Strain relief at the active site of phosphoserine aminotransferase induced by radiation damage

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Abstract

The X-ray susceptibility of the lysine-pyridoxal-5'-phosphate Schiff base in *Bacillus alcalophilus* phosphoserine aminotransferase has been investigated using crystallographic data collected at 100 K to 1.3 Å resolution, complemented by on-line spectroscopic studies. X-rays induce deprotonation of the internal aldimine, changes in the Schiff base conformation, displacement of the cofactor molecule, and disruption of the Schiff base linkage between pyridoxal-5'-phosphate and the Lys residue. Analysis of the "undamaged" structure reveals a significant chemical strain on the internal aldimine bond that leads to a pronounced geometrical distortion of the cofactor. However, upon crystal exposure to the X-rays, the strain and distortion are relaxed and eventually diminished when the total absorbed dose has exceeded 4.7×10^6 Gy. Our data provide new insights into the enzymatic activation of pyridoxal-5'-phosphate and suggest that special care should be taken while using macromolecular crystallography to study details in strained active sites.

Keywords: pyridoxal-5'-phosphate; Schiff base; phosphoserine aminotransferase; radiation damage

X-ray crystallography is the most widely used technique for solving three-dimensional structures of biological macromolecules. However, interaction of the ionizing radiation with the macromolecules and their surrounding solvent may result in a significant damage to the sample, even in cryo-cooled crystals (Henderson 1990; Gonzalez and Nave 1994). Nonspecific radiation damage causes the reduction of the crystal diffractive power and increases the crystal mosaicity, overall B factor, and the unit cell

volume (Ravelli and McSweeney 2000; Murray and Garman 2002; Ravelli et al. 2002). Furthermore, specific radiation damage, such as cleavage of the disulfide bonds, decarboxylation of acidic residues, loss of the tyrosine hydroxyl groups and methylthio groups of methionines may occur (Burmeister 2000; Ravelli and McSweeney 2000; Weik et al. 2000). For bacteriorhodopsin, exposure of the cryo-cooled crystals to a relatively low X-ray flux has been shown to induce the conversion of the protein into an orange species (Matsui et al. 2002). In addition, X-ray-induced reduction of the metal ions in protein crystals and the concomitant conformational changes at the active sites of metal-containing enzymes have been reported (Berglund et al. 2002; Adam et al. 2004; Wuerger et al. 2004).

Phosphoserine aminotransferase (PSAT; EC 2.6.1.52) is a vitamin B₆-dependent enzyme that catalyzes the reversible conversion of 3-phosphohydroxypyruvate to L-phosphoserine. The Schiff-base linkage between the cofactor, pyridoxal-5'-phosphate (PLP), and a protein

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Abbreviations: AAT, aspartate aminotransferase; BALC, *Bacillus alcalophilus*; CD, circular dichroism; PLP, pyridoxal-5'-phosphate; PSAT, phosphoserine aminotransferase.

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Lys residue (the internal aldimine) (Fig. 1A) is a conservative feature among all members of this protein family (Jansonius 1998; Schneider et al. 2000). In all PLP-dependent enzymes except glycogen phosphorylase, the internal aldimine is replaced by an external aldimine between PLP and the substrate amino group at the beginning of the catalytic cycle. The ionization state of the PLP-Lys Schiff base can be changed depending on the protonation of the imine nitrogen atom (Hayashi 1995). In the protonated Schiff base, the imine bond and the pyridine ring reside in the same plane (Fig. 1B), while deprotonation is accompanied by disruption of planarity (Fig. 1C) due to rotation around the C3-C4-C4'-NZ torsion angle (Mizuguchi et al. 2001). For a number of aminotransferases, including aspartate aminotransferase (Hayashi et al. 1998), aromatic amino acid aminotransferase (Islam et al. 2000), and histidinol phosphate aminotransferase, (Mizuguchi et al. 2003) presence of a chemical strain on the internal aldimine bond has been reported. This strain was proposed to distort the absolutely coplanar conformation of the protonated Schiff base (Fig. 1B) by increasing the C3-C4-C4'-NZ torsion angle and, hence, to lower the imine pK_a value by as much as 3–4 units (Hayashi et al. 2003; Mizuguchi et al. 2003).

