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Flash-Freezing Causes a Stress-Induced Modulation in a Crystal Structure of Soybean Lipoxygenase L3

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Abstract

A dynamic conformational flexibility of a protein might be a source of non-covalent structural heterogeneity, causing diminished diffracting ability of crystals and disorder in a crystal structure of soybean lipoxygenase L3. Room-temperature data, space group *C*2, correspond to a structure with large channels lined mostly or in part by disordered fragments of the molecule or flexible loops with an increased thermal vibration. A rapid change in temperature of ~200 K creates a wave of a stress-induced modulation that propagates in the crystal changing its reciprocal space into a three-dimensional quilt-like mixture of *C* and *P* intertwined lattices. Low-temperature data indicate a transformation from the dynamic to static disorder, leading to a primitive unit cell with 10% reduced volume. The molecules, formerly related by a twofold axis are rotated by ~7° and are shifted along the diagonal to be ~4 Å closer together. During a routine data collection for the flash-frozen crystals of similar properties such phenomena could easily go unnoticed leading to biased results because of such effects and possibly improper indexing of the data.

1. Introduction

Lipoxygenase catalysis is a key element in polyunsaturated fatty-acid metabolism. In mammalian cells, the enzyme is responsible for the biosynthesis of several important classes of eicosanoids, the leukotriens, lipoxins and HETE's (Samuelson, Dahlen, Lindgren, Rouzer & Serhan, 1987). In plants the substrates for the lipoxygenase catalyzed hydroperoxidation reaction are linoleic and linolenic acids. The conversions represent early steps in the biosynthesis of compounds with growth regulatory and pest-resistance properties (Siedow, 1991). Lipoxygenase has been extensively studied because of its relevance in the fields of medicine and agriculture.

