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On avoiding negative electron density in Gram-Charlier refinements of anharmonic motion: the example of glutathione

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Abstract: The structure of glutathione, γ -L-Glutamyl-L-cysteinyl-glycine ($C_{10}H_{17}N_3O_6S$), was studied by multi-temperature single-crystal X-ray diffraction. Residual density maps from conventional independent atom model refinement gave indication of anharmonic motion in the molecule. This was further investigated by invariom refinement with anisotropic displacement parameters for all atoms, which described asphericity due to chemical bonding and lone pairs; afterwards only the residual-density signal of anharmonic motion remained. Treating anharmonicity with third-order Gram-Charlier displacement parameters led to regions with unphysical negative electron density. In contrast, a maximum entropy method (MEM) determination of the electron density successfully takes the features into account. Respective difference electron density plots (MEM minus prior and [Invariom+GC] minus invariom) agree well with each other. Challenges in treating and understanding the phenomenon are discussed. A procedure is proposed how unphysical negative electron density can be avoided. It is closely related to the free lunch algorithm.

Keywords: anharmonic motion; invariom refinement; maximum entropy method.

Introduction

Glutathione (γ -L-glutamyl-L-cysteinyl-glycine) is a water-soluble pseudo-tripeptide of high biological relevance, which is synthesized in eukaryotic cells by a two-stage mechanism [1]. Glutathione plays an essential part in antioxidant defense, is involved in several metabolic processes and is a protagonist in cellular regulation processes [2]. It acts as physiological reservoir of cysteine and plays a role in regulation of protein function, signal transduction, gene expression and apoptosis [3]. The enzyme glutathione-S-transferase catalyzes the linkage of glutathione to organic compounds. Deviations from normal levels of glutathione in the body have been associated with different human diseases, e.g. cancer, acquired immune deficiency syndrome (AIDS), neuro-degenerative diseases or diabetes [4].

The focus of the present work is the single-crystal structure of the reduced form of glutathione, which had first been elucidated in 1958 [5, 6] and re-determined in 1987 [7]. More recently, the crystal structure of the oxidized form of glutathione has been reported [8]. We investigate the structure of the tripeptide, since the Fourier residual electron density after independent atom model (IAM) refinements with SHELXL [9], as generated using the graphical user interface SHELXLE [10], showed features characteristic of anharmonic motion¹ [12] that appear as alternating features of positive and negative residual electron density [13]. To further study this effect we collected high-resolution Bragg data $[(\sin \theta/\lambda)_{\max} = 1.16 \text{ \AA}^{-1}]$ at 100 K. Two further data sets up to $0.83 \text{ \AA}^{-1}$ and $0.8 \text{ \AA}^{-1}$ resolution were measured at temperatures of 6 and 295 K, respectively. In glutathione anharmonic motion [14] appears mainly around the protonated carboxyl group and involves both oxygen atoms, and, to a lesser degree, the sulfur atom. Using a disorder model fails to model these

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¹ The recommendation to avoid calling displacements ‘thermal’ [11] should also be applied to anharmonic displacement parameters, and thermal motion is just one possible displacement contribution. We therefore omit any classifiers about what causes anharmonicity.

features. Gram-Charlier (GC) type higher-order atomic displacement parameters [15, 16] improve the situation, but technical problems remain. Only the maximum entropy method (MEM) currently leads to a successful description of the vibrational behavior.

Studies of anharmonic motion were first carried out in the context of analyzing room-temperature neutron diffraction data [17]. The effect has regained attention [18] and relevance with the recent emphasis of unmodeled features in the residual electron density distribution of high-resolution X-ray data [19, 20]. A thorough study on the explosive 1,3,5-trinitroperhydro-1,3,5-triazine (RDX) provided a good example in this respect [21], and it demonstrated the importance of anharmonic atomic displacement parameters (ADPs) for the determination of an accurate molecular electron density distribution (EDD) $\rho(\mathbf{r})$.² Other more recent examples where anharmonicity was reported include the free radical p-(methylthio) phenyl nitronyl nitroxide [22], 1-(20-aminophenyl)-2-methyl-4-nitroimidazole [23] and the non-linear optical material 2-methyl-4-nitroaniline (MNA) [24].

