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Structure and ligand-binding mechanism of a cysteinyl leukotriene-binding protein from a blood-feeding disease vector

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Abstract

Blood-feeding disease vectors mitigate the negative effects of hemostasis and inflammation through the binding of small-molecule agonists of these processes by salivary proteins. In this study, a lipocalin protein family member (LTBP1) from the saliva of *Rhodnius prolixus*, a vector of the pathogen *Trypanosoma cruzi*, is shown to sequester cysteinyl leukotrienes during feeding to inhibit immediate inflammatory responses. Calorimetric binding experiments showed that LTBP1 binds leukotrienes C₄ (LTC₄) and D₄ (LTD₄) and E₄ (LTE₄) but not biogenic amines, adenosine diphosphate or other eicosanoid compounds. Crystal structures of ligand-free LTBP1 and its complexes with LTC₄ and LTD₄ reveal a conformational change during binding that brings Tyr 114 into close contact with the ligand. LTC₄ is cleaved in the complex leaving free glutathione, and a C₂₀ fatty acid. Chromatographic analysis of bound ligands showed only intact LTC₄, suggesting that cleavage could be radiation-mediated.

INTRODUCTION

Blood feeding by vector arthropods is required for the transmission of pathogenic microorganisms and viruses responsible for diseases such as malaria, leishmaniasis, Chagas' disease and dengue fever. Vector saliva is injected into the host during feeding and contains a mixture of proteins and small molecules that inhibit processes of hemostasis and inflammation in the skin and circulation¹. The importance of saliva in pathogen transmission is currently an area of active investigation, and salivary proteins themselves are being developed as vaccine antigens². An important mode of action of salivary proteins is the scavenging of small molecule mediators of vascular tone, platelet activation and inflammation^{3–8}. In the *Trypanosoma cruzi* (Chagas' disease) vector, *Rhodnius prolixus*, the ligand-binding lipocalin protein family has been greatly expanded in the saliva and includes forms that are adapted to transport nitric oxide⁹ and bind a variety of physiological agonists including histamine^{10, 11}, serotonin, norepinephrine⁴ and ADP⁷. Although the quantity of

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salivary protein injected is small, the feeding volume is also small and concentrations of these scavengers are sufficient to reduce the agonist level in the local skin and circulation⁶.

Eicosanoid mediators of inflammation and platelet function are released during blood feeding as a result of tissue damage at the bite site, and subsequent activation of mast cells by IgE-salivary protein complexes. Thromboxane A₂ is a product of the cyclooxygenase pathway secreted by platelets that modulates vascular tone and is a potent secondary agonist of platelet activation¹². Leukotrienes are 5-lipoxygenase products synthesized in leukocytes and mast cells that modulate leukocyte chemotaxis, smooth muscle contraction and immediate inflammatory responses¹³. The cysteinyl leukotrienes are conjugates of the eicosanoid fatty acid epoxide, leukotriene A₄ (LTA₄), with glutathione or glutathione cleavage products¹³. The primary cysteinyl leukotriene product, leukotriene C₄ (LTC₄), is secreted by activated mast cells and causes a rapid wheal and flare reaction in the skin of the type associated with insect bites¹⁴. After secretion, LTC₄ is converted to LTD₄ and LTE₄ by sequential peptidase digestion of the conjugated glutathione moiety. These compounds show generally similar activities to LTC₄, but have different receptor interaction selectivities¹³.

In this study we show that LTBP1 a novel lipocalin from *R. prolixus* saliva is a specific cysteinyl leukotriene scavenger that binds LTC₄, LTD₄ and LTE₄ that would act to prevent inflammatory responses that occur in the skin as a result of the bites. Crystal structures of the ligand-free protein and ligand complexes show that conformational changes in the protein act to bury and stabilize the ligand. Surprisingly, cleavage of LTC₄ at its thioether linkage occurs during binding. However, a markedly higher stability of LTD₄ in complex with LTBP1 and of LTC₄ in complex with a binding site mutant of LTBP1 suggest that the protein environment may affect this process.

RESULTS

LTBP1 is a cysteinyl leukotriene-binding protein

LTBP1, a previously uncharacterized salivary lipocalin¹⁵, was produced as a recombinant protein and examined for the binding of ligands promoting haemostasis and inflammation. Calorimetric measurements revealed no binding of the protein with any of the vasoactive, proinflammatory or procoagulant effectors previously shown to interact with other lipocalins from the *R. prolixus* salivary gland. The group of non-binding candidate ligands included serotonin, norepinephrine, histamine and adenosine diphosphate (ADP) (Data not shown). High affinity binding was indicated in ITC experiments for three cysteinyl leukotriene compounds LTC₄, LTD₄ and LTE₄ (Fig. 1A–C, 4A), while other physiologically important arachidonic acid derivatives were shown to be poor ligands for the protein, indicating that the primary function of LTBP1 is the removal of proinflammatory/vasoactive cysteinyl leukotrienes from the vicinity of the bite site. LTB₄, an eicosanoid compound related to the cysteinyl leukotrienes but having no conjugated peptide moiety, showed a very weak interaction with LTBP1 (Fig. 1F, 4A). The thromboxane A₂ analog U46619 also did not interact detectably with LTBP1, nor did arachidonic acid itself or prostaglandin D₂ (data not shown), indicating that LTBP1 does not act as a platelet activation antagonist, as does RPAI1. LTBP1 appears to serve as a physiological scavenger of cysteinyl leukotrienes, thereby preventing or delaying immediate host inflammatory responses in the skin that