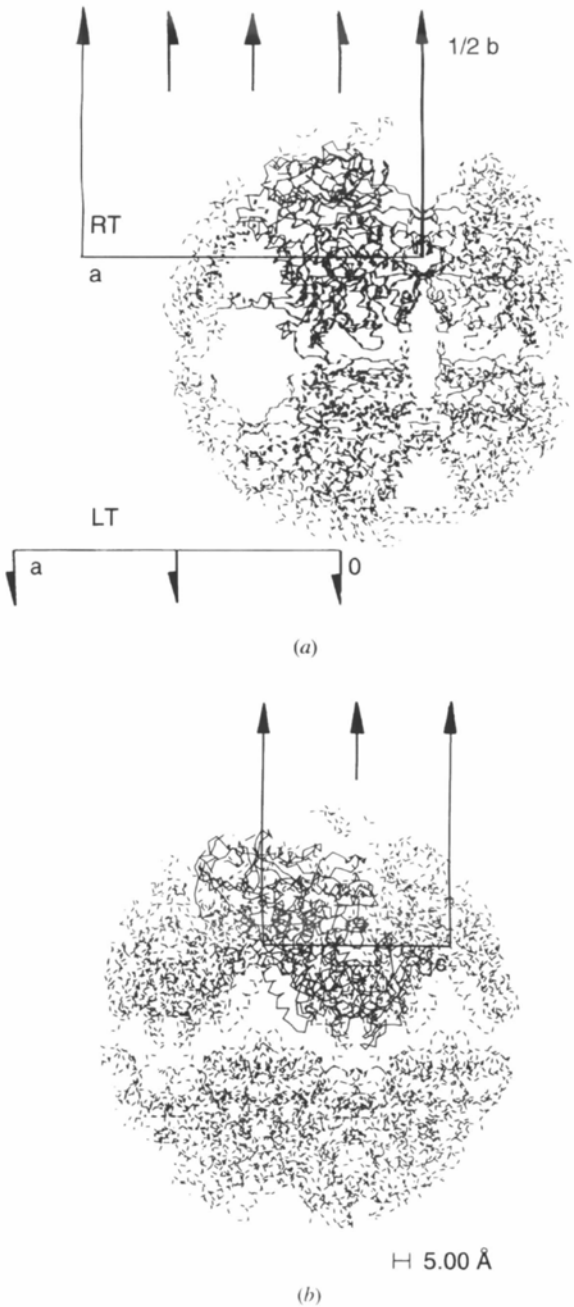
The lipoxygenases from soybeans have been the subject of numerous investigations including structure determinations (for a list of useful references see a review article by Nelson & Seitz, 1994). Soybean lipoxygenases exist as a family of isoenzymes referred to as L1–L3. These proteins, which share approximately 70% sequence identity, differ from each other on the basis of the regiochemical outcome of catalysis and pH optimum. Structural studies on the L3 isoenzyme (*cv. Provar*, MW 97 kDa, a monomer of 857 amino acids and a non-heme Fe^{II}) have been the subject of our investigation. Data used for structure solution and refinement were collected at room temperature (Skrzypczak-Jankun, Funk, Boyington & Amzel, 1996) and the refined model ($R = 17\%$) contains several features that require further elucidation (Skrzypczak-Jankun, Amzel, Kroa & Funk, 1996). Mainly, the insertion loop of the L3 isoenzyme (residues 33–45) is not defined in the electron density and some fragments of the molecule show a considerable thermal vibration. The insertion loops in L3 N-terminal domains (about 20 residues long) are in the immediate vicinity of the twofold axis and at the bottom of a large, conical cavity, approximately 65 Å long and 35–45 Å across at the base, where it connects to the channel running in the *x* direction (Fig. 1). Since the disordered regions of the molecule are exposed to the solvent, it was anticipated that diffusing glycerol into the lattice and freezing might alter the topology of this area by providing a linking agent to trap those fragments of the molecule into a hydrogen-bonding network. As a result, the channels along the twofold axis and between the *xz* layers of the molecules might be effected the most. Low-temperature experiments have been undertaken to see if some or all of the drawbacks in the L3 structure could be avoided and if the improved data extending to higher resolution would allow us to address them. There were some previous reports about cryogenic experiments with the L1 isoenzyme, indicating but not explaining a lack of consistency between the data obtained from different frozen crystals (Tesmer, Muchmore, Steczko, Axelrod & Smith, 1991). This report presents preliminary results of the low-temperature experiments for soybean lipoxygenase L3 that might provide an explanation for the former observations and be of interest to others using cryogenic conditions to study the structure of large macromolecules.

2. Experimental

Slow cooling was tested on crystals mounted in the capillaries, at temperatures ranging from 277 to 285 K on a dual area detector (San Diego Multiwire System)

with a Cu fine-focus rotating anode operating at 50 kV, 100 mA. The crystal-to-detector distance was approximately 800 or 600 mm depending on the orientation of the crystals.

