of syntaxin increases the number of clusters rather than their size, which is in agreement with previous observations (7). In contrast, a hypo-
thetical cylindrical syntaxin arrangement (Fig. 4C, right) would not explain our findings.

The self-organization model shows that a simple balance between weak homophilic inter-
actions and a repulsive component can explain all available experimental data on the com-
position and the dynamics of syntaxin clusters.

Syntaxin clustering via self-assembly is likely a paradigm that applies to a variety of membrane protein clusters. Like the syntaxins, many other membrane proteins are known both to form clusters and to homo-oligomerize in vitro via their cytoplasmic domain. Examples include structur-
ally diverse proteins such as synaptotagmins (13, 20) and receptors (4, 21) (containing single transmembrane domains), anion transporters [e.g., (22)] containing multiple transmembrane domains, and proteins such as reggie with lipid anchors [e.g., (23)]. The tendency to form hom-
ologomers in solution would be enhanced in the plane of a membrane where membrane anchoring orients the molecules ideally for oligomerization. Hence, in the membrane self-
assembly is enforced, leading finally to nanodomains containing many copies of the self-assembled membrane proteins. Thus, just as for the syntaxins, homo-oligomerization will likely lead to cluster formation for any membrane protein that can self-associate in solution.

The concept of clustering via self-assembly is not restricted to membrane proteins oligo-
merizing via their cytoplasmic domain. Rather, it can be widely applied to membrane proteins oligo-
merizing via their transmembrane or extracellular domains, with the only prerequisite being that in these cases there would be additional protein domains causing steric crowding or other repulsive forces, as, for example, accumulating charges.

As outlined above, the mechanisms underlying the high degree of membrane micropatterning are not well understood for most membrane proteins. With syntaxin 1 as an example, we present a conceptual framework for the description of protein domains in membranes. This biological principle is expected to explain a considerable part of the compositional variability of membrane protein clusters and hence will help to advance our understanding of membrane micropatterning.

References and Notes


15. Materials and methods are available on Science Online.
24. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Domain Architecture of Pyruvate Carboxylase, a Biotin-Dependent Multifunctional Enzyme

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Biotin-dependent multifunctional enzymes carry out metabolically important carboxyl group transfer reactions and are potential targets for the treatment of obesity and type 2 diabetes. These enzymes use a tethered biotin cofactor to carry an activated carboxyl group between distantly spaced active sites. The mechanism of this transfer has remained poorly understood. Here we report the complete structure of pyruvate carboxylase at 2.0 angstroms resolution, which shows its domain arrangement. The structure, when combined with mutagenic analysis, shows that intermediate transfer occurs between active sites on separate polypeptide chains. In addition, domain rearrangements associated with activator binding decrease the distance between active-site pairs, providing a mechanism for allosteric activation. This description provides insight into the function of biotin-dependent enzymes and presents a new paradigm for multifunctional enzyme catalysis.

In biochemical pathways, metabolites must be efficiently transferred between enzymes to avoid the energetic penalty associated with their loss to diffusion, degradation, or competing side-reactions. Particularly efficient transfer is afforded by multifunctional enzymes that directly transfer products from one reactive site to the next through tunnels and channels (1) or through the use of covalently attached prosthetic groups (2). Although the reactions catalyzed at the individual active sites of many multifunc-
tional enzymes are well understood, few studies have detailed their complete domain architecture. Consequently, descriptions of intermediate transfer between active sites in multifunctional enzymes remain largely incomplete.

The multifunctional enzymes of the biotin-dependent family use a covalently attached biotin prosthetic group to directly transfer an activated CO2 intermediate between distinct active sites in several essential metabolic reactions (3). Some members of this enzyme family, including acetyl-coenzyme A carboxylase (ACC) and pyr-
uvate carboxylase (PC), have recently attracted interest as potential targets in the treatment of obesity and type 2 diabetes (4, 5). Although several individual domain structures have been determined for various family members (6–9), the relative arrangement of these domains in a complete multifunctional enzyme remains unknown. PC is typically composed of three distinct functional domains arranged on a single 120- to 130-kD polypeptide chain. The three domains are an N-terminal biotin carboxylase (BC) domain, a central carboxyltransferase (CT) domain, and a C-terminal carboxylase (CA) domain.