include pain, irritation and swelling. The lack of high affinity LTB₄ binding indicates that that LTBP1 probably does not function as an inhibitor of neutrophil migration induced by this chemoattractant leukotriene¹⁶. The equilibrium constant (K_a) for LTC₄ binding was estimated to be $1 \times 10^9 \text{ M}^{-1}$ (K_D of 1 nM) using a limit of the c parameter of 500¹⁷, and the enthalpy of binding (ΔH) was approximately -25 kcal/mol (Fig. 1A). LTD₄ and LTE₄ were also bound with high affinity by LTBP1, but the ΔH of binding for these compounds was 7–10 kcal/mol less-favorable than with LTC₄, suggesting that a significant difference exists in the nature of the interaction between the protein and these ligands (Fig. 1A–C).

The structure of LTBP1

LTBP1 consists of an eight-stranded antiparallel β -barrel typical of the lipocalin protein family, with an additional helical region on the C-terminal side of the barrel and a long coiled region N-terminal to the barrel (Fig. 2A, Table 1)¹⁸. A broad, positively-charged binding pocket surrounded by loops connecting β -strands A-B, C-D, E-F and G-H is present at one end of the barrel, while the opposite end is closed by the crossing of the N-terminal coiled region of the protein (Fig. 2A, 5A–C). Three disulfide bonds are present in the LTBP1 structure, linking Cys 10 near the N-terminus with Cys 111 on β -strand G, Cys 45 at the end of β -strand B with Cys 155 at the C-terminal end of the protein, and Cys 67 on β -strand D with Cys 83 on β -strand E (Fig. 2A).

Lipocalins have undergone a rather startling degree of diversification in the salivary gland of *R. prolixus* and its relatives, with 20–75 different forms being present in the secretions of individual species. Furthermore, blood feeding has evolved multiple times within the insect family containing *R. prolixus*, and the lipocalin-rich salivary secretions of the group are known to contain lipocalin functional forms distinct to particular genera or species^{15, 19, 20}. Sequence comparisons show that LTBP1 belongs to the lipocalin group that contains RPAI1 (39% identity), an *R. prolixus* scavenger of the platelet agonist ADP⁷. The protein shows only about 20–25% identity with members of a second group of *R. prolixus* lipocalins containing the nitric oxide-binding nitrophorins and the biogenic amine-binding proteins, whose members have two disulfide bonds rather than three (Fig. 3D)^{11, 21}. Comparison of the structures of nitrophorin 4 (nitric oxide-binding), ABP (serotonin-binding) and LTBP1 (Fig. 3A–C) illustrates the differences between these proteins in the lengths of β -strand elements and the conformations of the loops surrounding the binding pockets. LTBP1 also shows little similarity in sequence to eicosanoid-binding lipocalins from ticks, which are derived from a different progenitor lipocalin and have evolved these binding functions independently^{8, 22, 23}.

The structure of the LTBP1-LTC4 complex

Binding of LTC₄ induces a number of structural changes in the ligand binding pocket and loop regions of LTBP1. The non-functionalized end of the fatty acid is inserted deeply into a narrow hydrophobic channel that is opened, relative to the ligand-free structure, by rotation of the side chain of Met 95 and an outward movement of β -strand C, resulting in a change in the position of the side chain of Leu 56 by approximately 2 Å (Fig. 2B,D). Both oxygen atoms of the fatty acid carboxylate group interact with the side chain of Arg 110 forming part of an extensive hydrogen bonding/salt bridge network (Fig 5D). The planar, conjugated

triene structure of the ligand passes over the guanidinium group of this residue in a direction nearly parallel to its face (Fig. 5D). The two cis double bonds at C11 and C14 of the fatty acid moiety, are clearly apparent in the electron density as the chain is bent in this location after passing over Arg 110 (Fig. 6A). In the deepest portion of the pocket, the fatty acid chain is surrounded at its terminus by a cluster of aromatic and other hydrophobic side chains (Fig. 2B, D).

As a result of LTC₄ binding, loop G–H of the protein, lying at the entry to the pocket, undergoes a large rearrangement relative to the ligand-free structure, with C α of Ser 116 at the apex of the loop moving toward the ligand by a distance of 7.8 Å (Fig. 2C, 4B). In the closed conformation, the side chain of Tyr 114 rotates toward the ligand and inserts itself next to C6 of the fatty acid (Fig. 2C, 5D,E). During closure, the phenolic hydroxyl group of Tyr 114 swings a distance of 14.1 Å from its position in the ligand-free structure and forms hydrogen bonds with the side chain of Ser 93 and an ordered water molecule that is in turn hydrogen bonded with the carboxyl group of the fatty acid (Fig 2C, 4B, 5D). The remainder of the protein structure changes little on ligand binding, with the rest of the loops surrounding the binding pocket being similar in conformation to their counterparts in the ligand-free structure (Fig. 2A).

