of the dimer absorbance of Fig. 2a,d and e, but at very different rates in different variants. We associate the slow appearance of the 290 nm absorbance with slow Fe(II) oxidation (this has been demonstrated by Mössbauer spectroscopy for one variant (Glu-62/Lys, His-65/Gly) in which oxidation took over 2.5 h to complete [7]). In contrast, oxidation is complete in about 1 min for variants with intact ferroxidase centres [7,11]. In protein-free controls, in the presence of BSA, Fig. 2f, or in rat or human L chain recombinant ferritins, Fe(II) oxidation is again very slow compared to that in the presence of human H chain ferritin.

We suggest on the basis of the present experiments (combined with the Mössbauer data) that, in wild-type ferritin, oxidation at the ferroxidase centre proceeds according to Scheme 1, with the binding of two Fe(II) in close proximity enabling 2-electron transfer to a dioxygen molecule. Oxidation is followed by the splitting of the dinuclear species (Scheme 2). The oxidative step of



Scheme 1. 2-Electron oxidation and production of peroxide.



Scheme 2. Splitting of μ-oxo-bridged Fe(III) dimer.

Scheme 1 is similar to that proposed for the binding of dioxygen to haemerythrin [9], but the reaction now proceeds to the formation of H_2O_2.

It is suggested that reversible oxygenation in preference to oxidation in haemerythrin is enabled by the non-polar character of its dioxygen binding site which retards the release of the negatively charged HOO^- ion [9] and the production of hydrogen peroxide (as in scheme 1) is slow compared with dioxygen binding and 2-electron transfer [9]. In H chain ferritin, however, the dimer site is relatively open and accessible to water. In haemerythrin dimer dissociation tends to be proton assisted [9]. Dimer dissociation (scheme 2) which in ferritin H chains may enable iron to be released from the ferroxidase centre for ferrihydrite nucleation in the cavity, is also favoured at lower pH. The formation of ferrihydrite, however, is favoured by pH values above 7 [12], due to the release of protons associated with hydrolytic polymerisation. This explains why iron-core formation proceeds in L chains homopolymers (lacking ferroxidase centres) only at pH 7 or above.

Consistent with our proposed mechanism, we note that evidence for the production of peroxide during the oxidation of 24 Fe(II) added to horse spleen apoferritin has been obtained by Xu and Chasteen [13]. Cheng and Chasteen [14] found that the rate of disappearance of Fe(II) was first, not second, order as might be expected from scheme 1, but, under the conditions used, dimer production may not have been rate-limiting.

A computer graphics drawing of the ferroxidase centre region of human H chain apoferritin is shown in Fig. 1. A μ-oxo-bridged Fe(III) dimer (Fe–Fe distance 3.25 Å, Fe–O–Fe angle 125°) has been placed in a space which is part of an inlet from the central cavity. Fe ligands are shown in Fig. 1. There is space for a dioxygen molecule to bind to FeA, with the possibility of hydrogen bonding from a μ-hydroxo hydrogen and this has been modelled. The site, which resembles that of ribonucleotide reductase [15] more closely in its ligands than that of haemerythrin [9], shows considerable side chain flexibility and the two Fe^{3+} atoms are modelled at similar, but not identical positions to those described previously for Tb^{3+} [5]. In the proposed model a single carboxylate, Glu-62, bridges the two Fe atoms. Bridging also by Glu-107 may be possible [5] if this residue and the two metal atoms are placed in somewhat different positions. FeA also has Glu-27 and His-65 as ligands and FeB has Glu-107 and Glu-61 (when appropriately orientated as in Fig. 1). Substitution of either site A or site B ligands seems to prevent dimer formation. In principle, monomer Fe(III) could be produced by $1e^-$ transfer to O_2 at either A or B since both are accessible to O_2. In one of the variants giving only the 290 nm absorbance (Glu-62/Lys, His-65/Gly), and in which monomer, but no dimer, Fe(III) has been observed by Mössbauer spectroscopy [7], ligands of both Fe atoms are altered and the position of the dimer is now occupied by a salt bridge between Lys-62 and Glu-107 (as found in L chains). Monomer Fe(III) cannot therefore be at either site A or B in this variant and its location is at present uncertain.

