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Assembly of an active enzyme by the linkage of two protein modules

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ABSTRACT **The feasibility of creating new enzyme activities from enzymes of known function has precedence in view of protein evolution based on the concepts of molecular recruitment and exon shuffling. The enzymes encoded by the *Escherichia coli* genes *pur*U and *pur*N, N^{10}-formyltetrahydrofolate hydrolase and glycinamide ribonucleotide (GAR) transformylase, respectively, catalyze similiar yet distinct reactions. N^{10}-formyltetrahydrofolate hydrolase uses water to cleave N^{10}-formyltetrahydrofolate into tetrahydrofolate and formate, whereas GAR transformylase catalyses the transfer of formyl from N^{10}-formyltetrahydrofolate to GAR to yield formyl-GAR and tetrahydrofolate. The two enzymes show significant homology (≈60%) in the carboxyl-terminal region which, from the GAR transformylase crystal structure and labeling studies, is known to be the site of N^{10}-formyltetrahydrofolate binding. Hybrid proteins were created by joining varying length segments of the N-terminal region of the PurN gene (GAR binding region) and the C-terminal (N^{10}-formyltetrahydrofolate binding) region of PurU. Active PurN/PurU hybrids were then selected for by their ability to complement an auxotrophic *E. coli* strain. Hybrids able to complement the auxotrophs were purified to homogeneity and assayed for activity. The specific activity of two hybrid proteins was within 100- to 1000-fold of the native *pur*N GAR transformylase validating the approach of constructing an enzyme active site from functional parts of others.**

The evolution of biosynthetic pathways is an intriguing problem because pathway end products are often used to modulate activity of enzymes early in the pathway. Several theories, including the retrograde hypothesis (1, 2), forward evolution (3), gradual accumulation of mutant enzymes (4), and molecular recruitment (5), have been proposed to explain how biosynthetic pathways have evolved. In Jensen's hypothesis new enzyme functions are created by recruitment of enzymes catalyzing analogous reactions. Support for this hypothesis has come from a comparison of the structure and activity of mandelate racemase with muconate lactonizing enzyme (6). These enzymes are structurally homologous and both catalyze abstraction of α-protons from carboxylic acids. Recently, another enzyme, galactonate dehydratase, has been identified, which shares significant sequence homology with mandelate racemase (7) and also catalyses a reaction initiated by the abstraction of an α-proton from a carboxylic acid. It is therefore likely that many enzymes are represented in modern cells by a number of homologous counterparts that have diverged considerably in substrate specificity.

An understanding of how nature recruited primitive enzyme functionalities and tuned them to be competent for other tasks promises insight into how protein engineering may be used to design enzymes with novel properties. Previous approaches to the design of proteins with novel activities have included catalytic antibodies (8, 9); introduction of metal ion binding sites, such as the one engineered into trypsin to allow either control of the proteolytic activity (10) or to regulate specificity (11); creation of hybrid enzymes through exchange of subunits to create hybrid oligomers (12); replacement of structural elements such as the DNA binding domain of GCN4 with that of C/EBP (13); mutation of multiple individual residues to change the cofactor specificity of glutathione reductase from NADPH to NADH (14), modulation of the substrate specificity of aspartate aminotransferase (15); and changing the specificity of subtilisin (16) and α-lytic protease (17) through mutation of single functional groups.

There are no examples, however, of an active enzyme created by fusion of domains from two proteins in which both domains are important for catalysis. Creation of new enzymes using such a "tinker toy" approach in which binding domains are treated as discreet modules is not without precedent. It has been proposed that in nature, new genes can be created by combining the exons of unrelated genes in a process known as "exon shuffling" (18, 19). This suggests one way by which specific functionalities can be recruited and combined with others to create novel enzymes. This paper describes the creation of such a hybrid enzyme from enzymes involved in *de novo* purine biosynthesis.

In *Escherichia coli*, the third committed step of *de novo* purine biosynthesis is catalyzed by glycinamide ribonucleotide (GAR) transformylase. This 23-kDa enzyme catalyses the transfer of the formyl group from N^{10}-formyltetrahydrofolate to the free amino of GAR to give formyl-GAR and tetrahydrofolate as products. The catalytic mechanism has been investigated and the regions involved in substrate and cofactor binding have been identified through labeling studies (20) and inspection of the high resolution crystal structure the enzyme (21).