For phosphoserine aminotransferase, strain on the internal aldimine has not been reported. However, our recent crystallographic studies on PSAT from *Bacillus alcalophilus* (BALC PSAT) have revealed a significant distortion in the planarity of the completely protonated Schiff base (Dubnovitsky et al. 2005). The C3-C4-C4'-NZ torsion angle in the BALC PSAT crystal structure (PDB accession code 1W23) is about 34°. Moreover, the distance between the imine nitrogen atom and the C4' atom of the cofactor in the BALC PSAT is 1.73 ± 0.05 Å, while the expected value for a typical double covalent bond is 1.33 Å (McMurry 1996). A long bond distance (1.58–1.65 Å) between PLP and Lys side chain can also be found in high-resolution structures of tryptophan synthase from *Salmonella typhimurium* (Weyand et al.

2002). In addition, crystal structures of cystalysin from *Treponema denticola* (Krupka et al. 2000), ArnB aminotransferase from *Salmonella typhimurium* (Noland et al. 2002), and AAT from *Thermus thermophilus* (Ura et al. 2001) at high resolution have shown similar BALC PSAT significant distortion in the planarity of the completely protonated internal aldimine with C3-C4-C4'-NZ torsion angle of 28°–63°. These results prompted us to analyze the susceptibility of the PLP-Lys Schiff base to the radiation damage during data collection.

Here, using crystallographic data up to 1.3 Å resolution and single-crystal on-line spectroscopy, we analyze the X-ray-induced structural changes at the active site of *B. alcalophilus* PSAT. Remarkably, the Schiff base undergoes radiation damage within a relatively short total exposure time, both at second- and third-generation synchrotron beamlines. Analysis of the “early” crystal structure reveals a short NZ-C4' distance and close to zero C3-C4-C4'-NZ torsion angle of the internal aldimine. This conformation harbors a significant chemical strain on the Schiff base that leads to geometrical distortion of the cofactor. In the “late” structures after X-ray exposure, the active site strain is relaxed and the NZ-C4' distance and C3-C4-C4'-NZ torsion angle are increased.

Results and Discussion

Data collection

Seven complete data sets (A–G) at 1.68–1.77 Å resolution with increasing total absorbed dose were collected from a single cryo-cooled BALC PSAT crystal at the ESRF beamline ID 14-4 with a 20× attenuated beam. In order to monitor dose-dependent changes in the BALC PSAT crystal, an on-line spectrophotometer (R.B.G. Ravelli, J.W. Murray, M. Weik, and E.F. Garman, unpubl.) was used to record UV/VIS absorption spectra on the X-ray exposed part of the crystal before and after each data set. The data collection strategy was chosen so as to provide a

Figure 1. The pyridoxal-5'-phosphate-lysine Schiff base; stereochemical structure and protonation states. (A) Definition of the atom names and *re*- and *si*-sides of the pyridine ring. (B) Protonated form of the Schiff base. Intramolecular hydrogen bond is formed between the imine nitrogen and the O3' atom of PLP. The internal aldimine is coplanar to the pyridine ring. (C) Deprotonated form of the Schiff base. The intramolecular hydrogen bond is absent. The aldimine bond is not coplanar to the pyridine ring due to rotation around the C3-C4-C4'-NZ torsion angle (shown with arrow).

Table 1. Data collection and refinement statistics for the data sets A–G collected at the ESRF beamline ID 14-4

Data set	A	B	C	D	E	F	G
Data collection							
Absorbed dose (Gy)	2.2×10^4	9.9×10^4	2.2×10^5	8.8×10^5	1.5×10^6	2.9×10^6	4.7×10^6
Resolution (Å)	15–1.68	15–1.69	15–1.69	15–1.69	15–1.73	15–1.73	15–1.77
Unit cell parameters (Å)	143.02 × 83.76 × 66.70	143.02 × 83.76 × 66.70	143.021 × 83.77 × 66.70	143.06 × 83.77 × 66.72	143.10 × 83.78 × 66.74	143.19 × 83.79 × 66.77	143.31 × 83.79 × 66.81
Unique reflections	92,486	91,813	91,818	90,981	85,266	85,006	79,249
$\langle I/\sigma(I) \rangle$ in the last shell	2.0	2.1	2.0	2.0	2.3	2.1	2.1
Completeness (%)	98.7 (99.3)	98.8 (99.8)	98.7 (99.8)	98.7 (99.8)	98.7 (99.9)	98.7 (100.0)	98.6 (99.9)
R _{sym} (%)	8.5 (52.2)	8.4 (52.7)	8.5 (53.9)	8.5 (55.1)	8.3 (48.2)	8.4 (52.9)	8.4 (53.3)
Wilson B-factor (Å ²)	16.5	16.8	16.9	17.0	17.4	18.1	19.2
Refinement							
Resolution (Å)	15–1.68	15–1.69	15–1.69	15–1.69	15–1.73	15–1.73	15–1.77
No. of reflections	86,075 + 4544	84,507 + 4461	84,619 + 4463	84,670 + 4472	78,964 + 4171	79,089 + 4178	73,936 + 3914
R _{cryst} (%), $F > 0\sigma$	18.1	18.1	18.1	18.1	17.9	18.0	17.9
R _{free} (%)	22.8	22.8	22.8	22.9	23.0	23.1	23.4
Deviations from ideal geometry							
Bond distances (Å)	0.007	0.007	0.007	0.007	0.007	0.007	0.006
Angle distances (Å)	0.024	0.024	0.024	0.024	0.023	0.024	0.023

