The 13-kDa protein p13suc1 has two folded states, a monomer and a structurally similar domain-swapped dimer formed by exchange of a β-strand. The refolding reaction of p13suc1 is multiphasic, and in this paper we analyze the kinetics as a function of denaturant and protein concentration and compare the behavior of wild type and a set of mutants previously designed with dimerization propensities that span 9 orders of magnitude. We show that the folding reactions of wild type and all mutants produce the monomer predominantly despite their very different equilibrium behavior. However, the addition of low concentrations of denaturant in the refolding buffer leads to thermodynamic control of the folding reaction with products that correspond to the wild type and mutant equilibrium dimerization propensities. We present evidence that the kinetic control in the absence of urea arises because of the population of the folding intermediates. Intermediates are usually considered to be detrimental to folding because they slow down the reaction; however, our work shows that intermediates buffer the monomeric folding pathway against the effect of mutations that favor the nonfunctional, dimeric state at equilibrium.

p13suc1 (referred to subsequently as suc1),1 a member of the Cks family of cell cycle regulatory proteins, has two native states: a monomer (1) and a domain-swapped dimer formed by exchange of a central strand, β4, of the β-sheet (2, 3) (Fig. 1). Each subunit of the dimer is highly superimposable on the monomer except for the so-called “hinge loop” that connects the swapping strand to the rest of the structure. The hinge loop forms a β-hairpin in the monomer and has an extended conformation in the dimer. In this work we present a global model of the folding and interconversion of monomeric and dimeric suc1. The description illustrates how domain swapping, by exploiting mostly native interactions, creates ambiguity in protein folding that can lead to misfolding and aggregation. Unexpectedly, however, we find that intermediates can protect the folding reaction and ensure that it leads to the correct oligomeric native product.

Recent theoretical and experimental work on folding of small proteins has shown that the native state topology defines the general features of protein folding pathways, and that sequence specificities modulate it to some extent (4–6). This view raises two broad questions: 1) How do protein sequences favor native-like interactions at each stage of the folding process and avoid misfolding? Do proteins possess gatekeeper residues that restrict the conformational space to native regions (7), or is the overall energy bias in favor of native interactions rather than non-native ones sufficient to guide folding? 2) Even if only native interactions are favored, it is still possible to make native interactions with residues from another polypeptide chain rather than intramolecularly by the process of domain swapping? How do proteins avoid these undesirable interactions and ensure folding to the correct oligomeric state?

Here we focus on the second question, which may be more significant than has previously been recognized. More than 40 proteins have now been crystallized in either a monomeric or domain-swapped form, showing that many proteins have a tendency to domain swap (8, 9); further, aggregation during refolding has been proposed in a number of proteins to result from domain swapping-mediated oligomerization (10, 11). Domain swapping might be even more of a problem within the crowded environment of the cell (12). One way to avoid misfolding and aggregation via domain swapping in the cell could be to assist folding with chaperones, thus providing an Anfinsen cage for monomers to fold in isolation. Our results on suc1 suggest another way; partly structured intermediates can limit misfolding by protecting the route to the biologically active state when mutations accumulate for functional reasons that energetically disfavor this state and instead favor domain-swapped misfolded states. This beneficial role of intermediates contrasts with the more commonly held negative view of intermediates as species that slow folding and reduce the cooperativity of the process, thereby acting as precursors to aggregation; in the latter case, however, misfolding is likely to be mediated by non-native intermolecular interactions rather than the native ones that characterize domain swapping.

EXPERIMENTAL PROCEDURES

Materials—High purity urea was obtained from Rose Chemicals Ltd. All other chemicals were from Sigma or BDH.

Protein Expression and Purification—The proteins were expressed in Escherichia coli and purified as described previously (13–16). Briefly, the harvested cells were resuspended in 50 mM Tris-HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA and then lysed by sonication, and the cell debris was removed by centrifugation. The supernatant was heat treated at 70 °C for 3–5 min, and the precipitate was removed by centrifugation. A 70% ammonium sulfate precipitation was
RESULTS

We showed previously that partitioning between monomer and domain-swapped dimer of suc1 is controlled by two prolines in the hinge loop that generate strain in the monomer (P90) and dimer (P92) hinge conformations (14, 15). Similar observations about the role of strained hinge loops in domain swapping have since been made in the B1 domain of protein L (17). The monomer-dimer equilibrium of suc1 can be monitored by analytical gel filtration chromatography, and the $K_d$ obtained for wild type was $1.8 \text{ mM}$; at protein concentrations in the $1–5 \text{ mM}$ range, suc1 is $>99\%$ monomeric at equilibrium (14). The mutants were designed previously by manipulating the strain with dissociation constants spanning 9 orders of magnitude (summarized in Table I). The mutant PA90 is an obligate monomer at protein concentrations in the micromolar to millimolar range. PA92 has a much lower $K_d$, but in the micromolar range it is also mainly monomeric. EP91 is mainly dimeric at low micromolar concentrations, and $\Delta \Delta T_{m}$ is $87–89$ is an obligate dimer.