A second enzyme capable of formylating GAR has been identified in *E. coli* (22). This enzyme, the product of the *pur*T gene bears little resemblance to the *pur*N GAR transformylase in terms of sequence similarity and substrate specificity. A similar enzyme has been found in *Bacillus subtilis*, although to date no eukaryotic homologue has been identified. The *pur*T GAR transformylase uses ATP to drive forward the formylation of GAR using formate as the formyl donor. Formate is thought to be generated, at least in part, by the activity of another unique prokaryotic enzyme, N^{10}-formyltetrahydrofolate hydrolase. Encoded by the *pur*U gene, the 32-kDa enzyme hydrolyses N^{10}-formyltetrahydrofolate to formate and tetrahydrofolate. At the DNA level there is a marked homology (≈60%) between the N^{10}-formyltetrahydrofolate binding domain of the *pur*N GAR transformylase and a region in the C-terminal portion of PurU. These two enzymes provide the

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Abbreviations: fDDF, 10-formyl-5,8-dideazafolate; GAR, β-glycinamide ribonucleotide; GAR transformylase, glycinamide ribonucleotide transformylase

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model system for investigation of molecular recruitment *in vitro*. In short, we propose to create a GAR transformylase activity by recruitment of the GAR binding domain from PurN and the region of PurU, which is responsible for the binding and hydrolysis of N^{10}-formyltetrahydrofolate.

MATERIALS AND METHODS

Materials. Restriction enzymes were obtained from Promega and New England Biolabs. T4 DNA ligase, *Taq* polymerase, and the Wizard PCR Preparation System were obtained from Promega. Ultrapure dNTPs were obtained from Pharmacia. Agarose for gel electrophoresis was obtained from Kodak and FMC. Ni-NTA agarose was obtained from Qiagen (Chatsworth, CA). Radiochemicals were purchased from New England Nuclear. The cofactor 10-formyl-5,8-dideazafolate (fDDF) was purchased from John Hynes (Medical University of South Carolina). All other materials were obtained from commercial sources and were of the highest available quality.

Bacterial Strains. DH-5α (GIBCO/BRL–Life Technologies), BL21(DE3) pLys S (Novagen), MW12 [*ara* Δ*(gpt-pro-lac) thi rbs-221 ilvB2102 ilvHI2202 purN'-lacZ*$^+$*Y*$^+$::*Kan*^{R} *purT* λ*(DE3)*] (26).

Plasmid Construction. PCR amplification was performed with 1 μg plasmid DNA template, 0.2 mM dNTPs, 1 × PCR buffer, 1.5 mM $MgCl_2$, 2 μM primer, and 2 units *Taq* polymerase in a total volume of 100 μl. Reaction conditions were to heat at 95°C for 5 min ("hot start" PCR) followed by 30 cycles of 1 min at 95°C; 2 min at 42°C; 2 min at 72°C; and a final cycle of 1 min at 95°C; 2 min at 42°C; 15 min at 72°C. PCR products were purified from Nusieve low melting point agarose using the Wizard PCR Preparation System following the manufacturer's directions. The two purified fragments were then combined using overlap extension under identical conditions to the first reaction. The resulting fragment was purified as before and then cloned directly into a T-vector (Promega). The fragment was then subcloned into a T7 expression vector, either pT7–7 (United States Biochemical) or pET28b (Novagen). Primers used for PCR were as follows: *pur*N forward, GAT ATA CAT ATG AAT ATT GTG GTG CTT ATT TCC; *pur*U reverse, ATC GAT AAG CTT TAC GTT GAG AAA AAT GAA C; *pur*N reverse (long fragment), CAG CTC ACG CAC GGA TCC ATG GGT GTG TAA TCC GG; *pur*N reverse (short fragment), CAG CTC ACG CAC GGA TCC GTC AAA CGC GCT GGC GA; *pur*U forward (long fragment), TTG CCC AGA AGG ATC CGT GCG TGA G; *pur*U forward (short fragment), CGC GGA TCC CGC AAC GAG CAC GAT CAA; *pur*U only, GAT ATA CAT ATG TTG CCC AGA AGG ATC CGT GCG TGA G.

