A Molecular Switch and Proton Wire Synchronize the Active Sites in Thiamine Enzymes

René A. W. Frank, Christopher M. Titman, J. Venkatesh Pratap, Ben F. Luisi,* Richard N. Perham*

Thiamine diphosphate (ThDP) is used as a cofactor in many key metabolic enzymes. We present evidence that the ThDPs in the two active sites of the E1 (EC 1.2.4.1) component of the pyruvate dehydrogenase complex communicate over a distance of 20 angstroms by reversibly shuttling a proton through an acidic tunnel in the protein. This "proton wire" permits the cofactors to serve reciprocally as general acid/base in catalysis and to switch the conformation of crucial active-site peptide loops. This synchronizes the progression of chemical events and can account for the oligomeric organization, conformational asymmetry, and "ping-pong" kinetic properties of E1 and other thiamine-dependent enzymes.

Enzymes that display so-called ping-pong kinetics catalyze two successive half-reactions: the first is associated with release of the first product and the formation of an enzyme-intermediate complex before the second substrate binds; the second reaction causes release of the second product and returns the enzyme to the starting state. Such enzymes are normally dimers or higher order oligomers and exhibit hysteretic activation of their cofactor (if any) or negative cooperativity in binding their substrate. In addition, many are structurally asymmetric (*1*), the functional significance and molecular basis of which is a long-standing puzzle.

Thiamine (vitamin B_1)–dependent enzymes are typical of ping-pong enzymes. Thiamine in the form of its diphosphate (ThDP, Fig. 1A) is used as a cofactor in the catalysis of a broad range of important reactions, including the decarboxylation of 2-oxo acids (*2*). The minimal quaternary structure of ThDP-dependent enzymes is that of a homodimer, with two well-separated active sites, each binding one ThDP. The first ThDP molecule is bound faster than the second by orders of magnitude (*3*), even though the two binding sites are initially equivalent, which suggests that the sites communicate.

The enzyme preorganizes the cofactor to favor the loss of its C2 proton from the thiazolium ring, thereby generating a carbanion that is the "activated" state (Fig. 1, A and B). In the half century since this pioneering discovery (*4*), the subsequent steps of the catalytic cycle have been identified (*5*, *6*), and in Fig. 1B, these are summarized for the oxidative decarboxylation of pyruvate by the E1 component of the pyruvate dehydrogenase (PDH) multienzyme complex. It has been suggested that the aminopyrimidine ring of ThDP in concert with a conserved glutamate side chain acts as a proton acceptor during formation of the activated ThDP C2-carbanion (Fig. 1A) (*7*) but that this occurs only when the substrate binds (*8*).

Several other steps in the catalytic reaction also require a proton donor and acceptor. Thus in the decarboxylation of 2-oxo acids, a general acid is thought to protonate the substrate to form a metastable covalent intermediate [reviewed in (*2*)]. A proton acceptor is required for the second half-reaction, and depending on the specificity of the enzyme, the intermediate can react with the dithiolane ring of a lipoyl group, a thiol, a primary amine, an aldehyde, a ketone, or a proton. This broad palette of substrates reflects the diverse repertoire of chemical transformations mediated by ThDP-dependent enzymes (*2*, *9*). The catalytic mechanism, as exemplified in Fig. 1B, satisfyingly explains the chemical process but does not account for the hysteresis of ThDP binding, the conserved oligomeric state, or the conformational asymmetry of ThDP-dependent enzymes.

The ThDP-dependent E1 component of the PDH multienzyme complex from *Bacillus stearothermophilus* has been intensively studied in this context (*10*, *12*–*15*). We have now solved the crystal structure of the subcomplex formed between the heterotetrameric ($\alpha_2\beta_2$) *B. stearothermophilus* E1 and the peripheral subunit-binding domain (PSBD) from the lipoate acetyltransferase (E2) chain of the PDH complex (*16*). The structure is shown in Fig. 2 with details reported elsewhere (*16*). Here, we provide evidence for a pathway of communication between the ThDP cofactors bound in the two physically remote active sites.

