Brain acetylhidrolase that inactivates platelet-activating factor is a G-protein-like trimer

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The platelet-activating factor PAF (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a potent lipid first messenger active in general cell activation, fertilization, inflammatory and allergic reactions, asthma, HIV pathogenesis, carcinogenesis, and apoptosis. There is substantial evidence that PAF is involved in intracellular signalling, but the pathways are poorly understood. Inactivation of PAF is carried out by specific intra- and extracellular acetylhidrolases (PAF-AHs), a subfamily of phospholipases A2 that remove the sn-2 acetyl group. Mammalian brain contains at least three intracellular isoforms, of which PAF-AH(1b) is the best characterized. This isoform contains a heterodimer of two homologous catalytic subunits α and α2, each of relative molecular mass 26K, and a non-catalytic 45K β-subunit, a homologue of the β-subunit of trimeric G proteins. We now report the crystal structure of the bovine α2 subunit of PAF-AH(1b) at 1.7 Å resolution in complex with a reaction product, acetae. The tertiary fold of this protein is closely reminiscent of that found in p21ras and other GTPases. The active site is made up of a tryptophan-like triad of Ser 47, His 195 and Asp 192. Thus, the intact PAF-AH(1b) molecule is an unusual G-protein-like (α2/β)6 trimer.

Mammalian brain contains significant levels of PAF, which acts as a synapse messenger and transcription inducer of the early-response genes c-fos and c-jun. It accumulates rapidly in neural tissue during seizures or ischaemia and the resulting brain damage can be reduced by PAF antagonists. PAF is also being implicated as a messenger in long-term potentiation, a cellular model of memory formation. The β-subunit of the PAF-AH(1b) complex is a product of the gene that is responsible for the onset of type-I lissencephaly, a developmental brain disorder caused by impaired neuronal migration, in which no cortex is formed. This suggests a potential role for PAF in early brain development and neuronal migration.

The two catalytic α-subunits of PAF-AH(1b) (this new notation is consistent with that accepted for the structurally related G-protein subunits) share extensive amino-acid sequence identity—63% in the bovine form—but the consensus sequence they share is unique among proteins. When these subunits are purified or overexpressed individually in Escherichia coli, they form catalytically competent homodimers. In vivo, however, α2 is preferentially labelled by [1,3-3H]fisopropyl fluorophosphate in the heterodimer, suggesting functional asymmetry.

The PAF-AH(1b) α2 subunit is a single polypeptide chain of 231 residues. The molecule contains a single α/β domain with a central, parallel, 6-stranded β-sheet. This fold is very like that found in GTPases (Fig. 1a, 2). The insertions and deletions in the
\( \alpha \) structure, compared to p21<sup>α</sup>, can be rationalized in functional terms: for example, the turn/β fragment unique to p21<sup>α</sup> and involved in GTP binding is absent in \( \alpha \), and the first β-strand found in the \( \alpha \)-subunit of the G protein transducin and in p21<sup>α</sup>, which is involved in interactions with other proteins, is also unique to these proteins. On the other hand, \( \alpha \) contains a unique N-terminal \( \alpha \)-helix which, together with the loop that follows, is part of the dimer-forming interface. Finally, the catalytic centre of \( \alpha \) is located on two \( \Omega \) loops that are significantly different from their analogues in p21<sup>α</sup>.

Serine 47 has been identified as the putative nucleophile<sup>7</sup>. In the crystal structure, Ser 47 is close to His 195; further, the imidazole ring of His 195 is hydrogen-bonded through its N<sub>ε</sub>1 atom to the side-chain carboxyl of Asp 192 (Fig. 1b). The stereochemistry of this triad is indicative of a catalytic function, which is supported by the inactivity of H195A and D192N mutants (data not shown). The chirality of the triad is the same as that found in the active sites of other esterases and neutral lipases, where nucleophilic attack is on the re face of the ester<sup>15</sup> (the Cahn–Ingold–Prelog nomenclature specified the topologically different faces of peptides and esters involved in nucleophilic attack as re faces, not tr and st<sup>15</sup>). The acetate bound in the active site interferes with the ground-state stereochemistry of the triad and disrupts the hydrogen bond that should link His 195 and Ser 47 in the active enzyme. The bound acetate also makes it possible to identify unambiguously the hydrogen-donor groups that constitute the oxyanion hole: the main-chain amides of Ser 47 and Gly 74, and N<sub>ε</sub>1 amide of Asn 104 (Fig. 1b). Cloned human plasma PAF-AH is also thought to work by means of a catalytic triad<sup>16</sup>.