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domain, and a C-terminal biotin carboxyl carrier protein (BCCP) domain (Fig. 1A). The enzyme uses a covalently attached biotin cofactor to catalyze the adenosine triphosphate (ATP)–dependent carboxylation of pyruvate to oxaloacetate in two steps (10). Biotin is initially carboxylated at the BC active site by ATP and bicarbonate. The carboxyl group is subsequently transferred by carboxybiotin to a second active site in the CT domain, where pyruvate is carboxylated to generate oxaloacetate. The BCCP domain transfers the tethered cofactor between the two remote active sites. To discover what distance is traversed by the BCCP domain and what domain motions facilitate the transfer of biotin between active sites, we have determined the crystal structure of the complete PC enzyme.

The crystal structure of PC from *Rhizobium etli* (RePC) was determined by single-wavelength anomalous diffraction and solvent flattening, followed by molecular replacement (11). The final model was refined to a resolution of 2.0 Å (see table S1 for refinement statistics and fig. S1 for representative electron density). The crystals contain a dimer in the asymmetric unit. Monomer A includes the complete polypeptide chain, composed of the three functional domains [BC (blue), CT (yellow), and BCCP (red)], and a central allosteric domain (green) that has not been previously described (Fig. 1B). Monomer B is nearly complete except for the C-terminal BCCP domain, which was disordered and could not be modeled (model completeness is described in table S2). The dimer is asymmetric, and the notable differences in the position and orientation of the BC domain between the two monomers have important implications in the mechanism of PC catalysis and regulation.

The two active sites of RePC were identified through structural homology with related enzyme subunits and from the position of bound ligands. The nonhydrolyzable ATP analog, adenosine 5′-O-(3-thiotriphosphate) (ATP-γ-S), is bound at the active site of the BC domain (Fig. 2A). The binding site consists of an ATP-grasp fold (12), typical of the arrangement reported for orthologous enzymes (6, 13). The overall BC domain fold is very similar [1.1 to 1.2 Å root mean square deviation (RMSD)] to the BC subunits from other family members (fig. S2A). However, the position of ATP-γ-S in the binding site of RePC reveals differences with the position of ATP in the BC subunit of ACC (13) (fig. S3). The position of ATP-γ-S in RePC is much more typical of other ATP-grasp binding sites (14, 15) [supporting online material (SOM) text]. The CT domain of RePC also shares high structural similarity (1.1 to 1.5 Å RMSD) with the equivalent subunits from orthologous enzymes (7, 9) (fig. S2B). This domain consists of a core αβαβ barrel capped by a funnel that centers on a structurally conserved metal ion at the active site. The metal ion in RePC superimposes closely with the active-site metal ions of orthologous enzymes. Metal-ion analysis indicates that Zn²⁺ is the active-site metal in RePC, which is consistent with the observed electron density. Mutations of residues directly ligating this Zn²⁺ ion either eliminate or substantially reduce the catalytic activity of PC (16).

PC is subject to a variety of allosteric control mechanisms (3), and acetyl–coenzyme A (acetyl-CoA) is an allosteric activator for PC enzymes from many species (17). Both acetyl-CoA and the nonhydrolyzable analog, ethyl-CoA, are activators of RePC, with activation constant $K_A$ values of 30.4 and 360 μM, respectively (table S3). RePC was co-crystallized with ethyl-CoA, and the partial electron density allowed the nucleotide portion of the activator to be modeled into the structure, revealing the location of the allosteric binding site. The binding site is located in a previously unrecognized domain that serves as a mediator among the BC, CT, and BCCP domains. The allosteric domain consists of four antiparallel β strands bracketing a central α helix (Fig. 1B, green) and superficially resembles the protein fold and acetyl-CoA binding of the GCN-5–related acetyltransferases (18) [secondary structure matching (19) gives a weak Q score of 0.053 with a GCN-5–related aminoglycoside 3′-N-acetyltransferase, 1bo4]. The central α helix
Fig. 3. (A) Model of the RePC tetramer showing the movement of the BCCP domain between neighboring active sites on opposing polypeptide chains. (B) Surface representation of the top face of the tetramer. For clarity, one of the two individual monomers has been outlined in black. The distance between ATP-γ-S in the BC active site and Zn²⁺ in the CT active site of the opposing polypeptide chain is 65 Å. (C) Surface representation of the bottom face of the tetramer, after a 180° rotation about the y axis. The BCCP domain is disordered in these monomers and could not be modeled. The distance between ATP-γ-S in the BC active site and Zn²⁺ in the CT active site of the opposing polypeptide chain increases to 80 Å as a result of the altered orientation of the BC domain.