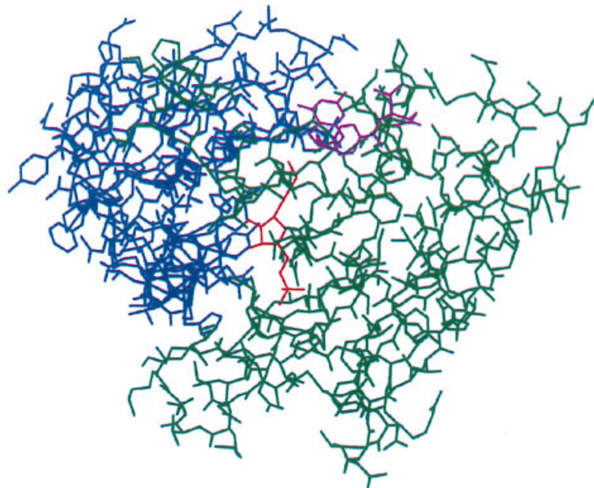
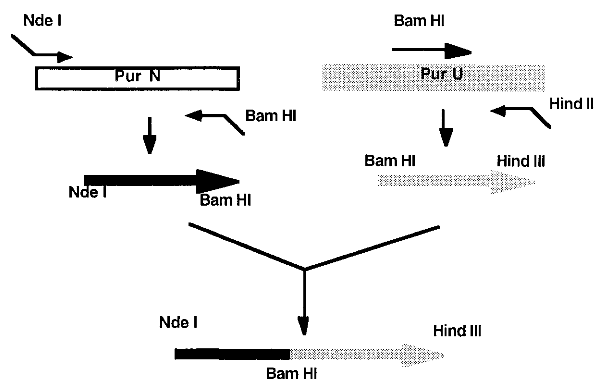


FIG. 1. Crystal structure of *pur*N GAR transformylase complexed with β-GAR and N^{10}-formyltetrahydrofolate. *pur*N is shown bound to β-GAR (red) and N^{10}-formyltetrahydrofolate (purple) and colored to show that the protein falls into two discrete domains—one responsible for binding GAR (green) and one domain that binds the N^{10}-formyltetrahydrofolate (blue). The active site is at the interface between the two domains.

Protein Preparation. Expression of the hybrid enzyme for purification was done in *E. coli* strain BL21(DE3) pLys S. Cells containing the expression vector were grown in Luria–Bertani media with 30 μg/ml kanamycin at 37°C until an OD_{600} of 0.4–0.6 was reached. Expression was induced by the addition of 1 mM of isopropyl β-D-thiogalactoside (IPTG). The cells were allowed to grow in the presence of IPTG for an additional 4 hr at 37°C before harvesting by centrifugation.

Cells were resuspended in a small volume of 50 mM Tris·HCl/1 mM EDTA (pH 7.5) and lysed by sonication.

A.



B.

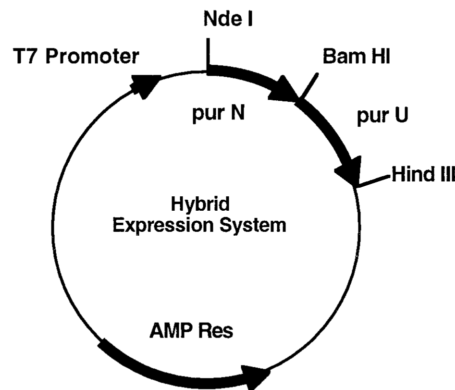


FIG. 2. Construction of hybrid enzymes. The hybrid enzymes were constructed using the PCR overlap extension method in the following way. The *pur*N fragment and *pur*U fragments were amplified separately. The *pur*N reverse primer and *pur*U forward primers were varied to change the size of each domain. A separate *pur*U primer was used to allow expression of the longer *pur*U domain only. This primer introduced an *Nde*I site to facilitate cloning and in so doing introduced a start codon. The *pur*N and *pur*U fragments were combined using overlap extension (*A*) and the resultant fragment was then cloned into a T7 expression vector, either pT7–7 or pET28b (*B*). The sequence of the fragment to be expressed was confirmed by double-strand sequencing using the Sequenase 2.0 kit (United States Biochemical).