The structure reveals a ThDP cofactor and a Mg^{2+} ion in each active site, with a

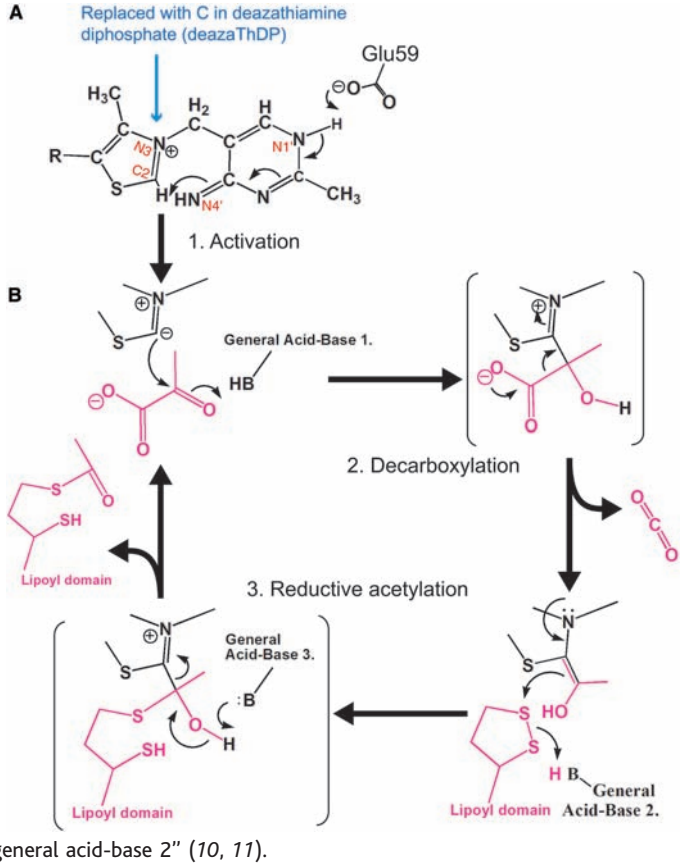

Fig. 1. (**A**) The structure of thiamine diphosphate ("R" corresponds to a diphospho-oxyethyl group). ThDP bound to E1 adopts the imino-tautomer of aminopyrimidine before forming the activated carbanion by deprotonation of its C2 atom (*6*). The C2 proton is relayed via the ThDP N4′ and N1′ to an invariant glutamate (βGlu^{59}). (**B**) The two half-reactions of the E1 ping-pong catalytic mechanism are decarboxylation (step 2) and reductive acetylation (step 3), in which the first substrate is pyruvate and the second is a lipoyl group attached to a pendant protein domain of E2 from the PDH multienzyme complex (*12*). The substrates, intermediates, and products (CO_2 and acetyl-lipoyl-E2) are shown in magenta. Three general acid-base catalysts are required. E1 βHis^{128} has been identified as "general acid-base 2" (*10*, *11*).

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge, UK.

*To whom correspondence should be addressed. E-mail: r.n.perham@bioc.cam.ac.uk or ben@cryst.bioc.cam.ac.uk

third Mg^{2+} ion lying at the center of the E1 tetramer within a solvated tunnel that links the active sites over a distance of 20 Å (Fig. 2, B and C). The tunnel is unusually rich in acidic side chains, containing four Asp and six Glu residues, plus other acidic residues around the aminopyrimidine ring, and has no basic residues to neutralize them (Fig. 2B). Residues from all four subunits (two α, two β) line the tunnel and hydrogen bond with entrained water. This highly acidic tunnel is conserved in all five E1 structures solved to date (*17*–*21*), and a similar cavity was noted for the dimeric ThDP-dependent transketolase from *Saccharomyces cerevisiae* (EC 2.2.1.1) (*22*). A similar channel is found in all crystal structures available of ThDP-dependent enzymes (*23*); however, despite its wide occurrence, the tunnel has been largely overlooked with respect to function.