Refolding to Monomer and Dimer Is under Kinetic Control

The proportions of suc1 monomer and dimer produced in the refolding reaction were measured by gel filtration analysis of samples immediately after refolding. The two forms of suc1 are separated by a large kinetic barrier, as is the case for many domain swapping proteins (18), and therefore the monomer-dimer ratio does not change during the analysis. We find that the relative amounts of monomer and dimer formed upon refolding are under kinetic rather than thermodynamic control (Fig. 2); refolding of $5 \text{ mM suc1}$ from the acid-denatured state produces only $93\%$ monomer, which is less than expected from the $K_d$. Subsequently, over a period of weeks, the $7\%$ dimer and $93\%$ monomer that is formed in the refolding reaction slowly converts to the equilibrium proportions of $1\%$ dimer and $99\%$ monomer.

The Monomer-Dimer Ratio Formed on Refolding of the Mutants Is Similar to That of Wild Type Despite Large Changes in Dissociation Constant

We next determined the monomer-dimer ratios formed on refolding the domain swapping mutants, PA90, EP91, and PA92. Surprisingly, we find that although wild type and the mutants vary greatly in the relative amounts of monomer and dimer populated at equilibrium, they show a striking uniformity in the proportions of monomer and dimer formed upon refolding from the denatured state (Fig. 3A). Interestingly, EP91 has a much greater dimerization propensity than wild type ($K_d = 0.1 \text{ mM}$) and populates only $10\%$ monomer at equilibrium at $5 \mu \text{M}$ protein concentration, but it forms even less dimer than does the wild type upon refolding. Monomeric EP91 is metastable, and equilibration to the more stable dimer species requires several weeks at room temperature. At the other extreme, $\Delta \Delta T_{m}$ (deletion of residues 87–89) is an obligate dimer.

Our results show that mutants with $K_d$ values spanning over 9 orders of magnitude all refold to approximately the same monomer-dimer ratio. Thus, the equilibrium partitioning between monomer and dimer can be altered by redesigning the hinge loop, but the folding reaction is unchanged. Even EP91 and $\Delta \Delta T_{m}$, mutants that are obligate dimers at equilibrium, refold predominantly to monomer; for these mutants, the monomer is a metastable state that is formed first and then is slowly converted to the more stable dimeric state.

Wild Type and Mutants Refold in Low Denaturant Concentrations to the Equilibrium Monomer-Dimer Ratios

When

...
wild type is refolded in buffer containing low urea concentra-
tions (1–2 mM). Control is no longer observed, and in-
stead the equilibrium proportions of monomer and dimer are
produced. Fig. 4A shows the proportion of dimer formed on
refolding plotted as a function of urea concentration in
the refolding buffer. At 5 mM protein concentration, 7% dimer
is formed in the absence of urea, whereas in 1–2 mM urea,
the equilibrium proportion of ~1% is formed. Similar behavior is
seen at 25 mM protein concentration (Fig. 4A). Likewise, the
mutants refold in low urea to the proportions of monomer and
dimer expected from each of their $K_d$ values. The mutant $\Delta$7879
illustrates this behavior most clearly. Refolding of 100 mM
$\Delta$7879 in the absence of urea yields 60% monomer, whereas
refolding in 1.5 mM urea yields >99% dimer (the equilibrium
proportion) (Fig. 4B).

An alternative explanation for the apparent switch from
kinetic control of the folding reaction in the absence of urea to
thermodynamic control in the presence of urea is that urea
speeds up the interconversion between monomer and dimer once
folding is complete; the interconversion process is known
to require significant unfolding (14), and therefore urea is
expected to speed it up. However, this explanation can be ruled
out because monomer-dimer interconversion is still very slow
(of the order of hours) at the low urea concentrations at
which the switch is observed.