The \( \alpha \) homodimer is formed by two molecules related by the crystallographic two-fold axis (Fig. 3a), oriented so that the carboxyl edges of their β-sheets are close to one another. In each monomer, a total of 1,150 Å<sup>2</sup> of solvent-accessible surface is lost when the dimer forms (Fig. 3b): 18 amino acids lose more than 20 Å<sup>2</sup> upon dimerization, and fourteen of these are conserved between the two catalytic subunits. Of the remaining four, only C55Y is a non-conservative substitution; this rationalizes the disorder between residues 53–56 (see Methods). Many of the interface-forming residues are hydrophilic and numerous hydrogen bonds and salt bridges mediate the contacts: for example, Gln 18–His 106, Asp 20–Arg 41, Ser 25–Gln 143 and Arg 29–Tyr 193; there are no buried water molecules within the interface.

The conserved character of the interface-forming residues indicates that the crystallographic dimer described here is a good model for the \( \text{in vivo} \) \( \alpha \)/\( \alpha \) heterodimer. It is a doughnut-shaped structure with a solvent-accessible gorge, some 15 Å deep. The two active sites are at the bottom of the gorge and only 12 Å from each other, as measured between the carboxyl carbons of the acetate. The proximity suggests that these sites do not function independently, consistent with the noted functional asymmetry of

![FIG. 1. A stereo plot of \( \alpha \)-carbon atoms of the final model; the residues that form the catalytic triad are shown in their entirety and are labelled. b. An 'omit' difference electron density map showing active-site residues (labelled) and the acetate molecule (Ace) after all these groups were deleted from phase calculation and the remainder of the protein was subjected to two cycles of crystallographic refinement using PROLSQ. The electron density is contoured at a 2σ level.](image-url)
the heterodimer in which the activity of the \( \alpha \) subunit can be suppressed by steric effects.

In general catalysis by neutral lipases and secretory phospholipases A2 is markedly enhanced at substrate concentrations above their critical micellar concentrations (CMCs): plasma PAF-AH is significantly more active at PAF concentrations (CMC\( \text{PAF} \)) above 5 \( \mu \)M (ref. 6). The structure of the \( \alpha \) homodimer shows that the entrance to the active gorge is not particularly hydrophobic, and there is no flexible secondary structure element that might function as a "lid", a structural motif in serine-dependent lipases that are active at interfaces\(^7\). The crystal structure is therefore consistent with the enzyme being active on a monomeric substrate.

The activity of all PAF-AHs depends on their recognition of a short acyl chain in the sn-2 position, but stringency varies between isoforms. The plasma enzyme prefers the sn-2 acetyl group but will hydrolyse acyl chains of up to five carbons\(^8\); the brain isoform II also has broader specificity\(^9\). However, PAF-AH (lb) is very selective, being 20 times less active towards propionyl-PAF than PAF itself\(^9\). The crystal structure can explain these effects. Three residues, all conserved in the \( \alpha \) subunit, come into contact with the acetate's methyl group: they are Leu 48, Leu 194 and Thr 103 (Fig. 4). Each donates one methyl group, that is, at C61 (Leu 194), C62 (Leu 48) and C72 (Thr 103), to form a hydrophobic pocket for the substrate's acetyl moiety. Analysis of the probe-accessible surface created by these groups, and structural comparison with other neutral lipases indicate that the

![Figure 2](image)

