Chronophin Dimerization Is Required for Proper Positioning of Its Substrate Specificity Loop

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Background: The role of homodimerization in the family of C2a-capped HAD phosphatases is unknown.

Results: Chronophin homodimerization is required for proper positioning of the substrate specificity loop and for substrate dephosphorylation.

Conclusion: The specificity of chronophin is allosterically controlled by a homophilic intermolecular interaction.

Significance: Our results reveal a general principle of how HAD hydrolase dimerization can contribute to substrate specificity.

Mammalian phosphatases of the haloacid dehalogenase (HAD) superfamily have emerged as important regulators of physiology and disease. Many of these enzymes are stable homodimers; however, the role of their dimerization is largely unknown. Here, we explore the function of the obligatory homodimerization of chronophin, a mammalian HAD phosphatase known to dephosphorylate pyridoxal 5′-phosphate (PLP) and serine/threonine-phosphorylated proteins. The exchange of two residues in the murine chronophin homodimerization interface (chronophinA194K,A195K) yields a constitutive monomer both in vitro and in cells. The catalytic activity of monomeric chronophin toward PLP is strongly impaired. X-ray crystallographic studies of chronophinA194K,A195K revealed that dimer formation is essential for an intermolecular arginine-arginine-tryptophan stacking interaction that positions a critical histidine residue in the substrate specificity loop of chronophin for PLP coordination. Analysis of all available crystal structures of HAD hydrolases that are grouped together with chronophin in the C2a-type structural subfamily uncovered a highly conserved mode of dimerization that results in intermolecular contacts involving the substrate specificity loop. Our results explain how the dimerization of HAD hydrolases contributes to their catalytic efficiency and substrate specificity.

Enzymes of the haloacid dehalogenase (HAD) type constitute a large and ancient superfamily whose members are present in all three kingdoms of life. The majority of HAD enzymes are phosphatases known to cover an exceptionally broad substrate space, ranging from metabolites to macromolecules such as DNA and serine/threonine (Ser/Thr)- or tyrosine (Tyr)-phosphorylated proteins (1–3). A number of HAD phosphatases have been causally linked to human diseases, including cancer and cardiovascular, metabolic, and neurological disorders (4); however, very little is currently known about the regulation of these enzymes.

Contrasting their structurally highly diverse substrates, HAD phosphatases are remarkably similar in terms of topology and active site architecture even though their overall amino acid sequence identities are very low (3). A canonical, modified Rossmann fold positions the catalytic core residues that are distributed over four HAD motifs. The first aspartate in the strictly conserved DxDx(V/T) HAD phosphatase signature motif serves as the nucleophile and phosphoryl group acceptor that forms a phosphoaspartate intermediate during catalysis. This aspartate also coordinates the catalytically essential Mg2+ ion (1).

HAD phosphatases are additionally equipped with so-called cap domains. Unlike the structurally stereotypical build-up of the catalytic domain, caps are highly diversified modules that can be grouped into four classes, C0, C1, C2a, or C2b, according to their size, structure, and insertion site in the core domain (3). A primary cap function is to mediate solvent occlusion/inclusion during the catalytic cycle. In general, C1/C2-capped HAD phosphatases process small metabolites that can be sequestered within the active site by cap closure, thus ensuring efficient dephosphorylation. In contrast, macromolecules themselves can provide the necessary active site shielding and are preferentially processed by C0 (“capless”) phosphatases. Besides contributing to catalytic efficiency, caps supply substrate specificity determinants with residues that engage in substrate recognition and thereby establish phosphatase specificity (5–8). Interestingly, caps can also mediate the commonly found HAD phosphatase oligomerization: among the 20 structurally characterized human HAD phosphatases alone, 10 are oligomeric, and in four of these phosphatases, oligomerization is mediated by cap-cap interactions (4).
Chronophin is a homodimeric, C2a-capped HAD phosphatase (9–11) known to target Ser\(^3\)-phosphocofilin (12–16) and the Ser/Thr-phosphorylated steroid receptor co-activators 1 and 3 (17). In addition to its emerging protein phosphatase activities, chronophin (gene name, pyridoxal phosphate (PDXP)) acts as a small molecule phosphatase dedicated to the metabolism of pyridoxal 5\(^{-}\)-phosphate (PLP) (10, 11). PLP is the biologically active form of vitamin B\(_6\) that functions as a cofactor in the catalysis of \(>160\) different enzymatic reactions, in particular in the biosynthesis of neurotransmitters (18, 19).

Chronophin has been suggested to link pathological alterations in vitamin B\(_6\) metabolism with cofilin-dependent actin dynamics in the rat hippocampus following status epilepticus (20, 21).

In the present study, we show that chronophin homodimerization is a prerequisite for its proper enzymatic function as a PLP phosphatase. These findings can be extended to dimeric C2a-capped HAD hydrolases in general and indicate a paradigmatic role for their dimerization in substrate recognition and thus in the control of catalytic efficiency and substrate specificity.

**EXPERIMENTAL PROCEDURES**

*Database Searches*—The Protein Data Bank was searched for structures of haloacid dehalogenase-like hydrolases using the Pfam entries PF13419, PF00702, PF13344, PF13242, PF08282, and PF12710. The search was conducted with a 90\% sequence identity cutoff to reduce the number of multiple entries and mutant proteins and yielded 177 unique entries. Cap domain subtypes were determined manually and cross-validated using a recently published data set (22). The PISA online tool (23) was used to determine the oligomeric state and for dimer interface calculations (see supplemental Table S1).