To investigate the role of this acidic, hydrated tunnel, we changed two of the acidic residues to Ala (Asp180αAla and Glu183αAla) or formed their amide equivalents (Asp180Asn and Glu183Gln), which we refer to as E1 (AA) and E1 (NQ), respectively [see Materials and Methods in (*23*)]. These double mutants cause a net loss of four acidic side chains across the two-fold axis of symmetry in the tunnel (Fig. 2, B and C). The E1 mutants were incorporated into a PDH complex as efficiently as the wild-type E1 (*23*). Moreover, a 2.3 Å resolution crystal structure of the E1 (NQ) mutant in complex with the PSBD shows the active sites to be intact and the quaternary structure unchanged compared with the wild-type E1 (*23*).

To evaluate the effects of mutations in this tunnel, we monitored decarboxylation in the first half-reaction catalyzed by E1 using dichlorophenol indophenol (DCPIP) as an artificial electron acceptor (*23*). The rate of decarboxylation by the E1 (AA) mutant was 31% of that of wild-type E1 (Fig. 3A). Likewise, the PDH complex assembled with the E1 (AA) mutant exhibited even less activity (12%) relative to wild-type (Fig. 3A), indicating that the overall E1 reaction (decarboxylation and the subsequent reductive acetylation of the lipoyl substrate) is seriously impaired by this mutation. These catalytic activities were even lower for the E1 (NQ) mutant, in which decarboxylation fell to 7% and PDH complex activity to 3% relative to the wild-type (*23*). Asp^{180} and Glu^{183} are remote from the E1 active sites (≥7 Å), yet their replacement causes a major loss of catalytic activity. The acidic tunnel in which these amino acid residues are located must therefore play an important role in the catalytic mechanism.

The reported crystal structures of E1 have indicated that two conserved peptide loops close down on the active site to form a hydrophobic funnel-shaped entrance that probably protects the activated ThDP C2-carbanion against aqueous or nonspecific electrophiles (*17*–*20*). It has been suggested that ThDP-dependent enzymes are only active in the "closed" conformation (*24*). In the crystal structure of the *B.*