Most publications on accurate EDDs employ the multipole model [25, 26] for an experimental charge density study, which is currently also the underlying model in invariom refinements. Alternatively, EDDs can be determined experimentally by X-ray wavefunction refinement [27] and the maximum entropy method [28], the latter of which has successfully been applied to peptides and amino acids [29–32] as well as to macromolecular data [33]. For our study the MEM is especially well suited, where negative electron density is intrinsically avoided. This is not the case for GC modeling of anharmonic motion. Based on the results from the MEM a generally applicable procedure on how to successfully model such features with the GC model is described.

Experimental, refinement and theoretical computations

Experimental

Glutathione was crystallized from a mixture of 95% ethanol/water by slow evaporation. Three single-crystal X-ray diffraction experiments (Table 1) were carried out on the title compound.³ Two of these, at temperatures of 100 K and 295 K with Mo K α radiation, were performed

² For a distinction between static and dynamic EDD and the relationship to nuclear probability distributions see Section “Least-squares refinement”.

³ The first experiment at 100 K used a different but very similarly sized crystal than the other two measurements.

on a Bruker D8 diffractometer with an APEX II CCD detector, an Incoatec microsource with mirror optics and a nitrogen gas-stream cooling device (100 K data). An overall coverage of 99.8% (100 K) and 99.9% (295 K) was obtained for full resolution $[(\sin \theta/\lambda)_{\max} = 1.16 \text{ \AA}^{-1}$ at 100 K, $(\sin \theta/\lambda)_{\max} = 0.8 \text{ \AA}^{-1}$ at 295 K]. Both experiments lasted 4 days and a total number of 182,146 (100 K) and 53,430 (295 K) reflections were collected. No significant intensity decay was observed. SADABS [34] was used for scaling and multi-scan absorption correction [36]. A correction factor for low-energy contamination of 0.0045 was also applied to take this source-specific systematic error into account [37].

The third experiment was carried out at 6 K using a Cryosystems of America helium open-flow low-temperature device at the P11 BioImaging and Diffraction beamline of the PETRA III synchrotron at DESY in Hamburg, Germany [38]. The wavelength of 0.5636 Å (22 keV) in combination with a Pilatus 6M detector and the minimal crystal-detector distance of 156 mm permitted to reach a resolution of $(\sin \theta/\lambda)_{\max} = 0.83 \text{ \AA}^{-1}$ with 95% coverage.⁴ The measurement time was less than 3 min and a total of 36,183 reflections were collected. Integration was carried out with XDS [40], SADABS was again used for scaling. Full crystallographic details for all three experiments are given in Table 1. CCDC 1422209 (100 K), 1500579 (6 K) and 1500651 (295 K) contain the corresponding depositions of crystal structures (invariom model) including structure factors embedded in the CIF files.

Least-squares refinement

In conventional charge density studies with the multipole formalism [26, 41], population parameters are least-squares adjusted against high-resolution diffraction data to observe EDD experimentally. Undetected or un-modeled disorder [42] and anharmonic motion [43] can lead to unreliable or wrong results in a charge density study. Since EDD and thermal motion are correlated, they cannot always be successfully de-convoluted when such effects are present [44].

In an attempt to circumvent such technical problems for the modeling of the title compound, we have carried out invariom refinements [45] that use fixed pseudoatom scattering factors to the hexadecapolar level derived from density functional theory (DFT) computations. Invariom scattering factors facilitate de-convolution by including features of bonding and lone pair electron densities in the structure factor. At the same time such refinements do not permit to adjust multipole parameters, and limit model flexibility, which helps to avoid overfitting.⁵ Table 2 lists invariom scattering-factor names, the respective local-atomic site symmetry and the model compounds that provide the local chemical environment from which these scattering factors were computed.