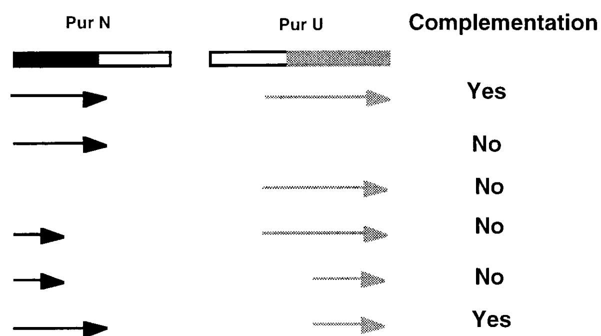


FIG. 3. Functional complementation screen of hybrid enzymes. The functional complementation screen for GAR transformylase activity was essentially as described (26). The auxotrophic *E. coli* strain, MW12, was transformed with plasmid containing the hybrid enzyme constructs plated onto LB-Amp plates and allowed to grow overnight. Colonies from this plate were picked onto minimal media plus ampicillin plates and allowed to grow. The constructs that allowed growth of the auxotrophic *E. coli* strain on the minimal media are indicated.

Inclusion bodies were collected as the pellet after centrifugation at 40,000 rpm in a Beckman 70 Ti rotor for 1 hr. The inclusion body pellet was washed three times with 8 M urea and then resuspended in 8 M urea/50 mM Tris·HCl (pH 8.0). This was then loaded onto a 4-ml Ni-NTA agarose column. The column was washed at a flow rate of ≈0.2 ml/min with 40 ml of 8 M urea/50 mM Tris·HCl (pH 8.0) and with 8 M urea/50 mM Tris·HCl (pH 6.5) until an $A_{280} < 0.01$ of the flow though was reached. Elution was with a stepwise gradient of 20 ml of 8 M urea/10 mM Tris·HCl (pH 5.9), followed by 20 ml of 8 M urea/10 mM Tris·HCl (pH 4.5), and finally 20 ml of 6 M guanidinium hydrochloride/0.2 M acetic acid.

Eluted hybrid enzyme was dialyzed against refolding buffer plus 8 M urea, 20 mM Tris·HCl, 5 mM EDTA, 5 mM DTT, 0.1 mg/ml PEG (pH 8.0) for ≈4 hr. The urea concentration was decreased in 1 M steps every 5–8 hr. After dialysis in refolding buffer only, the refolded protein was centrifuged to remove any insoluble matter and quantitated using a commercial Bradford reagent (Bio-Rad) using bovine gamma globulin as standard before storage at −70°C.

Enzyme Assays. Enzyme activity was determined by monitoring the deformylation of 10-formyl-5,8-dideazafolate ($\Delta\varepsilon$ = 18.9 $mM^{-1}\cdot cm^{-1}$ at 295 nm) resulting from the transfer of the formyl group to GAR (23). Assays were performed in 50 mM Tris, 1 mM EDTA at pH 7.5 and 20°C using a Gilford 252 spectrophotometer. For specific activity determination the concentration of GAR was 350 μM and fDDF concentration 195 μM. For determination of the steady-state parameters hybrid enzyme concentration was 2 μM, GAR concentration was varied between 4 and 153 μM, and fDDF concentration was varied between 2.5 and 195 μM.

Product Partioning. Hybrid enzyme (1 μM) was incubated with 0.7 mM [^{14}C]fDDF alone or in addition to 0.8 mM GAR for 3 hr at 37°C. Reaction products were separated by TLC using PEI–Cellulose plates (EM Separations, Gibbstown, NJ) eluting with 50 mM K_2HPO_4 (pH 7.0) and quantitated using a Molecular Dynamics PhosphorImager.

RESULTS

To increase the likelihood of catalytically active constructs varied segments of PurN and PurU were joined. Two fragments of each gene were prepared; for simplicity these will be referred to as long or short. From the crystal structure of GAR transformylase (Fig. 1) it is clear that the enzyme has two distinct domains; the amino-terminal domain binds GAR and the carboxyl-terminal domain binds N^{10}-formyltetrahydrofolate. By visual inspection of the crystal structure an estimation of residues that were important in creation of the GAR binding domain was made; this defined the longer PurN fragment (residues 1–115). PurN and PurU sequences were then compared using FASTA (24) to identify the N^{10}-formyltetrahydrofolate binding site. This analysis indicated that residues 63–157 in PurN were ≈60% homologous to residues 144–237 in PurU. For each region of homology a secondary sequence plot was created using the Genetics Computer Group program PEPPLOT; when compared, the plots showed significant homology with only two regions of disparity. This served to define the limit of the shorter fragment of PurN (from residue 1 to the beginning region of homology, residue 63) and also the shorter fragment of PurU (from the beginning of the region of homology, residue 144 to the carboxyl-terminus, residue 280). The longer PurU fragment (residues 85–280) was chosen based upon the report that that this region is homologous to the N^{10}-formyltetrahydrofolate binding site of *pur*N (25).