**FIG. 2** A comparison of the tertiary structures of PAF-AH (lb)\( \alpha \), and GTPases: a, p21\(^{C}\)s (entry SP21 in the Protein Data Bank); b, \( \alpha \) subunit of PAF-AH (lb); c, catalytic domain of the transducin \( \alpha \)-chain (entry 1GA). Red, C\textalpha{} atoms used for least-squares overlap of PAF-AH (lb)\( \alpha \), with either of the GTPases; green, the most significant insertions or deletions; blue, the partly disordered residues (55–61) in PAF-AH (lb)\( \alpha \). Figure generated using RIBBONS (M. Carson). Secondary structure elements of PAF-AH (lb)\( \alpha \), H0, residues 22 to 36; H1, 47 to 55; H2, 77 to 90; H3, 110 to 127; H4, 147 to 164; H5, 198 to 215; S1, 41 to 45; S2, 67 to 70; S3, 96 to 101; S4, 131 to 136; S5, 169 to 173.

### Table 1: Summary of crystallographic analysis

<table>
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<th>X-ray data collection</th>
<th>Native</th>
<th>Mercury</th>
<th>Lead</th>
<th>Gold</th>
<th>Cryo</th>
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<tr>
<td>Beam line (( \lambda ))</td>
<td>BW7B (0.895)</td>
<td>X31 (0.95)</td>
<td>X31 (0.93)</td>
<td>X31 (0.95)</td>
<td>BW7B (0.895)</td>
</tr>
<tr>
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<td>RT</td>
<td>RT</td>
<td>RT</td>
<td>120K</td>
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<tr>
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<td>201,462</td>
<td>68,970</td>
<td>122,863</td>
<td>387,544</td>
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<td>15,029</td>
<td>13,983</td>
<td>15,006</td>
<td>33,716</td>
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<td>20–2.19</td>
<td>20–2.24</td>
<td>20–2.20</td>
<td>20–1.65</td>
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<tr>
<td>Completeness (%)</td>
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<td>100</td>
<td>98.9</td>
<td>99.9</td>
<td>99.7</td>
</tr>
<tr>
<td>( R_{\text{sym}} ) (%)(^*)</td>
<td>8.4</td>
<td>8.2</td>
<td>9.1</td>
<td>7.3</td>
<td>6.3</td>
</tr>
<tr>
<td>( R_{\text{cal}} ) (%)(^\ddagger)</td>
<td>11.9</td>
<td>17.2</td>
<td>15.6</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Overall phasing results**

- \( R_{\text{free}} \) (acentric/centric): 0.83/0.76
- Phasing power (acentric/centric): 1.20/0.90
- Number of sites (major/minor): 1/2

**Final refinement results (120 K)**

| (data in parenthesis relate to a \( \Delta 55–61 \) model) |
| Resolution limit | 8.5–1.7 \( \AA \) |
| Bond distance (1–2) r.m.s. | 0.174 (0.195) |
| Angle distance (1–3) r.m.s. | 0.024 (0.015) |
| Planar (1–4) r.m.s. | 0.044 (0.034) |
| Peptide planes r.m.s. | 0.060 (0.047) |
| Main-chain average B-factor r.m.s. | 8.0 (5.7) |
| Side-chain average B-factor r.m.s. | 20.3 \( \AA^2 \)/1.4 \( \AA^2 \) |
| Residues in most favoured regions of Ramachandran plot\(^*\) | 99.6% |
| Residues in additionally allowed regions | 8.8% |

\( R_{\text{free}} \) = \( \sum \left| F_{\text{obs}} - F_{\text{calc}} \right| / \sum \left| F_{\text{calc}} \right| \), where \( F_{\text{calc}} \) and \( F_{\text{obs}} \) are the structure factor amplitudes for native and derivative crystals respectively.

\( R_{\text{sym}} \) = \( \sum \left| \left| F_{\text{sym}} \right| - \left| F_{\text{calc}} \right| \right| / \sum \left| F_{\text{calc}} \right| \), where \( F_{\text{sym}} \) is the intensity of the \( l \)-th observation and \( l \) is the mean intensity of the reflection.

