haploids with an autonomously replicating plasmid containing the cognate *STE* gene and a selectable marker. The resulting fertile transformants were crossed to produce a diploid; strains that had lost the plasmid were obtained as mitotic segregants.
17. Suppression of pseudohyphal growth is defined by failure to observe pseudohyphae after 3 days. A further test was to wash away surface colony cells and observe whether any cells in the colony had penetrated the agar. Wild-type and Ste2$^-$, Ste3$^-$, Ste4$^-$, and Ste18$^-$ mutants left a florid network of long pseudohyphal filaments. The Ste20$^-$ mutants were completely suppressed; cells did not penetrate the agar but grew on the surface as elliptical cells. Null mutations in *STE11*, *STE7*, and *STE12* are somewhat leaky. Washing away surface colony cells exposed a few cells that penetrated the agar. Some formed short pseudohypha-like chains made up of round cells; rarely was a long cell observed. The presence of these short structures in kinase cascade mutants suggests the possibility of an alternate pathway for activation of pseudohyphal growth.
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28. The failure to detect a morphological phenotype for kinase cascade mutations in haploid cells is probably a consequence of the inability of wild-type haploid cells to form filaments. Only diploid cells have a polar budding pattern that allows filament formation; most haploid strains have an axial pattern that is inconsistent with filament formation. However, some haploid strains in standard use (*32*), as well as some *bud* mutants, produce a high proportion of polar budding cells. These polar budding haploid strains are capable of producing short filaments. It is likely that in these haploid strains, the kinase cascade is also required for filament formation.
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Mutations That Allow Disulfide Bond Formation in the Cytoplasm of *Escherichia coli*

Alan I. Derman, William A. Prinz, Dominique Belin, Jon Beckwith*

Disulfide bonds are rarely found in cytoplasmic proteins. Mutations were selected for in *Escherichia coli* that allow disulfide bond formation in the cytoplasm. In the presence of these mutations, export-defective versions of alkaline phosphatase and mouse urokinase were able to fold into their enzymatically active conformations in the cytoplasm because their disulfide bonds were formed. The mutations were mapped to the gene for thioredoxin reductase and diminish or eliminate the activity of this enzyme. Thioredoxin itself was found to be unnecessary for this disulfide bond formation. Thioredoxin reductase, but not thioredoxin, is thus implicated in keeping cysteines reduced in cytoplasmic proteins.

Disulfide bonds are important for the folding and structure of many proteins (*1*), most of which are exported from the cytoplasm (*2*). The cytoplasmic proteins that do have disulfide bonds are typically sulfhydryl oxidoreductases, which undergo redox interconversion between free sulfhydryl and disulfide-bonded conformations (*3*). The absence from the cytoplasm of proteins with stable disulfide bonds and their presence among exported proteins is generally attributed to the differing reducing environments of subcellular compartments (*4*, *5*). The reducing potential of the cytoplasm is substantially greater than that of the lumen of the endoplasmic reticulum or the extracellular environment (*5*). Indeed, the formation of disulfide bonds in exported proteins occurs only during or after their export from the cytoplasm (*6*, *7*). However, an oxidizing environment is not itself sufficient for the formation of disulfide bonds in exported proteins. A system consisting of at least two cellular envelope proteins, DsbA and DsbB, is necessary for the formation of disulfide bonds in exported proteins in *E. coli* (*8*).

With this background in mind, one can imagine two possible explanations for the scarcity of disulfide bonds in cytoplasmic proteins. The absence from the cytoplasm of a system such as the Dsb proteins may suffice to explain the fact that disulfide bonds are rarely found in cytoplasmic proteins. Alternatively, or in addition, a mechanism may exist that actively prevents the formation of disulfide bonds in the cytoplasm. We have taken a genetic approach in order to assess these possible explanations. We have obtained mutants of *E. coli* that allow the formation of disulfide bonds in the cytoplasm. That we have been able to obtain these mutants argues for the existence of a mechanism that actively prevents the formation of disulfide bonds in the cytoplasm. Characterization of these mutants provides insight into how disulfide bond formation is ordinarily prevented in the cytoplasm.