Refolding Occurs via an Intermediate—The product of refold-
ing suc1 at 1 mM is >99% monomer, and the major refolding
phase measured by stopped flow fluorescence was shown previ-
ously to have a rate constant at low denaturant concentrations
that deviates from that expected for a two-state mechan-
ism (Fig. 5A) (13). This downward curvature in the refolding
arm of the chevron plot can be interpreted as arising from the
population of an intermediate before the rate-limiting transi-
tion state,

$$U - I_M - \frac{1}{2} M$$  \hspace{1cm} (Eq. 1)

where $U$ denotes the denatured state, $I_M$ is the intermediate,$\frac{1}{2} M$ is the rate-determining transition state, and $M$ is the
monomer native state. The brackets indicate a rapid pre-equili-

rium between the denatured state and the intermediate. (There is also slight curvature in the unfolding arm of the
chevron plot that has been ascribed to Hammond-like move-
ment of the transition state (13). However, the chevron plot for
two-state folding, given by the solid line in Fig. 5A and from
which the observed rate constant for refolding deviates, was
calculated assuming a smooth movement of the transition state
across the whole denaturant range.) Further evidence for an
intermediate comes from experiments performed with the hydro-
phobic dye ANS that indicate that there is a species formed in the
refolding reaction within the dead time of the stopped flow that
binds ANS and that subsequent release of ANS occurs on the
time scale of the major phase in the refolding reaction monitored
by intrinsic protein fluorescence (Fig. 6). Measurement of the
rate of refolding of suc1 at protein concentrations between 0.2
and 2 mM revealed no significant protein concentration depend-
ence of the rate or relative amplitude of the major refolding phase
(13). Thus, the major refolding phase does indeed correspond to
the formation of the monomer form rather than the oligomeric
forms of the protein. Further, we know that dimer suc1 forms
directly from the unfolded state and that association occurs early
in the reaction (14). Therefore, the following minimal model
applies to the folding pathways of monomeric and dimeric suc1,

$$2M - 2I_M = [2I_M - 2U] - \frac{1}{2} D + D$$  \hspace{1cm} (Eq. 2)

where $\frac{1}{2} D$ is the rate-determining step for formation of the
dimer (D).

Below we show data consistent with a model in which 1) the
folding pathway to dimeric suc1 proceeds through a distinct
intermediate that is dimeric and 2) the monomeric and dimeric
intermediates are responsible for the kinetic control of the
folding of suc1.
Kinetic control in the folding of suc1. The proportions of monomer and dimer formed in the reaction are different from those expected at equilibrium. Shown are absorbance-monitored elution profiles of wild type suc1 on an analytical Superdex 75 gel filtration column in 50 mM Tris pH 7.5, 1 mM EDTA, 300 mM NaCl. Monomer elutes at 12.3 ml, and dimer elutes at 10.5 ml. A, sample taken immediately after refolding from the acid-denatured state at 10 M protein concentration.

The proportion of suc1 monomer and dimer formed in the refolding reaction, and consequently the intermediate on the dimeric folding pathway. Further, the roll-over at low urea concentrations (Fig. 5B), indicating an intermediate in the amplitudes of the major and middle kinetic phases from the stopped flow fluorescence (Fig. 5D). However, less than 5% of dimer is formed during refolding at this low protein concentration, whereas the amplitude of the middle phase from the stopped flow is 30%. Without knowing the fluorescence properties of the various species, it is not possible to use the amplitude of the kinetic phase to estimate its occupancy, and therefore the lack of agreement between the two percentages cannot be interpreted. Our assignment of the middle phase to dimer formation is, however, supported by the change as a function of protein concentration in the amplitudes of the major and middle refolding phases and the correspondence with the change in the relative amounts of monomer and dimer formed (Fig. 7, C and D). The decrease in amplitude of the middle phase is paralleled by a decrease in the amount of monomer product; likewise, the increase in amplitude of the middle phase is paralleled by an increase in the amount of dimer product. Therefore a simple model can be proposed for the folding of suc1 to monomer and dimer,

\[ 2M + 2_{\text{dimer}} \rightarrow (2M + 2U + I_{\text{dimer}}) \rightarrow \text{D} \]  

where \( I_{\text{dimer}} \) is the dimeric intermediate.
We cannot exclude the possibility that the dimeric intermediate is in fact a mixture of various oligomeric species. However, at lower protein concentrations any ensemble is likely to be dominated by dimeric species, whereas at higher concentrations higher order oligomers may form. Higher order end products are indeed observed when refolding is performed at very high protein concentrations, as discussed below.