The glutathione moiety of LTC₄ is bound at a site with more surface exposure than the fatty acid, and the terminal carboxyl group of its glycine residue is stabilized by hydrogen bonds with two ordered water molecules and a single hydrogen bond with the amide nitrogen atom of Lys 61 of the protein. The glutamate portion of glutathione contacts the G-H loop of LTBP1, particularly the carbonyl oxygen of Tyr 114. Surprisingly, the thioether linkage of LTC₄ appears completely cleaved, leaving a separate, free glutathione molecule and a well-ordered fatty acid with no visible substitution at C6, which is approximately coplanar with C5 and C7 (Fig. 5D, 6A). The sulfur atom lies 3.5 Å from C6, shows no sign of oxygenation, and apparently exists as a free sulfhydryl or thiolate group which forms a hydrogen bond (3.0 Å) with the C5 hydroxyl group of the fatty acid (coordinate error of 0.12 Å, Table 1, Fig. 5E, 6A). The ligand structure is essentially identical in crystals harvested and flash frozen either 24 hr or 3 weeks after setting up hanging drops at room temperature. Radiation-mediated cleavage of thioether links in protein crystals has been previously observed²⁴ and may be the cause of the cleavage observed here. To address this issue, a diffraction data set was collected from a LTBP1-LTC₄ complex crystal to a resolution of 2.0 Å using an in-house sealed-tube X-ray source producing radiation of far lower intensity than the synchrotron source. The resulting electron density maps show cleavage of the thioether, but with the refined position of C6 being closer to the sulfur atom of glutathione (refined distance 3.1 Å, with a coordinate error of 0.20 Å, Table 1, Fig. 6B) than in the model described above. This suggests that a smaller fraction of the C6-S bond was cleaved, at lower radiation intensities, and supports the idea that cleavage is, at least in part, radiation-mediated.

Chromatographic analysis of ligands and complexes

To further examine the fate of LTC₄ after binding with LTBP1, solutions containing LTC₄ by itself or incubated with LTBP1 were analyzed by reversed phase HPLC which separates the

bound ligand from the protein. Chromatography of an LTC₄ standard in ethanol, LTC₄ in aqueous buffer, or a 1.2:1 LTBP1:LTC₄ complex showed a peak at the retention time of intact LTC₄ when eluted with a gradient of increasing acetonitrile in water in the presence of 0.1 % trifluoroacetic acid (Fig. 6A, B). An equal quantity of LTC₄ was seen with both the protein complex and buffer control samples, indicating that ligand recovery is quantitative, and none is lost to cleavage (Fig. 7A, B). The identity of the released product was confirmed using diode array detection of the eluent (Fig. 7D). The optical spectra of the ligands and standards obtained in this manner are identical to that of authentic LTC₄ with an absorbance maximum at 282 nm and shoulders at 273 and 290 nm.

In a second experiment, crystals of a 0.8:1 LTC₄-LTBP1 complex were collected by centrifugation from the mother liquor of hanging drops 14 days after complex formation and dissolved in water. These also showed LTC₄ as the only significant product after chromatography, demonstrating that cleavage products cannot be isolated from crystals in this way, and that cleavage is not an artifact of crystallization (Fig. 7C). Finally, as a means of detecting any changes to the conjugated triene structure in the bound form of the ligand, the spectrum of the protein-bound LTC₄ was measured by difference spectrophotometry revealing only a small bathochromic shift relative to the free ligand that could be due to the different solvent environment of the binding pocket (Fig. 7E). The maximum was shifted to 287 nm and contained the characteristic shoulders of the cysteinyl leukotriene spectrum indicating that the conjugated triene structure is retained in the bound ligand. Together, the chromatographic and spectral data suggest a radiation-mediated cleavage mechanism.

The structures of the LTBP1-LTD₄ and Y114A LTBP1-LTC₄ complexes

The difference in binding enthalpy for LTC₄ and LTD₄/LTE₄ measured in ITC experiments may be explainable by differences in the structures of the various ligand complexes. Additionally, the large movement of Tyr 114 with its insertion directly adjacent to C6, make this residue likely to play an important mechanistic role in ligand binding. To examine these issues, we crystallized LTBP1 in the presence of LTD₄, and the Tyr 114-alanine (Y114A) mutant of LTBP1 in the presence of LTC₄. In both cases, the crystals were isomorphous to those of the wild-type LTBP1-LTC₄ complex, and showed clear electron density attributable to bound ligands. In the refined structure of the LTBP1-LTD₄ complex, the fatty acid moiety adopts a conformation different than seen in the LTC₄ complex, and unexpectedly shows a largely intact thioether linkage (Fig. 5F, 6C). The conjugated triene system (C6–C13), is tilted relative to Arg 110, placing C6 within covalent binding distance of the sulfur. Consequently, C7 and C8 are moved 0.9 and 0.6 Å from their positions in the LTBP1-LTC complex, while C6 is moved 1.6 Å and exhibits sp^{3.4} hybridization (Fig. 5F, 6C, E). The C6-S bond is present, and the absolute configurations at C5 (S) and C6 (R) match those for natural LTD₄ (Fig. 5F, 6C). The tetrahedral geometry and positioning of C6 and C5 allow an extended conformation for C3–C5 relative to the LTC₄ complex, although the location of the C5 hydroxyl is similar in the two structures. The carboxylate group of the fatty acid is situated similarly to its position in the LTC₄ complex and forms the same electrostatic interactions with Arg 110 (Fig. 5F). The side chain of Lys 61 is well ordered in the LTD₄ complex and is hydrogen bonded to the C5 hydroxyl group of the fatty acid ligand. The

structure of the protein itself, including the G-H loop, is almost identical to that of the LTBP1-LTC₄ complex.