We have carried out a genetic selection for mutants in which disulfide bonds are formed in a protein localized to the cytoplasm. Alkaline phosphatase (AP), a nonspecific phosphomonoesterase, is normally found in the *E. coli* periplasm (*9*). AP has two intrachain disulfide bonds that are required for its native structure (*10*) and therefore for its enzymatic activity (*11*). However, when AP is retained in the cytoplasm, as when expressed without its signal sequence, its disulfide bonds are not formed (*6*, *12*). The inability of these disulfide bonds to be formed is the most likely explanation for the finding that AP is enzymatically inactive when retained in the cytoplasm (*6*, *13*). Selection for mutants in which AP that is localized to the cytoplasm acquires enzymatic activity is therefore equivalent to selection for the formation of disulfide bonds in the cytoplasm.

We required that AP substitute for a phosphomonoesterase that is normally present in the cytoplasm and whose activity is required for a particular metabolic function. Fructose-1,6-bisphosphatase is a phosphomonoesterase that is required for growth of *E. coli* on gluconeogenic carbon sources such as glycerol (*14*). If in a mutant lacking fructose-1,6-bisphosphatase (*fbp*$^-$), AP were able to acquire enzymatic activity in the cytoplasm, AP could substitute for fructose-1,6-bisphosphatase and restore growth on glycerol (*15*).

We constructed a strain that carries a deletion in the *fbp* gene (*16*) and expresses AP without its signal sequence under control of the *tac* promoter (*12*). We isolated 10 independent spontaneous mutants of this strain that were able to grow on glycerol (Glyc$^+$) at 37°C only if expression of the signal-sequenceless AP was induced with isopropyl *thio*-β-D-galactopyranoside (IPTG). These mutants satisfied another selection for active cytoplasmic AP as well.

A. I. Derman, W. A. Prinz, J. Beckwith, Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115.
D. Belin, Département de Pathologie, Centre Médical Universitaire, Geneva, Switzerland.
*To whom correspondence should be addressed.

The *serB* gene codes for the enzyme that catalyzes the final step in serine biosynthesis, the dephosphorylation of phosphoserine (*17*). When we introduced a deletion of the *serB* gene (*18*) into the mutants, we found that expression of the signal-sequenceless AP rendered them Ser^+ again.

Hfr mapping and P1 transduction revealed that a mutation at a locus between 19 and 20 min on the *E. coli* chromosome was responsible for the selected phenotype in all 10 mutants. The *trxB* gene, which codes for thioredoxin reductase (*19*), maps at 19.3 min (*20*). Because this enzyme is thought to contribute to the sulfhydryl reducing potential of the cytoplasm (*21, 22*), the *trxB* gene seemed a likely candidate for the site of the mutations. A disruption of the *trxB* gene (*trxB::kan*) (*23*) behaved like the selected mutations and rendered the *fbp*$^-$ strain $Glyc^+$ and the *serB*$^-$ strain Ser^+ when the signal-sequenceless AP was expressed. When these strains and the original mutants were transformed with a plasmid carrying only the *trxB* gene (*24*), the $Glyc^+$ and Ser^+ phenotypes were abolished. Thus, all of the mutations that we obtained lie in the *trxB* gene and result in a deficiency in or elimination of thioredoxin reductase.

In these *trxB* mutants, the AP acquired enzymatic activity in the cytoplasm. In the parent strain, about 1% of signal-sequenceless AP is enzymatically active owing to a small amount of export (*12*). In the mutants and in the *trxB::kan* strain, up to 25% of the signal-sequenceless AP was enzymatically active (Table 1), and this AP activity was present in the cytoplasm (Table 2).

The presence of AP activity in the cytoplasm suggested that disulfide bonds could form in the cytoplasm of *trxB* mutants. Indeed, all or nearly all of the signal-sequenceless AP produced in a pulse-labeling eventually acquired disulfide bonds (Fig. 1). As only a fraction of the protein was enzymatically active (Table 1), it is possible that some of these disulfide bonds were not native. As expected, though, the disulfide-bonded AP was in the cytoplasm (Fig. 2).