The Nature of the Dimeric Intermediate—It is likely that the dimeric intermediate is similar in structure to the monomeric intermediate except that some of the interactions are intermolecular rather than intramolecular. 1) The structure of the transition state for folding/unfolding of the dimer was shown to be very similar to that of the monomer (19). 2) The part of the protein that mediates domain swapping ($\beta$-strand 4) is part of...
the folding nucleus both in monomeric and dimeric suc1. Thus, folding is organized in the same way in both forms. 3) $\beta$-strand 4 forms a substantial amount of interactions with the rest of the protein in the monomeric intermediate early in the folding reaction (16) and even in the denatured state as revealed by molecular dynamics simulations (20) and SDS-PAGE (14). Therefore, association of $\beta$-strand 4 is likely also to occur early in the folding reaction of the dimer. Considering the similarity in structure of the monomeric and dimeric native states and the similarity of the two folding transition states including the location of the folding nucleus, it is reasonable to assume that the structure of the dimeric intermediate is likewise similar to that of the monomeric intermediate. Thus, the folding pathway of the dimer can be considered as a bimolecular version of the folding pathway of the monomer.

Stable Trimeric and Tetrameric Refolding Products at High Protein Concentrations—At protein concentrations of 50 $\mu$M and above, higher order oligomers are formed in the folding reaction (Fig. 8). These species are trimers and tetramers as indicated by gel filtration. No species of a higher order than tetramer were detected even at a protein concentration of 500 $\mu$M. The oligomers are stable at room temperature, and dissociation occurs to monomer and dimer over a period of weeks, suggesting that they are indeed intertwined species that are formed by domain swapping. The explanation for the absence of a higher order than tetramer may lie in the fact that the
Fig. 5. Kinetic data for the refolding of wild type and mutants as a function of urea. A, chevron plot of the rate constants for folding and unfolding of monomeric suc1, measured by stopped flow fluorescence. The major phase of the folding reaction (60% of the total fluorescence change) is shown with filled circles. The solid line represents the rate constants expected for two-state folding, determined by combining the equilibrium and kinetic unfolding data (taken from Rousseau et al.) (13). The middle phase (30%) is also shown (open triangles). B, comparison at 25 μM protein concentration of the rate constants for folding of monomer (circles) and the apparent rate constant for folding of dimer (squares). The
trimers and tetramers must form cyclic structures to have the observed stability (open ended linear trimers or tetramers have noncomplemented ends that would make them short-lived and/or prone to aggregation). The entropic cost of closing an oligomer loop consisting of more than four protein molecules is likely to be prohibitively high; hence the absence of such states. It is also possible that the conformation of the hinge loop is unfavorable in these high order cyclic oligomers. The folding of intertwined trimers and tetramers, as for dimers, is likely to be organized in a similar manner to the folding of monomers with the same folding nucleus being used in an intermolecular fashion to assemble the higher order species.

**Mutations That Shift the Monomer-Dimer Equilibrium Do Not Affect the Folding Mechanism**—Analysis of mutants PA90, PA92, EP91, and Δ8789 displaying extremes of domain-swapping behavior at equilibrium confirms the kinetic model for folding and interconversion of suc1 described above. First, as stated previously, the relative amount of monomer formed upon refolding is similar to wild type (Fig. 3A). Second, the $m$ value for the unfolding kinetics, $m_0$, for wild type and mutant monomers, are similar (Fig. 5C), indicating that no gross structural changes have occurred in the transition state, and the same is true for wild type and mutant dimers (Fig. 5E). Third, monomeric and dimeric forms of the mutants also fold via a three-state mechanism, populating intermediates before the rate-limiting transition state on the monomeric and dimeric folding paths as shown by the roll-over in the refolding arm of the chevron plots (Fig. 5C and Table I). Finally, like wild type, refolding of the mutants is under kinetic control; for these mutants, the ratio of monomer and dimer formed upon refolding is different from what is seen at equilibrium. For EP91 and Δ8789 this leads to the extreme situation where refolding produces mainly monomer, whereas the dimeric form is the most populated at equilibrium (Fig. 3A).