*DNA Constructs*—Murine chronophin cDNA was reverse transcribed from adult mouse brain tissue. Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer’s instructions, and cDNA was obtained with the High Fidelity RNA PCR kit (Takara Bio Inc.) and oligo(dT) primers. The PCR product was subcloned into the BamHI and EcoRI restriction sites of pcDNA3 (Invitrogen) to construct untagged chronophin, into the KpnI and XhoI sites of pENTR3C (Invitrogen) followed by insertion via homologous recombination into the KpnI and XhoI sites of pENTR3C (Invitrogen) followed by insertion via homologous recombination into pDEST27 (Invitrogen) to produce GST-tagged chronophin for expression in mammalian cells, or into the bacterial expression vector pETM11 (European Molecular Biology Laboratory) to create N-terminally His\(_6\)-tagged chronophin for *in vitro* studies. The chronophin\(_{A194K-A195K}\) (chronophin\(_{KK}\)) construct was generated by site-directed mutagenesis.

*Protein Expression and Purification*—His\(_6\)-tagged chronophin wild type or chronophin\(_{KK}\) in pETM11 was transformed into BL21(DE3) cells (Stratagene) and expressed for 18 h at 20 °C after induction with 0.5 mM isopropyl \(\beta\)-D-1-thio-galactopyranoside. To increase solubility, chronophin was co-expressed with chaeroperones from the pG-Tf2 plasmid (Takara Bio Inc.) according to the instructions of the manufacturer. Cells were harvested at 8,000 \(\times\) g for 10 min and lysed in 100 mM triethanolamine (TEA), 500 mM NaCl, 20 mM imidazole, 5 mM MgCl\(_2\), pH 7.4 in the presence of protease inhibitors (EDTA-free protease inhibitor tablets, Roche Applied Science) and 150 units/ml DNase I (Applichem) using a cell disruptor (Microfluidizer Processor M-110 P, Microfluidics). Cell debris was removed by centrifugation for 30 min at 30,000 \(\times\) g. For purification, cleared supernatants were loaded on a HisTrap HP column operated on an ÄKTA liquid chromatography system (GE Healthcare) in binding buffer (50 mM TEA, 500 mM NaCl, 20 mM imidazole, 5 mM MgCl\(_2\), pH 7.4), and His\(_6\)-tagged proteins were eluted using a linear gradient up to 50\% elution buffer (50 mM TEA, 250 mM NaCl, 500 mM imidazole, 5 mM MgCl\(_2\), pH 7.4). Fractions containing His\(_6\)-tagged chronophin were pooled, and the His\(_6\) tag was cleaved with tobacco etch virus protease for 2 days at 4 °C. Subsequently, cleaved protein was separated from uncleaved protein and from the His\(_6\)-tagged tobacco etch virus protease on a HisTrap HP column. Untagged chronophin was further purified on a HiLoad 16/60 Superdex 200 prep grade size exclusion chromatography column (GE Healthcare) in buffer A (50 mM TEA, 250 mM NaCl, 5 mM MgCl\(_2\), 5\% (v/v) glycerol, pH 7.4).

**Analytical Size Exclusion Chromatography**—Globular proteins of known molecular weight (Gel Filtration LMW Calibration kit, GE Healthcare) were used to calibrate a Superdex 200 10/300 GL column (GE Healthcare), and blue dextran was used to determine the column void volume. Protein elution volumes were measured by monitoring the absorption at 280 nm. The elution volumes were used to calculate the partition coefficient (\(K_d\)) with the formula \(K_d = (V_v - V_o)/(V_t - V_o)\) where \(V_v\) is the elution volume, \(V_o\) is the void volume, and \(V_t\) is the total volume of the column. The apparent molecular weight was then derived from the inverse logarithm of the partition coefficient.

**Analytical Ultracentrifugation**—Sedimentation velocity analytical ultracentrifugation was carried out using a Beckman Optima XL-I analytical ultracentrifuge (Beckman Coulter) with an eight-hole An-50 Ti rotor at 40,000 rpm at 20 °C. Four hundred microliters of dialyzed, purified recombinant protein dialyzed against buffer A without glycerol and reference buffer solution were loaded in standard double-sector charcoal-filled Epon centerpieces equipped with sapphire windows. Protein concentration corresponded to an \(A_{280}\) of 0.5–0.8. Data were collected in continuous mode at a step size of 0.003 cm using absorption optical detection at a wavelength of 280 nm. Data were analyzed using the NIH software SEDFIT to determine continuous distributions for solutions to the Lamm equation \(c(s)\) as described previously (24). Analysis was performed with regularization at confidence levels of 0.68 and floating frictional ratio \(ff = 1.32 ± 0.02\) for both chronophin wild type and mutant, suggesting a globular conformation, time-independent noise, baseline, and meniscus position to root mean square deviation (r.m.s.d.) values between 0.007 and 0.012. Consistent results were obtained in three independent experiments.