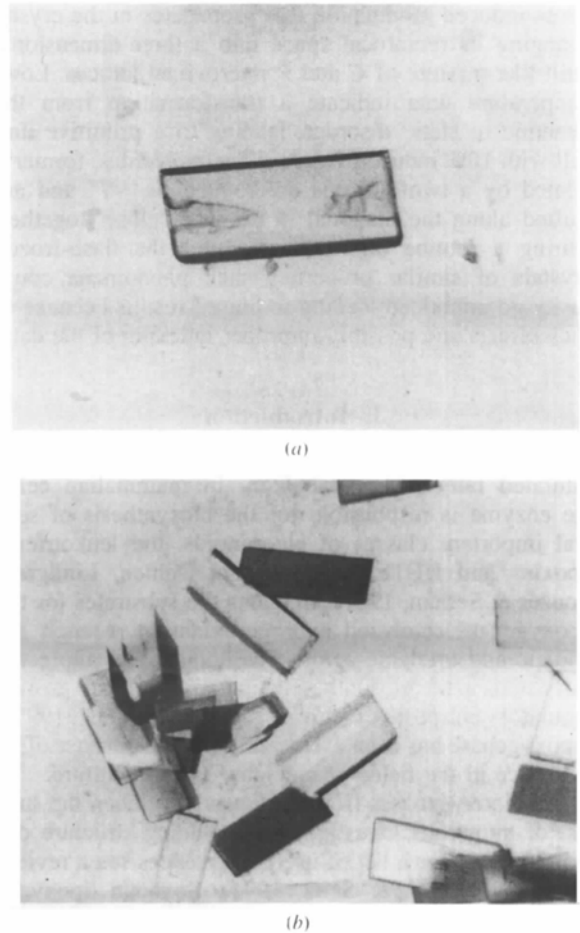
Flash freezing was tested on crystals mounted in a loop made from a polystyrene fiber 10–15 μm thick. The smallest crystal dimension was 60 μm. Freezing conditions were studied on crystals transferred from the original crystallization conditions to the soaking solution. The concentration of polyethylene glycol, presence of cryoprotectant and soaking time were tested. Since the crystals grew from polyethylene glycol (PEG) solution the effect of higher concentrations of PEG 8000 on shock freezing of the crystals was investigated first. Solutions of PEG 20–40%(*w*/*v*) in increments of 5%(*w*/*v*) were tested. The best results were obtained at 20–25%(*w*/*v*) but the crystals slowly turned opaque and no improvement was observed upon prolonged soaking to allow for better diffusion of PEG into the crystals. This observation called for the application of some cryoprotectant to maintain a glassy structure of solvent and transparency of crystals. Among cryoprotectants described in the literature (Rogers, 1994) glycerol is the most widely used and it has been successfully applied to lipoxygenase L1, a highly homologous enzyme of the same tertiary structure (Minor *et al.*, 1994). So the next step was to study a series of solutions containing



(*a*)

(*b*)

Fig. 1. Crystal packing of soybean lipoxygenase L3 (a sphere of 70 Å radius), room-temperature data (RT), a dashed line represents the symmetry-related molecules: (*a*) *xy* projection, (*b*) *yz* projection. The symmetry elements and the unit-cell edges are marked for comparison.



(*a*)

(*b*)

Fig. 2. Typical crystals of soybean lipoxygenase L3: (*a*) with the screw dislocations and (*b*) without defects.

Table 1. *Comparison of the unit-cell dimensions (Å, °) for soybean lipoxygenase L3*

	Room temperature	93 K	
Crystallographic	Monoclinic		
Space group	$C2$	$P2_1$	
a	112.8	106.4	
b	137.4	133.5	
c	61.9	60.7	
β	95.5	97.3	
V (Å^3)	954954	855218	10% reduction
No. of molecules in the asymmetric unit	1	2	

PEG from 18–26%(*w*/*v*) in increments of 2%(*w*/*v*), and including glycerol from 0 to 20%(*v*/*v*) in increments of 5%(*v*/*v*). A soaking time of up to 15 h was used to allow for better equilibration and penetration of glycerol into the crystals. The pH and other ingredients were kept the same as in the mother liquor for the crystallization conditions. Glycerol, enzyme grade, was purchased from Fisher Biotech. The crystals (Fig. 2) were transferred to dialysis buttons (50 or 100 µl), washed with 20% PEG without glycerol to remove any debris, closed with the dialysis membrane with cutoff M_r = 12 000 or 50 000 and left to soak for a designated period of time. The best results were obtained for crystals soaked for 12–15 h in 20% PEG 8000 with 15% glycerol. Such crystals remained transparent and intact after placing in a nitrogen stream at 93 K and remained this way during the time of data collection.