Initial hydrogen-atom positions for invariom refinement were generated with SHELXL using AFIX commands with the exception of the thiol hydrogen, for which the position located in the difference Fourier map was chosen. All X–H bond distances were then elongated to

⁴ Higher resolutions with full coverage could not be reached due to the beam-line setup, which does not include the possibility of a 2 θ movement or rotation of the detector. In addition there was shading of the helium cold-stream device on the detector. A comparably high R_{int} was caused by the scan speed. Data quality is still acceptable due to the high redundancy (or multiplicity) of the data. R_{pim} [39] might be a more useful criterion than R_{int} here.

⁵ Overfitting can also be studied and quantified by R_{complete} [46].

Tab. 1: Experimental, crystallographic and refinement details.

Crystal data			
Chemical formula		C ₁₀ H ₁₇ N ₃ O ₆ S	
<i>M</i> _r		307.32	
Crystal system, space group		Orthorhombic, <i>P</i> 2 ₁ 2 ₁ 2 ₁	
Temperature (K)	6	100	295
<i>a</i> (Å)	5.6131(11)	5.6278(1)	5.6216(4)
<i>b</i> (Å)	8.720(2)	8.7401(2)	8.7855(6)
<i>c</i> (Å)	27.940(5)	27.9446(7)	28.0552(18)
<i>V</i> (Å ³)	1367.6(5)	1374.53(5)	1385.61(16)
<i>Z</i>		4	
Radiation type	Synchrotron	Mo Kα	Mo Kα
Wavelength Å	0.5636	0.7107	
μ (mm ^{−1})	0.146	0.27	0.263
Crystal size (mm)	0.19 × 0.14 × 0.11		
Data collection			
Diffractometer	P11 single axis goniostat	Bruker D8 APEX II	
Absorption correction		Empirical [34]	
<i>T</i> _{min} , <i>T</i> _{max}	0.8097, 0.8359	0.668, 0.692	0.698, 0.747
No. of meas. refl.	36,183	182,446	53,221
No. of indep. refl.	6500	18,083	5942
No of obs. [<i>I</i> > 2σ(<i>I</i>)] refl.	6228	16,872 (9436)	4952
<i>R</i> _{int}	0.083	0.033	0.048
(sin θ/λ) _{max} (Å ^{−1})	0.83	1.16	0.80
Refinement			
Absolute structure [35]	2584 quotients	7203 quotients	1834 quotients
Absolute structure parameter	−0.01(4)	0.023(6)	0.03(3)
H-atom treatment	x, y, z-parameters constrained, <i>U</i> _{ij} estimated		
Invariom			
<i>R</i> ₁ (<i>F</i>), <i>wR</i> (<i>F</i> ²), <i>S</i>	0.032, 0.076, 1.27	0.026, 0.031, 3.34	0.041, 0.061, 2.46
No. of reflections	6228	16,872 (9436)	4952
No. of parameters	181/198		
Δρ _{max} , Δρ _{min} (e Å ³)	0.50, −0.53	0.95, −0.81	0.50, −0.57
Invariom + Gram-Charlier			
<i>R</i> ₁ (<i>F</i>), <i>wR</i> (<i>F</i> ²), <i>S</i>	0.028, 0.070, 1.19	0.019, 0.016, 2.39	0.033, 0.032, 1.74
No. of parameters	262	262	262
Δρ _{max} , Δρ _{min} (e Å ³)	0.32–0.47	0.29, −0.23	0.21, −0.24

invariom database values and constrained in a riding-hydrogen model. Anisotropic ADPs for hydrogen atoms were estimated (see Section “Theoretical computation of ADPs”) with the TLS+ONIOM method [47]. Constraints and input files for refinement with aspherical scattering factors with the program XD2006 [48] were generated with the preprocessor program INVARIOMTOOL [49]. After invariom refinement anharmonic motion in the Fourier residual map was the only significant remaining signal and parameter correlations did not exceed 67%.