In summary the work presented here suggests that 'ferroxidase activity' may be accounted for by fast $2\text{-}e^-$ transfer to a pair of Fe(II) at the ferroxidase centre, followed by Fe(III) dimer dissociation and migration into the cavity. When dimer formation is prevented, a slow, presumably $1e^-$ oxidation is possible. Formation of Fe dimers is co-operative since even when only 4 Fe(II) atoms are added to the H chain 24-mer, all or most of the iron appears in this form immediately after oxidation. The precise steps following dimer dissociation and the location of the early products remain to be elucidated. However, we know that monomer Fe(III) [7,11,16] as well as the 290 nm absorbance [17] gradually diminish as ferrihydrite clusters grow. Once the iron core has formed, it provides an alternative surface for Fe(II) oxidation which allows the reduction of 4 Fe(II)/O_2 and the reduction of dioxygen to water [13,18].

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REFERENCES

[1] Harrison, P.M., Andrews, S.C., Artymiuk, P.J., Ford, G.C., Guest, J.R., Hirzmann, J., Lawson, D.M., Livingstone, J.C., Smith, J.M.A., Treffry, A. and Yewdall, S.J. (1991) Adv. Inorg. Chem. 36, 449–486.

[2] Andrews, S.C., Arosio, P., Bottke, W., Briat, J.-F., von Darl, M., Harrison, P.M., Laulhère, J.-P., Levi, S., Lobreaux, S. and Yewdall, S.J. (1992) J. Inorg. Biochem. (in press).

[3] Levi, S., Luzzago, A., Cesareni, G., Cozzi, A., Franceschinelli, F., Albertini, A. and Cortese, R. (1988) J. Biol. Chem. 163, 18086–18092.

[4] Lawson, D.M., Treffry, A., Artymiuk, P.J., Harrison, P.M., Yewdall, S.J., Luzzago, A., Cesareni, G., Levi, S. and Arosio, P. (1989) FEBS Lett. 254, 207–210.

[5] Lawson, D.M., Artymiuk, P.J., Yewdall, S.J., Smith, J.M.A., Livingstone, J.C., Treffry, A., Luzzago, A., Levi, S., Arosio, P., Cesareni, G., Thomas, C.D., Shaw, W.V. and Harrison, P.M. (1991) Nature 349, 541–544.

[6] Wade, V.J., Levi, S., Arosio, P., Treffry, A., Harrison, P.M. and Mann, S. (1991) J. Mol. Biol. 221, 1443–1452.

[7] Bauminger, E.R., Harrison, P.M., Hechel, D., Nowik, I. and Treffry, A. (1991) Biochim. Biophys. Acta 1118, 48–58.

[8] Treffry, A., Harrison, P.M., Luzzago, A. and Cesareni, G. (1989) FEBS Lett. 247, 268–272.

[9] Sanders-Loehr, J. (1989) in: Iron Carriers and Iron Proteins (T.M. Loehr, ed.) pp. 373–466, VCH Publishers Inc., New York.

[10] Que Jr., L. and True, A.E. (1990) in: Progress in Inorganic Chemistry, vol 38 (S.J. Lippard, ed.) pp. 97–200, Wiley, NY.

[11] Bauminger, E.R., Harrison, P.M., Heche, D., Nowik, I. and Treffry, A. (1991) Proc. R. Soc. Lond. B 244, 211–217.

[12] Paques, E.P., Paques, A. and Crichton, R.R. (1979) J. Mol. Catal. 5, 363–375.

[13] Xu, B. and Chasteen, N.D. (1991) J. Biol. Chem. 266, 19965–19970.

[14] Cheng, Y.G. and Chasteen, N.D. (1991) Biochemistry 30, 2947–2953.

[15] Norland, P., Sjoberg, B.-M. and Ekland, H. (1990) Nature 345, 593–598.

[16] Bauminger, E.R., Harrison, P.M., Nowik, I. and Treffry, A. (1989) Biochemistry 28, 5486–5493.

[17] Treffry, A. and Harrison, P.M. (1984) J. Inorg. Biochem. 21, 9–20.

[18] Treffry, A., Sowerby, J.M. and Harrison, P.M. (1978) FEBS Lett. 95, 221–224.

[19] Jones, T.A. (1978) J. Appl. Cryst. 11, 268–272.

[20] Kraulis, P.J. (1991) J. Appl. Cryst. 24, 946–950.