Binding of LTD₄ clearly induces the same conformational changes of the G-H loop as does LTC₄, but not extensive cleavage of the thioether, even though the data for both complexes were collected under similar conditions (Fig. 5F). Some disorder, as indicated by the electron density surrounding the sulfur atom, suggests that a small fraction of the ligand may be cleaved, but no movement of C6 away from its bonded position or movements of C3–C5 are apparent, indicating that the extent of cleavage is small (Fig. 6C). The cysteinylglycine fragment of the LTD₄ complex is essentially superimposable with the corresponding part of the glutathione tripeptide in the LTC₄ complex, suggesting that in the LTC₄ complex, only the fatty acid changes position as a result of ligand cleavage, while the peptide retains its position (Fig. 6A, C, E).

Mutation of Tyr 114 of LTBP1 to alanine reduced the affinity of the protein for LTC₄ and LTD₄, and resulted in a much less favorable ΔH of binding as measured by ITC (Fig. 1D, E). In the crystal structure of the LTC₄-Y114A complex, binding induces closure of the protein despite the absence of the aromatic side chain, with C α of Ala 114 moving toward the LTC₄ ligand and occupying a similar position to C α of Tyr 114 in the wild-type protein (Fig. 6F). Two well-ordered water molecules are present in the space normally occupied by the side chain of Tyr 114, with one positioned at the site of the phenolic hydroxyl group and having similar hydrogen bonding interactions with the Ser 93 hydroxyl (Fig. 6F). Because of this, the ligand-protein hydrogen bonding network seen in the wild-type structure is maintained. However, the structure of the bound ligand differs from that in the wild-type LTBP1-LTC₄ complex, and very closely resembles the LTD₄ complex in having an intact thioether linkage and the same tilted orientation of the conjugated triene system toward the glutathione that puts C6 into position for covalent bonding to sulfur (Fig. 6D). The cysteine and glycine residues of the glutathione moiety are nearly superimposable with the wild type structures, while the glutamate residue of the peptide is disordered toward its terminus in the mutant structure (Fig. 6D). It is not clear why LTD₄ in the wild-type protein or LTC₄ in the Y114A mutant would be less prone to radiation-mediated cleavage than LTC₄ in the wild-type protein, since the ligands are oriented in the binding pocket in a nearly identical manner, and are chemically very similar or identical.

DISCUSSION

R. prolixus is a blood feeding insect that acquires its meal by penetrating the skin of the host with a sharp, tube-like mouthpart and sucking blood directly from the vasculature and from the skin surrounding the site²⁵. The mouthpart also contains a second channel through which a salivary secretion is injected that contains modulators of hemostasis and inflammation¹⁵. Most of these factors function to maximize blood flow for rapid ingestion, while inhibiting immediate skin responses that result in plasma extravasation and discomfort associated with the bite. The latter point is important in that inhibition allows uninterrupted feeding through the prevention of host defensive responses. *R. prolixus* is a large insect that consumes a relatively large blood meal, yet a sleeping bite victim rarely wakes during feeding presumably due to inhibition of pain and inflammation by salivary components. *R. prolixus*

is a vector of *Trypanosoma cruzi*, the causative agent of a serious parasitic disease, Chagas' disease²¹. Other blood feeders such as mosquitoes, ticks, sand flies and fleas use mechanistically similar secretions to acquire blood, and during the process of feeding transmit infectious pathogens including *Plasmodium* (malaria), *Leishmania* (leishmaniasis), *Yersinia pestis* (bubonic plague), dengue virus, West Nile virus and Zika virus^{2, 26, 27}. Interactions at the host-vector interface have been shown to be important in the efficient transmission and establishment of some of these agents.

The diverse group of lipocalins present in *R. prolixus* saliva plays many roles in feeding biology, most commonly in the binding and sequestration of small molecule agonists of hemostatic and inflammatory responses^{4, 6, 9, 28}. The nitrophorins are lipocalins that contain a single heme molecule bound to the protein through iron coordination with a specific histidine imidazole. In the *R. prolixus* salivary gland, the protein is loaded with nitric oxide (NO), which forms a sixth heme iron ligand in the distal pocket¹¹. In the host circulation, NO is released on dilution of the protein, where it serves as a potent vasodilator and inhibitor of platelet function. After release of NO, histamine is bound in the distal pocket through iron coordination of its imidazole nitrogen and specific hydrogen bonding with other binding pocket residues^{10, 11}. Histamine is released from skin mast cells in response to feeding and is in part responsible for the itching response associated with insect bites. A similar *R. prolixus* lipocalin, ABP, does not bind heme, and instead is a scavenger of the biogenic amines serotonin, and the catecholamines norepinephrine and epinephrine^{4, 21}. These compounds are released in response to wounding and cause rapid vasoconstriction and diminution of blood flow to the feeding site. They also act as potentiators of platelet activation initiated by primary agonists such as exposed collagen. A third lipocalin form, given the name RPAII, binds purine nucleotide triphosphates, diphosphates, monophosphates and free nucleotides. ADP is released from platelet dense granules as a secondary agonist resulting in a stronger and more complete activation and aggregation response. Binding of this compound by RPAII helps to prevent formation of a platelet plug, which is the earliest physical barrier to blood loss as well as forming a membrane substrate for the assembly of coagulation factor complexes^{6, 7}. One member of the nitrophorin group of lipocalins, nitrophorin 2, acts as a potent inhibitor of coagulation by binding to coagulation factor IX (anti-hemophilia B agent) and IXa with low nanomolar affinity, thereby blocking the action of the intrinsic factor Xase complex, a critical component of the pathway to thrombin, the ultimate protease in the coagulation cascade^{29, 30}.