\( R_{\text{cal}} \) = \( \sum \left| F_{\text{cal}} - F_{\text{calc}} \right| / \sum \left| F_{\text{calc}} \right| \).

\( R_{\text{free}} \) the analysis of the Ramachandran plot was done using PROCHECK\( ^{20} \).
methyl of Thr 103 is critical.

The different specificities of PAF-AHs may be important. Membrane phospholipids can be oxidized to molecules containing shorter, frequently oxidized groups in the sn-2 position, and these molecules can mimic the cell-damaging effects of PAF. Hence, PAF-AHs with broader specificity and preference towards insoluble substrates—in particular the plasma isoform—may act as general scavengers of PAF and PAF-like inflammatory phospholipids. Conversely, enzymes with high specificity, like PAF-AH(1b), respond selectively to PAF, as would be expected if they form part of a signal transduction pathway.

The β-subunit of PAF-AH(1b) shows limited but significant sequence homology with the β-subunits of G proteins, one of which—transducin G_{t}—has the tertiary fold of a seven-blade β-propeller\(^{23}\). Sequence alignment of the two proteins (not shown) indicates that, except for the N-terminal fragments—nearly 100 residues in the β-subunit and 46 in G_{t}—both proteins share a common tertiary scaffold. Thus, the intact PAF-AH(1b) trimer is essentially a G-protein-like (α_1/β_2) β molecule; the β-subunit appears to be associated exclusively with the α chain\(^{15}\), supporting the idea of a functional asymmetry for the catalytic heterodimer.

The PAF-AH(1b) β-subunit is important in brain development, being selectively expressed in the developing brain in regions undergoing neuronal migration in the cortex and cerebellum\(^{22,23}\). This pattern supports a role for this subunit in neuronal migration. Its importance is underscored by its remarkable conservation: there is only one substitution among its 410 amino acids in the bovine and human species\(^{8}\), and only one between the murine and human forms\(^{22}\). There is one known fungal homologue of PAF-AH(1b) β—45% amino-acid identity—the NudF gene product of Aspergillus nidulans\(^{34}\), which is essential for the movement of nuclei along the hyphae of filamentous fungi, a process requiring microtubules and cytoplasmic dynein. An evolutionarily ancient phenomenon, nuclear movement, may have been recruited in the developing brain to initiate neuronal migration through as-yet unidentified interactions of PAF-AH(1b) β with the cytoskeleton, as suggested by the binding of WD-repeat proteins, including PAF-AH(1b) β, to pleckstrin-homology (PH) domains, including those of β-spectrin and dynamin\(^{31}\).

Why does the catalytic α/β dimer associate with the β-subunit to form a heterotrimer? Messenger RNA expression of PAF-AH(1b) α indicates that in the human fetus the transcript is found predominantly in the brain, although it can be detected in several tissues in the adult\(^{26}\). Hydrolysis of PAF may induce conformational changes in the trimer that affect the ability of the β-subunit to interact with cytoskeletal proteins. We are investigating the nature of the interactions between the subunits of the PAF-AH(1b) complex.

**Methods**

Purification of the enzyme has been described\(^{21}\). The protein was crystallized using 3 μl of the protein at 4.3–6.3 mg/ml\(^{-1}\) in 10 mM Tris-HCl, pH 7.5, 2 mM DTT, mixed 1:1 with well solution (16% saturated ammonium sulphate in 200 mM sodium acetate buffer, pH 6.5) and equilibrated in a hanging drop at room temperature. Trigonal crystals, space group P₃2₁, a = b = 82.3 Å, c = 72.9 Å, grew to 0.4 × 0.5 × 0.6 mm after 3–4 weeks, giving a diffraction pattern extending to ~2.5 Å on a rotating anode X-ray source, Hg, Pb- and Au-derivatized crystals were prepared by transferring native crystals to solutions containing 1 mM tinctures, 500 mM trimethyl lead acetate and 5 mM sodium gold acetate, respectively, and equilibrating for 36 h. Data were collected at the EMBL Outstation in Hamburg. Native data at room temperature were collected on the Wiggler beamline BW7B; all data for derivatized crystals were collected on the Wiggler beamline BW7B; all data for derivatized crystals were collected on