In a next step GC parameters of third order⁶ were added to the model, but this described the effect only partly, since noticeable regions of (unphysical) negative electron density [50] remained around O31. Seven of the GC parameters of O31 and O32 now showed correlations exceeding 70%, of which three exceeded 80%.

⁶ Fourth order parameters were neither required, nor could they probably have been refined to physically meaningful values with the data resolution we could reach. Nevertheless, the conservative estimates of resolution required for GC refinement [16] were based on the reflection/parameter ratio, which is more favorable when multipole parameters are fixed to invariom-database values.

The EDD in a molecule can, by definition, only be positive. This should hold for static EDD as well as dynamic EDD, where only the former underwent de-convolution from dynamic effects like thermal motion. In least-squares modeling, parameters describing (static) EDD correlate to a certain degree with ADPs (describing dynamics), since EDD is convoluted with physical effects that might smear it [51]. De-convolution requires a model for both EDD and atomic displacements, and might therefore not be successful or may remain incomplete, which we see in glutathione. In single-crystal X-ray diffraction only the dynamic EDD can directly be measured. A successfully de-convoluted static EDD would correspond to the probability distribution of the nuclei, which should therefore also be positive everywhere. In the case of glutathione it is neither the static nor the dynamic electron density that becomes negative in some places, but the combination of the static model density and the GC displacements.

That GC parameters can cause negative regions in areas of overall low electron density is known [16] and is caused by subtracting or shifting electron density by third (odd) order functions. The importance of negative electron density should be judged in perspective

Tab. 2: Details of invariom refinement [45].

Atom name	Invariom assigned	Local-atomic site symmetry	Model compound
S(2G)	S1c1h	<i>mz</i>	Methanethiol
O(1E, O2)	O1.5c[1.5n1c]	<i>mm2</i>	Acetamide
O(11,21)	O1.5c[1.5o1c]	<i>mz</i>	Acetic acid anion
O(31)	O1c1h	<i>mz</i>	Methanol
O(32)	O2c	<i>mm2</i>	Formaldehyde
N(1)	N1c1h1h1h	3	Methylammonium cation
N(2,3)	N1.5c[1.5o1c]1c1h	<i>mz</i>	N-methylacetamide
C(1)	C1.5o1.5o1c	<i>mz</i>	Acetic acid anion
C(1A,2A)	C1n1c1c1h	<i>mz</i>	2-aminopropane
C(1B)	C1c1c1h1h	<i>mm2</i>	Propane
C(1D,2)	C1.5o1.5n[1c1h]1c	<i>mz</i>	N-methylacetamide
C(1G)	C1c1c1h1h	<i>mm2</i>	Propane
C(2B)	C1s[1h]1c1h1h	<i>mz</i>	Ethanethiol
C(3)	C2o1o1c	<i>mz</i>	Acetic acid
C(3A)	C1n1c1h1h	<i>mz</i>	Ethylamine
H(2G)	H1s[1c]	6	Methanethiol
H(31)	H1o[1c]	6	Methanol
H(1A,B,C)	H1n[1c1h1h]	6	Methylammonium cation
H2,3	H1n[1.5c1c]	6	N-methylformamide
H(11A)	H1c[1n1c1c]	6	2-aminopropane
H(11B,12B,11G,12G)	H1c[1c1c1h]	6	Propane
H(2A)	H1c[1n1c1c]	6	2-aminopropane
H21B,22B	H1c[1s1c1h]	6	Ethanethiol
H(31A, 31B)	H1c[1n1c1h]	6	Ethylamine

with the respective standard uncertainty of these parameters [52]. Negative regions of electron density after GC refinement have also been discussed by Sørensen et al. [53].

In our attempts to arrive at a reliable description of anharmonic-ity and EDD in glutathione we also tried a disorder model instead of the GC description for the high-resolution data measured at 100 K. As to be expected the situation is clearly less satisfactory here. When split atom sites were introduced for O31 and O32, bond distances refined to unphysical values and DFIX restraints became mandatory to enforce a similar bond length between C3 and the respective split oxygen site. Moreover, SIMU/RIGU [54] restraints were necessary to maintain a reasonable shape of the ADPs that otherwise become extremely elongated in one direction. When cutting the resolution to the Acta Crystallographica Section C limit of $d=0.84$ Å ADPs became non positive definite. We therefore discarded disorder models altogether for the 100 K data. For the 6 K data refinement of split positions is possible (see Section “Results and discussion”).