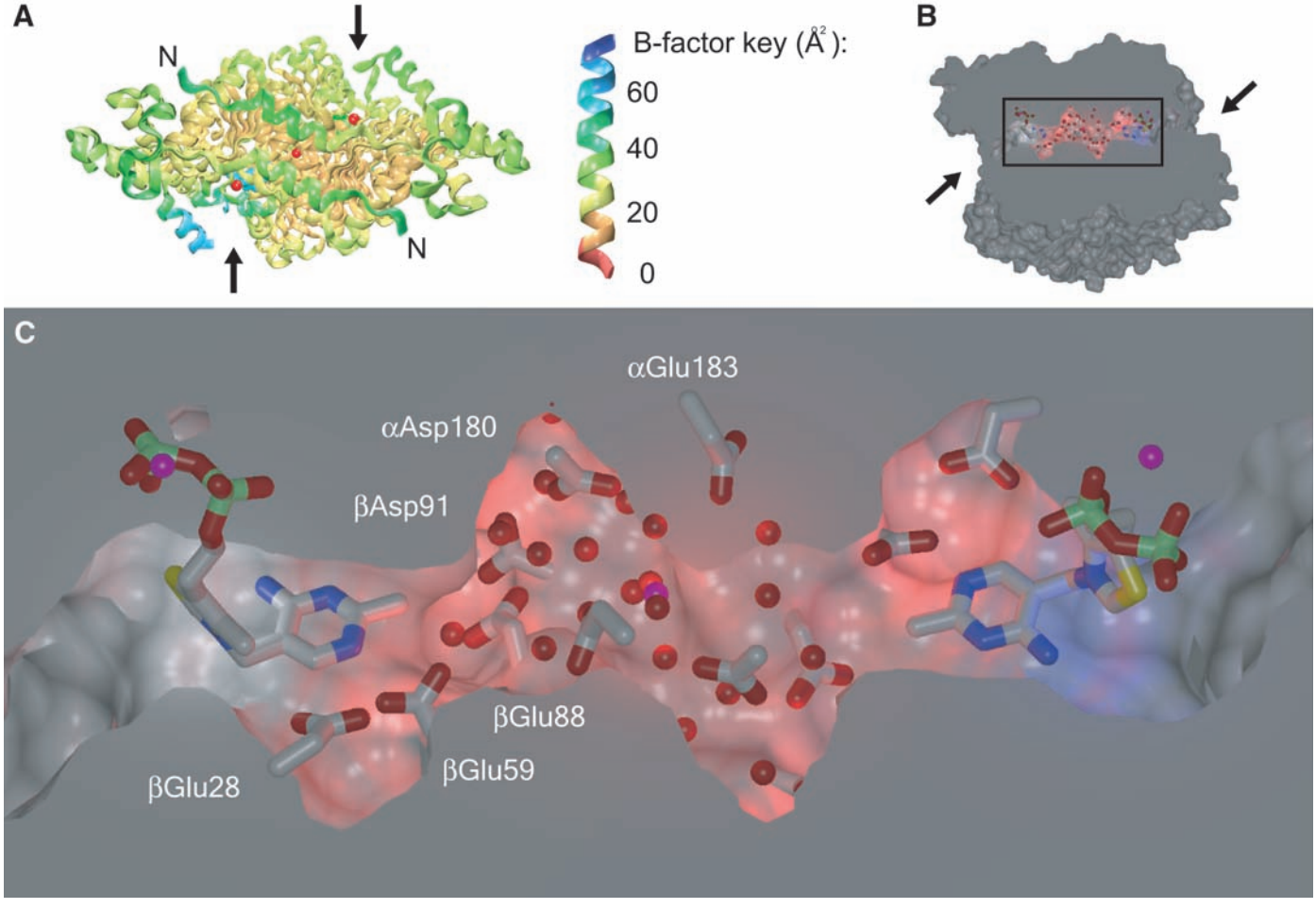

Fig. 2. *B. stearothermophilus* E1 active-site asymmetry and proposed proton wire. (**A**) Ribbon diagram of E1 taken from its complex with the PSBD of E2 colored by temperature (B-) factor. The ThDP cofactors are represented by space-filling atoms and three Mg^{2+} ions (red spheres), one in each of the two active sites and one midway in the protein tunnel–like cavity that links them. The dyad axis of E1 is oriented vertically. E1 is symmetric except for residues in the active sites, which are non-equivalent. Two peptide loops (α275 to 293 and α203 to 212) at the entrance to one of the two active sites are disordered. Two arrows indicate the entrance to the active sites, and the N-termini of the two α subunits are labeled. Image (**B**) is a close-up of image (**C**). A solvent-accessible surface is shown for E1, which is clipped with a bounding plane to expose the interior. The figure was made with VMD, MSMS, and Raster3D (*23*).

stearothermophilus E1, we observed a striking nonequivalence in these conserved loops at the two E1 active sites; the two active-site loops (inner, α203 to 212 and outer, α275 to 293) were found to be well ordered in one α subunit but were disordered in the other of the $\alpha_2\beta_2$ E1 (Fig. 2A). Moreover, when *B. stearothermophilus* E1 is subjected to limited proteolysis, it is the outer of these loops (α275 to 293) that is the principal site of cleavage, but only half the E1 α chains appear to be susceptible, which implies a conformational asymmetry (*14*), as now observed in the crystal structure. In many other enzymes, active-site communication can be achieved through allosteric changes and subunit rearrangements (*25*); however, we did not find any significant structural differences between the subunits of *B. stearothermophilus* E1, other than the active-site loops. We considered whether an alternative means of communication might occur involving the conduction of charge.