**Atomic Force Microscopy (AFM)**—Proteins were diluted between 30- and 100-fold from 30 \(\mu\)M stock solutions in AFM deposition buffer (25 mM HEPES, 50 mM KCl, 10 mM MgCl\(_2\), pH 7.5), immediately deposited onto freshly cleaved mica, rinsed with deionized water, and dried in a gentle stream of nitrogen. All images were collected on an MFP-3D-BIO atomic force microscope (Asylum Research) in oscillating mode using Olympus OMCL-AC240 silicon probes with spring constants of \(~2\) newtons/m and resonance frequencies of \(~70\) kHz.
Images were captured using Asylum Research software on Igor Pro at a scan size of $2 \times 2 \, \mu m^2$, a scan rate of 0.5 Hz, and a resolution of 1024 $\times$ 1024 pixels. For analysis, AFM images were flattened to third order. AFM volumes were measured using the NIH Image-based software ImageSXM and used to calculate protein molecular weights as described (25) with the formula $V = 1.2 \times (MW) - 5.9$ where $V$ is the AFM volume and MW is the molecular weight. Molecular weights (and error ranges) were derived from the center positions (and two standard deviations) of Gaussian fits with $R^2 > 0.79$ or 0.96 for chronophinWT or chronophinKK, respectively) to the distributions of measured volumes using the software Origin (Origin-Lab, version 8.6). Consistent results were obtained from triplicate experiments for both chronophinWT and chronophinKK.

Isothermal Titrination Calorimetry (ITC)—ITC experiments were performed on a MicroCal ITC200 microcalorimeter (GE Healthcare) at 25 °C and were analyzed using MicroCal Origin software. Prior to all ITC experiments, protein samples were extensively dialyzed overnight at 4 °C against filtered and degassed buffer A. Each titration experiment consisted of 2.5–10 μl injections of 300–600 μM BeF$_3$ diluted in buffer A into the 280–μl sample cell containing 25–75 μM chronophin. Heats of dilution measurements were carried out as mentioned above by injecting BeF$_3$ into buffer A. For each experiment, the binding enthalpy was directly measured, whereas the stoichiometry (N) and the dissociation constant ($K_d$) were obtained using the analysis software, assuming a single site binding model.

Crystallization and Data Collection—Proteins were concentrated to 8–10 mg/ml (as determined by absorption at 280 nm using a calculated molar extinction coefficient of 18,450 M$^{-1}$cm$^{-1}$) in buffer A (chronophinKK) or in 10 mM TEA, 100 mM NaCl, 1 mM MgCl$_2$ (chronophinWT) using 10,000 molecular weight cutoff centrifugal filter devices (Amicon Ultra-15, Millipore). All crystals were grown at 20 °C using the hanging drop vapor diffusion method by mixing equal volumes of protein solution with reservoir solution. ChronophinKK crystals were grown in 0.1 M MES at pH 6.5 with 25% (w/v) polyethylene glycol monomethyl ether 550 and appeared as thin plates after 3–4 days. ChronophinWT was crystallized in 0.1 M imidazole, 0.2 M NaCl, 1 mM sodium tartrate supplemented with 1 mM BeF$_3$ to obtain the chronophinWT-BeF$_3$ structure, and cubic crystals appeared within 24 h. All crystals were cryoprotected for flash cooling in liquid nitrogen by soaking in mother liquor containing 30% (v/v) glycerol. All data sets were collected at beamline 14.1 (Berlin Elektronenspeicherring-Gesellschaft für Synchrotronstrahlung (BESSY), Berlin, Germany). Data were processed using iMOSFLM (26) and Scala from the CCP4 program suite (27). All three structures were solved by molecular replacement with the program Phaser (28) using human pyridoxal 5’-phosphate phosphatase/chronophin (Protein Data Bank code 2OYC) as a search model. The chronophinWT/chronophinWT-BeF$_3$ structures were refined at 2.2-Å resolution, and the chronophinKK structure was refined at 1.75-Å resolution with Phenix (29), incorporating torsion angle non-crystallographic symmetry restraints. Structural representations were generated with PyMOL (The PyMOL Molecular Graphics System, version 1.5.0.4, Schrödinger, LLC). PyMOL was also used to determine the r.m.s.d. of structural alignments.

In Vitro Phosphatase Assays—PLP dephosphorylation assays were conducted in 96-well microtiter plates. ChronophinWT or chronophinKK (100 nM/well) was preincubated for 10 min at 22 °C in buffer A supplemented with 0.001% (v/v) Triton X-100. The reactions were started by the addition of PLP (final concentrations ranging from 0 to 1,000 μM in a total volume of 50 μl) and stopped after 2 min by the addition of 100 μl of Biomol Green (Enzo Life Sciences). Color was allowed to develop for 10 min before the absorbance of the resulting phosphomolybdate complex was read at 620 nm on an Envision 2104 multilabel microplate reader (PerkinElmer Life Sciences). Free phosphate release was quantified using phosphate standard curves, and $V_{max}$ and $K_m$ values were calculated using GraphPad Prism version 6 (GraphPad Software Inc.). The lines were fitted by nonlinear regression using the least square fitting method.

Cell Transfection and Pulldown Assays—HEK293-AD cells (Strategene) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) fetal calf serum (FCS), 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were plated at a density of 4.5 × 10$^5$ cells in 6-well plates and transfected the next day using TransIT-LT1 (Mirus) with a total amount of 1 μg of DNA. The amount of transfected DNA was kept constant with empty vector. Cells were lysed in 50 mM TEA, 250 mM NaCl, 5 mM MgCl$_2$, 1% (v/v) Triton X-100, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 50 μg/ml aprotinin, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (Pefabloc) for 15 min at 4 °C under constant rotation. After centrifugation at 15,000 x g for 15 min at 4 °C, the lysates were incubated for 1.5 h under constant rotation with glutathione-Sepharose 4B beads (GE Healthcare) pre-equilibrated with washing buffer (50 mM TEA, 250 mM NaCl, 5 mM MgCl$_2$, 1% (v/v) Triton X-100). The beads were washed four times with washing buffer using a vacuum manifold, bead-associated proteins were eluted with Laemmli sample buffer, and eluates were separated by SDS-PAGE and blotted onto nitrocellulose membranes (Hybond C, Amersham Biosciences). Chronophin dimerization was assessed by Western blot analysis with chronophin-specific antibodies (clone C85E3, Cell Signaling Technology).