minimal total exposure time per data set. This was achieved using a previously described method of “composite” data sets (Berglund et al. 2002; Adam et al. 2004), benefited from a large crystal and a small beam size used (see Materials and Methods). Each data set consists of 100 frames of 0.75°/frame. The crystal was translated after every 20 frames to a previously unexposed region. In between the data sets, the rotating crystal was exposed to an unattenuated beam for increasing time intervals of 5 sec, 10 sec, 1 min, 1 min, 2 min, and 3 min, respectively. The total absorbed dose and data collection statistics for each data set are presented in Table 1.

For a more detailed description of the subtle X-ray-induced structural changes obtained in BALC PSAT crystals, a complete data set (H) at 1.3 Å resolution was collected on the beamline BW7A at EMBL/DESY, Hamburg. The above described data-collection strategy was used. After a 1-h exposure of the rotating crystal, another complete data set (I) was collected from the same “long-exposed” crystal location. Data collection statistics are shown in Table 2.

X-rays induce deprotonation of the Schiff base

The Lys-PLP Schiff base can exist in two ionization states, depending on whether the imine nitrogen atom is protonated or deprotonated (Hayashi 1995). Changes in the protonation state of the internal aldimine in PLP-containing enzymes can be monitored by absorption spectroscopy (Metzler 1977). In solution, the protonated form of the Schiff base in BALC PSAT has an absorption maximum at 404–407 nm, while the deprotonated form exhibits maximum absorption at 335–340 nm (Fig. 2A). The pK_a

value of the internal aldimine in BALC PSAT is ~9.2 as determined by spectrophotometry (Dubnovitsky et al. 2005).

During crystallographic data collection at the ESRF beamline ID 14-4, the on-line UV/VIS absorption spectra of the BALC PSAT crystals were recorded. Before exposure to the X-rays, only one maximum at 406–410 nm is observed (Fig. 2B). This maximum corresponds to the protonated Schiff base in solution at 295 K ($\lambda_{\text{max}} = 406\text{--}408\text{nm}$) (Fig. 2A) and suggests a complete protonation of the imine nitrogen in the cryo-cooled crystal, as expected at pH 8.2 used in crystallization.

Table 2. Data collection and refinement statistics for the data sets H–I collected at the EMBL/DESY beamline BW7A

Data set	H	I
Data collection		
Resolution (Å)	12–1.3	12–1.3
Unit cell parameters (Å)	143.73 × 84.27 × 67.21	143.81 × 84.30 × 67.26
Redundancy	5.9	6.3
$\langle I/\sigma(I) \rangle$	15.4 (2.2)	96.4 (3.6)
Completeness (%)	96.2 (95.0)	93.7 (94.7)
R _{sym} (%)	5.9 (48.7)	3.2 (25.7)
Wilson B-factor (Å ²)	12.9	12.4
Refinement		
Resolution (Å)	12–1.3	12–1.3
No. of reflections	183,099 + 9361	178,369 + 9397
R _{cryst} (%), $F > 0\sigma$	13.1	11.7
R _{free} (%)	16.4	15.6
Deviations from ideal geometry		
Bonds distances (Å)	0.012	0.013
Angle distances (Å)	0.031	0.031

Figure 2. Absorption spectra of BALC PSAT in solution and in crystalline form. (A) Spectra of BALC PSAT in solution were measured in 100 mM TRIS-HCl (pH 8.0) (solid line), 100 mM CHES (pH 9.1) (dotted line), or 100 mM CABS (pH 10.6) (dashed line). (B) On-line spectra of the BALC PSAT crystal before collection of the data sets A–G (shown with corresponding letters). The total absorbed dose is increasing from data set A–G. The initial spectrum, A, is highlighted in bold. (C) Changes in the absorption of the BALC PSAT crystal at 340 nm (open symbols) and 407 nm (filled symbols) during continuous exposure to an unattenuated beam.