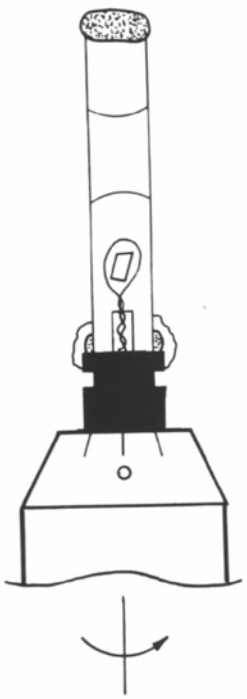
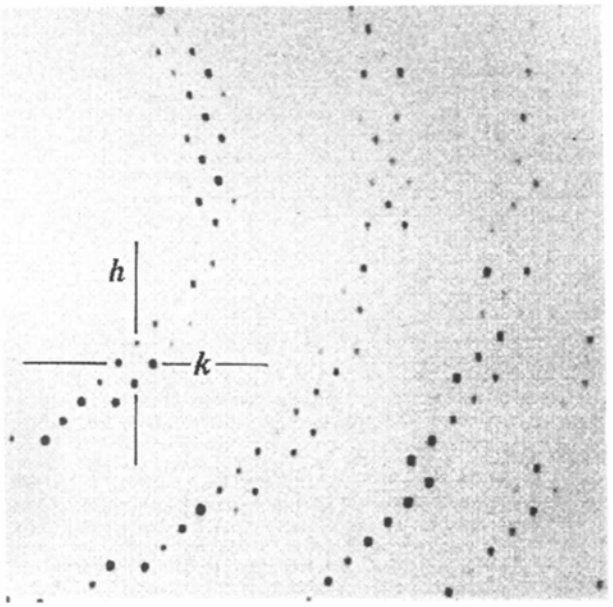
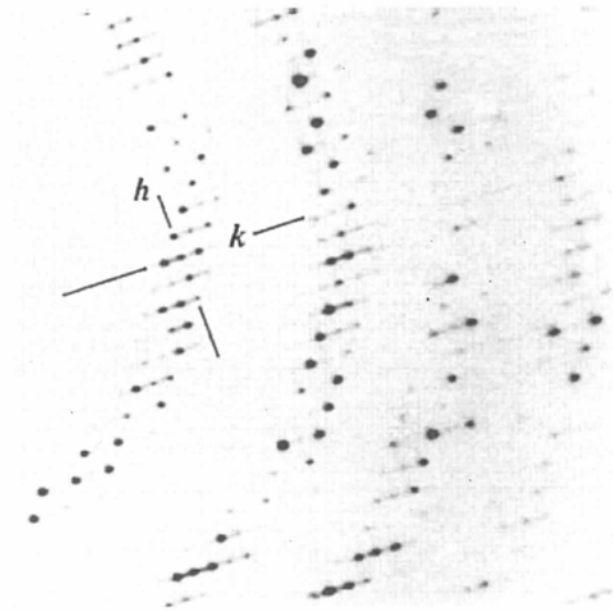


Fig. 3. Crystal mounting for the experiment to compare the data of the same crystal at room temperature and upon freezing.

The following categories of crystals were studied to distinguish between the influence of glycerol *versus* freezing upon the crystal structure of lipoxygenase L3: (i) 'native' crystals, (ii) crystals soaked in glycerol solution and mounted in the capillary at room temperature, (iii) crystals soaked then mounted in the loop and frozen, and (iv) room- and low-temperature study of the same crystal. In the last case a crystal soaked in glycerol solution was mounted in the loop and immediately protected



(*a*)



(*b*)

Fig. 4. Fragments of the oscillation pictures from the detector: (*a*) at room temperature, no glycerol; (*b*) at 93 K, 15% glycerol.