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**Fig. 3a** Structure of the α₁ homodimer, looking down the entrance to the active-site gorge; black dotted line denotes the boundary between the monomers, and selected residues that line the entrance to the gorge are labelled. Bound acetate molecules are shown in yellow, and the surfaces accessible to probe are coloured according to their electrostatic potential. b, Dimer-forming interface of the α₁ subunit. The view here is of the right monomer shown in a, rotated 90° around the vertical axis. The residues indicated are those that lose at least 20 kT of solvent-accessible surface upon dimerization, as well as catalytic Ser 46 and His 195 (magenta), shown for clarity. They are coloured as follows: amino acids conserved between α₁ and α₂ are in green; non-conserved residues are shown in gold. Figure generated by GRASP\(^{21}\).

**Fig. 4** The specificity pocket, as illustrated by probe (1.4 Å)-accessible surfaces of amino acids Leu 48, Leu 194 and Thr 103. The three critical methyl groups are shown in purple, green and blue, respectively. Figure generated by GRASP\(^{25}\).

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the tunable bending magnet beamline X31 where the wavelength was adjusted to maximize the anomalous signal from the heavy atoms. In all experiments, only one crystal was needed for a complete set. A single native crystal was flash-frozen after being transferred to a cryoprotectant solution of 30% glycerol in the crystallization medium. The unit cell dimensions after freezing were $a = b = 81.3\,\AA, c = 72.6\,\AA$. Data were reduced and scaled using the HKL package containing DENZO and SCALEPACK programs (Z. Otwinowski and W. Minor). Unless specified otherwise, all software was used as implemented in the CCP4 suite of programs (Daresbury Laboratory, SERC, UK). Phases for room-temperature data were refined using MOLPHARE and further enhanced using density modification, as implemented in SQUEEZE.2. The resulting map was of sufficient quality to allow 50% of the main chain to be traced as polyalanine. This partial model was used in combination in SISMAA (R. Read) to improve the phasing. The structure was traced using program O (ref. 28) and the model was refined at 2.1 Å resolution using PROLSQ, initially with residues 5–219. Water molecules were then added and refinement was continued with 13 residues (53–65) deleted from the model owing to intrinsic disorder in that region. When the 1.7 Å data were introduced, the orientation of the molecule was optimized using AMORE (J. Navaza) and X-PLOR (A. Brünger) version 3.1; only a small deviation in rotation and translation parameters was observed; the resulting R-factor for this model against all data was 0.33. A parallel calculation was done using only 90% of data to monitor the $R_{	ext{free}}$ value for 10% of randomly chosen reflections. The 2.1 Å model was subjected directly to two rounds of slow cooling using X-PLOR, followed by PROLSQ and REFMAC refinement of positional and thermal parameters; no manual revision of the model was done. As a result, the $R$ factor was reduced for a model including 141 water molecules to 0.22, while the $R_{	ext{free}}$ was 0.27. At this point, refinement using all data was resumed at the point at which it was left. Several cycles of PROLSQ reduced the $R$ factor to below 0.20. A number of side chains were corrected manually in program O and the acetate, now clearly defined in the active site, was included in the refinement. Of the 13 residues deleted from the previous model, only 4 had to be modelled into ambiguous density. The C-terminal amino acids 216–219 were deleted owing to the absence of interpretable density. Solvent structure and the model (consisting of residues 5–216; an acetate ion bound in the active site, and 241 water molecules) were refined using REFMAC, a maximum-likelihood refinement program. Because of the partial disorder of residues 55–61, their stereochemistry was relatively poor. A final refinement calculation was therefore done for a 255–61 model from which they were deleted. Stereochemical parameters for both models are listed in Table 1.

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CORRESPONDENCE and requests for materials should be addressed to Z.S.D. (e-mail: zsd@virginia.edu). Atomic coordinates have been deposited in the Brookhaven Protein Data Bank, entry code 2WAB, where they will be held for a year before release.