Table 1. Specific activity of hybrid enzymes

Enzymes	Specific activity saturating conditions, mmol per min/mg
Wild type	50 ± 2
"Long" hybrid	0.43 ± 0.03
"Short" hybrid	0.03 ± 0.002

Hybrid constructs were created by overlap extension with a *Bam*HI restriction site marking the boundary between the two domains (Fig. 2*A*). The PCR fragments were cloned into pT7–7, which uses the T7 promoter to drive protein expression (Fig. 2*B*). The hybrids were screened for activity by their ability to complement an auxotrophic *E. coli* cell line in a manner previously described (26). Two constructs were able to complement the auxotrophs (Fig. 3) with growth observed in 18–24 hr. This is somewhat slower than the rate observed with wild-type GAR transformylase (18 hr). The properties of these constructs were further investigated using purified protein.

To maximize expression of hybrid enzyme, the *E. coli* strain BL21(DE3) pLys S was used. Standard growth conditions of temperature and media composition produced recombinant protein, of which >95% was in the form of inclusion bodies. Techniques described in the literature which prevent or minimize inclusion body formation (27, 28) were unsuccessful. Preparation of soluble hybrid enzymes therefore began with the insoluble inclusion body pellets. Although in many cases no further purification is required following solubilization of the protein from inclusion body pellets, SDS/PAGE analysis of the pellet suggested that a subsequent purification step would be required. Because of protein aggregation, purification of the hybrid enzyme was performed under denaturing conditions using affinity chromatography. The DNA encoding the hybrid enzymes was subcloned into the expression vector pET 28b (Novagen), which adds an "His-Tag" at the N terminus. Purification was then accomplished by resolubilizing the pellet in urea and passing the denatured protein over a Ni-NTA column (Qiagen). His-tagged proteins were eluted from the column with a stepwise pH gradient. The hybrid enzymes eluted in 8 M urea (pH 5.9), and by SDS/PAGE were found to be >80% pure (Fig. 4).

Table 2. Steady-state characterization of hybrid enzyme

Enzyme	k_{cat} (s^{-1})	K_m (GAR)	K_m (fDDF)	k_{cat}/K_m (fDDF)
*pur*N	16 ± 1	19 ± 4	17 ± 3	0.94
*pur*U	0.026	ND	7 ± 0.3	0.0037
Hybrid	0.016 ± 0.0005	16 ± 2	35 ± 3	0.00046

ND, not determined.

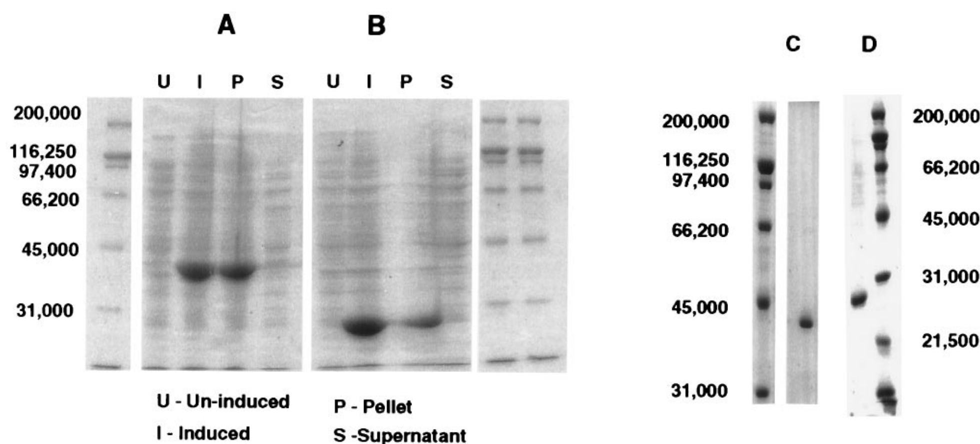


FIG. 4. Expression of hybrid enzymes. SDS/PAGE analysis of expression and purification of the hybrid enzymes. *A* and *B* show uninduced, induced, pellet, and supernatant for the long and short hybrid enzymes, respectively. *C* and *D* show the purified long and short hybrid enzymes, respectively. Gels were scanned, the images captured and resized using ADOBE PHOTOSHOP 3.0.

