Supplemental Data

Structural Basis for Inactivation of the Human

Pyruvate Dehydrogenase Complex by Phosphorylation:

Role of Disordered Phosphorylation Loops

Masato Kato, R. Max Wynn, Jacinta L Chuang, Shih-Chia Tso, Mischa Machius, Jun Li, and David T. Chuang

SUPPLEMENTAL RESULTS AND DISCUSSION

Disordered Phosphorylation Loops Are Present in Apo Y89F-α Mutant E1p

The Y89F-α mutation results in apo-E1p, as indicated by the absence of the reconstituted PDC activity without added ThDP and reduced affinity for ThDP (Table 3). It is noteworthy that with DCPIP as an electron acceptor, Y89F-α E1p shows 10.4-fold higher E1p-catalyzed decarboxylation activity than the wild type. We showed previously that the corresponding Tyr113-α in the related E1b component of the human BCKDC functions as a molecular switch that down-regulates E1b-catalyzed decarboxylation rate (Machius et al., 2006). The Y113F-α E1b mutant, in which the Tyr113-α ligand to cofactor ThDP is disrupted, shows a similar increase to the Y89F-α E1p variant in the rate of decarboxylation. The increased decarboxylation rate in Y113F-α E1b was detected both in the presence and absence of the artificial electron acceptor DCPIP (Machius et al., 2006). In parallel, the Y89F-α E1p variant shows similar fold increases in the decarboxylation rate, when assayed either with or without DCPIP (data not shown). Thus, the elevated E1p-catalyzed decarboxylation rate is intrinsic to the Y89F-α E1p variant, and does not result from an enhanced accessibility of DCPIP to the E1p active sites.

To investigate the Ph-loop conformations of apo E1P, we determined the structure of Y89F-α mutant E1p co-crystallized with the subunit-binding domain (SBD) of E2p without ThDP supplement. Since the asymmetric unit contains one E1p heterodimer (αβ), a model of SBD could not be built due to averaging of electron density for SBD (Machius et al., 2006). The overall structure of Y89F-α E1p is virtually identical to that of wild-type E1p (r.m.s.d. = 0.3 Å for 567 equivalent Cα atoms in E1p αβ heterodimer). Since ThDP was not supplemented in crystallization, no electron density for ThDP is visible in the structure. As expected, both Ph-loops A and B are largely disordered (Figure S1B). The N-terminus of the symmetry-related molecule extends into the empty active site (not shown). Since the N-terminal region harbors exogenous residues (His_6-tag plus thrombin recognition sequences) from the cloning vector, this crystal packing is of no physiological significance. Importantly, in the wild-type E1p structure, Ph-loops are well ordered with the bound ThDP (Figure 3A). Thus, the apo Y89F-α E1p structure supports the thesis that the bound ThDP induces a disorder-to-order transition of the Ph-loops, analogous to that observed in the related E1b component of BCKDC (Li et al., 2004; Nakai et al., 2004).

Phosphorylated S1-E1p Is in the Apo Form When Crystallized in the Presence of Mg-ThDP

Phospho-S1-E1p was crystallized in the presence of 10 mM Mg-ThDP. The crystals exhibit the symmetry of space group P1 with eight E1p heterotetramers in the asymmetric unit. The overall structure of phospho-S1-E1p is very similar to that of wild-type and S1-E1p. However, none of the E1p heterotetramers contains the cofactor Mg-ThDP in the active sites. The crystals thus contain apo phospho-S1-E1p. This result is in good agreement with 100-fold weaker binding of Mg-ThDP to phospho-S1-E1p compared to the wild type (Table 3). All of the 16 E1p-α subunits

in the asymmetric unit show disordered Ph-loops that can be classified into two groups according to the degree of disorder.