Here we describe the identification, characterization and structural analysis of LTBP1, a new component of the salivary mixture that binds the cysteinyl leukotrienes LTC₄, LTD₄ and LTE₄ with very high selectivity and affinity, strongly suggesting that it functions as an anti-inflammatory protein in the skin and circulation of the mammalian host. The protein acts together with known histamine, serotonin, norepinephrine, and ADP binding proteins to limit the extent of platelet activation, vasoconstriction, edema and itching associated with feeding. Furthermore, the LTBP1-LTD₄ and Y114A-LTC₄ complexes represent the first crystallographic views of intact cysteinyl leukotriene ligands bound to any protein target. These ligands are extremely important modulators of smooth muscle contraction and are most notably involved in airway constriction in anaphylaxis and asthma³¹. In the skin, IgE responses to antigens from the insect cause activation and degranulation of mast cells. The

synthesis and secretion of LTC₄ is also strongly induced. After secretion, peptidases act on the glutathione moiety of LTC₄ to produce LTD₄ and LTE₄. When injected into the skin, all three of these compounds rapidly produce a wheal and flare reaction resulting in redness, discomfort and edema¹⁴. Increases in vascular permeability cause erythrocyte-poor plasma to leak into the tissues. This liquid is deficient in protein-rich red blood cells and provides a lower-quality blood meal for the insect²⁵. By binding all three cysteinyl leukotrienes, LTBP1 would slow or prevent this process, reducing the likelihood of sensation by the host and improving the quality of the meal.

Comparison of the ligand-free and ligand-bound forms of LTBP1 reveals an opening of a hydrophobic channel, and a large loop rearrangement on binding that closes the protein around the ligand. Cysteinyl leukotrienes are bulky molecules which contain a long hydrophobic fatty acid chain and a highly functionalized hydrophilic peptide portion. The conformational changes appear to allow entry of the ligand into the protein while maximizing the number of stabilizing contacts in the final complex. Curiously, binding of LTC₄ is accompanied by cleavage of its thioether linkage, leaving glutathione and a well-ordered free fatty acid as products. Chromatographic analysis of the bound ligand showed the presence of only intact LTC₄, with no indication of any cleavage products, suggesting that the C6-S bond may be particularly susceptible to radiation-mediated cleavage during diffraction data collection. Radiation-mediated cleavage is also supported by an apparently smaller fraction of cleaved ligand obtained when data were collected using lower-intensity copper radiation from an in-house source rather than synchrotron radiation. However, the stability of the thioether linkages in the LTD₄-wild-type and the LTC₄-Y114A complexes suggests that the degree of radiation-mediated lysis of the thioether may depend on the protein and ligand environment. The electron density of LTC₄ indicates that C5 of the fatty acid remains tetrahedral, while C6 becomes approximately coplanar with C5 and C7 suggesting sp² hybridization (Fig. 5D, 6A). This arrangement would rule out a stable ketone or enol, but would be consistent with a carbocation or carbon radical at C6, either of which would normally be unstable and short-lived. In the closed conformation of the complex, the face of the Tyr 114 aromatic ring lies directly adjacent to C6 and is in position to stabilize a solvent-excluded C6 carbocation by means of a cation- π interaction, and could facilitate the elimination of glutathione resulting in a thiolate-carbocation pair or a thiol-fatty acid carbocation pair (Fig. 5D, E, 6D, F). This might explain why the complex of the Tyr 114-Ala mutant is more stable to X-ray-mediated cleavage. In the LTD₄ complex, which must resemble the pre-cleavage state of LTC₄, C6 lies 3.8 Å from CZ of Tyr 114 and 4.3 Å from the phenolic hydroxyl, placing it over the plane of the aromatic ring (Fig. 5F). The fatty acid shows the expected tetrahedral geometry at C5 and C6 and an intact C-S bond. Additionally, the peptide fragment is positioned in virtually the same position as its counterpart in the LTC₄ complex. If cleavage in the LTC₄ complex is radiation induced, this structure provides an unusually complete, and well-ordered example of specific radiation damage in a protein crystal.

MATERIALS AND METHODS

Protein production

A synthetic cDNA for LTBP1 (genbank accession AAQ20818) was codon optimized for expression in *Escherichia coli* and cloned into pET17b without a signal sequence and with an initiator methionine codon added to the 5' end. The plasmid was moved into the *E. coli* strain BL21(DE3)pLysS and the resulting transformant was grown and induced as described previously³. Inclusion bodies were harvested and prepared as described previously and the protein was solubilized in 6 M guanidine HCl, pH 8, reduced with 10 mM DTT, and refolded by dilution into an excess of 40 mM Tris HCl, pH 8, 300 mM arginine, and 2 mM cystamine. After concentration by ultrafiltration, recombinant LTBP1 was purified by gel filtration chromatography on Sephacryl S100 and ion exchange chromatography on Q-Sepharose. Production of selenomethionine-substituted protein followed a similar procedure except that the protein was produced in *E. coli* strain B834(DE3)pLysS grown in SelenoMet (Molecular Dimensions) medium supplemented with selenomethionine.