The charge state of the cofactor changes when activated, from a positively charged thiazolium to a zwitterion. In deazathiamine diphosphate (deazaThDP), a ThDP-dependent enzyme inhibitor, the quaternary amine (N3) responsible for the positive charge on the thiazolium is replaced with a carbon (Fig. 1A) (*23*, *26*). DeazaThDP has a neutral thiazolium-like group, which makes it an analog of the activated ThDP. ThDP and deazaThDP are isoelectronic and differ by only 2 Dalton in one constituent atom, yet they exercise markedly different effects on the accessibility of the active-site loops. Replacement of ThDP with deazaThDP led to the protection of the active-site loops (α275 to 293) against limited proteolysis in both active sites, compared with ThDP bound-E1 in which half of the loops were cleaved (Fig. 3B) as observed previously (*14*). These results suggest that the cofactor's charge state and its transition from a dormant to an activated state is the "molecular switch" underlying the conformation of loops surrounding the active sites. The cofactor may control the conformation of the active-site loop in the folded state via interactions with the inner loop, which interacts with and stabilizes the outer loop (Fig. 3C). Thus, the crystal structure presented here suggests that one active site is closed and therefore contains an activated ThDP before substrate is engaged, which contradicts a substrate-driven mechanism for ThDP activation proposed earlier (*8*). However, NMR data that show a protonated C2 in the bound ThDP (*8*) are consistent with our proposal that one site is activated while the other remains protonated before substrate binding.