The first group comprises half of the 16 E1p-α subunits, which feature completely disordered phosphorylation loops. The disordered regions include residues Gly262-α to Arg282-α in phosphorylation loop (Ph-loop) A and residues Gly198-α to Glu205-α in Ph-loop B (Figure S1C). There is no symmetry-related molecule near the completely disordered Ph-loops in this group. The remaining half of the E1p-α subunits exhibit a partially disordered Ph-loop A and a completely disordered Ph-loop B (Figure S1D). Five to nine residues starting from Gly262-α toward the C-terminus in Ph-loop A of this group do not have corresponding electron densities. Interestingly, the α-helix in Ph-loop A of this group is extended by 2 turns toward the N-terminus in comparison to the corresponding α-helix in wild-type Ph-loop A (compare Figure 3A and S1D). As a result, the phosphorylation site 2 is located in this extended α-helix. This alternative conformation of the Ph-loop A is stabilized by interactions between the α-helix formed in this Ph-loop and a symmetry-related E1p molecule (not shown).

In the non-phosphorylated wild-type E1p structure, cofactor ThDP communicates with the Ph-loops of E1p (Figure 5), which explains the disordering of the Ph-loops in the apo Y89F-α E1p structure (Figure S1B). Since Mg-ThDP is absent from all heterotetramers in the apo phospho-S1-E1p structure, it is impossible to discern whether the phosphorylation of site 1, the absence of bound ThDP or a combination of both impart the disordering of the Ph-loops. To approach this problem, phospho-S1-E1p was crystallized in 10 mM ThDP and 10 mM $MnCl_2$ (Mn-ThDP). Mn-ThDP has a significantly higher binding affinity than Mg-ThDP for phospho-S1-E1p (Figure S3). See the main text for details.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Construction, Expression and Purification of Recombinant Proteins

Site-directed mutagenesis was carried out on the expression plasmid for N-terminally His_6-tagged wild-type E1p using the QuickChange mutagenesis kit from Stratagene (La Jolla, CA). Each of the mutant plasmid and the pGroESL plasmid for over-expressing chaperonins GroEL and GroES were co-transformed into *Escherichia coli* BL-21(DE3) cells; the cells were selected against both kanamycin and chloramphenicol. Expression of the wild-type and mutant E1p proteins was carried out similar to that described previously for the E1b protein of human BCKDC (Chuang et al., 2000). Bacterial lysates were obtained by sonication, and recombinant E1p proteins were extracted by Ni-NTA resin. The isolated proteins were dialyzed against 50 mM potassium phosphate buffer, pH 7.5, 10 mM KCl, 5% glycerol, 20 mM β-mercaptoethanol and 2 mM EDTA. The dialyzed protein was further purified by anion exchange chromatography (Resource Q, GE Healthcare, Piscataway, NJ) and size exclusion chromatography (Superdex 200, GE Healthcare, Piscataway, NJ).

Preparation of the subunit-binding domain of human E2p (pSBD) was carried out as described previously from a recombinant fusion protein LBD-pSBD, which contained LBD from the E2b of human BCKDC, followed by a tobacco-etch virus (TEV) protease recognition sequence (LENLYFQG) and pSBD (residues 346-401 of E2p) (Brautigam et al., 2006). Production of the E2p/E3BP core was carried out as described previously (Kato et al., 2006). The lipoylated L2 domain was produced also as described previously (Kato et al., 2005). The production of SUMO-PDK4 is reported elsewhere (Wynn et al., 2008).

Phosphorylation of E1p

The phosphorylation reaction mixture contained 2.0 μM S1-E1p or S2-E1p and 1.0 μM SUMO-PDK4 in 20 mM potassium phosphate (pH 7.5), 0.1 mM EDTA, 1 mM $MgCl_2$, 2 mM DTT. The concentration of E1p is calculated based on the molecular mass of the E1p heterotetramer (156,564 Da), unless otherwise indicated. The concentration of SUMO-PDK4 is according to the calculated dimer molecular mass of 116,562 Da. The reaction was initiated by adding [γ^{32}P]ATP to 0.4 mM. Aliquots were taken from the reaction mixture at different time points and mixed with 4x SDS-PAGE sample buffer (volume ratio 3: 1) to terminate the reaction. Samples were subjected to electrophoresis in 12% SDS-PAGE gels. The degree of phosphorylation of E1p was

estimated by phosphorimaging of the band corresponding to the E1p-α subunit. The amount of [^{32}P] phosphate incorporated per E1p heterotetramer was converted to % phosphorylation.