Since the description of anharmonic motion in glutathione with the multipole model in combination with GC parameters (concerning free refinement of multipole parameters see the results section) always led to rather pronounced negative values of the probability density function (P.D.F.), ultimately a MEM calculation using the program BAYMEM [55] was performed. Here we started from a dynamic prior density based on invariom refinement with harmonic ADPs. The latter density was computed using the program PRIOR [56]. The MEM calculations were stopped at different χ^2_{aim} values ranging from 6.0 down to 2.0, leading to continuous improvements in the residual density, as was validated by an evaluation of the fractal dimension (“Meindl”) plot [57]. However, at $\chi^2_{\text{aim}}=2.0$ the derived EDD contains spurious maxima and only choosing a higher χ^2_{aim} value of 5.3 avoids

this overfitting.⁷ The MEM model so-obtained permits to extend the experimental resolution in analogy to the free-lunch algorithm [59]. When GC parameters are fitted using these data generated by the MEM algorithm, negative EDD can be avoided also with the invariom/multipole model description, although the fit then remains inferior to free invariom + GC refinement and MEM modeling.

Theoretical computation of ADPs

For computing hydrogen ADPs by the TLS+ONIOM method [47] two-layer ONIOM [60, 61] computations were carried out with Gaussian09 [62]. Input files were prepared with the preprocessor program BAERLAUCH [63] using the invariom refinement result with X–H bond distances set to the values from DFT [B3LYP/D95++(3df,3pd)] optimized model compounds listed in Table 2. All molecules within a distance of 3.75 Å to any atom of the central molecule were included in a 15 molecules cluster with 555 atoms altogether. For both the central molecule (the high layer) and the surrounding molecules (the low layer) the same B3LYP functional was used; the basis set was 6-31G(d,p) for the high and 6-31G for the

⁷ For the MEM Bindzus et al. [58] suggest to generally use the featurelessness of the Meindl-plot as a termination criterion. Since this did not work for glutathione it does not seem to be a generally valid criterion against overfitting. An alternative criterion are low-value iso-surfaces that cease to be smooth in the overfitted region, although a numerical criterion what ‘smooth’ means exactly can not be provided.

low layer. Only the central molecule was geometry-optimized. Frequencies of normal modes were then transformed into ADPs with a locally modified version of the XDVB1/2 programs of the XD package [64]. 3N vibrational modes from the MO/MO computation (111 ones) were converted, using a scale factor of 0.9945 as recommended by Scott and Radom [65]. Estimated anisotropic displacement parameters of hydrogen atoms were included in final invariom refinements at all three temperatures and the abovementioned invariom prior used for MEM at 100 K.

Results and discussion

Deconvolution of anharmonic motion and electron density

As illustrated in Figure 1 the residual EDD is stepwise reduced, starting with the IAM, improving with invarioms and becoming close to featureless with the invariom+Gram Charlier model. Therefore the invariom models were chosen for the deposition of the CIF file. However, only the MEM is able to avoid non-physical negative electron density still arising in this best multipole-based model with freely refined GC parameters.