Fig. 4. Ethyl-CoA binding alters the position and orientation of the BC domain. The allosteric domain of monomer A was aligned with the allosteric domain of monomer B. The superposition shows minor deviations in the CT domain but substantial deviations in the BC domain. The BC domain begins to deviate precisely at the three residues forming tight hydrogen-bonding interactions with the 5′-α- and β-phosphates of ethyl-CoA: Arg⁴⁷² and the backbone amides of Gln⁴⁷⁰ and Asp⁴⁷¹. Where ethyl-CoA is unbound in monomer B, the side chain of Arg⁴⁷² is pointed away from the position occupied by the activator, and the backbone of Gln⁴⁷⁰ and Asp⁴⁷¹ threads from a radically different direction.

includes Asp⁴⁷¹ to His⁴⁰⁸ and connects the BC and CT domains, whereas Asp¹⁰¹⁸ to Asp¹⁰⁶⁵ fold around this helix in an antiparallel (β₄) sheet and connect the CT domain to the BCCP domain. The allosteric domain displays no sequence similarity with other members of the biotin-dependent enzyme family, suggesting that it is specific to PC. Ethyl-CoA is bound at the N-terminal end of the central α helix, near the BC-BC dimer interface (Fig. 2B). Residues interacting with the nucleotide portion of CoA are highly conserved among PC enzymes, explaining why PC from *Corynebacterium* (20) and subunit-type PCs (17) that have mutations or deletions in these residues are not activated by acetyl-CoA.

A critical feature of multifunctional enzyme catalysis is the requisite transfer of intermediates between remote active sites. The structure of RePC monomer A includes a complete BCCP domain relative to the individual catalytic domains of the enzyme, which advances our understanding of intermediate transfer. Although the electron density for the biotin cofactor was disordered, the global position and structure of the BCCP domain provide a powerful restraint on the arrangement of the prosthetic group carrier (SOM text). It is clear from the structure that the distance between active sites greatly exceeds the ~16 Å length of the biotin arm. Thus, the BCCP domain itself must move as it carries biotin between the active sites. The 34 Å linker connecting BCCP to the allosteric domain is highly flexible (8, 21), facilitating domain movement. The linker is similar to those described for the lipoyl domains of 2-oxo acid dehydrogenase (22). If the BCCP domain were to transfer carboxybiotin between active sites on the same polypeptide chain, a dramatic domain movement would be required. Alternatively, serving between active sites on opposing polypeptide chains necessitates substantially less motion. The quaternary structure and kinetic evidence described below support the latter scenario.

The RePC crystal lattice contains a tetramer, composed of a dimer of dimers. The tetramer is stabilized by conserved dimerization interfaces between neighboring BC domains and between neighboring CT domains. PC exists predominantly as a tetramer in solution, and only the tetramer catalyzes the overall reaction (23). The tetramer is asymmetric, with ethyl-CoA bound to each of the two (monomer A) monomers on the top face, whereas the two (monomer B) monomers on the bottom face are unbound. On the top face, the BC active site is positioned ~65 Å from the CT active site of an opposing polypeptide chain, with the biotinylated BCCP domain positioned equidistant between the two. Based on the distance and orientation between these opposing active sites, the BCCP domain is predicted to swing between active-site pairs on opposing polypeptide chains rather than between active sites on the same chain (Fig. 3).