To scale up production of the fully phosphorylated protein, S1-E1p was incubated with tag-free PDK4 at the molar ratio of S1-E1p: PDK4 = 4: 1 at room temperature for 120 min. The phosphorylated S1-E1p was separated from PDK4 by Ni-NTA extraction and further purified by anion exchange chromatography (Resource Q, GE Healthcare, Piscataway, NJ). The purified S1-E1p with complete phosphorylation at Ser264-α (site 1) was concentrated and used for kinetic and crystallographic studies.

Enzyme Assays

To assay for activity of the overall reaction catalyzed by the PDC (Reaction 1), the reaction mixture (0.5 ml) contained 30 mM potassium phosphate (pH 7.5), 100 mM NaCl, 3 mM NAD^+, 0.4 mM CoA, 2 mM $MgCl_2$, 2 mM DTT, 2 mM ThDP and the PDC reconstituted with 10 nM E1p, 1.9 nM lipoylated E2p/E3BP and 24 nM E3. The oxidative decarboxylation of pyruvate at 23 °C was initiated by the addition of various concentrations of pyruvate (0.4 to 4,000 μM). The rate of NAD^+ reduction was monitored spectrophotometrically at 340 nm.

To assay for activity of the E1p-catalyzed decarboxylation (Reaction 2), the reaction mixture in 1 ml contained 50 mM potassium phosphate (pH 7.5), 1 mM ThDP, 2 mM $MgCl_2$ and 150 nM E1p in the presence of 0.05 mM 2,6-dichlorophenolindophenol (DCPIP) as an artificial electron acceptor. The reaction at 23 °C was initiated by the addition of pyruvate (0.4 to 4,000 μM). The reduction of DCPIP was monitored spectrophotometrically at 600 nm.

To assay for the E1p-catalyzed reductive acetylation of the lipoylated L2 domain (Reaction 3), the reaction mixture in 0.25 ml contained 50 mM potassium phosphate (pH 7.5), 1 mM ThDP, 2 mM $MgCl_2$, 2.0 mM [2-^{14}C]pyruvate (specific activity 7,097 cpm/nmol), 260 nM E1p, and 20 μM lip-L2. The reaction was initiated by the addition of the radiolabeled pyruvate. After incubation at 22 °C for 5 min, the reaction was terminated by adding trichloroacetic acid to a final concentration of 10% (w/v), followed by the addition of 0.2% (w/v) bovine serum albumin as a protein carrier. The pellets were washed three times with 10% trichloroacetic acid to remove excess [2-^{14}C]pyruvate and any radiolabeled ThDP intermediate released from the denatured E1p protein. The radioactivity incorporated into the lip-L2 domain was counted in a scintillation counter.

