Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Soybean lipoxygenase-3 in complex with 4-nitrocatechol

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Received 14 November 2003
Accepted 12 January 2004

PDB Reference: lipoxygenase-3:4NC complex, 1no3, r1no3sf.

4-Nitrocatechol (4NC) is a known inhibitor of lipoxygenase. This work presents the X-ray structure of soybean lipoxygenase-3 in complex with 4NC refined at 2.15 Å resolution. The X-ray analysis shows 4NC near iron with partial occupancy, blocking access to Fe but not covalently bound to it. The two hydroxyl groups interact with the C-terminus (4-OH) and His523 ND1 (3-OH). The residues surrounding the iron cofactor, His518*, His523, His709, Ile857* COO^- and water, form a trigonal bipyramid with the residues marked with asterisks in the axial positions. The water bound to iron and the presence of the inhibitor seem to be responsible for the rearrangements and changes in the geometry of the ligand distribution and confirm the displacement of His518 from iron coordination. A description of the catechol binding contributes to the understanding of lipoxygenase inhibition and the participation of its co-oxidative activity in the utilization of natural flavonoids.

1. Introduction

Lipoxygenases (LOXs) are known for their ability to metabolize polyunsaturated fatty acids (PUFAs). They also have a great ability to oxidize other chemicals. This aerobic reaction is called 'co-oxidation' (peroxidase-like, pseudo-peroxidase or hydroperoxidase activity) and the chemicals undergoing this process are called 'co-substrates'. It has been found that many xenobiotics and endobiotics may undergo a biotransformation and be utilized in the body *via* processes mediated by LOXs (Kulkarni, 2001). Recent studies by X-ray analysis have provided new examples of LOX-mediated transformations of natural polyphenolic compounds such as curcumin (Skrzypczak-Jankun *et al.*, 2003) and quercetin (Borbulevych *et al.*, 2004). These compounds are natural medical remedies and are commonly present in the typical Eastern diet as ingredients of spices or tea. X-ray analysis has shown that in the presence of soy LOX-3 both compounds can be degraded to a catechol derivative with only one aromatic moiety; in these particular cases the degradation products are 4-hydroperoxycatechol and protocatechuic acid, respectively. Like every experimental method, X-ray analysis has its shortcomings. The limited resolution of the data does not allow us to describe a small molecule buried within a large protein as accurately as the same small molecule alone at atomic resolution. Also, the occupancy of the binding site can only be guessed at when there is no proof provided by other more reliable methods. These limitations prompted us to revisit the structure of 4-nitrocatechol complexed with soybean lipoxygenase, for which the accurate stoichiometric ratio was measured experimentally and the binding was proven by X-ray analysis (at 2.6 Å resolution), but the structure of which was not refined (Pham *et al.*, 1998). This work presents a description of the structure refined at 2.15 Å resolution.

2. Experimental

Isothermal titration calorimetry, crystallization, data collection and preliminary X-ray analysis have been described in Pham *et al.* (1998). X-ray data to only 2.6 Å resolution were used for molecular replacement and rigid-body refinement, with calculations performed in *X-PLOR* v.3.85 and deposited as PDB entry 1byt. Our present work provides the results of structure refinement performed for all data to 2.15 Å using the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). The occupancy of 4-nitrocatechol was refined using the program *SHELXTL-NT* v.2001 (Bruker AXS Inc, Madison, WI, USA), which gave a result of 0.53; this result was applied in subsequent cycles of refinement without further adjustments. The refinement with program *REFMAC*5 (from *CCP*4) intertwined with map calculations and model adjustments to the electron density allowed substantial improvement in the protein geometry compared with the native molecule determined at 2.6 Å resolution (PDB code 1lnh; Skrzypczak-Jankun *et al.*, 1997). No restraints have been applied to

Table 1
Data-collection and refinement statistics for the LOX-3:4-nitrocatechol structure.

Values in parentheses are for the last resolution shell.