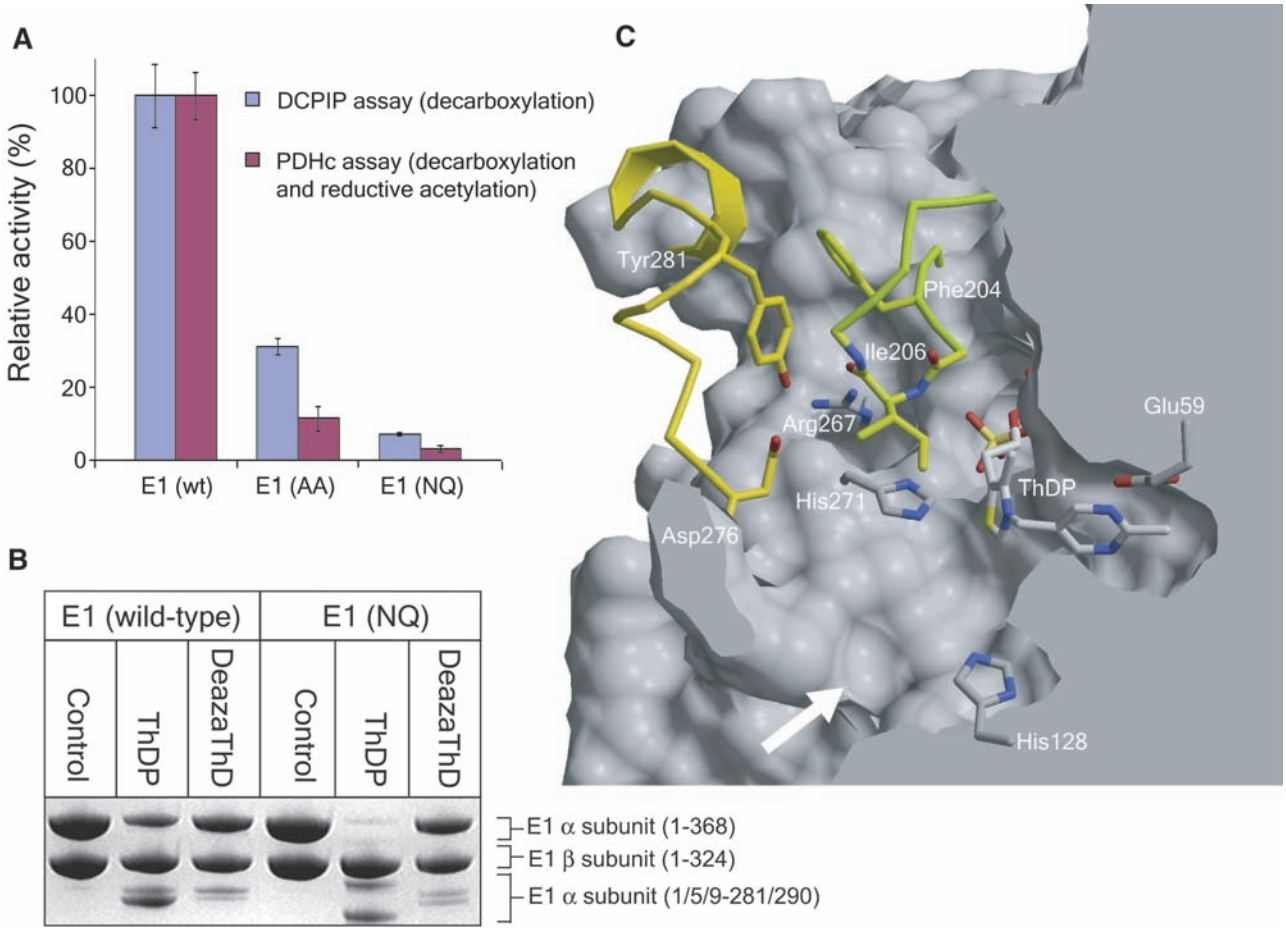

Fig. 3. (**A**) Assays of catalytic activity for wild-type E1 and two acidic cavity mutants: E1 (Asp180Ala and Glu183Ala) and E1 (Asp180Asn and Glu183Gln) are labeled E1 (AA) and E1 (NQ), respectively. The blue bars show the rate of pyruvate decarboxylation, measured with DCPIP as an artificial electron acceptor (*23*). The magenta bars show the activities of assembled PDH complexes (PDHc), whereby the overall E1 reaction (decarboxylation and reductive acetylation) is being measured (*23*). The error bars correspond to 1.0 SD. (**B**) SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (Coomassie-stained) showing stable products from limited proteolysis with chymotrypsin of wild-type E1 and the E1 (NQ) mutant after prior saturation with ThDP or deazaThDP. The fragments are all derived from E1α and are the result of proteolysis at Tyr^{281} and Trp^{290} in an exposed active-site loop (*14*). E1α cut at the active-site loop may also have secondary trimming at N-terminal Phe^{5} and Phe^{9}. (**C**) A molecular switch in the active site of *B. stearothermophilus* E1. Vital catalytic residues of the active site are shown in stick format. Two loops (outer, α275 to 293 in yellow and inner, α203 to 212 in lime green) are disordered before ThDP activation. Activation of ThDP stabilizes packing of βHis^{271} and αIle^{206} against ThDP, and a binding pocket for αTyr^{281} of the outer loop is formed by αPhe^{204}, αAsp^{276}, αArg^{276}, and the backbone carbonyl of αIle^{206}. A white arrow indicates the entrance to the active site. The figure was made using MOLSCRIPT, MSMS, XtalView, and Raster3D (*23*).

How is it that the ThDP cofactors in both active sites are not activated? The answer may lie in the fate of the proton removed from the first ThDP when it binds to the apo-E1 and the closure of this active site. The acidic tunnel could act as a proton wire, by shuttling a proton from the closed active site to the opposing open active site in the apo-enzyme. Perhaps, this is accomplished by a "Grotthus-like" mechanism (*27*), in which the proton is displaced along a chain of neighboring donor-acceptor groups provided by the preponderance of entrained water and Asp and Glu residues (Fig. 4A). Accordingly, when the second ThDP is bound and activated, the first site will be reprotonated, and so the active site asymmetry is maintained (Fig. 4B). It is a formal possibility that, in solution, the active sites are in dynamic equilib-