Crystallization and Data Collection

Wild-type E1P crystals were obtained using the hanging drop vapor diffusion technique by mixing 2 μl of the protein solution (11.8 mg/ml wild-type E1p in 50 mM potassium phosphate buffer, pH 7.5, 5% (v/v) glycerol) and 2 μl of the reservoir solution (8-11% (w/v) PEG-8000, 0.15-0.3 M sodium acetate, 5-10% (w/v) glucose, 0.05 M taurine, 10 mM tris(2-carboxyethyl)phosphine, 10 mM $MnCl_2$ and 0.1 M Bis-Tris, pH 6.5). Crystals were transferred to a reservoir solution supplemented with 25% (v/v) glycerol and 10 mM Mn-ThDP for 30 min before flash-cooling by plunging into liquid propane. S1-E1P crystals were obtained by mixing 2-μl of the protein solution (9 mg/ml protein in 50 mM HEPES, pH 7.5, 50 mM KCl, 5% (v/v) glycerol, 10 mM DTT and 10 mM Mg-ThDP) with 2 μl of the reservoir solution. Crystals were transferred to the reservoir solution supplemented with 25% (v/v) glycerol and 10 mM Mg-ThDP and flash-cooled by plunging into liquid propane. Apo phospho-S1-E1P crystals were produced by mixing 2 μl of the protein solution (3.7 mg/ml protein in 50 mM Hepes, pH 7.5, 50 mM KCl, 5% (v/v) glycerol, 10 mM Mg-ThDP and 10 mM DTT) with 2 μl of the reservoir solution. Crystals were transferred to reservoir solution supplemented with 10 mM Mg-ThDP and 25% (v/v) glycerol and flash-cooled by plunging into liquid propane. Phospho-S1-E1P crystals with Mn-ThDP were obtained by mixing 2 μl of the protein solution (3.7 mg/ml protein in 50 mM HEPES, pH 7.5, 50 mM KCl, 5% glycerol, 10 mM DTT and 10 mM Mn-ThDP) and 2 μl of the reservoir solution. Crystals were transferred into reservoir solution supplemented with 20 mM Mn-ThDP and 25% (v/v) glycerol and flash-cooled by plunging into liquid propane. The Y89F-α E1p/pSBD protein complex was obtained by mixing the E1p mutant with pSBD protein in a 1: 2 molar ratio. Crystals were obtained using the vapor diffusion method by mixing 2 μl E1p/pSBD (16 mg E1p/ml) with 2 μl well solution containing 10% PEG 6000, 1.5 M NaCl and 0.1 M Bis-

Tris, pH 6.5. Crystals were cryoprotected in 12% PEG 6000, 1.5 M NaCl, 0.1 M Bis-Tris, pH 6.5 and 20% glycerol.

Diffraction data were collected using synchrotron radiation at beamline 19ID of the Structural Biology Center at the Advanced Photon Source, Argonne National Laboratory (Argonne, IL) or in-house using X-rays from an FR-E SuperBright rotating anode and an R-AXIS IV detector (Rigaku, The Woodlands, TX). All data were processed with HKL2000 (Otwinowski and W. Minor, 1997).

SUPPLEMENTAL REFERENCES

Brautigam, C.A., Wynn, R.M., Chuang, J.L., Machius, M., Tomchick, D.R., and Chuang, D.T. (2006). Structural insight into interactions between dihydrolipoamide dehydrogenase (E3) and E3 binding protein of human pyruvate dehydrogenase complex. Structure *14*, 611-621.

Chuang, J.L., Davie, J.R., Wynn, R.M., and Chuang, D.T. (2000). Production of recombinant mammalian holo-E2 and E3 and reconstitution of functional branched-chain alpha-keto acid dehydrogenase complex with recombinant E1. Methods Enzymol *324*, 192-200.

Kato, M., Chuang, J.L., Tso, S.C., Wynn, R.M., and Chuang, D.T. (2005). Crystal structure of pyruvate dehydrogenase kinase 3 bound to lipoyl domain 2 of human pyruvate dehydrogenase complex. EMBO J *24*, 1763-1774.

Kato, M., Wynn, R.M., Chuang, J.L., Brautigam, C.A., Custorio, M., and Chuang, D.T. (2006). A synchronized substrate-gating mechanism revealed by cubic-core structure of the bovine branched-chain alpha-ketoacid dehydrogenase complex. EMBO J *25*, 5983-5994.

Li, J., Wynn, R.M., Machius, M., Chuang, J.L., Karthikeyan, S., Tomchick, D.R., and Chuang, D.T. (2004). Cross-talk between thiamin diphosphate binding and phosphorylation loop conformation in human branched-chain alpha-keto acid decarboxylase/dehydrogenase. J Biol Chem *279*, 32968-32978.

Machius, M., Wynn, R.M., Chuang, J.L., Li, J., Kluger, R., Yu, D., Tomchick, D.R., Brautigam, C.A., and Chuang, D.T. (2006). A versatile conformational switch regulates reactivity in human branched-chain alpha-ketoacid dehydrogenase. Structure *14*, 287-298.