Space group	*C*2
Unit-cell parameters (Å, °)	*a* = 112.9, *b* = 137.5, *c* = 61.9, *β* = 95.5
V (Å^3)	956512.6
Resolution limits (Å)	10.0–2.15 (2.23–2.15)
Unique reflections	49989
Completeness	99.1 (99.30
R_{merge}	0.079 (0.346)
Refinement (*REFMAC*5 from *CCP*4)	
No. protein atoms	6810
No. inhibitor atoms	11
No. water atoms	494
R_{work}	0.186
R_{free}	0.233
Mean *B* values (Å^2)	
Overall	26.8
Protein	25.4
Inhibitor	37.7
R.m.s. deviations from ideal geometry	
Bond lengths (Å)	0.016
Bond angles (°)	1.979
Torsion angles (°)	7.451
R.m.s.d. (Å) from native LOX-3 (PDB code 1lnh)	
Main chain only	0.594
All atoms	0.912

the geometry of the iron site. A graphical evaluation was performed using *XTAL-VIEW* (McRee, 1999). *CHAIN* v.7.2 (Sack, 1988) was used to prepare Fig. 1. The statistical results for the model, consisting of 850 amino acids (7–857), an Fe cofactor, a 4-nitrocatechol molecule and 494 water molecules, are summarized in Table 1.

3. Results and discussion

Evidence for coordinated water and catechol binding has been discussed previously, but concerned only ferric LOX-1 (Nelson *et al.*, 1995). The withdrawal of one histidine from the coordination sphere of iron and catechol binding in a bidentate mode involving both OH groups was suggested. An isothermal calorimetric titration (Pham, 1998) performed at pH 8 showed the LOX:4NC stoichiometric ratio for LOX-3 to be 1:1.3 for Fe^{3+}, 1:0.4 for Fe^{2+} and 1:0.5 and 1:0.1 for ferric and ferrous LOX-1, respectively. The microcalorimetric titration provided evidence that different isozymes react differently and that the stoichiometry of the complex formed depends upon the oxidation state of the iron cofactor. In our crystallographic work, X-ray analysis was performed for crystals of native soybean lipoxygenase-3 (with Fe^{2+}) grown at pH 5.3 and soaked with 4-nitrocatechol. It is rather unlikely that catechol would be deprotonated under such circumstances. Therefore, finding 4NC at a non-binding distance is perfectly logical. Fig. 1 shows the residues surrounding the iron cofactor in the soybean LOX-3:4NC complex. His518* (conformer *A*), His523, His709, Ile857* COO^- and water (1197 in PDB entry 1no3) form a somewhat distorted trigonal bipyramid (the average distance is 2.2 Å and the average angles are 91 and 120°, respectively, with the axial residues making an angle of 155°), with the residues marked with an asterisk in the axial positions. Ligand withdrawal has been also suggested as a response to catechol binding to protocatechuate 3,4-dioxygenase (Vetting *et al.*, 2000; PDB code 1eoc). In that case (X-ray analysis reported at pH 8.5), the cofactor is Fe^{3+} and the 4NC withdraws one amino-acid ligand (Tyr) from the axial position in the trigonal bipyramid and binds in a bidentate fashion, taking one axial and one equatorial position in an octahedron. Although we did not observe either mono- or bidentate attachment of catechol as the iron ligand for LOX-3:4NC (under the described experimental conditions), our results did confirm the displacement of one residue, His518 (the distance to iron is 2.4 and 2.9 Å for the *A* and *B* conformers, respectively). Small shifts of residues His518, Asn713 and Gln514 (in relation to the native enzyme; PDB code 1lnh) introduced changes into the hydrogen bonding that provided support for the water (or OH) ligand and for the nitro group in the 4NC molecule. The 3-OH group in the catechol is pointing toward His523 and not Ile773 as assigned previously (based on the 2.6 Å