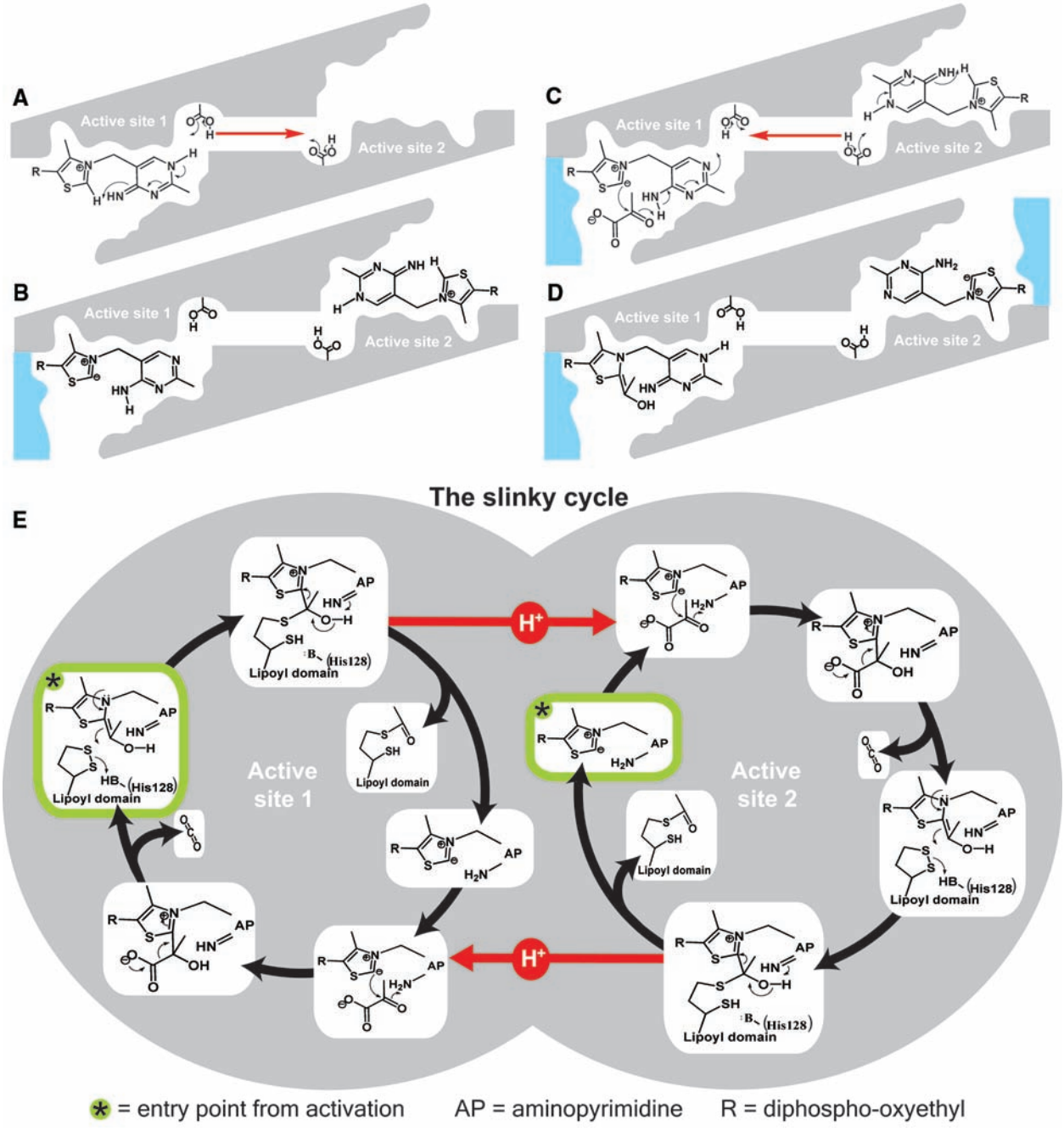

Fig. 4. Catalytic mechanism of ThDP-dependent enzymes. (**A** to **D**) are the steps of ThDP activation in both active sites and (**E**) is the slinky cycle. (A) ThDP binds fast/tightly to the first site and is activated to generate a ThDP C2-carbanion. The ThDP C2 proton is relayed via the amino N4′ group to the N1′ atom of the ThDP aminopyrimidine ring and onward through a proton wire to the open, apo-active site. (B) Two loops (represented by a blue shape) preorganize the active site by folding around the zwitterionic thiazolium. After the first site is activated, the second site has no route for abstracting a second proton, so its ThDP binds but remains dormant. (C) Substrate (in this example, pyruvate) reacts with the activated C2. The ThDP of the second active site is a general acid, donating a proton to the first site. (D) This results in decarboxylation at the first site (not shown, see Fig. 4E), which forms the enamine intermediate, and activation of the second site. Both active sites now gain entry into the slinky cycle, shown in panel (**E**). The entry points into the slinky cycle are nonequivalent for each active site and are highlighted by an asterisk and a green border. At the first and last steps of ping-pong catalysis, both ThDPs separated by a 20 Å proton wire are mutually obligated as general acid-base catalysts in a slinky-like progression of chemical events. The dithiolane ring of the lipoyl domain is the second substrate in this example and requires activation by βHis^{128} in the active site (*10*, *11*).

rium, each exchanging between the activated and dormant state. Once the holoenzyme has been formed, with both ThDPs bound in place, this will be the state of the enzyme in vivo at the start of each catalytic cycle, as it is in our crystal structure.