To confirm this prediction, we created two RePC mutants. In one construct, the biotinylated Lys¹¹¹⁹ residue was mutated to prevent biotinylation of the BCCP domain, and, in the second construct, a carbamylated Lys⁷¹⁸ in the CT active site was mutated to impair the second half-reaction.
When assayed individually, the mutant enzymes Lys1119→Gln1119 (K1119Q) and K718Q exhibited 0.1 and 4% wild-type activity, respectively (table S4). Hybrid tetramers were created by mixing and mutating the mutants together before assaying for enzyme activity. Dilution of PC promotes equilibration among monomers, dimers, and tetramers (23) and allows a mixed heterotetramer population to reassociate. The mixed population of heterotetramers exhibited 20% wild-type activity (table S3), nearly five times as much as that observed with either mutant homotetramer and near to the maximum predicted activity of 26% (fig. S4). The recovery of activity on reassociation is possible only if the hybrid tetramers recombine to restore a functional pair of neighboring active sites, capable of transferring the tethered carboxybiotin intermediate between two opposing chains. The transfer of a carboxybiotin intermediate between active sites on separate polypeptide chains is a previously unrecognized feature of PC catalysis. Several multi-functional enzymes have similarly been shown to transfer their tethered intermediates between active sites on opposing polypeptide chains (24, 25), suggesting that intermolecular intermediate transfer is a common and essential feature of catalysis.

Ethyl-CoA is bound to only one monomer of the RePC asymmetric dimer, permitting a direct comparison of the consequences of activator binding on domain arrangement and orientation. A superposition of the two monomers reveals a 40° rotation and a translational shift of nearly 40 Å in the BC active site, centered at the ethyl-CoA binding site of the allosteric domain (Fig. 4). In the tetramer, ethyl-CoA is bound to both monomers on the top face, and the BC active site is positioned ~65 Å from its opposing CT active-site pair (Fig. 3B). On the bottom face of the tetramer, ethyl-CoA is unbound, and the distance between the opposing active-site pairs increases to ~80 Å (Fig. 3C). The rotation in the BC domain inhibits acetyl-CoA binding on the bottom face of the tetramer. Thus, both binding sites are available per tetramer, which is consistent with the Hill coefficient observed for yeast PC (26) and with the observation that only 50% of acetyl-CoA binding sites are occupied in yeast PC (27). The structure suggests that acetyl-CoA activates PC by decreasing the distance between neighboring active sites. While the active-site pairs on the top face of the tetramer are pushed closer together, the active-site pairs on the bottom face are pulled farther apart. This is a rare example of allosteric activation paired with negative cooperativity and implies that half of the active-site pairs are more active than the others. Recent kinetic studies and numerical simulations support half-sites reactivity for the BC subunit of ACC (28), suggesting that this mechanism is conserved among enzymes of the biotin-dependent family. Such half-sites reactivity may permit PC to affect catalysis more efficiently while maintaining its in vivo association with other metabolic enzymes (29).

The allosteric binding site in PC offers a target for modifiers of activity that may be useful in the treatment of obesity or type 2 diabetes, and the mechanistic insights gained from the complete structural description of RePC permit detailed investigations into the individual catalytic and regulatory sites of the enzyme. Furthermore, as a consequence of its fully defined domain architecture, PC represents a paradigm for understanding interdomain arrangement and allosteric regulation in multifunctional enzymes.

References and Notes
11. Materials and methods are available as supporting material on Science Online.
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When Fear Is Near: Threat Imminence Elicits Prefrontal–Periaqueductal Gray Shifts in Humans

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Humans, like other animals, alter their behavior depending on whether a threat is close or distant. We investigated spatial imminence of threat by developing an active avoidance paradigm in which volunteers were pursued through a maze by a virtual predator endowed with an ability to chase, capture, and inflict pain. Using functional magnetic resonance imaging, we found that as the virtual predator grew closer, brain activity shifted from the ventromedial prefrontal cortex to the periaqueductal gray. This shift showed maximal expression when a high degree of pain was anticipated. Moreover, imminence-driven periaqueductal gray activity correlated with increased subjective degree of dread and decreased confidence of escape. Our findings cast light on the neural dynamics of threat anticipation and have implications for the neurobiology of human anxiety-related disorders.

Critical to an organism’s survival is the ability to switch flexibly between defensive states in response to threat. Within behavioral ecology, a key component of defensive switching is the “predatory imminence continuum” where distinct threat states are configured according to whether a predator is distal or proximal to the prey (1–5). This continuum