**The Kinetic Intermediates Buffer the Effect of Mutation on the Folding Pathway**—The difference free energies upon mutation of the hinge loop can be calculated for all the species on the monomer and dimer folding pathways (based on the model proposed above). This is done by using the equilibrium free energies of unfolding for wild type and mutant monomers, the $K_u$ values for wild type and mutant dimers, and the unfolding and refolding rate constants of wild type and mutant monomers and dimers (Table I). It is clear from these difference energies that the stabilities of the monomer and dimer folding intermediates are affected by mutation to almost the same extent as the transition states. Therefore, the large effect of mutation on the native monomer-dimer equilibrium is almost completely buffered on the refolding part of the energy landscape when the intermediates are populated. This can be quantified with a term $\Delta \Delta G$ ratio refolding, the change upon mutation in the ratio of monomer-dimer formed on refolding, which is a measure of the relative heights of the rate-determining steps of monomer and dimer refolding pathways.

$$\Delta \Delta G_{\text{ratio refolding}} = (\Delta \Delta G_{\text{dimer transition}} - \Delta \Delta G_{\text{dimer intermediate}}) - 2 \times (\Delta \Delta G_{\text{monomer transition}} - \Delta \Delta G_{\text{monomer intermediate}}) \quad \text{(Eq. 5)}$$

Because $\Delta \Delta G_{\text{dimer intermediate}}$ is very similar to $\Delta \Delta G_{\text{dimer transition}}$ and $\Delta \Delta G_{\text{monomer intermediate}}$ is very similar to $\Delta \Delta G_{\text{monomer transition}}$, the rate-determining steps are insensitive to mutation, and so the effect of mutation on the ratio of monomer and dimer formed upon refolding is very small; $\Delta \Delta G_{\text{intermediate}}$ buffers $\Delta \Delta G_{\text{transition}}$. In contrast, in the absence of intermediates, the change in the refolding product ratio upon mutation is given by the following equation.

$$\Delta \Delta G_{\text{ratio refolding}} = \Delta \Delta G_{\text{dimer transition}} - 2 \times \Delta \Delta G_{\text{monomer transition}} \quad \text{(Eq. 6)}$$

The rate-determining steps are now sensitive to mutation. Therefore the effect of mutation on the ratio of monomer and dimer formed upon refolding is much bigger, and the refolding behavior is affected to approximately the same extent as the equilibrium.

Because the difference in the extent of structure present in the native states compared with the respective transition states is much greater than the difference between the intermediates and the respective transition states, mutation has more of an effect on the unfolding rates than on the refolding rates. Consequently the rate of interconversion between monomer and dimer (which we showed previously requires substantial unfolding (14, 16, 19, 21, 22)) is greatly affected by the mutations. Thus, mutations that stabilize the dimer and destabilize the monomer, such as Δ8789 and EP91, stabilize the dimer transition state less than the dimer native state and destabilize the monomer transition state less than the monomer native state, thereby slowing down dimer unfolding and speeding up monomer unfolding. The result is that, although it takes several months for wild type monomer at 1 μM to convert to dimer, EP91 monomer converts to dimer within days, and Δ8789 converts within hours.

**DISCUSSION**

**A Model for Folding of suc1 Monomer and Domain-swapped Dimer**—suc1 has two folded states, a monomer and a domain-swapped dimer in which two molecules exchanges a β-strand. Here we have measured the fluorescence-detected folding kinetics and the ratio of monomer-dimer refolding products as a function of urea and protein concentrations and have compared the behavior of wild type and a set of mutants with domain swapping propensities spanning 9 orders of magnitude. A minimal scheme that accounts for all the data involves two distinct folding pathways from the denatured state leading to monomeric and dimeric suc1. Monomeric suc1 folds via an intermediate state, according to a three-state mechanism. The folding apparent rate constant for folding of dimer was calculated as described in the text. C, chevron plots for mutant monomers, with wild type shown for comparison. The data are fitted assuming a three-state folding reaction. D, rate constants for folding versus urea concentration at 5 μM protein concentration measured by stopped flow fluorescence. Circles, $k_u$; large squares, $k_{u1}$; small squares, $k_{u2}$; diamonds, apparent dimer refolding rate. E, unfolding rate constants of wild type and mutant dimers plotted as a function of urea concentration.
pathway to the dimeric native state is a bimolecular copy of the monomeric pathway; two molecules associate in the unfolded state and subsequently fold with the same three-state mechanism to the dimeric state. Monomer-dimer interconversion under native conditions is very slow, because it requires complete unfolding from one state and subsequent refolding to the alternative state. Therefore, once folded in a given state, the protein is kinetically trapped. When suc1 is refolded at protein concentrations above 50 μM, stable trimeric and tetrameric products are also observed, and their folding paths may also be higher order domain-swapped copies of the monomeric folding pathway.