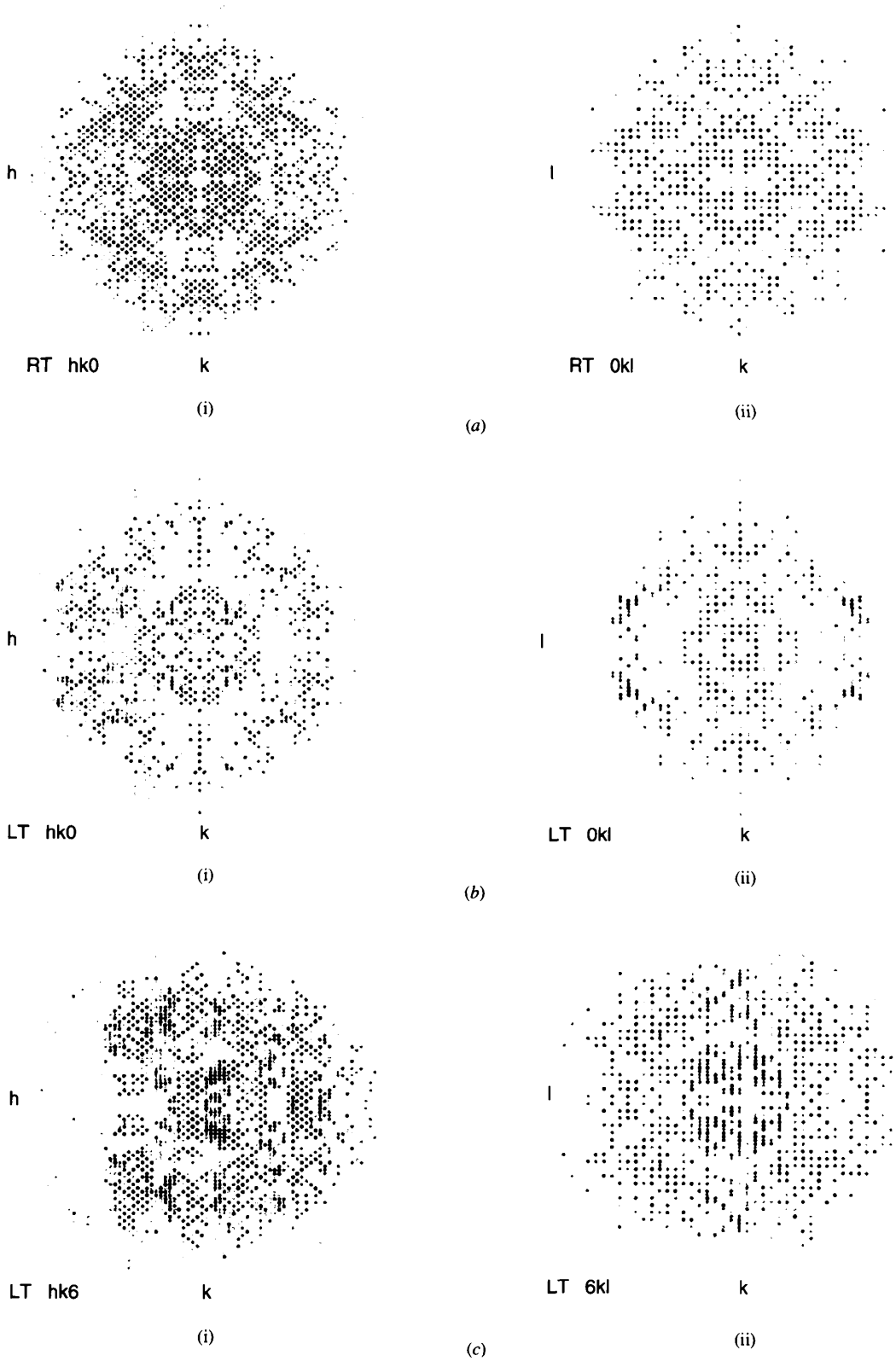


Fig. 5. Simulated roentgenograms: (*a*) the room-temperature data, *C* lattice, *hk*0 and 0*kl*, and the low-temperature data showing the intertwined *P* and *C* lattices; (*b*) *hk*0, 0*kl*; and (*c*) *hk*6, 6*kl*.

Table 2. *Summary of data distribution for the room- (RT) and low-temperature (LT) data*

Crystals were from the same crystallization batch and the data were processed in the same manner.