Nakai, T., Nakagawa, N., Maoka, N., Masui, R., Kuramitsu, S., and Kamiya, N. (2004). Ligand-induced conformational changes and a reaction intermediate in branched-chain 2-oxo acid dehydrogenase (E1) from Thermus thermophilus HB8, as revealed by X-ray crystallography. J Mol Biol *337*, 1011-1033.

Otwinowski, Z., and W. Minor, W. (1997). Processing of X-ray Diffraction Data Collected in Oscillation Mode. In Methods in Enzymology, J.R.M.S. C.W. Carter, ed. (New York: Academic Press), pp. 307-326.

Wynn, R.M., Kato, M., Chuang, J.L., Tso, S.C., Li, J., and Chuang, D.T. (2008). Pyruvate dehydrogenase kinase-4 structures reveal a metastable open conformation fostering robust core-free basal activity. J Biol Chem.

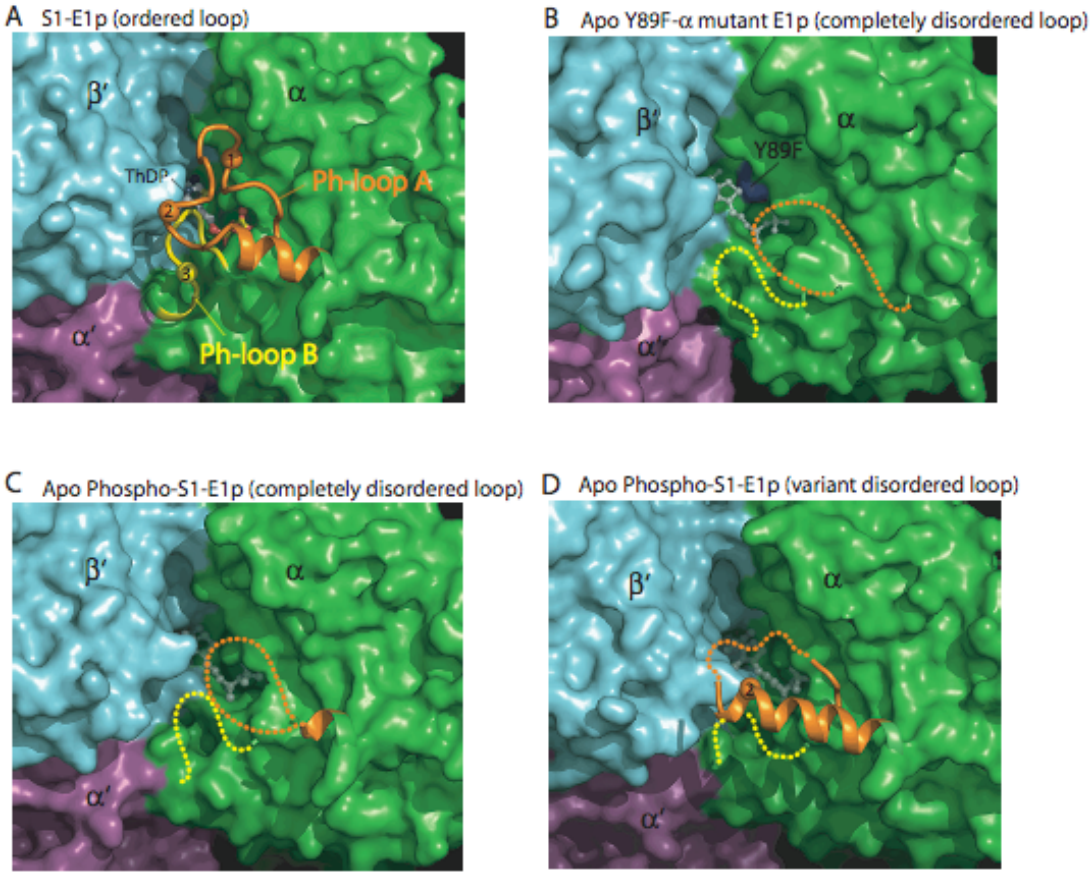

Figure S1. Structures of the Phosphorylation Loops in S1-E1p and Apo Phospho-S1-E1p
Structures of the Ph-loops in E1p-α subunits are shown as ribbon models against other parts of the protein in surface representation. Phosphorylation loop A (Ph-loop A) (residues from 259-α to 282-α) is in orange, and Ph-loop B (from 198-α to 205-α) is in yellow. The three phosphorylation sites are indicated by spheres and labeled according to the site numbers.
(A) Fully ordered Ph-loops in S1-E1p bound to Mg-ThDP. The bound ThDP is shown as a ball-and-stick model.
(B) The completely disordered Ph-loops in apo Y89F-α mutant E1p. The position of the Y89F mutation is indicated in blue. ThDP bound to the wild-type E1p structure is superimposed for comparison and shown as a white ball-and-stick model.
(C) Completely disordered Ph-loops in apo phospho-S1-E1p.
(D) Variant disordered Ph-loops in apo phospho-S1-E1p. This conformation is maintained through interactions with a symmetry-related molecule similar to the variant Ph-loops in phospho-S1-E1p with bound Mn-ThDP (*cf.* Figure 3D).
ThDP is shown as a white ball-and-stick model for comparison between panels (C) and (D).