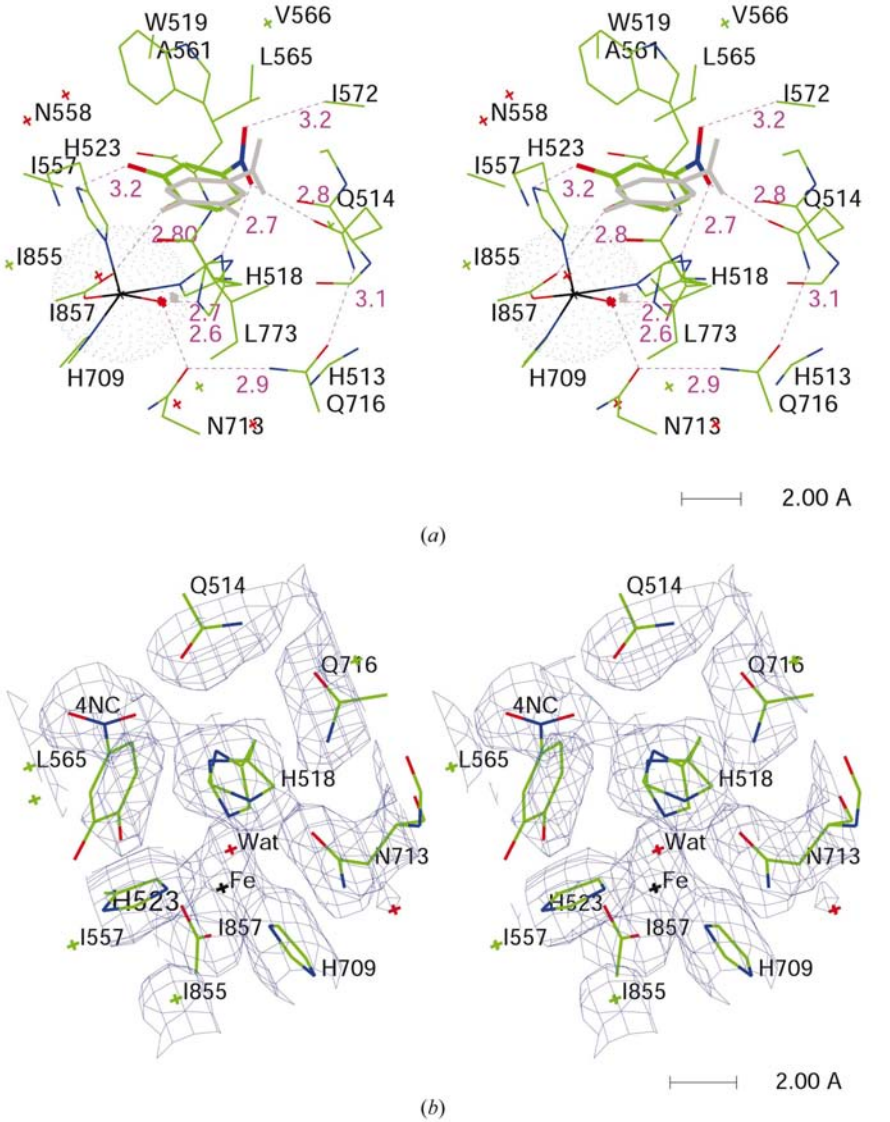

Figure 1
A stereo drawing of the 4-nitrocatechol-binding site in LOX-3 (PDB code 1no3). (*a*) Adjacent residues within 8 Å radius, where the dotted ball represents the iron coordination sphere of 2 Å radius, the gray molecules depict 4NC and water as found at 2.6 Å resolution (PDB code 1byt); the purple dashed lines mark the selected short contacts in this area. (*b*) $2F_o - F_c$ electron-density map contoured at 0.8σ clearly showing the orientation of 4NC in the central cavity but not the 3-OH positioning.

resolution unrefined model). Owing to the low occupancy of the catechol site, the maps provide no indication that would prefer either orientation even at 2.15 Å resolution and so both could be possible (Fig. 1*b*). However, the refined model can be better stabilized by 3-OH interactions with the electron pair on His523 ND1. As for the description of the iron coordination, it should be noted that even when the iron site is reported with the given geometry, it does not automatically mean that it possesses the number of ligands defined by this geometrical figure. Often, the spatial distribution of the iron neighbors corresponds to the given geometrical description but one or two of the neighbors may be at non-binding distances, meaning that they are not ligands *per se*. Our observations and those made by others (Vetting *et al.*, 2000) provide evidence of the easy rearrangement and the natural flexibility in the structure of the non-heme iron site and can explain difficulties in the interpretation of spectroscopic data for this type of metalloenzyme (Nelson *et al.*, 1995; Schenk *et al.*, 2003).

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