The hybrid enzymes were refolded using a stepwise dialysis method and then assayed for transformylase activity using fDDF as cofactor (18, 23). Specific activities of the two hybrid enzymes were determined and are shown in comparison to the wild-type activity in Table 1. The highest specific activity, obtained with the longer hybrid, was at only 100-fold lower than that of the wild type. The second and only other of the hybrid constructs able to complement the auxotrophs had an activity ≈1000-fold lower than the wild-type activity. Because of the low activity only the more active hybrid was characterized fully and these data are shown in Table 2. Steady-state characterization allowed binding affinities to be compared between the hybrid PurN and PurU enzymes. The K_m for GAR of the hybrid is similar to that of PurN, whereas the K_m for fDDF of the hybrid is approximately twice that of PurN and five times that of PurU from which the domain was derived. The k_{cat} of the hybrid enzyme was ≈1000-fold lower than the naturally occurring GAR transformylase.

To determine whether the hybrid enzyme was actually producing formyl-GAR and not merely deformylating fDDF, the hybrid enzyme was incubated with an excess of either [^{14}C]fDDF alone or [^{14}C]fDDF and GAR for 3 hr at 37°C. Separation of the products and subsequent quantitation indicated that the hybrid enzyme has a high hydrolytic activity compared with the transformylation reaction; the ratio of the two reactions is ≈40:1, for the wild-type GAR transformylase this ratio is ≈1:10,000. A consequence of the high hydrolysis: transformylase ratio is that the transformylase activity of the hybrid is reduced by a factor of 40.

DISCUSSION

Given that for many enzymes even the most subtle amino acid changes can affect activity and stability it is remarkable that we have been able to create an active enzyme using the approach described here. In the absence of a crystal structure we must speculate as to the physical arrangement of the domains in the hybrid GAR transformylase and whether they are organized in a manner similar to that of the *pur*N GAR transformylase. Since large amounts of each binding domain were used in creation of the hybrids it is likely that the domains retain their overall structural character, and activity in the case of the PurU fragment, but it is the correct alignment of these domains that is critical if the enzyme is to exhibit transformylase activity. A basic requirement for this to occur is that the amino group of GAR must be located proximal to the formyl group of the cofactor.

The longer N^{10}-formyltetrahydrofolate binding domain, which extends some 60 amino acids beyond the start of the region of homology, yields the hybrid with greatest activity. This suggests that, although the *pur*U N^{10}-formyltetrahydrofolate binding domain presents a similar surface to the GAR binding domain as the equivalent N^{10}-formyltetrahydrofolate domain from PurN, a linker needs to be present to optimize the interdomain contacts and also the orientation of the two domains for catalysis. Such improvement of the orientation of the two domains by varying the length of a linker peptide between the two domains is one way in which the overall stability and catalytic efficiency of the hybrid enzyme described here could be improved.

Examples exist in the literature where chimeric proteins have been created by domain swapping (29, 30), but in general, these proteins have few if any interdomain contacts. An attempt to produce a chimeric enzyme by fusion of domains of the ornithine and aspartate transcarbamoylases did not produce an active enzyme (31), possibly due to the absence of extensive interdomain contacts required to produce the active trimeric form of the enzyme.

Use of protein modules in the creation of novel protein and enzymatic activities offers almost limitless possibilities. Using an approach that is an *in vitro* extension of the ideas of molecular recruitment and exon shuffling we have created a hybrid GAR transformylase. The work described here suggests that the PurN GAR transformylase may have arisen through recruitment of a GAR binding domain into a protein capable of binding, and hydrolysis of, N^{10}-formyltetrahydrofolate. Also, it provides an example of what we think of as Stage 1 in protein catalyst design, that is, creation of a catalyst by fusion of a catalytic domain with a domain that orientates an acceptor substrate. Other examples in the class would be changing an enzyme's activity either by a series of point mutations or addition/deletion of secondary structural elements. Stage 2 would be to engineer catalytic residues into a protein with an existing binding activity, such as using a sugar binding/transport protein to create a hydrolase. Stage 3, the most difficult level in our scheme, is to engineer both residues that confer binding and catalysis into a structural framework, such as provided by the α/β barrel proteins.

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