Crystallization, data collection and structure determination

LTBP1 was crystallized using the hanging drop-vapor diffusion method from 30% PEG 6000 in 100 mM Tris HCl pH 8.0. The best crystals were obtained with 3 μ L drops at a 2:1 protein:precipitant ratio, and flash frozen prior to data collection in the precipitant solution containing 12% glycerol. For crystallization of leukotriene complexes, sufficient ligand to form a 1:1 complex with the protein was added to a glass vial in ethanol. After evaporation of the solvent, the protein solution was added to the vial to suspend the ligand and form the complex. The preformed complex was then combined with precipitant solution in a hanging drop at a 2:1 protein to precipitant ratio.

Diffraction images were collected at beamlines 22-ID and 22-BM of the Southeast Regional Collaborative Access Team (SER-CAT) at the Advanced Photon Source, Argonne National Laboratory. Images were processed using HKL2000 or XDS^{32, 33} (Table 1). The structure of LTBP1 was determined by single anomalous diffraction methods using data from the selenomethionine derivative in Phenix AutoSol³⁴ (Table 1). A relatively complete model was built using ARP/wARP³⁵. The remainder of the model was built manually using data collected from the wild-type ligand-free protein in Coot³⁶. Refinement was performed using Phenix³⁴. The structure of the LTC₄ complex was determined by molecular replacement with Phaser using the ligand-free model for searching³⁷. The ligand complex models were also refined with Phenix. To examine the degree of radiation damage to the crystal using lower-intensity radiation, a data set of the LTC₄ complex was collected at 100 K using a Rigaku sealed-tube X-ray source coupled with an R-Axis IV detector (Table 1). The coordinate and structure factor data for this study were deposited with the RCSB-PDB data bank under the accession numbers: 5H9K (ligand-free LTBP1), 5H9L (LTBP1-LTC₄ complex), 5HAE (LTBP1-LTC₄ complex collected with an in-house source), 5HA0 (LTBP1-LTD4 complex), 5H9N (LTBP1 Y114A mutant-LTC₄ complex).

Isothermal titration calorimetry and chromatographic measurements

Isothermal titration calorimetry was performed using a VP-ITC (Malvern Instruments) calorimeter at 30°C and a buffer consisting of 20 mM Tris HCl, pH 7.5, 150 mM NaCl. Solutions of the eicosanoid ligands were prepared by sonication with buffer after removal of ethanol. After a course of 10 µL injections, the data were analyzed by fitting to a single binding site model using the Microcal Origin analysis software.

Ligands and ligand complexes were prepared as described above and analyzed using reversed phase chromatography on a 0.5 × 150mm, XDB C₁₈ column (Agilent) with an Agilent 1100 series capillary HPLC. The column was equilibrated in 0.1% TFA/30% AcCN and developed with a linear gradient to 0.1% TFA/80% AcCN over 30 minutes at 10 µl/min. The sample injection volume was 1 µl. Photodiode array spectral acquisition was done from 210–400 nm along with single wavelength monitoring at 280nm. Difference spectrophotometry was carried out on a Varian Cary 100 Bio double beam spectrophotometer. Equal volumes of 30 µM LTBP1 in 20 mM Tris HCl, pH 7.4, 0.15 M NaCl were added to 1 mL sample and reference cells followed by addition of LTC₄ (to a concentration of 15 µM) to the sample cell to observe the bound form of the ligand in difference spectra recorded at 25°C.

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Fig 1.

Measurement of ligand binding by LTBP1 using ITC with the top plot of each panel showing the resulting heats produced by each injection and the bottom plot showing injection enthalpies as points with a continuous line showing the fit to a single-site binding model. All combinations were tested at a protein concentration in the calorimeter cell of 2 μM and a ligand concentration in the syringe of 20 μM , at a temperature of 30°C. A) Binding of LTC₄. Thermodynamic parameters: $H = -25.4 \pm 0.2 \text{ kcal/mol}$, $K_a = 1 \times 10^9 \text{ M}^{-1}$. Binding stoichiometry (N) = 1.2. B) Binding of LTD₄. Thermodynamic parameters: $H = -15.6 \pm 0.2 \text{ kcal/mol}$, $K_a = 1 \times 10^9 \text{ M}^{-1}$. Binding stoichiometry (N) = 1.1. C) Binding of LTE₄. Thermodynamic parameters: $H = -18.3 \pm 0.2 \text{ kcal/mol}$, $K_a = 4.7 \pm 1.6 \times 10^8 \text{ M}^{-1}$,

T Δ S = -6.3 kcal/mol, N = 1.1. D) Binding of LTC₄ with the Y114A mutant of LTBP1. Thermodynamic parameters: Δ H = -15.7 $\pm$ 0.3 kcal/mol, K_a = 2.1 $\times$ 10⁷ M⁻¹, T Δ S = -5.4 kcal/mol, N = 1.3. E) Binding of LTD₄ with the Y114A mutant of LTBP1. Thermodynamic parameters: Δ H = -12.9 $\pm$ 0.3 kcal/mol, K_a = 2.1 $\pm$ 0.5 $\times$ 10⁷ M⁻¹, T Δ S = -2.7 kcal/mol, N = 1.3. F) Binding of LTB₄ with wild-type LTBP1. Thermodynamic parameters: Δ H = -8.3 $\pm$ 1.2 kcal/mol, K_a = 4.2 $\pm$ 2.4 $\times$ 10⁶ M⁻¹, T Δ S = 0.9 kcal/mol, N = 0.8.