Accession Codes—The x-ray crystal structures of murine chronophinWT and murine chronophinKK have been deposited in the Protein Data Bank under accession codes 4BX3 (murine chronophin), 4BX2 (murine chronophin in complex with BeF$_3$), and 4BX0 (murine chronophin A194K,A195K).

Statistical Analysis—Pulldown and ITC experiments were analyzed with the two-tailed unpaired t test using GraphPad Prism version 6.

RESULTS

Homooligomer Formation Is a Common Feature of C2a-type HAD Hydrolases—We analyzed a data set comprising all available HAD hydrolase structures from various species (see “Experimental Procedures” for details). Of the 177 unique entries, 104 HAD hydrolases (59%) are likely to form homooligomers as compared with 72 structures that are assigned as monomers and with one heterooligomer. As shown in Table 1, C2a-type enzymes are particularly noteworthy because all available structures represent dimers (12 entries) or tetramers (five entries). However, the function of HAD hydrolase...
dimerization is unclear as all catalytic core residues are encoded in a single polypeptide chain, and the available structures of oligomeric HAD hydrolases show that the adjacent protomer does not contribute active site residues. To understand the role of HAD dimerization, we investigated mammalian chronophin as a representative C2a-type family member.

Crystallization and Structure Determination of Murine Chronophin—We determined the three-dimensional structure of murine chronophin by x-ray crystallography and refined it by molecular replacement with human chronophin (Protein Data Bank code 2OYC and Ref. 9) to 2.2-Å resolution with an R work of 17.1% and an R free of 20.7% (Protein Data Bank code 4BX3). Data collection and refinement statistics are given in Table 2.

The alignment of human and murine chronophin structures (Protein Data Bank codes 2OYC and 4BX3) shows an almost perfect superposition with a r.m.s.d. of 0.61 Å determined for the Ca atoms of residues 1–290. Both orthologs crystallize as homodimers via the cap domain as shown for murine chronophin by x-ray crystallography and refined it by r.m.s. root mean square.

### Table 1

| Oligomeric states of structurally characterized HAD-type hydrolases |
|------------------|
| Structural HAD subfamily | C0 | C1 | C1 + C2 | C2a | C2b | Structures/oligomeric state |
|------------------|
| Monomers         | 11 | 40 | 6       | 12  | 15  | 72                             |
| Dimers           | 3  | 43 | 6       | 11  | 11  | 75                             |
| Trimmers         | 1  | 2  | 1       | 1   | 1   | 2                              |
| Tetraders        | 10 | 4  | 5       | 1   | 20  |                                |
| Hexamers         | 4  | 1  | 5       | 1   | 5   | 1                              |
| Octamers         | 4  | 1  | 1       | 1   | 1   | 1                              |
| Dodecamers       | 1  | 1  |         |     |     |                                |
| Heterodimers     | 28 | 93 | 12      | 17  | 27  | 177                            |

### Table 2

Data collection and refinement statistics

|                      | Chronophin<sup>WT</sup> | Chronophin<sup>WT</sup>-BeF<sub>3</sub> | Chronophin<sup>KK</sup> |
|----------------------|--------------------------|----------------------------------------|--------------------------|
| **Data collection**  |                          |                                        |                          |
| Wavelength (Å)       | 0.91841                  | 0.91841                                | 0.91841                  |
| Space group          | 123                      | 123                                    | P2                       |
| Unit cell parameters |                          |                                        |                          |
| a, b, c (Å)          | 167.10, 167.10, 167.10   | 166.83, 166.83, 166.83                 | 36.13, 91.82, 39.19      |
| α, β, γ (°)          | 90.00, 90.00, 90.00      | 90.00, 90.00, 90.00                    | 90.00, 90.00, 90.00      |
| Resolution range (Å)<sup>a</sup> | 44.66–2.19 (2.31–2.19) | 44.59–2.19 (2.31–2.19) | 33.32–1.75 (1.84–1.75) |
| R<sub>work</sub>     | 0.082 (0.858)            | 0.116 (1.12)                           | 0.093 (0.658)            |
| R<sub>free</sub>     | 0.034 (0.358)            | 0.048 (0.472)                          | 0.049 (0.347)            |
| R<sub>free</sub>     | 12.9 (2.2)               | 10.2 (1.8)                             | 10.2 (2.3)               |
| Completeness (%)     | 99.9 (99.7)              | 100 (100)                              | 99.8 (99.9)              |
| Multiplicity         | 6.8 (6.7)                | 6.7 (6.6)                              | 3.5 (3.5)                |
| Total reflections    | 269,875                  | 266,023                                | 89,076                   |
| Unique reflections   | 39,743 (5,736)           | 39,582 (5,743)                         | 25,494 (3,709)           |