Exposure of the BALC PSAT crystal to an unattenuated beam results in very quick spectral changes (Fig. 2B). Continuous exposure leads to the gradual decay of the absorption at 405–420 nm, while the absorption at 335–340 nm is rising (Fig. 2C). Since similar spectra can be obtained during pH-titration of the BALC PSAT solution (Fig. 2A), we assume that X-rays induce deprotonation of the internal aldimine in the BALC PSAT crystals. So far, we do not have any explanation for the “red” shift of the long-wavelength absorption maximum

and its increment that initially occurs within several seconds of crystal exposure.

Structural changes induced by X-rays

Figure 3 shows the difference Fourier maps derived from the data sets A–G. No significant structural changes were observed between the initial data set A and the data sets B and C, respectively (Fig. 3A,B). However, further exposure leads to the loss of definition

Figure 3. X-ray-induced structural changes in the active site of BALC PSAT. SigmaA-weighted difference Fourier maps ($wF_A - wF_n$) between the first data set, A, and the successive data sets, B–G, respectively, are shown. Superimposed atomic coordinates from the model A are presented. The green density represents maps contoured at 6.5σ , and the blue density represents maps contoured at -6.0σ . The figure was produced using BOBSCRIPT (Esnouf 1997) and Raster3D (Merritt and Murphy 1994). (A) $wF_A - wF_B$ map. (B) $wF_A - wF_C$ map. (C) $wF_A - wF_D$ map. (D) $wF_A - wF_E$ map. (E) $wF_A - wF_F$ map. (F) $wF_A - wF_G$ map.

Figure 4. Movement of pyridoxal-5'-phosphate in the BALC PSAT active site under X-ray exposure. Eighteen active site protein residues (subunit B) were superimposed, but only two of them are shown for clarity purposes. Black coordinates correspond to the model A (before exposure), and gray coordinates correspond to the model G (after exposure). The figure was produced using BOBSCRIPT (Esnouf 1997) and Raster3D (Merritt and Murphy 1994).

of the internal aldimine bond and several water molecules situated nearby (Fig. 3C–F). At the same time, the correlated appearance of positive and negative electron density peaks indicates a movement of the C2', C4', and O3' atoms of the cofactor. These results suggest that the conformation of the internal aldimine and the relative position of PLP molecule in the active site of BALC PSAT change in response to the X-ray exposure.

Indeed, comparison of the BALC PSAT models refined against data sets A–G has revealed expected conformational differences (Fig. 4). After exposure, the plane of the pyridine ring is rotated around the C2–C5 axis by more than 5° and the axis C2–C5 itself is rotated in the ring's plane by ~3°. As a result of this movement, the refined positions of the C4' atom in the models A and G differ by as much as 0.6 Å (the mean coordinate error for the models A–G assessed by Luzzati plots is 0.10–0.15 Å). Using the empirical formula of Cruickshank (1999) for a better estimation of the mean coordinate error, the obtained values are 0.10–0.12 Å. Residue Lys196 also undergoes slight conformational changes. Finally, several geometrical parameters of the PLP–Lys Schiff base are gradually altered in the models

A–G (Table 3). These include the distance between C4' and the imine nitrogen, the torsion angle C4–C4' and the distance between C4' and the CA atom of the Lys196.

The torsion angle of the internal aldimine (Fig. 1B,C) increases by 25°–35° between the data sets A and G (Table 3). Disruption of the coplanar conformation of the aldimine bond in relation to the PLP pyridine ring was suggested to destabilize greatly the protonated form of the internal aldimine and to stabilize slightly the unprotonated form (Hayashi et al. 1998). Thus, our crystallographic data are consistent with the results obtained by single-crystal absorption spectroscopy of the BALC PSAT (Fig. 2B). Taken together, these observations suggest that deprotonation of the Lys–PLP Schiff base and accompanying changes in the Schiff-base geometry are taking place under X-ray exposure. This could be an explanation for the unexpectedly high values of the aldimine torsion angle (28°–63°) observed in crystal structures of other PLP-dependent enzymes including cystalysin from *T. denticola* (Krupka et al. 2000), ArnB aminotransferase from *S. typhimurium* (Noland et al. 2002), and AAT from *T. thermophilus* (Ura et al. 2001).