Murine urokinase, which is exported from a variety of mouse tissues and is exported to the periplasm when expressed in *E. coli* (*25*), contains a serine protease domain with six disulfide bonds (*26*) that resembles AP in that it is enzymatically inactive if expressed in the cytoplasm of wild-type *E. coli*. When expressed in the cytoplasm of the *trxB::kan* strain, however, the serine protease domain was enzymatically active. The activity was not present in growing cells, but accumulated gradually in nongrowing cells after the induction of urokinase expression during exponential growth (Fig. 3).

Table 1. Activity of signal-sequenceless AP in *trx* mutants. Strains were grown in M63 minimal medium containing 0.2% each of ribose and glycerol and supplemented with 50 μg/ml each of 18 amino acids (no cysteine or methionine), and appropriate antibiotics. Expression of signal-sequenceless AP was induced with 5 mM IPTG for 1 hour, and cells were then added to chilled iodoacetamide to give a final concentration of 100 mM. After 20 min on ice, the cells were washed twice (*12*), resuspended at one-tenth volume in 10 mM EDTA, 20 mM tris-HCl (pH 8.0), incubated with chicken egg white lysozyme (2 mg/ml) for 30 min on ice, and then lysed by three cycles of freezing in a dry ice–ethanol bath and thawing in a 28°C water bath. The volume of the lysates was then restored to that of the original culture by the addition of a buffer consisting of 10 mM $MgCl_2$, 10 mM $ZnCl_2$, 1 M tris-HCl (pH 8.0). The basis for the iodoacetamide treatment and the enzymatic assay for AP have been described (*12*). The *trxB36* and *trxB41* strains are thioredoxin reductase mutations obtained from the selection; Δ*trxA* is a chromosomal deletion that eliminates nearly all of the *trxA* gene, which codes for thioredoxin (*31*). Activities reported are the mean and standard error of the mean for three trials. In each of these trials, five independent isolates of the Δ*trxA* strain and one Δ*trxA*, *trxB::kan* derivative from each of these five isolates were assayed. The percentages in the last column were calculated by taking the activity measured in the *trx*$^+$ strain to represent an activation of 1% of the AP protein (*12*).

trx genotype	AP activity	Percent of total AP protein activated
trx$^+$	27 ± 8.4	1
trxB36	699 ± 154	26
trxB41	563 ± 225	21
trxB::kan	666 ± 168	25
Δ*trxA*	58 ± 6.4	2
Δ*trxA*, *trxB::kan*	553 ± 68.4	20

Table 2. Subcellular fractionation of AP activity of signal-sequenceless AP in *trxB* mutants. Cultures were grown, induced, treated with iodoacetamide, and washed as in Table 1 except that induction was for 2 hours and cultures were washed only once. Subcellular fractionation was carried out as described (*12*) except that (i) a spheroplast buffer of 18% sucrose, 33 mM tris-HCl (pH 8.0) was used; (ii) spheroplasts were prepared by 15-min incubation on ice with 1 mM EDTA and chicken egg white lysozyme (10 μg/ml); and (iii) instead of cells being set aside to be lysed for the whole-cell control, two tubes of spheroplasts were prepared per strain, one was separated into pellet and supernatant fractions as described, and one was lysed directly. The spheroplasts (pellet) were resuspended in spheroplast buffer before lysis. Enzymatic assays were carried out as described (*6, 12*). The data represent the average of two experiments. The *trxB36* and *trxB41* mutations were obtained from the selection, and *prlA4* is a mutation in the cellular export machinery that promotes export of signal-sequenceless AP (*12*). ND, not determined.