| **Refinement**       |                         |                                        |                          |
| Wilson B-factor (Å<sup>2</sup>) | 47.0                     | 41.1                                    | 19.1                     |
| Average B-factor (Å<sup>2</sup>) | 48.4                     | 45.5                                    | 28.6                     |
| Macromolecules       | 48.7                     | 45.8                                    | 28.0                     |
| Solvent              | 40.6                     | 39.2                                    | 34.2                     |
| R<sub>cryst</sub>    | 0.1707                   | 0.1678                                  | 0.1913                   |
| R<sub>free</sub>     | 0.2067                   | 0.2123                                  | 0.2363                   |
| Number of non-hydrogen atoms | 4,645                  | 4,676                                    | 2,496                    |
| Macromolecules       | 4,453                    | 4,460                                    | 2,247                    |
| Ligands              | 14                       | 28                                      | 7                       |
| Water                | 178                      | 188                                     | 242                      |
| r.m.s. deviations in |                          |                                        |                          |
| Bond lengths (Å)     | 0.015                    | 0.014                                   | 0.005                    |
| Bond angles (°)      | 1.640                    | 1.567                                   | 1.006                    |
| Planar groups (Å)    | 0.010                    | 0.010                                   | 0.004                    |
| Dihedral angles (°)  | 15.91                    | 15.31                                   | 13.14                    |
| Coordinate error (Å)<sup>b</sup> | 0.25                   | 0.27                                   | 0.28                     |
| Ramachandran statistics<sup>c</sup> | 96.92                  | 98.29                                   | 99.32                    |
| Favored (%)          | 2.22                     | 1.54                                    | 0.34                     |
| Allowed (%)          | 0.85                     | 0.17                                    | 0.34                     |
| Outliers (%)         | 30.9                     | 11.13                                   | 11.63                    |

<sup>a</sup> Numbers in parentheses refer to the respective highest resolution data shell in the data set.
<sup>b</sup> R<sub>cryst</sub> = Σ(|F<sub>i</sub>| - |F<sub>c</sub>|) / Σ|F<sub>c</sub>|, where F<sub>i</sub> and F<sub>c</sub> are the observed and calculated structure factor amplitudes.
<sup>c</sup> Ramachandran statistics indicate the fraction of residues in the favored, allowed, and disallowed regions of the Ramachandran diagram as defined by MolProbity (54).
Role of Chronophin Dimerization

Characterization of chronophinKK.

Creation of a Monomeric Murine Chronophin Variant—To study the functional relevance of chronophin homodimerization, we exchanged the short, uncharged side chains of Ala194 and Ala195 in the murine chronophin dimerization interface (see Fig. 1A, inset) for the longer and charged side chains of Lys (chronophinKK) and compared the oligomeric states of recombinantly expressed, purified chronophinWT and chronophinKK by size exclusion chromatography. Fig. 1B shows that chronophin (molecular mass, 31.8 kDa) has a peak elution volume that corresponds to a calculated molecular mass of 60.7 kDa, indicating that the protein indeed forms a stable dimer in solution. In contrast, chronophinKK has a peak elution volume corresponding to a calculated molecular mass of 33.7 kDa, equivalent to chronophin in a monomeric state. Analytical ultracentrifugation sedimentation velocity experiments confirmed a monomeric molecular mass for chronophinKK (~32 kDa), whereas wild type chronophin showed a sedimentation behavior consistent with a dimeric state (~56 kDa) (Fig. 1C). Furthermore, in AFM imaging experiments, chronophinKK showed protein volumes that are consistent with a monomeric state of this variant (30 ± 13 kDa for chronophinKK compared with 63 ± 18 kDa for chronophinWT) (Fig. 1D).

To determine the oligomeric states of chronophinWT and chronophinKK in mammalian cells, we simultaneously expressed GST-tagged and untagged versions of chronophinWT and chronophinKK in HEK293 cells, performed pulldown binding experiments with glutathione-Sepharose beads, and subsequently probed bead-associated chronophin by Western blotting. The expectation is that GST-chronophinWT will pull down untagged chronophinWT, whereas the monomerized chronophinKK will prevent co-precipitation. Fig. 1E demonstrates that GST-chronophinWT (detectable at ~60 kDa after separation of the bead eluates by SDS-PAGE and immunoblotting with α-chronophin antibodies) indeed associated with molecular masses of ~56 and ~32 kDa, respectively. D, atomic force microscopy images of chronophinWT and chronophinKK. Top, images are 500 × 500 nm with a height scale of 0.75 nm. Bottom, Gaussian fits to statistical volume distributions for chronophinWT and chronophinKK give maxima of 70 nm³ (337 particles) and 30 nm³ (1,409 particles), respectively, which translate into a molecular masses of 63 and 30 kDa, respectively. A fraction of chronophinWT also shows protein volumes consistent with a monomeric state. E, GST pulldown experiments. GST-tagged chronophinWT/chronophinKK (GST-chron. W/GST-chron. KK) and untagged chronophinWT/chronophinKK (chronophin WT/chronophin KK) were co-expressed in HEK293-AD cells as indicated. GST-chronophin was precipitated with glutathione-Sepharose beads, and subsequently probed bead-associated chronophin by Western blots. The optical density of the signal corresponding to chronophinWT precipitated with GST-chronophinWT (Fig. 1F) was normalized to the GST-chronophin signal in each lane and compared. **p < 0.01 (2-tailed t-test). The mean values ± S.E. (error bars) of the relative intensities are shown (n = 4). mAU, milliabsorbance units; AU, absorbance units.
chromophin<sup>WT</sup> (detectable at ~32 kDa) in pulldown binding assays, whereas an interaction of GST-chromophin<sup>KK</sup> with chromophin<sup>KK</sup> was not detectable. The results of four independent experiments are quantified in Fig. 1F. Together, these data clearly show that chromophin<sup>KK</sup> is a monomer both in vitro and in mammalian cells.