With increased total absorbed dose, the refined bond distance of the internal aldimine (a double covalent bond) increases from 1.37–1.40 Å to 1.51–1.66 Å distance (Table 3) that is more suitable for a single covalent bond. These results can be interpreted either as an X-ray-induced reduction of the internal aldimine, or as a disruption of the chemical bond in part of the molecules. In light of the available data, however, it is not possible to distinguish between the two possibilities. The first explanation seems to be more probable, as no electron density for the unbound conformation of the Lys196 was observed in any map. Nonetheless, in our previous crystallographic studies of the BALC PSAT at atomic resolution (Dubnovitsky et al. 2005), the bond distance NZ–C4' was even longer (1.73 ± 0.05 Å), while it was restrained to 1.7 Å, since the electron density for the Schiff base in the final map was insufficient for unequivocal unrestrained refinement. If the restraint has been removed, the refined bond distance of 1.97 ± 0.05 Å was observed (data not shown). Such a

Table 3. Structural changes at the active site of BALC PSAT under X-ray exposure

Data set		A	B	C	D	E	F	G	H	I
Distance C4'–NZ (Å)	Chain A	1.40	1.43	1.44	1.43	1.45	1.45	1.51	1.31	1.52
	Chain B	1.37	1.39	1.41	1.42	1.43	1.48	1.66	1.35	1.53
Torsion angle C3–C4–C4'–NZ (°)	Chain A	2.72	1.9	5.9	7.5	9.1	22.0	27.9	–7.2	45.2
	Chain B	19.6	11.9	20.5	27.0	28.8	48.9	54.0	–4.6	7.7
Distance C4'–CA–(Lys197) (Å)	Chain A	6.65	6.71	6.82	7.02	7.06	7.22	7.33	6.69	7.42
	Chain B	7.35	7.32	7.34	7.38	7.44	7.55	7.65	7.17	7.44

large value suggests that in the structure obtained after ~ 20 h exposure at the EMBL/DESY beamline BW7A, the PLP in some protein molecules is not anymore covalently bound to the Lys196. In other words, the final model would represent the averaged coordinates for the disrupted and the chemically intact aldimine bond. These results therefore support the second explanation, e.g., X-rays induce disruption of the PLP-Lys Schiff base in the BALC PSAT crystals. In this case, however, it is still possible that reduction and disruption of the internal aldimine occur either simultaneously or consequently.

Interestingly, the two active sites of the BALC PSAT dimer show a certain degree of asymmetry. Indeed, such a difference between the theoretically identical and independent active sites has been previously reported for several PLP-containing enzymes (Eliot and Kirsch 2004, and references therein). The initial geometry of the Schiff base and the quantitative changes during the X-ray exposure do not match very well in subunits A and B (Table 3). Analysis of the electron density maps in the active site of the monomer A suggests that Lys196 is partially disordered already in the initial structure (data not shown). For this reason, mainly subunit B was used in comparison. Although the two active sites seem to be differentially susceptible to the radiation damage, all structural changes described above coincide qualitatively in both subunits.

Due to the relatively low resolution of the data sets A–G (1.68–1.77 Å) (Table 1), the mean coordinate error is relatively high (up to 0.12 Å). The data-to-parameter ratio at such a resolution is pretty low for anisotropic refinement of atomic temperature factors. Thus, some degree of uncertainty could be introduced into the final models (DePristo et al. 2004). In order to describe the

subtle structural changes in the BALC PSAT crystals under X-ray exposure more reliably, we collected two high-resolution data sets at the EMBL/DESY beamline BW7A (Table 2). The mean coordinate errors assessed by a Luzzati plot or using Cruickshank's empirical formula for data sets H and I do not exceed 0.08 Å and 0.04 Å, respectively. Moreover, the number of observations at the resolution 1.3 Å is high enough to introduce a full anisotropic refinement of the model. Analysis of the difference Fourier maps (Fig. 5A) and the final BALC PSAT structures refined against data sets H and I have shown X-ray induced structural changes similar to our previous findings at ~ 1.7 Å resolution. Strong positive and negative peaks in difference Fourier maps confirm the movement of the whole PLP molecule and suggest concomitant changes in the position of the Lys196, including both the side and the main chain (Fig. 5A). The relative position of the PLP molecule in the active site, the torsion angle, and the bond length of the internal aldimine are changed even after a relatively short total exposure time (~ 90 min; unfortunately, no estimate of the flux and the dose could be given) at the second-generation synchrotron beamline (Table 3). Further, comparison of the anisotropic displacement parameters of the active site residues shows an increased degree of disorder in the BALC PSAT structure after an X-ray exposure (Fig. 5B,C). The mean equivalent isotropic temperature factor for all atoms in the residue Lys196 (subunit B) is increased from 13.6 Å² in the model H to 20.1 Å² in the model I. The corresponding values for the imine nitrogen (NZ) and the C4' atom of PLP are rising from 13.5 to 26.5 Å² and from 13.6 to 20.4 Å², respectively, while the other atoms of the cofactor are less affected. The average temperature factors for all protein atoms in models H and I are 16.4 Å and