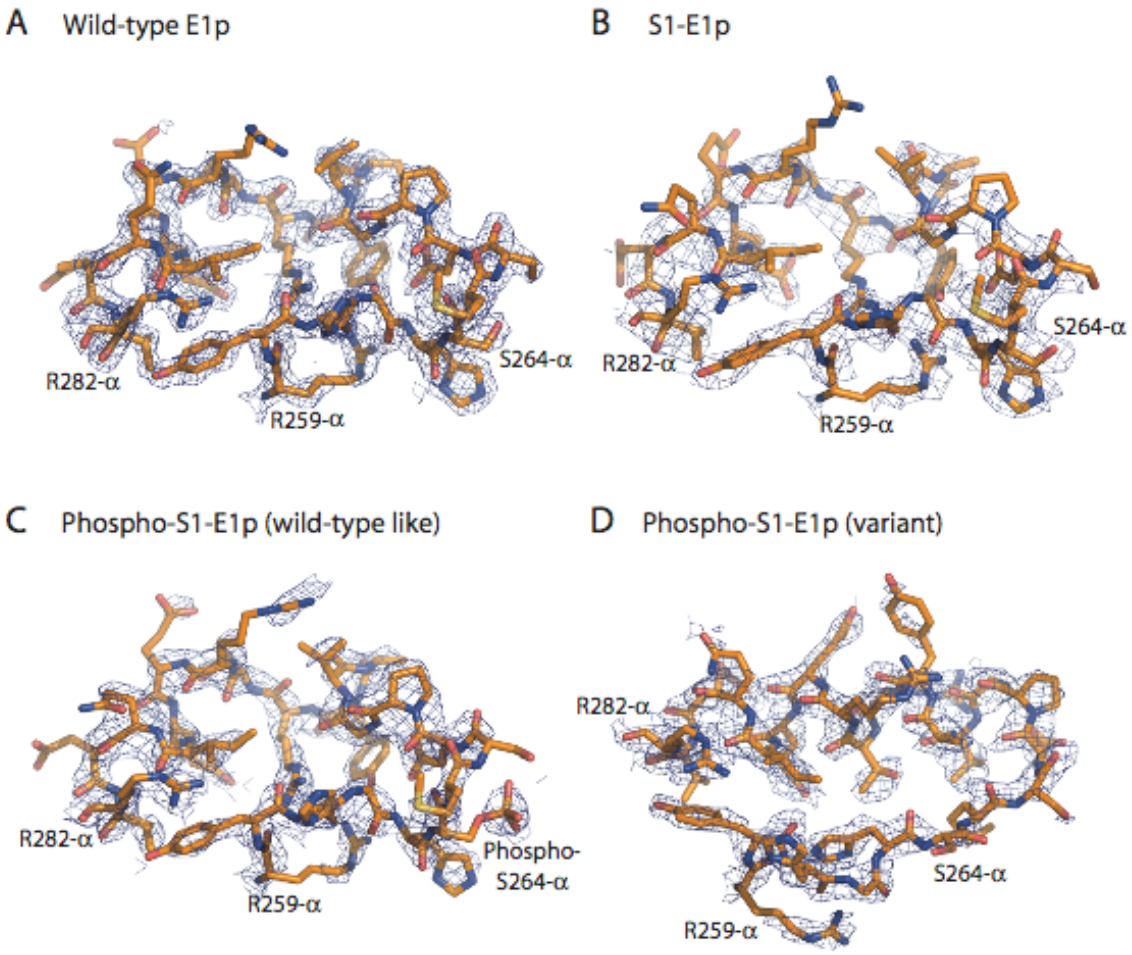

Figure S2. Omit Maps for Ph-loop A in the Ordered Conformation
(A–D) Omit maps are shown for the ordered Ph-loop A in the structures of (A) wild-type E1p, (B) S1-E1p, (C) phospho-S1-E1p for the wild-type conformation, and (D) phospho-S1-E1p for the variant conformation. To calculate the omit maps, the residues in Ph-loop A were removed from the models, and random shifts (±0.1 Å average) were introduced in all of the remaining atoms, followed by the refinements. The complete refined models are superimposed as stick models. Omit maps are contoured at a 1-σ level.