**Enzymatic Properties of Monomeric Chromophin**—We next investigated the effect of chromophin monomerization on its enzymatic activity toward PLP. Fig. 2 demonstrates that whereas homodimeric chromophin efficiently dephosphorylates PLP, the activity of the monomeric chromophin<sup>KK</sup> variant is strongly impaired. This is mainly due to a ~65-fold increase in <i>K<sub>m</sub></i><sup>WT</sup>, whereas the calculated <i>V<sub>max</sub></i><sup>WT</sup> remains largely unaffected. As a result, the catalytic efficiency (<i>k<sub>cat</sub>/K<sub>m</sub></i>) of chromophin<sup>KK</sup> is reduced to ~3.5% compared with chromophin<sup>WT</sup>. The catalytic constants of chromophin<sup>WT</sup> and chromophin<sup>KK</sup> toward PLP are summarized in Table 3.

**Isothermal Titrregation Calorimetry of BeF<sub>3</sub> Binding to Chronophin<sup>WT</sup> and Chronophin<sup>KK</sup>**—To test whether the impaired catalytic efficiency of chromophin<sup>KK</sup> is due to rearrangements in catalytic core residues, we measured the binding of chromophin to BeF<sub>3</sub>. BeF<sub>3</sub> structurally mimics the phosphosparatate transition state of HAD phosphatases by coordinating the catalytic core residues and the catalytically essential Mg<sup>2+</sup> as a phosphate analog (30–36). Fig. 3 shows that the interaction of BeF<sub>3</sub> with chromophin<sup>WT</sup> and chromophin<sup>KK</sup> can be optimally fitted to a one-site binding model. The stoichiometry of BeF<sub>3</sub> binding is ~0.85 for chromophin<sup>WT</sup> and chromophin<sup>KK</sup>, indicating an equimolar interaction in both cases (Table 4). The apparent deviation from the 1:1 binding ratio is likely due to protein precipitation issues during the experiment. Thus, chromophin<sup>WT</sup> and chromophin<sup>KK</sup> bind BeF<sub>3</sub> with a comparable stoichiometry. Surprisingly, however, the BeF<sub>3</sub> binding constant of chromophin<sup>KK</sup> is about 3-fold higher than that of chromophin<sup>WT</sup>. Furthermore, the quantity of released heat (∆H), the entropic contribution (∆S) upon BeF<sub>3</sub> binding, and the resulting free Gibbs free energy (∆G) values also differ significantly between chromophin<sup>WT</sup> and chromophin<sup>KK</sup> (Table 4). Together, the in vitro phosphatase and ITC experiments suggest that the chromophin homodimer interface in the cap domain exerts allosteric effects on the catalytic cleft of the enzyme.

**Crystallization and Structure Determination of Chronophin<sup>KK</sup>**—Therefore, we solved the structure of the monomerized chromophin variant. Chromophin<sup>KK</sup> crystallized in the space group P2<sub>1</sub> and could be refined to 1.75-Å resolution with an R<sub>work</sub> of 19.1% and an R<sub>free</sub> of 23.6% (Protein Data Bank code 4BX0 and Table 2). The r.m.s.d. of 0.47 Å for the Ca atoms of residues 1–290 between chromophin<sup>WT</sup> and chromophin<sup>KK</sup> clearly shows that the replacement of Ala<sup>194</sup> and Ala<sup>195</sup> with Lys residues in the cap domain had no impact on the overall fold of a chromophin protomer (Fig. 4A). However, the substrate specificity loop in chromophin<sup>KK</sup> is tilted by ~25° compared with chromophin<sup>WT</sup> (measured between the Ca atoms of Asp<sup>182</sup> and Pro<sup>187</sup> of the respective molecules). The enlarged areas in Fig. 4A show that in chromophin<sup>WT</sup> residues Trp<sup>177</sup> and Arg<sup>186</sup> in
Role of Chronophin Dimerization

the substrate specificity loop (Pro\textsuperscript{176}–Pro\textsuperscript{187}) of protomer A stack together with Arg\textsuperscript{163} of protomer B, which itself forms a hydrogen bond with the backbone carbonyl oxygen of Gly\textsuperscript{183} in protomer A. Importantly, the imidazole ring of His\textsuperscript{178} in the substrate specificity loop coordinates the pyridine ring of PLP by \(\pi\)-electron stacking. PLP has been introduced from Protein Data Bank code 2P69 to visualize purposes. The altered substrate specificity loop positioning in chronophin\textsubscript{KK} causes a reorientation of His\textsuperscript{178} with its imidazole side chain now being almost perpendicular to the PLP pyridine ring. Right panels, back views of the structures. The detailed views in the lower panels reveal that in chronophin\textsubscript{KK}, Trp\textsuperscript{177} and Arg\textsuperscript{185} in the substrate specificity loop of protomer A stack with Arg\textsuperscript{163} of protomer B (which is not shown in the upper panels). Arg\textsuperscript{163} additionally forms a hydrogen bond to the backbone carbonyl oxygen of Gly\textsuperscript{183} in the substrate specificity loop of protomer A. These interprotomer interactions are absent in monomeric chronophin\textsubscript{KK}. B, electron density maps of residues 170–190. Shown are the refined 2\(F_\text{c}\) − \(F_\text{c}\) electron density maps contoured at an r.m.s.d. of 1 overlaid on the models of chronophin\textsubscript{WT} (gray) and chronophin\textsubscript{KK} (red). C, superposition of the active sites of chronophin\textsubscript{WT} (gray; Protein Data Bank code 4BX3) and chronophin\textsubscript{KK} (red; Protein Data Bank code 4BX2). Chronophin\textsubscript{KK} is additionally shown in the BeF\textsubscript{3}\textsuperscript{−}-liganded state (cyan; Protein Data Bank code 4BX2). Catalytically essential residues of the HAD motifs I–IV (roman numerals) are indicated.