It could be assumed that the crystal-field effect, which is in principle included in a refinement that freely adjusts the multipole parameters, would also need to be included in the model, although the effect has been shown to be small and in the order of experimental uncertainty. The obvious way to include the crystal-field effect would be by multipole refinement, which we have attempted using the 100 K data with the same parameter set that also constitute fixed aspherical scattering factors in invariom refinement. Unfortunately multipole parameters refine to non-physical values as can be immediately seen from the deformation electron density map. One can obviously not distinguish between anharmonic motion and electron density [43, 44] also in this molecule. Finally this refinement also led to pronounced negative electron density (despite of improvements of the fit) and hence does not solve the technical problem of negative electron density after GC refinement. We therefore did not further consider the results of the free multipole+Gram-Charlier refinement. Table 3 summarizes selected figures of merit for all four remaining models for the 100 K data. Averages of the difference of the mean square displacement amplitudes (DMSDA) along bond vectors [51] are also given. Maximal values for the DMSDA only occur for the atoms affected by anharmonic motion and exemplarily amount to $19 \cdot 10^{-4}$ (6 K), $17 \cdot 10^{-4}$ (100 K) and $82 \cdot 10^{-4} \text{ \AA}^2$ (295 K) for the best GC model.

Figure 2 shows iso-surfaces of the anharmonic displacive motion of the two glycine carboxyl oxygen atoms and the cysteine sulfur atom generated with MOLE-COOLQT [66]; green iso-surfaces emphasize the deviation of anharmonic motion from the harmonic anisotropic displacements.

Figure 3 shows the improvement of the fit by the maximum entropy method stopped at different values of χ^2 . The MEM density obtained at smaller χ^2 fits the data better, but the number of spurious maxima in these densities rises, which is an indicator of overfitting. To avoid this we therefore rely on the $\chi^2=5.3$ curve for further analysis, which is almost everywhere below the curve of the invariom refinement including Gram-Charlier displacement parameters, thus indicating a slightly better fit (as is also reflected in better figures of merit in Table 3). Only the 100 K data have been evaluated with the MEM, since only at this temperature is the resolution fulfilling requirements for charge density analysis.

Concerning the interpretation of the MEM results a difference electron density plot between prior and MEM ($\chi^2=5.3$) is illuminating (Figure 4). Resulting features are qualitatively similar to the difference electron density between invariom+Gram-Charlier and the invariom models (Figure 2). The anharmonicity of the vibration becomes apparent in both cases by the shashlik-like features visible for all three atoms [13]. These features cannot be fitted with a harmonic function. The fact that flexible electron density models are challenged in distinguishing between electron density and vibrational smearing becomes most apparent for sulfur. The MEM model also permitted to solve the technical problem of negative EDD when using the multipole model. In addition the MEM permits to predict phase consistent high-resolution data, to which GC parameters can be fitted. These can then be used as fixed parameters in the invariom+GC refinement to avoid negative EDD, albeit with a penalty of 0.5% in the R-Factor compared to the free invariom+GC refinement.

Hydrogen bonding in glutathione

Hydrogen bonding (HB) can be a contributing factor to anharmonic motion. In glutathione the atoms affected are in quite different HB environments (see Table 4): O21 is an acceptor of the peptide H2 in a strong HB and the ammonium H1C in a weaker HB, whereas O31–H31 form an even stronger HB to O1E. Another energetic contribution in this respect is a short H...H contact in between H12B of the glutamyl and H32A of the glycine residue. It is likely that these hydrogen atoms in neighboring

Fig. 1: Fourier residual electron density plot ($0.15 \text{ e}/\text{\AA}^3$) generated with MOLECOOLQT, and respective Meindl-plot [57] from residual density analysis for the 100 K data. (a) After IAM refinement, (b) after invarium refinement with anisotropic ADPs for hydrogen and (c) after adding Gram-Charlier displacement parameters for the sulfur and the carboxyl oxygen atoms in the invarium model. Even in unconstrained refinement (c) negative static EDD features remain.

Tab. 3: Figures of merit for different refinement models of glutathione measured at 100 K.

	IAM	Invarium	Invarium + GC 3 rd order	MEM $\chi^2 = 5.3$
R(F)	3.26%	2.60%	1.92%	1.84%
wR(F)	3.21%	2.23%	1.61%	1.32%
S	4.81	3.34	2.39	n.a.
No. of parameters	198	198	228	n.a.
H-atom positions	Constrained to parent site			Fixed positions
H-atom displacements	U_{iso} refined	U_{ij} estimated		n.a.
Average DMSDA	$4.3 \cdot 10^4 \text{ \AA}^2$	$2.4 \cdot 10^4 \text{ \AA}^2$	$2.3 \cdot 10^4 \text{ \AA}^2$	n.a.