Intermediates Buffer the Energetic Effect of the Mutation on the Folding Pathway—The most striking result in this paper is that wild type and the domain swapping mutants all refold to approximately the same monomer-dimer ratio, whereas in the presence of low urea concentrations the refolding products are those expected from the equilibrium monomer-dimer ratios. These observations can be accounted for by the monomeric and dimeric folding intermediates. Both intermediates have a substantial amount of native interactions and are almost as compact as their respective transition states; therefore a mutation affecting the energy of a rate-limiting transition state will

![Fig. 7. Effect of protein concentration on the folding reaction of wild type suc1. A, stopped flow fluorescence trace of refolding of acid-denatured suc1 wild type at 1 and 100 μM final protein concentrations. B, plot of the rate constant for refolding versus protein concentration for the two major refolding phases of wild type. C, comparison of relative amplitudes of refolding kinetic phases with proportions of monomer and dimer formed in the reaction, plotted as a function of urea concentration. Shown are the percentage of monomer formed (open circles) and the relative amplitude of the main refolding phase (filled circles). D, percentage of dimer formed (open circles) and the relative amplitude of the middle refolding phase (filled circles).](http://www.jbc.org/)

![Fig. 8. Refolding of wild type suc1 at high protein concentration produces higher order species. A gel filtration elution profile of a sample of protein refolded at 150 μM protein concentration is shown.](http://www.jbc.org/)
affect the associated intermediate to a similar extent. Consequently, there is only a very small net effect of mutation on the folding kinetics when the intermediates are populated. A similar uncoupling of folding and stability by intermediates is seen in the comparison of refolding of chymotrypsin inhibitor 2 and barnase in the presence of GroEL (23, 24).

Implications for Misfolding—Structural and functional data indicate that only the monomer form of suc1 is active. It is therefore crucial that folding of suc1 is directed toward the correctly folded, active state and that inappropriate folding routes leading to inactive forms such as the dimer are avoided. However, folding of suc1, like several other proteins, is initiated around a nucleus of partly formed native-like tertiary interactions (5, 25) that have the potential to form intermolecularly rather than intramolecularly. Although polypeptide sequences may be optimized to prevent the formation of nonnative contacts that lead to aggregation (26), misfolding is enhanced by functional loops that have the potential to form intermolecular interactions (5, 25) that have the potential to form intermolecular interactions (27). Chaperones provide one way to assist folding; our results show how folding intermediates can be exploited to hardwire access to the correct oligomeric state via native contacts, diverging could give rise to new functions by "exaptation," i.e., as a side effect of evolutionary pressures on the existing function of the monomer but without disrupting it. Intermediates could ensure the kinetic accessibility of the monomer even when it accumulates unfavorable interactions in an active site loop during evolution that favor a domain-swapped form. In this way, the more stable dimeric and initially nonfunctional counterpart can co-evolve without interfering with the monomeric function, waiting to be seized by evolution to fit in a different cellular context.

Evolutionary Implications—Intermediates slow down the folding of many proteins (25), and in suc1 they lower the efficiency of the folding reaction as they cause a slight overproduction of the dimeric species (compared with folding in the absence of intermediates). However, we see that this initial disadvantage could ultimately ensure folding to the correct oligomeric state during evolution; immediately after the discovery of domain swapping, Eisenberg and co-workers (18) realized that the phenomenon could provide a means for the evolution of oligomers from monomers by successive small mutational steps; even a single point mutation can be sufficient to switch from a monomeric to an oligomeric state. Domain swapping can add allostery to the pre-existing monomeric function or introduce a new binding site at the interface between subunits (18). In further mutational steps the protein can then diverge from the initial swapped structure and evolve more diverse functions. The work on suc1 shows how domain-swapping could give rise to new functions by "exaptation," i.e., as a side effect of evolutionary pressures on the existing function of the monomer but without disrupting it. Intermediates could ensure the kinetic accessibility of the monomer even when it accumulates unfavorable interactions in an active site loop during evolution that favor a domain-swapped form. In this way, the more stable dimeric and initially nonfunctional counterpart can co-evolve without interfering with the monomeric function, waiting to be seized by evolution to fit in a different cellular context.

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Intermediates Control Domain Swapping during Folding of \( p13^{\text{sucI}} \)

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