RT data
Number of reflections

Resolution (Å)	$\langle I \rangle$	$\langle \sigma(I) \rangle$	Observed	Theoretical	(%)	Cumulative values Observed	Theoretical	(%)
15.00	721.44	52.30	152	176	86.4	152	176	86.4
10.00	871.92	50.82	366	387	94.6	518	563	92.0
7.50	554.29	32.11	704	742	94.9	1222	1305	93.6
5.00	284.58	19.42	2841	2992	95.0	4063	4297	94.6
3.50	277.96	22.70	7564	8003	94.5	11627	12300	94.5
3.00	90.21	15.71	6473	7115	91.0	18100	19415	93.2*
2.75	42.83	13.80	4880	5711	85.4	22980	25126	91.5
2.50	27.52	13.18	6552	8237	79.5	29532	33363	88.5

LT data (93 K)
Number of reflections

Resolution (Å)	$\langle I \rangle$	$\langle \sigma(I) \rangle$	Observed	Theoretical	(%)	Cumulative values Observed	Theoretical	(%)
15.00	312.65	20.27	241	333	72.4	241	333	72.4
10.00	384.06	23.37	589	726	81.1	830	1059	78.4
7.50	300.98	18.09	1118	1354	82.6	1948	2413	80.7
5.00	175.46	11.51	4554	5443	83.7	6502	7856	82.8
3.50	236.16	15.75	11068	14476	76.5	17570	22332	78.7
3.00	100.30	12.14	9146	12818	71.4	26716	35150	76.0
2.75	47.93	9.85	7120	10304	69.1	33836	45454	74.4
2.50	30.34	9.36	10240	14818	69.1	44076	60272	73.1
2.25	20.39	9.45	13877	22008	63.1	57953	82280	70.4
2.10	14.92	9.59	7080	18614	38.0	65033	100894	64.5

*Steczko *et al.* (1995) reported 3.0 Å data for soybean lipoxygenase L3 from *cv. Hawkeye*: the number of observed reflections 11 384 corresponds to approximately 60% completeness.

by the outer capillary (Fig. 3). After collecting some data at room temperature, the capillary was carefully removed before flash freezing and the same data were recollected for the frozen sample.

All flash-freezing experiments and data collection at sub-zero and room temperatures (for comparison) were performed using an R-AXIS II image-plate detector, with a Cu rotating anode, operating at 55 kV, 180 mA, and equipped with a cryostat set to operate at 95–93 K. The measured intensities were processed using standard R-AXIS II software and were taken into consideration only if they showed excellent agreement between predicted and observed positions of the reflections. The unit-cell dimensions are given in Table 1. The data for one crystal were collected to 2.1 Å resolution, with a crystal-to-detector distance of 120 mm, an oscillation angle of 1.5° and an exposure time of 30 min per frame. A summary of the data distribution is given in Table 2, the reflection was accepted if $[I/\sigma(I)] > 0.5$, $R_{\rm merge}$ was 7%. Figs. 4(*a*) and 4(*b*) show fragments of the oscillation photographs (frames) from the detector with the crystal *c* axis positioned approximately along the beam. Fig. 5 shows the simulated diffraction patterns (Dixon, 1994) for the different layers of the reciprocal space, for the data collected at room (RT) and low (LT) temperatures, where the gray scale in the PostScript file corresponds to the intensity of the observed reflections. The LT structure of L3 was solved by molecular replacement using the coordinates of the L3 molecule from a room-temperature study (Skrzypczak-Jankun, Amzel *et al.*, 1996), the program *AMoRe* (Navaza, 1994) and 25 748 reflections from 10 to 3 Å resolution. A high correlation coefficient (0.79) from the rotation–translation calculations and a low discrepancy factor ($R = 0.33$) for an unrefined model assure the correctness of the positioning of the molecules. There are two molecules in the asymmetric unit and it is our ultimate goal to correctly position and refine all 1714 amino acids. A detailed account of this work will be published upon completion of a crystallographic refinement. The packing of the molecules has been checked using the program *O* (Jones, Zou, Cowan & Kjeldgaard, 1991), the drawings (Fig. 1) were prepared in *TOM* (Cambillau & Horjales, 1987).