Fig. 2: Representations of the probability distribution function (P.D.F.) of O(31), O(32) and S(2G) for the high-resolution 100 K dataset generated with MOLECOOLQT. Left column: red and blue iso-surfaces show negative and positive regions of the 3rd order Gram-Charlier part of the displacements. Orange and cyan meshes represent results from difference Fourier synthesis of multipole-model structure factors with and without Gram-Charlier coefficients. Right column: green iso-surface at 50% of the P.D.F.; red iso-surfaces indicate regions with negative P.D.F. values at a level of 99%. As S(2G) does not show a negative P.D.F. and hardly any visible deformations of the 50% ellipsoid, the 50% iso-surface is color mapped with values of the harmonic P.D.F. to highlight differences.

molecules repel each other, and that this repulsion competes with attractive hydrogen bonding involving the carboxyl group.

Such a degree of “destructive interference” (using language for wave-like behavior) between force constants of ‘competing’ interactions as assumed for the HB situation in glutathione might be underlying anharmonic behavior

in general. Explanations involving different force constants of intra- and intermolecular interactions are plausible also in the thiomethyl group in p-(methylthio) phenyl nitronyl nitroxide [22], where the anharmonically vibrating sulfur atom is rigid due to its higher mass compared to the methyl hydrogens, and would be consistent with observing anharmonic displacements in the

Fig. 3: Meindl-plots [57] of the MEM calculations at various χ^2 values starting from the harmonic invariom prior. A Meindl-plot of invariom refinement including Gram-Charlier displacement parameters is shown for comparison.

explosive RDX [21] as well. In RDX different force constants could be due to attractive hydrogen bonding and $O \cdots O$ repulsion. A disturbance in the interplay between

Tab. 4: Hydrogen-bond geometry for the structure of glutathione measured at 100 K.

D–H...A	D–H	H...A	D...A	D–H...A
N1–H1A...O2 ⁱ	1.02	1.84	2.8051(3)	156
N1–H1B...O11	1.02	2.18	2.6654(3)	107
N1–H1B...O11 ⁱⁱ	1.02	1.97	2.8423(3)	142
N1–H1C...O11 ⁱⁱⁱ	1.02	2.55	3.2355(3)	124
N1–H1C...O21 ⁱⁱⁱ	1.02	1.72	2.6985(3)	158
N2–H2...O21 ⁱⁱⁱ	1.01	1.97	2.9078(3)	154
S2G–H2G...O11 ^{iv}	1.34	2.18	3.4702(3)	159
N3–H3...N2	1.01	2.29	2.7489(3)	106
N3–H3...O32 ^v	1.01	2.00	2.8629(8)	142
O31–H31...O1E ^{vi}	0.96	1.65	2.6011(6)	171
C1A–H11A...N2	1.10	2.62	3.3699(3)	125
C1G–H11G...O21 ⁱⁱⁱ	1.09	2.52	3.3832(4)	135
C2B–H21B...S2G ^{vii}	1.09	2.83	3.8705(3)	159
C3A–H31A...O1E ⁱⁱⁱ	1.10	2.57	3.3874(4)	130

Symmetry codes: (i) $x, y+1, z$; (ii) $x+\frac{1}{2}, -y+\frac{3}{2}, z$; (iii) $x+1, y, z$; (iv) $x+1, y1, z$; (v) $x+2, y+\frac{1}{2}, z+\frac{1}{2}$; (vi) $x+1, y-\frac{1}{2}, z+\frac{1}{2}$; (vii) $x-\frac{1}{2}, y+\frac{1}{2}, z$. Distances are given in Å and angles in degree.