Figure 5. Structural changes in the BALC PSAT crystal at 1.3 Å resolution. (A) SigmaA-weighted difference Fourier map between the data sets H and I contoured at 8 σ (green) and -6.5 σ (blue). The figure was produced using BOBSCRIPT (Esnouf 1997) and Raster3D (Merritt and Murphy 1994). (B) Atomic coordinates and thermal ellipsoids of PLP and Lys196 (subunit B) in the model H (before exposure). The thermal ellipsoids are shown at the 50% probability level. The figure was produced using XtalView (McRee 1999) and Raster3D (Merritt and Murphy 1994). (C) Atomic coordinates and thermal ellipsoids of PLP and Lys196 (subunit B) in the model I (after exposure). The thermal ellipsoids are shown at the 50% probability level. The figure was produced using XtalView (McRee 1999) and Raster3D (Merritt and Murphy 1994).

Figure 6. Distortion of pyridoxal-5'-phosphate in the active site of BALC PSAT. Atomic coordinates of PLP and Lys196 side chain from the model H are presented in black. In gray, the superimposed planar conformation of the PLP molecule from the atomic resolution structure of BALC PSAT is shown (PDB accession code 1W23). The figure was produced using MOLSCRIPT (Kraulis 1991) and Raster3D (Merritt and Murphy 1994).

15.9 Å², respectively. Thus, the increase of the atomic temperature factors serves as additional evidence of the enhanced susceptibility of the BALC PSAT active site to radiation damage.

Strain of the internal aldimine

The conformation of the internal aldimine in the BALC PSAT structure H is suitable for the formation of the intramolecular hydrogen bond between the imine nitrogen and the O3' atom of PLP. The aldimine torsion angle is $< 10^\circ$ (Table 3). However, such a low value is achieved not only by rotation around the C4-C4' axis, but also by a significant distortion of the cofactor. At the time the internal aldimine bond is formed, the PLP molecule loses its planarity (Fig. 6). The entire ring is slightly distorted, but the most deviating part is the C4' atom directly involved in the Schiff-base linkage. It is displaced from the ring's plane by 0.50–0.55 Å toward the *si*-side. Consequently, the C4 and C2' atoms of PLP are also pressed out from the plane, although their deviations are much smaller. However, after the internal aldimine had been damaged by the X-rays, the cofactor planarity is restored. In the model I obtained after a relatively short total exposure time, some degree of distortion is still present, but only a residual effect was observed in the atomic resolution structure of the BALC PSAT (Dubnovitsky et al. 2005). These results, and especially the amplitude of the observed structural changes, suggest the presence of the significant chemical strain on the internal aldimine bond in BALC PSAT. For the other aminotransferases, the strain on the internal aldimine was shown to lower the imine pK_a values by as much as 3–4 units (Hayashi et al. 2003; Mizuguchi et al. 2003). However, in the studied to-date

phosphoserine aminotransferases (Hester et al. 1999; Dubnovitsky et al. 2005), the imine pK_a values are 2–2.5 units higher compared with other PLP enzymes where the chemical strain has been reported (Hayashi et al. 1998; Islam et al. 2000; Mizuguchi et al. 2003). Thus, it remains an open question whether the conformational strain on the internal aldimine in the BALC PSAT affects the imine pK_a value.