In support of these ideas, we found that limited proteolysis of the inactive E1 (NQ) acidic tunnel mutant leads to almost complete cleavage of the loops in both the active sites (Fig. 3B). However, in the presence of the deazaThDP, the E1 (NQ) behaves like the wild-type E1, in which the active-site loops are all protected from attack (Fig. 3B). These observations suggest that the charge state of ThDP is sufficient to control the conformation of active-site loops, but also that replacing acidic residues in the tunnel severs communication between active sites and dissipates the active-site asymmetry.

The involvement of the proton wire in the activation of ThDP provides a molecular basis for the hysteretic properties of this enzyme. It also resolves the puzzle of why the first substrate, pyruvate, exhibits apparently conflicting characteristics with respect to ThDP activation. On the one hand, pyruvate induces positive cooperativity of ThDP activation (*28*), yet several cocrystal structures of ThDP-dependent enzymes show that substrate analogs are bound in only one of the two active sites (*17*, *29*). Substrate binding exclusively to only one of the active sites is an extreme form of negative cooperativity sometimes referred to as "half of the sites' reactivity" and is common among many ping-pong enzymes (*30*). These apparently contradictory properties can be reconciled by the molecular switch and proton-wire model, which holds that the first ThDP is activated by binding; in contrast, activation of the second site is coupled to decarboxylation of pyruvate in the first site (Fig. 4, A to D) (*31*).

As shown schematically in Fig. 4, the activation and subsequent catalytic steps of this "slinky cycle" are dependent on the push or pull of a proton: while one site requires a general acid, the other requires a general base, and via the proton wire, they reciprocate their catalytic needs. This mechanism also permits the switching of active-site loops to coordinate the uptake of substrates and release of products, which is particularly important in E1, because the specificity of lipoyl domain recognition underlies the molecular mechanism of substrate-channeling in the PDH complex (*12*, *15*).

In the homologous E1 from eukaryotes, serine residues in the outer loop of the active site are the targets of phosphorylation by a specific kinase (EC 2.7.1.99), which regulates the catalytic activity. Phosphorylation at only one of the two active sites is sufficient to inactivate the entire enzyme (*32*), which demonstrates that coupling between the two active sites is obligatory. Additionally, kinetic evidence accumulated for a close relative of E1, the yeast ThDP-dependent pyruvate decarboxylase (EC 4.1.1.1) [(*33*, *34*) and references therein], suggests the active sites of the "catalytic dimer" alternate. These observations can readily be explained by the dependence of E1 activity on the communication between active sites envisaged in the molecular switch and proton-wire model (Fig. 4E). It will be interesting to see how far these proposals extend to other dimeric ping-pong enzymes, particularly those requiring an activated cofactor for catalysis.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/306/5697/872/DC1
Materials and Methods
SOM Text
Figs. S1 to S5
Tables S1 and S2
References and Notes

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Abnormal Cytokinesis in Cells Deficient in the Breast Cancer Susceptibility Protein BRCA2

Matthew J. Daniels,* Yunmei Wang,* MiYoung Lee, Ashok R. Venkitaraman†

Germ-line mutations inactivating *BRCA2* predispose to cancer. BRCA2-deficient cells exhibit alterations in chromosome number (aneuploidy), as well as structurally aberrant chromosomes. Here, we show that BRCA2 deficiency impairs the completion of cell division by cytokinesis. BRCA2 inactivation in murine embryo fibroblasts (MEFs) and HeLa cells by targeted gene disruption or RNA interference delays and prevents cell cleavage. Impeded cell separation is accompanied by abnormalities in myosin II organization during the late stages in cytokinesis. BRCA2 may have a role in regulating these events, as it localizes to the cytokinetic midbody. Our findings thus link cytokinetic abnormalities to a hereditary cancer syndrome characterized by chromosomal instability and may help to explain why BRCA2-deficient tumors are frequently aneuploid.

Inherited mutations affecting the *BRCA2* tumor suppressor predispose to breast, ovarian, and other epithelial cancers with high penetrance (*1*). BRCA2-deficient cells accumulate gross chromosomal rearrangements, including translocations and large deletions