To elucidate whether all catalytic core residues in monomeric chronophin are oriented correctly, we compared the HAD motif I–IV catalytic core residues of chronophin\textsubscript{KK} and chronophin\textsubscript{WT} with chronophin\textsubscript{WT} in complex with BeF\textsubscript{3}\textsuperscript{−}. The chronophin\textsubscript{WT}-BeF\textsubscript{3}\textsuperscript{−} structure was refined to 2.2-Å resolution with an \(R_{\text{work}}\) of 16.8% and an \(R_{\text{free}}\) of 21.2% (Protein Data Bank code 4BX2; see Table 2 for data collection and refinement statistics). (Note that we were unable to crystallize chronophin\textsubscript{KK} in complex with BeF\textsubscript{3}\textsuperscript{−}. Co-crystallization attempts failed due to protein precipitation issues, and soaking experiments resulted in broken crystals). Fig. 4C shows that the positioning of these amino acids does not differ substantially between chronophin\textsubscript{WT} and chronophin\textsubscript{KK} and further confirms that the cofactor Mg\textsuperscript{2+} is properly coordinated. We conclude from these results that the impaired binding of BeF\textsubscript{3}\textsuperscript{−} to monomeric chronophin (see Fig. 3 and Table 3) is not due to conformational changes in the active site residues of chronophin\textsubscript{KK}.

Together, the results of the steady-state enzyme kinetics, the ITC experiments, and the structural analyses support the conclusion that the core catalytic machinery of the monomeric chronophin\textsubscript{KK} variant is unaltered compared with homodimeric chronophin. However, the tilting of the substrate specificity loop and the subsequent reorientation of the PLP-binding His\textsuperscript{178} residue in the cap domain of the monomer lead to a marked increase in the \(K_\text{m}\) value toward the substrate PLP. The reduced binding affinity (albeit identical binding stoichiometry) of chronophin\textsubscript{KK} to BeF\textsubscript{3}\textsuperscript{−} compared with chronophin\textsubscript{WT} may thus indicate a diminished accessibility of the active site for BeF\textsubscript{3}\textsuperscript{−} due to the tilted substrate specificity loop.

Potential General Role of Dimerization for the Function of C2-capped HAD Phosphatases—Are the mode and function of chronophin dimerization for the positioning of the substrate specificity loop/\(\beta\)-hairpin a unique feature of this phosphatase or a characteristic trait of C2a-capped HAD hydrolases in general? We addressed this question by performing a DALI structure similarity search with human chronophin (Protein Data Bank code 2OYC) as a search model. The results were manually curated to select members of the C2a subfamily of HAD hydrolases. In accordance with the results shown in Table 1, we found 16 structures in addition to human chronophin and the newly solved structure of murine chronophin. The r.m.s.d. values for structural alignments of all Cα atoms of these family members with murine chronophin indicate high structural homology, although the amino acid sequence identities between these proteins and murine chronophin are very low (ranging from 16.1 to 28.3% with the exception of human chronophin that is 91.2% identical with murine chronophin). PDBePISA analysis indicates that all 18 molecules are highly likely to exist as homodimers as judged by the buried surface areas of \(\sim 1,000 \text{ Å}^2\) at the respective dimer interfaces. Five of them possibly form tetramers (dimers of dimers). The C2a-capped HAD hydrolases, r.m.s.d. values, and results of the PDBePISA analysis are listed in Table 5.

Fig. 5 shows the dimer interfaces of the structurally characterized homodimeric C2a-type HAD hydrolases identified in this analysis. All dimer interfaces are composed of two homol-
TABLE 5
Oligomeric states of structurally characterized C2a-type HAD phosphatases

| UniProtKB (Protein Data Bank code) | Organism                        | r.m.s.d. compared with 4BX3 | Dimer interface (Å) | ∆G (kcal/mol) |
|-----------------------------------|---------------------------------|-----------------------------|---------------------|---------------|
| Q96GD0 (2OYC)                     | Homo sapiens                    | 0.67                        | 0.136 (−16.4)       |               |
| P60487 (4BX3)                     | Mus musculus                    | −                           | 0.992 (−14.7)       |               |
| Q9H0R4 (3HLT)                     | H. sapiens                      | 2.47                        | 1.315 (−13.9)       |               |
| Q8VGR5 (2HO4)                     | M. musculus                     | 3.08                        | 1.300 (−13.6)       |               |
| Q9H09S (2X4D)                     | H. sapiens                      | 2.17                        | 1.307 (−17.9)       |               |
| P36151 (3RF6)                     | Saccharomyces cerevisiae        | 10.61                       | 1.866 (−25.7)       |               |
| Q52125 (3PDW)                     | Bacillus subtilis               | 2.69                        | 1.203 (−15.3)       |               |
| Q11556 (2HX1)                     | Cytophaga hutchinsonii         | 2.44                        | 0.980 (−13.7)       |               |
| ATCC 33406                        |                                 | −                           | 1.273 (−5.2)        |               |
| Q856C7 (1YV9)                     | Enterococcus faecalis V583     | 1.66                        | 1.151 (−12.3)       |               |
| P0AF24 (2C4N)                     | E. coli K-12                    | 2.02                        | 1.097 (−23.8)       |               |
| L7N4Y2 (419F)                     | Mycobacterium tuberculosis     | 2.31                        | 1.072 (−6.6)        |               |
| Q95622 (1ZJ1)                     | Pyrococcus horikoshii OT3      | 1.78                        | 1.146 (−19.6)       |               |
| Q8EO44 (3EPR)                     | Streptococcus agalactiae       | 1.69                        | 1.162 (−18.9)       |               |
| Q8D476 (1WVI)                     | Streptococcus mutans UA159     | 1.94                        | 1.143 (−12.9)       |               |
| Q97Q24 (1YDF)                     | Streptococcus pneumoniae       | 2.13                        | 1.048 (−5.8)        |               |
| Q99Z4W4 (1Y59)                    | Streptococcus pyogenes         | 1.67                        | 1.157 (−15.3)       |               |
| Q9X264 (1VJR)                     | Thermotoga maritima            | 1.91                        | 1.127 (−14.1)       |               |
| Q29873 (3QGM)                     | Archaeoglobus fulgidus         | 1.71                        | 1.086 (−4.8)        |               |
|                                 |                                 |                             | 1.315 (−23.8)       |               |