Enzyme	Fraction	*phoA Δ2-22*					*phoA*$^+$
		trx$^+$	*trxB36*	*trxB41*	*trxB::kan*	*trx*$^+$ (*prlA4*)	*trx*$^+$
Alkaline phosphatase	Lysed cells	46	400	551	366	722	6293
	Spheroplasts	2	365	501	334	14	74
	Supernatant	46	61	98	61	723	6013
β-lactamase	Lysed cells	2192	595	909	628	1646	6
	Spheroplasts	48	22	35	20	33	ND
	Supernatant	2129	572	853	577	1575	6
β-galactosidase	Lysed cells	208	146	100	107	211	413
	Spheroplasts	218	159	106	107	204	282
	Supernatant	15	8	9	10	22	19

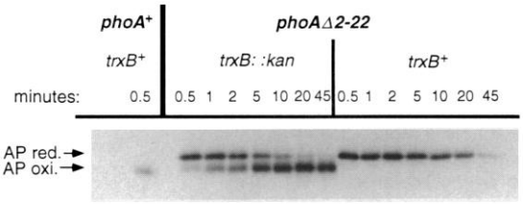

Fig. 1. Kinetics of disulfide bond formation in signal-sequenceless AP in the *trxB::kan* strain (*35*). Strains were grown in M63 minimal medium containing 0.2% each of ribose and glycerol and supplemented with 50 μg/ml each of 18 amino acids (no cysteine or methionine), and appropriate antibiotics. Expression of wild-type or signal-sequenceless AP was induced with 5 mM IPTG for 10 min, and cultures were pulse-labeled with [^{35}S]methionine for 1 min and chased for 45 min. At times during the chase, cells were added to chilled iodoacetamide to give a final concentration of 100 mM. After 20 min on ice, trichloroacetic acid was added to a final concentration of 5%. The precipitates were washed, the cells were lysed, and the cell contents were immunoprecipitated with a rabbit antibody to AP as described (*6, 12*). Nonreducing SDS–polyacrylamide gel electrophoresis (SDS-PAGE), which allows for the redox state of the AP species to be identified by electrophoretic migration, was carried out as described (*6*). The strain producing wild-type AP has a single chromosomal copy of the *phoA* gene (which codes for AP) under *tac* promoter control (*12*). Abbreviations: *phoA*$^+$, wild-type AP; *phoA*Δ2-22, signal-sequenceless AP; AP red., reduced AP; AP oxi., oxidized AP.

Our data suggest, then, that one function of thioredoxin reductase is to keep the cysteines of cytoplasmic proteins reduced. Thioredoxin reductase, in conjunction with a flavin cofactor, can reduce the redox-active cysteines of thioredoxin by using the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) as a source of reducing potential (*27*). But thioredoxin reductase is capable of reducing only thioredoxin (*28, 29*) and should not be able to reduce the cysteines of other cytoplasmic proteins directly. Its only known substrate, thioredoxin, is a general protein sulfhydryl reductase (*21, 22, 30*), and so a mechanism was suggested. If thioredoxin ordinarily keeps the cysteines of cytoplasmic proteins reduced, then the oxidized thioredoxin that accumulates in the absence of its reductase might be oxidizing these proteins instead. However, the AP activity of double mutants lacking both thioredoxin reductase and thioredoxin itself (*31*) was nearly as elevated as that of the single thioredoxin reductase mutants (Table 1). Thus, thioredoxin was either not required at all for, or contributed only minimally to, the cytoplasmic disulfide bond formation that occurred in thioredoxin reductase mutants.

Nevertheless, the rapid kinetics and high efficiency of disulfide bond formation (Fig. 1) suggest a facilitated process. Glutaredoxin, the other known general protein sulfhydryl reductase in the *E. coli* cytoplasm, is unlikely to be the facilitator because glutaredoxin cannot be reduced by thioredoxin reductase (*32*). Thus, the cytoplasm may contain another thioredoxin-like protein that can be reduced by thioredoxin reductase. In the absence of thioredoxin reductase, the oxidized form of this protein accumulates and promotes disulfide bond formation in the cytoplasm. This takes place, unexpectedly, in the presence of a ratio of reduced to oxidized glutathione that appears to be incompatible with the formation and maintenance of disulfide bonds in the cytoplasm (*5, 33*).