In the “undamaged” PLP-Lys Schiff base in BALC PSAT, the cofactor molecule is distorted and the ring's plane is sloped toward the side chain of Lys196 (Figs. 4,6). This conformation seems to be the only suitable for the formation of the stable intramolecular hydrogen bond and a short double covalent bond C4'-NZ in the conditions of very tight PLP binding in the BALC PSAT active site (Dubnovitsky et al. 2005). The chemical strain does not change the torsion angle C4-C4', as suggested for other aminotransferases (Hayashi et al. 1998; Islam et al. 2000; Mizuguchi et al. 2003). Therefore, the free energy gain of the intramolecular hydrogen-bond formation seems to be high enough to cause a geometrical distortion of the PLP pyridine ring. This, in turn, should lead to the distortion of the conjugated π -electron system of the pyridine ring and the internal aldimine. Such a distortion was confirmed by the CD spectroscopy of BALC PSAT at a different pH (Fig. 7). While the internal aldimine is protonated at pH 7.4, a single positive CD band at 410–415 nm is observed, but it is shifted to 345–350 nm (pH 10.0), when the internal aldimine is deprotonated, and hence, the strain on the aldimine bond should be relaxed. Although the interpretation of these results is not straightforward, the above-described positive CD signal was previously associated with the π - π^* transition (Kochendoerfer et al. 1996) and has been suggested to arise partially from the torsion angle of the Schiff base (Hayashi et al. 1998).

Figure 7. Circular dichroism spectra of BALC PSAT in solution. Spectra were recorded at pH 10.0 in 20 mM CABS (solid line) or at pH 7.4 in 20 mM HEPES (dashed line).

The electron sink properties of the cofactor play an important role in PLP catalysis (Jansonius 1998; Eliot and Kirsch 2004). Thus, we propose that strain and distortion of the conjugated π -electron system of PLP and the internal aldimine in BALC PSAT are implemented in the catalytic mechanism. It was shown that the free PLP alone can catalyze slowly many of the possible reactions catalyzed by the PLP-dependent enzymes (Christen and Metzler 1985). This innate catalytic potential of the cofactor is enhanced upon binding to the protein apoenzyme (Eliot and Kirsch 2004). Since breakage of the PLP-Lys Schiff base is an early event of the catalytic cycle, the conformational strain on the internal aldimine may increase significantly the reactivity of this bond and, hence, may play a key role in the enzymatic activation of PLP in phosphoserine aminotransferase.

Conclusions

We have described the radiation damage of the Lys-PLP Schiff base in *B. alcalophilus* PSAT crystals during crystallographic data collection at 100 K. The active site geometry changes quickly in response to the X-ray exposure. Several previously reported crystal structures of other PLP-containing enzymes at 1.5–1.96 Å resolution (Krupka et al. 2000; Ura et al. 2001; Noland et al. 2002; Weyand et al. 2002) have revealed unexpected conformation of the internal aldimine that should be reinspected based on our results. Our data suggest that crystallographic mechanistic studies of enzymes with strained active sites, in general, and PLP-dependent enzymes, in particular, should be done with great care. Moreover, if our present findings are confirmed on any other PLP-containing enzyme, it should become a common practice in further crystallographic studies of the PLP enzymes to optimize the data-collection strategy in order to minimize the total absorbed dose as much as possible without a significant loss in resolution. The on-line monitoring of UV/VIS spectral changes in protein crystals could be of general use for finding the maximum allowed dose to avoid X-ray-induced structural changes in the active sites of chromophore-containing enzymes.

Materials and methods

Purification and crystallization

B. alcalophilus PSAT was expressed in *Escherichia coli* cells, purified, and crystallized as previously described (Dubnovitsky et al. 2003). Orthorhombic crystals of space group $P2_12_12$ were grown from 30% (v/v) PEG 400, 200 mM magnesium chloride hexahydrate, 5% (v/v) glycerol, and 100 mM TRIS (pH 8.2).

Crystallographic data collection

A series of seven complete data sets (A–G) were collected from a single crystal at 100 K on the undulator beamline ID14-4 at the ESRF using a Q4R ADSC CCD detector, with the synchrotron running in 16-bunch mode. A beam size of $50 \times 250 \mu\text{m}^2$ was used at a wavelength of 0.939 Å. Each “composite” data set consists of 100 frames of 0.75°. Every 20 frames were collected from a fresh crystal location after a 60- μm translation (the crystal dimensions were $1.0 \times 0.2 \times 0.1 \text{ mm}^3$). A similar technique was previously used for data collection from a single and from multiple crystals (Berglund et al. 2002; Adam et al. 2004). In order to minimize the total absorbed dose per data set, each frame was collected with a 2-sec exposure to a $19.2\times$ attenuated beam. As a result of the short exposure time, the resolution was limited to 1.68–1.77 Å, although the BALC PSAT crystals diffract to atomic resolution (Dubnovitsky et al. 2005). After the first data collection, the rotating crystal was “burnt” with an unattenuated beam for 5 sec, 10 sec, 1 min, 1 min, 2 min, and 3 min. After every “burning”, the same 15° were collected. Finally, seven complete composite data sets with an increasing total absorbed dose were obtained (Table 1). The total exposure time spent for each individual data-set collection was 40 sec with a $19.2\times$ attenuated beam that allowed us to minimize structural changes during data-collection course.