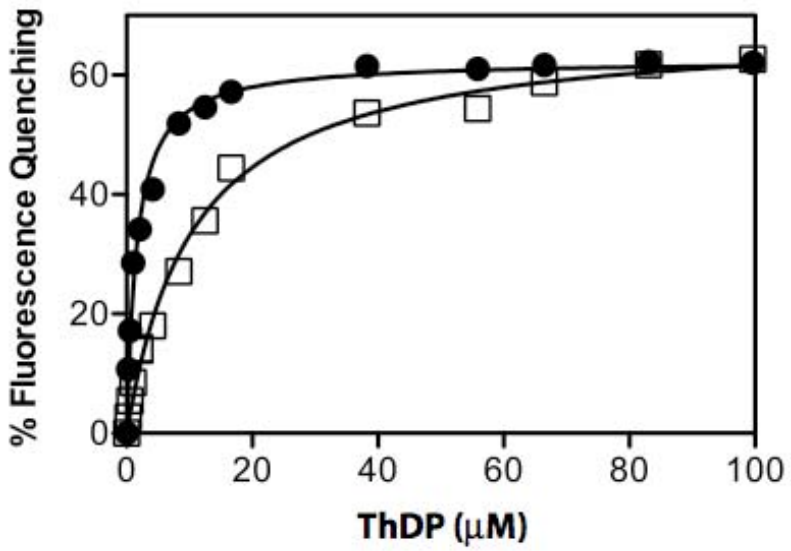

Figure S3. Fluorescence Quenching Measurements for ThDP Binding to Phospho-S1-E1p in the Presence of Either Mg^{2+} or Mn^{2+}

Tryptophan fluorescence quenching was measured as described in Experimental Procedures. Phospho-S1-E1p (0.23 μM) was titrated with ThDP in the presence of 1 mM Mn^{2+} (●) or Mg^{2+} (□). Curve fitting was carried out with the Prism program (GraphPad Software, San Diego, CA). The estimated K_d values of phospho-S1-E1p for ThDP are 1.56 ± 0.07 μM with Mn^{2+} and 10.6 ± 0.7 μM with Mg^{2+}.