The *trxB* mutants grew as well as wild-type strains on a variety of media, even when synthesis of the signal-sequenceless AP was induced. This observation is surprising for two reasons. First, one might expect that if disulfide bonds are formed in AP, they would form in proteins that are ordinarily localized to the cytoplasm and thereby disrupt the function of these proteins. It is possible that cytoplasmic proteins fold rapidly enough that their cysteines are buried and oxidation cannot occur. Alternatively, the normal folding pathway of these proteins may not involve intermediates in which their cysteines achieve the appropriate orientations for disulfide bond formation. In contrast, signal-sequenceless AP folds slowly in the cytoplasm (*12*), and the folding pathway of AP is entirely compatible with disulfide bond formation. Second, the broad enzymatic specificity of AP, demonstrated by its ability to substitute for both the *fbp* and *serB* gene products, might be expected to bring about the dephosphorylation of numerous species [such as adenosine 5′-triphosphate (*34*)] and thereby critically upset cellular metabolism. That no growth liability comes of active AP in the cytoplasm is enigmatic.

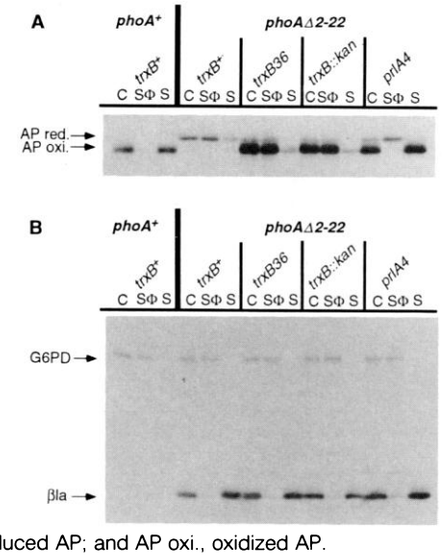

Fig. 2. Subcellular fractionation of signal-sequenceless AP in *trxB* mutants. The fractionation was done as in Table 2 except that after induction, cultures were pulse-labeled with [^{35}S]methionine for 1 min and chased for 30 min, at which time the cells were treated with iodoacetamide. Trichloroacetic acid precipitation of fractions, immunoprecipitations, and nonreducing SDS-PAGE was carried out as described (*6, 12*). The strain producing wild-type AP has a single chromosomal copy of the *phoA* gene (which codes for AP) under *tac* promoter control (*12*). The *trxB36* mutation was obtained from the selection, and *prlA4* is a mutation in the cellular export machinery that promotes export of signal-sequenceless AP (*12*). (**A**) Wild-type or signal-sequenceless AP was immunoprecipitated. (**B**) The cytoplasmic marker glucose-6-phosphate dehydrogenase (G6PD) and the periplasmic marker β-lactamase (βla) were immunoprecipitated. C, lysed cells; Sφ, spheroplasts (pellet); S, spheroplast supernatants; *phoA*$^+$, wild-type AP; *phoA*Δ2-22, signal-sequenceless AP; AP red., reduced AP; and AP oxi., oxidized AP.