Fig. 2.

The structure of LTBP1. A) Ribbon diagram of the superimposed structures of ligand-free LTBP1 (magenta) and the LTBP1-LTC₄ complex (green). The β -strands are labeled A-H and the loops surrounding the entry to the binding pocket are labeled AB-GH. The three disulfide bonds are indicated as stick diagrams (with sulfur as yellow) and the N- and C-termini of the protein are indicated. B) The models shown in A with a space filling representation of bound LTC₄ to show the large ligand-binding interface. Glutathione is indicated by GSH, and the C₂₀ fatty acid is indicated by FA. Carbon is colored light grey,

oxygen is colored red and nitrogen blue. C) Ligand-induced movements of loop GH. The backbones of the ligand-free (magenta) and LTC₄-bound (green) structures are shown in ribbon representation with the side chains shown as stick diagrams. Dashed lines represent movements of the loop as indicated by C α positions of Tyr 114 and Ser 116. The distances are indicated for each path. D) Side chain interactions with the fatty acid in the binding pocket of LTBP1. The pocket is lined with numerous hydrophobic and aromatic side chains. The protein structure is colored in green and the fatty acid structure is colored in light grey. The relatively large movements of the side chains of Leu 56 and Met 95 on ligand binding are indicated by showing the positions of these residues from the ligand-free structure in red.

Fig. 3.

Structural comparisons and amino acid alignment of LTBP1 with lipocalin sequences of known function in the salivary secretion of *R. prolixus*. A) Ribbon diagram of LTBP1 with LTC4 bound oriented in the same way as panels B and C. The N-terminus is labeled NT and the C-terminus is labeled CT. The loops surrounding the entry to the binding pocket are labeled AB, BC, CD, EF and GH. The LTC4 ligand is colored in light grey with oxygen colored red, nitrogen blue and sulfur yellow. B) Model of nitrophorin 4 (1NP4) oriented and labeled as in panel A. Heme is colored red with nitrogen colored blue. C) Model of the ABP-tryptamine complex (4GE1) oriented and labeled as in panel A. The tryptamine ligand is colored magenta with nitrogen colored blue. D) Amino acid alignment of the proteins shown in A–C along with RPAI1, whose crystal structure has not been determined. The positions of β -strands A–H are shown above the alignment, and the conserved cysteine positions are shaded. RPAI1 (39 % identity with LTBP1) binds ADP and inhibits the activation of platelets. ABP (24 % identity with LTBP1) binds biogenic amines, and prevents vasoconstriction and the activation of platelets. NP4 (nitrophorin 4, 20 % identity with LTBP1) binds nitric oxide and histamine and acts as a vasodilator, platelet activation inhibitor and anti-histamine.

Fig. 4. Structures of cysteinyl leukotrienes and details of the conformational change in loop G-H of LTBP1. A) Structures of cysteinyl leukotrienes (top left). R groups (top right) correspond to the three different forms. $R_1=\text{LTC}_4$, $R_2=\text{LTD}_4$, $R_3=\text{LTE}_4$. Structure of LTB_4 (bottom left). B) Stereoview stick diagram of a superposition of loop G-H in the ligand-free LTBP1 and the LTC_4 -complex. Ligand-free LTBP1 is shown in magenta and the LTC_4 complex is shown in green. In both cases, oxygen is shown in red, nitrogen in blue and sulfur in yellow.

Fig. 5. Details of the ligand binding interaction of leukotrienes with LTBP1. A–C) Surface representations of LTBP1 colored according to electrostatic potential with blue representing positively charged regions and red negatively charged (the ± 9 kT/e electrostatic potential is shown on a solvent-accessible surface). Panel A shows the ligand-free open form of the protein. Panel B shows the changes in binding pocket architecture on binding of LTC₄ and C shows the surface represented in panel B with a space filling model of the ligand occupying the binding pocket. In the ligand model carbon is colored green, oxygen is red, nitrogen is blue and sulfur is yellow. D) Electrostatic and hydrogen bonding interactions of the ligand in the structure of the LTBP1-LTC₄ complex. Protein side chain carbon atoms are colored in green, with oxygen in red and nitrogen in blue. Ligand carbon atoms are colored light grey. Hydrogen bonds are shown as red dashed lines. GSH is glutathione, FA indicates the fatty acid, and Wat indicates an ordered water molecule. E–F) Interactions of the ligand with the side chain of Tyr 114. Panel E shows the cleaved LTC₄ structure with distances of C6 to CZ and the hydroxyl group of the phenolic ring as indicated by black dashed lines. The hydrogen bond between the glutathione sulfhydryl and the C5 hydroxyl group of the fatty

acid is shown as a red dashed line. Panel F shows the same view of the intact LTD₄ structure with the distances shown as in panel E.