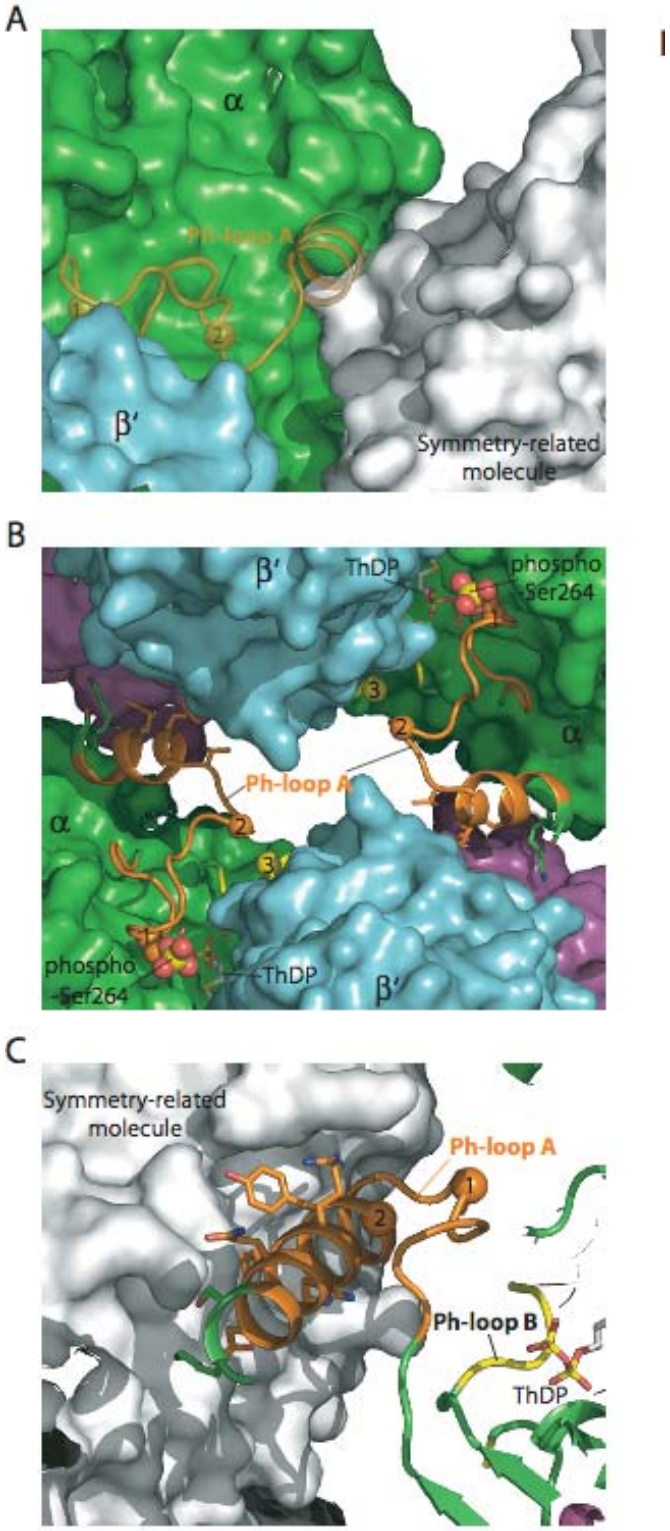

Figure S4. Interactions of the Ph-loops with Symmetry-Related Molecules in Phospho-S1-E1p

(A) The completely disordered Ph-loops in phospho-S1-E1p harboring bound Mn-ThDP. For reference, the wild-type ordered Ph-loops are shown as a transparent ribbon model. The distal symmetry-related molecule (white) from Ph-loop A is shown.

(B) The ordered Ph-loops of phospho-S1-E1p in complex with Mn-ThDP. Two E1p heterotetramers (only upper and lower halves of each molecule are shown) are related by two-fold non-crystallographic symmetry. Ph-loop A from one heterotetramer interacts with the surface of the other heterotetramer.

(C) The variant ordered Ph-loops in phospho-S1-E1p with bound Mn-ThDP. A symmetry-related molecule (white) interacts with Ph-loop A from another E1p heterotetramer.