* Buried surface areas and ∆G were calculated using the PDBePISA online tool. ∆G, solvation-free energy gain upon formation of the interface (not taking into account the effect of satisfied hydrogen bonds and salt bridges across the interface). Negative ∆G values correspond to hydrophobic interfaces or to positive protein affinity (23).

* Values in italics correspond to potential tetramer interfaces.

DISCUSSION

Substrate specificity in capped HAD phosphatases is accomplished by cap movements that facilitate active site solvent exclusion and by specificity determinants encoded in the cap domain. We identify homodimerization via C2a-type caps as a previously unrecognized factor involved in substrate specificity control. We demonstrate that the constitutive homodimerization of chronophin is a prerequisite for the proper positioning of the substrate specificity loop and consequently for efficient PLP dephosphorylation. Thus, the specificity of chronophin toward PLP as a substrate depends on an allosteric effect induced by a homophilic intermolecular interaction. Crystal structure analyses of oligomeric C2a-type HAD hydrolases from different species reveal that the positioning of the β-hairpin/resumeable substrate specificity loop via homodimerization is a conserved and common feature in this structural subfamily. Our results therefore suggest a general principle of how the dimerization of C2a-capped HAD hydrolases can contribute to substrate specificity.

Although the role of homodimerization in the C2a family has not been investigated so far, the relevance of oligomerization for C1- and C0-type HAD hydrolase functions was addressed in previous studies. For example, homodimerization of the...
C1-type Haemophilus influenzae P4 acid phosphatase is important for catalysis because side chains of one protomer stabilize the conformation of catalytic loop IV in the active site of the other protomer. Such intersubunit contacts that reach into the catalytic site may also be important for substrate recognition (37). The C1-capped mammalian cytosolic 5′-nucleotidase (cN-II) exists as a tetramer in its native form. Although a dimeric mutant of this protein is still active, monomeric cN-II is inactive, suggesting that enzymatic activity may be controlled by switching the oligomeric state, although the underlying mechanism is unknown (38, 39). Structural analysis of the tetrameric, C0-type (capless) HAD phosphatase KdsC from E. coli has revealed that protomers can act as cap surrogates to shield the active site of an adjacent protomer and to supply residues involved in substrate recognition (40). A similar tetramerization organization was reported for the Bacteroides thetaiotaomicron KDN-9-P phosphatase (41). Homooligomeric interfaces may also contribute to proper folding or stability of the active site as has been proposed for the bifunctional C0-type T4 polynucleotide kinase/phosphatase PNKP (42). Taken together, the constitutive dimer formation of oligomeric HAD phosphatases appears to be generally required for appropriate catalytic activity and can also contribute to substrate coordination. Our finding that homodimerization is necessary for the proper orientation of the β-hairpin/substrate specificity loop in the C2a-capped HAD phosphatase chronophin further advances our mechanistic understanding of HAD phosphatase specificity control.

Self-association of proteins to form dimers or higher order oligomers is frequently observed (43). Oligomerization provides a simple way to increase protein complexity and can result in structural (e.g. improved stability) and functional advantages (e.g. control over active site accessibility and specificity) (44). In some oligomeric proteins, protomers must be stably assembled to build a functional protein; this may be the case for the constitutively oligomeric HAD phosphatases characterized to date. Some “classical,” non-HAD-type phosphatases such as E. coli alkaline phosphatase are also known to form obligate homodimers and to lose structural stability and catalytic activity upon monomerization (45). In other homologous phosphatases, the association and dissociation of protomers is reversible and can serve as a (concentration-sensing) mechanism to regulate enzyme activity (43, 46–48). Some receptor-like protein-tyrosine phosphatases utilize this mechanism of dynamic regulation. For example, protein-tyrosine phosphatase α, CD45, and SAP-1 are reversibly inhibited by dimer formation, which can be controlled by ligand binding (49), phosphorylation (50), or oxidative stress (51). Here, dimerization leads to a reciprocal occlusion of the catalytic site of each phosphatase protomer (52, 53). It is currently unknown whether those HAD phosphatases that are assigned as monomeric can associate to form homo- or heterooligomers in a stimulus-dependent manner in cells. Conversely, it is unclear whether oligomeric C2a-type HAD hydrolases such as chronophin can also be present in a monomeric form in vivo, for example below a threshold concentration, in particular subcellular compartments, or upon posttranslational modification.

Acknowledgments—We thank Anna-Karina Lamprecht for technical assistance and the BESSY staff at beamline BL14.1.

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VOLUME 289 • NUMBER 5 • JANUARY 31, 2014