Data sets H and I (Table 2) were collected to 1.3 Å resolution from a single crystal at 100 K on the EMBL/DESY beamline BW7A equipped with a MarCCD 165-mm detector. A beam size of $100 \times 200 \mu\text{m}^2$ was used at a wavelength of 0.921 Å. A composite data set H consists of seven subsets, including 20 frames each. The oscillation range was 0.5° per frame. Each subset was collected after $\sim 150 \mu\text{m}$ translation of the crystal, which was $1.2 \times 0.2 \times 0.1 \text{ mm}^3$ in size. Data were collected in the dose mode with the mean exposure time of ~ 8 sec per frame. Thus, the total exposure time for the data set H was ~ 160 sec. Data set I was collected from a single crystal location after it had been exposed to the X-rays for 60 min in total. Using an oscillation range of 0.3°, 187 frames were collected with the mean exposure time of ~ 10 sec. Thus, the total exposure time for data set I including a preliminary “burning” and data collection is ~ 90 min.

Data were processed with the HKL suite (Otwinowski and Minor 1997). The intensities were converted to amplitudes using the TRUNCATE program (Collaborative Computational Project Number 4 1994).

Structure refinement

Structures of the BALC PSAT were refined using the restrained conjugate least-squares method as implemented in SHELX-97 (Sheldrick and Schneider 1997). XtalView (McRee 1999) was used for manual modelling. The atomic resolution structure of the BALC PSAT (PDB accession code 1W23) was used as an initial model. Default effective standard deviations were used for all stereochemical restraints. No restraints were given for the torsion angle or the bond length of the internal aldimine, but pyridoxal-5'-phosphate was refined with stereochemical restraints applied. The PLP planarity restraint was released in the refinement against the 1.3 Å resolution data (data sets H and I). Models H and I were subjected to full anisotropic refinement and addition of hydrogen atoms at riding positions after the refinement converged. The final models were inspected with the PROCHECK program (Collaborative Computational Project Number 4 1994). In

the Ramachandran plots, 91%–92% of all nonglycine and nonproline residues were found in the most favored regions, and no residue was observed in the disallowed regions for all models. The refinement statistics for models A–G and H and I are summarized in Tables 1 and 2, respectively.

Absorbed dose calculation

The total absorbed doses for the data sets A–G were calculated with the program RadDose (Murray et al. 2004) on the basis of measured crystal size ($1.0 \times 0.2 \times 0.1 \text{ mm}^3$), photon flux (about 3.7×10^{11} photons/sec in $50 \times 250 \mu\text{m}^2$) and energy (13.2 keV), and calculated crystal absorption and density. Other input parameters used by RadDose were the number of amino-acids per molecule (360), number of molecules per unit cell (eight), number of Cys and Met residues in the protein (12), and non-C/H/N/O components of the mother liquor (200 mM MgCl_2).

Spectroscopic analysis in solution

Absorption spectra of BALC PSAT in solution were recorded at room temperature in a 1-cm cuvette using PharmaciaBiotech Ultrospec2000 spectrophotometer. The final protein concentration was 0.8 mg/mL. The CD spectra of BALC PSAT were recorded using a Jasco J-715 CD spectropolarimeter in a 0.2-cm cuvette at a protein concentration of 1.9 mg/mL. The averaged spectra of four scans were corrected for the buffer blank. Samples for absorption and CD spectroscopy were prepared by dilution of the concentrated protein solution (10–20 mg/mL) in the appropriate buffers and were equilibrated before measurements for 1 h at room temperature.

Protein Data Bank accession codes

Atomic coordinates and structure factors have been deposited with the Protein Data Bank under identification codes 2BHx, 2BI1, 2BI2, 2BI3, 2BI5, 2BI9, 2BIA, 2BIE, and 2BIG for the structures A–I, respectively.

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