3. Results and discussion

Extensive studies have been performed on crystallization and data-collection conditions of soybean lipoxygenase L1 that served as a model for a structural study of L3 isoenzyme. The lipoxygenase L1 isoenzyme was crystallized from two very different environments. Boyington, Gaffney & Amzel (1990) reported that among thousands of conditions studied only crystals obtained with a high salt content (4.6 *M* sodium formate, 1.0 *M* ammonium acetate and 0.6 *M* lithium chloride mixed together in 0.1 *M* MES pH 7.0) showed good stability

and diffraction to at least 2.6 Å resolution (Boyington, Gaffney & Amzel, 1993). Steczko, Muchmore, Smith & Axelrod (1990) reported crystals of the same enzyme (L1) obtained from solutions lower in salt content and with polyethylene glycol present (0.2 *M* sodium acetate, 8–10% PEG 3400, pH 5.2–5.6), that were characterized as mechanically robust and not unusually sensitive to X-radiation. In a subsequent communication (Tesmer *et al.*, 1991), those crystals were described as 'short-lived at room temperature'. It was further reported that 'the unit cell of each frozen crystal was distinct not only from those at room temperature, but also from those of other frozen crystals'. Minor *et al.* (1994) have reported obtaining data for L1 that extend to 1.4 Å resolution by using synchrotron radiation and a crystal frozen to 100 K. The unit-cell parameters reported agreed with those cited before (Steczko *et al.*, 1990; Tesmer *et al.*, 1991) both at room and at low temperature without any noticeable shrinkage on cooling to 100 K.

Crystals of the L3 isozyme (0.05 *M* phosphate–citrate buffer pH 4.6, 20% PEG 8000, 0.2% NaN_3) are very delicate mechanically, their diffracting ability diminishes with time, they have to be mounted relatively 'wet', and they decay rapidly in the X-ray beam losing both intensity and resolution. For these practical reasons the processed data do not extend beyond 2.5 Å. Recently, Steczko, Minor, Stojanoff & Axelrod (1995) reported preliminary crystallographic data for the same isoenzyme but from a different cultivar (*cv. Hawkeye*). The crystals (crystallization conditions as for Steczko *et al.*, 1990), grown at a higher salt concentration and different ionic composition, PEG of a lower molecular weight and similar pH, belong to the same space group and have the same unit-cell dimensions (within 1 Å) as L3 from *cv. Provar* reported here. However, they diffracted to 3.0 Å (synchrotron radiation and room temperature) and the oscillation picture indicated that the crystal might have been fairly mosaic. Those independent observations confirmed our suspicions that the diffracting ability of those crystals depends not so much on the crystallization conditions but on the molecule itself and the way it packs into a crystal lattice. There are no indications, from the biochemical point of view, for the L3 isoenzyme to be lacking purity. The protein samples used for the crystallization do not show any signs of a chemical heterogeneity. Although, to form well ordered crystals the protein should be not only chemically but also structurally homogenous. Molecules with flexible surface-exposed loops, disordered termini, clefts and crevices within the protein that might undergo interdomain motions, *etc.*, are known as a source of a non-covalent structural heterogeneity in crystals (Sousa, 1995, and references therein). Molecules of soybean lipoxygenase possess all the features listed above and might easily pack in crystals in a structurally non-homogenous manner. Cooling the crystals during data collection and/or introduction of some stabilizing agent were therefore explored in an attempt to improve the diffracting abilities of the L3 crystals.

Slowly cooled crystals (temperature 277–285 K) showed greater mosaicity and increased errors during indexing and unit-cell refinement, in comparison with the data collected at room temperature. The unit-cell dimensions remained fairly reproducible and the values obtained from different crystals and at different temperatures oscillated within ±1 Å. There was no noticeable improvement in the resolution or decay.