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Fig. 6.

Ligand structure of various complexes of leukotrienes with LTBP1. A–D) Electron density surrounding ligands from four complexes. Panel A shows the LTC₄-LTBP1 complex obtained from data collected using synchrotron radiation. Panel B shows the same complex collected using copper radiation from a laboratory source. Panel C shows LTD₄ in complex with LTBP1 collected using synchrotron radiation. Panel D shows LTC₄ in complex with the Y114A mutant of LTBP1 collected using synchrotron radiation. In panels A and B the model was refined as a cleaved ligand, while in panels C and D the models were refined using a ligand containing an intact thioether linkage. Electron density maps (2Fo-Fc, blue) were contoured at 1 σ . In panel A, the double bonds of the fatty acid are indicated as Z-11 and Z-14, and the position of C6 is also shown. The other structures are shown from the same perspective. (E) Superposition of structures from panels A and C with electron density for cleaved LTC₄ shown in blue and for LTD₄ shown in orange. The stick diagram for LTC₄ is shown with carbon colored green, while LTD₄ carbon is colored light grey. The positions of C5 and C6 are labeled. (F) Superposition of LTC₄ complexes of wild-type (green) and Y114A (light blue) LTBP1. The LTC₄ ligand is modeled as cleaved in the wild-type structure and intact in the mutant structure. The water molecules W1 and W2 are seen in the

mutant structure and partially fill the space occupied by the tyrosine aromatic ring in the wild-type structure.

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Fig. 7.

Chromatographic and spectral analysis of bound LTC₄. A–C) Reversed phase chromatography of protein bound and free LTC₄. Panel A shows chromatogram of protein bound LTC₄ with an early-eluting LTBP1 peak and a major leukotriene peak. Panel B shows an equal amount of protein-free LTC₄ eluted in an identical manner. In panel C crystals of the LTC₄ complex were collected by centrifugation, dissolved and analyzed as in panel A. D) Optical spectrum of eluted peak from panel A obtained by diode array detection. The spectra from panels B and C showed precisely the same maxima. E) Optical difference spectra showing the LTBP1-bound form of LTC₄. Both the reference and sample cells contained LTBP1 at a concentration of 30 μ M. LTC₄ was added to the sample cell to a concentration of 15 μ M and spectra were recorded immediately (yellow) and after 1 minute (green). The orange recording shows a spectrum of LTC₄ taken in the absence of protein for comparison.

Table 1

Data collection, phasing and refinement statistics for LTBP1 (ligand free), its LTC4 complex collected with synchrotron radiation (LTC4A), its LTC4 complex collected on an in-house source (LTC4B), its LTD4 complex (LTD4), and the LTC4 complex of the Tyr 114-Ala mutant (LTC4-MUT).

Crystal	Selenium	Ligand Free	LTC4A	LTC4B	LTD4	LTC4-MUT
Resolution (Å)	32.6–1.39	33.0–1.35	33.3–1.37	35.0–2.00	33.4–1.44	28.8–1.28
Beamline	22-BM	22-BM	22-ID	–	22-ID	22-ID
Wavelength (Å)	0.97910	1.0000	0.97910	1.5418	1.0000	1.0000
Completeness (total/high resolution shell)	97.8/95.9	97.7/92.8	99.9/98.6	96.3/93.7	98.9/96.0	94.6/85.0
Average Redundancy (total/high resolution shell)	6.2/5.4	5.3/4.2	4.6/3.9	3.9/3.8	4.5/3.5	4.6/4.1
R _{merge} (total/high resolution merge shell)	4.2/11.8	3.5/12.1	9.3/51.5	6.4/13.6	6.7/24.7	4.8/16.4
I/sigI (total/high resolution shell)	26.0/10.44	30.5/10.7	9.2/3.1	20.6/14.7	9.3/4.4	15.1/11.1
Observed reflections	144,408	152,423	135,841	35,561	111,921	155,314
Unique Reflections	25,606	28,469	29,309	9,370	24,704	35,407
Space group	P2 ₁	P2 ₁	P2 ₁	P2 ₁	P2 ₁	P2 ₁
Unit cell dimensions (Å)						
a	35.11	35.13	34.27	34.27	34.40	34.23
b	58.62	58.70	58.39	58.39	58.30	58.09
c	35.44	35.48	36.29	36.02	36.04	36.15
beta (°)	111.90	111.78	103.69	103.75	104.14	104.16
Phasing statistics						
Number of selenium sites	3					
FOM (Phaser)	0.51					
RMS deviations						
bond lengths (Å)		0.006	0.006	0.006	0.006	0.006
bond angles (°)		1.08	1.08	1.08	1.08	1.15
Ramachandran plot (Favored/Allowed)		95.5/100	95.5/100	95.5/100	95.5/100	94.9/100
Mean B value for all atoms		15.0	18.5	20.7	16.9	20.0
Coordinate error ML (Å, Phenix)		0.11	0.12	0.20	0.12	0.11
R _{cryst} /R _{free}		16.0/18.0	16.4/19.1	14.6/20.2	16.0/18.8	16.2/18.7