Fig. 4: Difference electron density plot between MEM and prior density in selected regions emphasizing anharmonic motion in glutathione.

intra- and intermolecular vibrational modes would also constitute a situation where competing interactions can occur. A quantitative analysis of the respective force

constants is however currently only possible with the most sophisticated computations and beyond the scope of the present work.

Fig. 5: Top: Fourier residual electron density plot ($0.15 \text{ e}/\text{\AA}^3$) generated with MOLECOOLQT at 295 K (left) and 6 K (right) after invariom + Gram-Charlier refinement. Bottom: Meindl-plot from residual density analysis. (a) After IAM refinement, (b) after invariom refinement with hydrogen ADPs and (c) after adding Gram-Charlier displacement parameters for the sulfur and the carboxyl oxygen atoms in the invariom model.

Temperature dependence of anharmonicity in glutathione

While MEM analysis requires high-resolution data, invariom refinement can be used for the two other datasets not fulfilling charge-density resolution criteria. As can be expected the 295 K data show much stronger anharmonicity than at 100 K as illustrated in Figure 5 in the same sequence as in Figure 1; at 6 K the effect is hardly noticeable. Only a weak signal of anharmonic motion remains, as can be seen from the maximal and minimal residual electron density in Table 1, the residual density and the Meindl-plots in Figure 5. At 6 K these features can now also be modeled by split positions, in contrast to the situation at 100 K. A look at the ellipsoids of the disorder model is instructive: their longest elongations are perpendicular to each other (see inset in Figure 5). This seamlessly leads to a tentative explanation of anharmonic thermal motion in glutathione: at very low temperatures we have static disorder of two conformers that are packing together in the same space group with respective occupancies differing only for O21 and O32. At higher temperatures there is a dynamic exchange between these conformers where affected atoms become indistinguishable and their EDD smeared within the resolution of the diffraction experiment. For distinguishing static and dynamic disorder energetic considerations provide a solid foundation [67].

Discussion

The technical problem of modeling anharmonicity, and to simultaneously avoid negative EDD in refinement, appears at all three temperatures. It is anticipated that other, more sophisticated atomistic electron-density models like the quantum chemical basis-set description in Hirshfeld-Atom refinement [68, 69] will lead to the same problem unless the proposed free-lunch like procedure is used. MEM refinement does lead to the best model, but the interpretation in terms of atomic electron density plus vibration is more difficult, since associated atomic electron density in the MEM is not de-convoluted from atomic motion.

Conclusion and outlook

Experimental single crystal X-ray diffraction measurements were carried out on the structure of glutathione at three temperatures at 6, 100 and 295 K. Invariom refinement facilitates the detection of anharmonicity, since

problems with de-convolution between electron density and Gram-Charlier displacement parameters are considerably reduced – in contrast to classical multipole refinements where correlation between multipole and GC parameters is likely to be a problem. However, refinement of Gram-Charlier parameters with fixed invariom electron density still leads to areas of negative electron density in glutathione. To provide an alternative we have carried out an electron density determination with the MEM. While this refinement led to the best model, its interpretation in terms of atomic electron density on one hand and atomic vibration on the other remains difficult. To overcome technical problems when using the Gram-Charlier model in accurate structure determinations the MEM model was used for the high-resolution data measured at 100 K to predict data (here to a resolution of $1.73 \text{ \AA}^{-1}$). This in turn permits refinement of physically more meaningful GC parameters that can be fixed in a final refinement using experimentally observed data only. This, or analogous free-lunch like procedures, is recommended for future implementation or use in similar situations.

Concerning our physical understanding of the effect more insight can be provided by computation. When we assume that anharmonicity at a particular temperature is due to partially frozen out conformers of similar energies that are conformationally similar enough to crystallize in the same space group, their temperature-dependent interchange could be predicted. Respective conformer energies would hence be useful. Since every vibration is connected to a particular energy, it would be particularly interesting to take into account internal molecular and external lattice vibrations and their different temperature dependence. This generally holds for other molecules more amenable to computation that are also affected by anharmonic motion. We therefore encourage other authors to report anharmonic motion when it is being noticed in a crystal structure.

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