Crystals for the flash-freezing experiments were transferred to a soaking solution containing glycerol that served as the stabilizing agent and cryoprotectant. Some crystals developed cracks with time, obviously as a result of changes caused by glycerol diffusing into the crystal lattice. The crystals that showed the presence of screw dislocations (Fig. 2*a*) were especially vulnerable, usually cracked in the middle where the defect originated, and developed many cracks running perpendicular to the axis of the fastest growth of the crystal (*c* axis). Only crystals with no visual defects were used for flash freezing. The crystals examined at 93 K showed an easily recognized, new diffraction pattern (see Figs. 4*a* and 4*b*). The data indexing and unit-cell refinement confirmed that they underwent a transition that caused a loss of face-centering leading to *P* rather than *C* lattice. Comparison of the room-temperature data for the crystals with and without glycerol, provided the evidence that soaking in glycerol is not sufficient to induce the *C* to *P* transition of the unit cell. However, penetration of glycerol into the crystal channels and freezing might under favorable conditions produce a stable crystal with changes in the tertiary and quaternary structures subtle enough not to break the crystal, but having an effect on the symmetry of the unit cell. The simulated roentgenograms for the low-temperature data show that the changes occurring in the crystal lattice are expressed in the reciprocal space as a wave propagating throughout the crystal. The blocks of *C* and *P* lattices are intertwined, they are 5–12 *d* spacings thick and elongated in the *y* direction (Fig. 5). Close examination of the LT data shows that the '*C* blocks' comply to either $h + k = 2n$ or $h + k = 2n + 1$. However, in both cases there are some very weak, general reflections proving the *C* centering to be pseudo only. These reflections are to faint to show in Fig. 5 and they would be rejected in a routine indexing procedure, which we tend to base on the strong measurements. In the transition from $C2$ to $P2_1$ the molecules or their domains have to move causing the loss of the face centering and the twofold axis relating the molecules a shift of origin of $\frac{1}{4}$ along the *a* axis (Fig. 1). The evaluation of the molecular packing in the $P2_1$ cell shows, that the molecules in the former crystallographic dimer are rotated about 7° and they moved about 4 Å closer along the Fe—Fe vector that approximates the *xz* diagonal. The L3 molecules occupy a pseudo special position in the *C* cell of $\frac{1}{4},0,\frac{1}{4}$. After a thermal shock-

induced transition, the bulk of the molecules still comply to a non-crystallographic twofold symmetry, while the details – the 'soft loops' on the molecule surface and the channels – do not. This explains the smudges on the oscillation pictures in the *b* direction (Fig. 4*b*), a quilt-like pattern throughout the reciprocal space (Figs. 5*b* and 5*c*) and a relatively low completeness of the LT data (Table 2). The cryogenic experiments with the L3 isoenzyme indicate that the dynamic disorder observed in the L3 structure at room temperature, changes to a static disorder when the crystals are flash frozen. Slow cooling to 10–20 K below room temperature was not sufficient to trap the more mobile parts of the structure into a regular pattern and the changes in the data quality (increased mosaicity, no improvement in intensity or resolution) were opposite to what was anticipated.

The flash-freezing technique causes a very rapid change in temperature of a large magnitude (200 K in this case). Crystals mounted on a detector are usually in a random orientation and the stress-induced transition changes might be hard to notice. The preliminary evaluation of the crystals is normally carried out on the basis of several still or oscillation pictures. Sometimes one is sufficient to find a unit cell and index the data. However, in the case of a modulated structure, such limited sampling of the reciprocal space can lead to various and sometimes biased results depending on the patch of data recorded and the treatment of the very weak reflections in indexing. This might be the cause of the inconsistent results reported for the L1 isoenzyme (lack of reproducibility, different unit cells for the different crystals, Tesmer *et al.*, 1991) and our lack of success in indexing some limited sets of the LT data for the L3 crystals. The completeness of the data is deceptive as well, because the theoretical number of reflections is calculated according to the overall symmetry of a new space group, that does not correspond entirely to the physical nature of the crystal. These phenomena are important, since they might be encountered in cryogenic experiments with other large macromolecules, containing sculptured surfaces and spacious channels in the crystals.

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