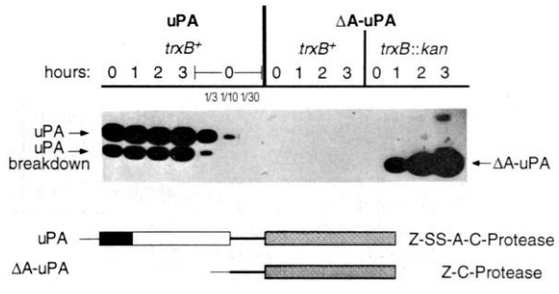

Fig. 3. Folding of the serine protease domain of uPA in the cytoplasm of the *trxB*::*kan* strain. Strains were grown in LB medium with appropriate antibiotics to exponential phase. Expression of the wild-type murine uPA or a variant lacking export information (ΔA-uPA) was induced with 5 mM IPTG for 1 hour (*36*). The cells were pelleted and resuspended to a concentration of 10 absorbance units (at 600 nm) per milliliter in 50 mM glucose, 10 mM EDTA, 25 mM tris-HCl (pH 8.1) and then held on ice for the indicated times. SDS-PAGE sample preparation buffer (2×, no β-mercaptoethanol) was added, the cells were lysed by two cycles of freezing and thawing, and the lysates were cleared by centrifugation. Nonreducing SDS-PAGE was carried out, and the serine protease activity in the gel was assayed by zymography with casein plasminogen agar underlays as described (*37*). The zymogram was allowed to develop for 10 hours at 37°C and then for 16 hours at room temperature. Z, the NH_2-terminal amino acids of the α fragment of β-galactosidase (thin line); SS, signal sequence of uPA (shaded box); A, noncatalytic A domain of uPA (open box); C, connecting peptide of uPA (thick line); Protease, the catalytic (serine protease) domain of uPA (stippled box). The serine protease domain is intact in the uPA breakdown product.

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The Learning of Categories: Parallel Brain Systems for Item Memory and Category Knowledge

Barbara J. Knowlton and Larry R. Squire

A fundamental question about cognition concerns how knowledge about a category is acquired through encounters with examples of the category. Amnesic patients and control subjects performed similarly at classifying novel patterns according to whether they belonged to the same category as a set of training patterns. In contrast, the amnesic patients were impaired at recognizing which dot patterns had been presented for training. Category learning appears to be independent of declarative (explicit) memory for training instances and independent of the brain structures essential for declarative memory that are damaged in amnesia. Knowledge about categories can be acquired implicitly by cumulating information from multiple examples.

Memory is not a single mental faculty but is composed of multiple and separate abilities that are mediated by distinct brain systems (*1*). The major distinction is between declarative or explicit memory, which depends on limbic and diencephalic structures (*2*) and provides the basis for conscious recollections of facts and events, and various nonconscious or implicit memory abilities, which support skill and habit learning, simple conditioning, and the phenomenon of priming (*3*, *4*).

Declarative memory typically refers to memory for recent single encounters and is usually assessed by tests of recall or recognition for specific items. However, when encountering a series of items, a subject not only learns about each item in the series but also accrues information about what all the items have in common. In this way, a subject learns about the category that is defined by the items that are presented. The question of interest is: What kind of memory supports the acquisition of category-level knowledge (*5*)? One view holds that category-level knowledge is acquired in the form of information about prototypes (average instances) or information about the statistical properties of the training items, and this knowledge is represented separately from knowledge about the training items themselves (*6*). Another view is that category-level knowledge has no special status but emerges naturally from item memory (*7*). Thus, a novel item would be endorsed as belonging to a particular category as a function of the similarity between the new item and the exemplars of that category already stored in memory.

Studies of amnesic patients could illuminate these issues, because these patients have severely impaired declarative (explicit) memory (due to limbic or diencephalic brain damage), but they are fully intact at tasks of nondeclarative (implicit) memory (*8*). Recently, amnesic patients exhibited normal classification learning (*9*) when category membership was defined by adherence to the rules of an artificial grammar (*10*). In the present study, we examined the ability of amnesic patients to learn to classify items on the basis of natural categories, that is, categories such as birds or furniture for which membership is based on family resemblance rather than on adherence of items to fixed rules.

Examples of study items and test items are illustrated in Fig. 1 (*11*). First, 12 control subjects and 10 amnesic patients (*12*) were presented with 40 training patterns (*13*). Then subjects were instructed that these patterns all belonged to a single category of patterns, in the same sense that, if a series of dogs had been presented, every item would belong to the category "dog." Five minutes later, subjects were tested with 84 new patterns and were asked to judge in each case whether the pattern did or did not belong to the same category as the training patterns (*14*). The two subject groups made category judgments with similar accuracy (Fig. 2A). An analysis of variance (ANOVA) indicated an effect of item type on classification [$F(3, 63) = 33.9$, $P < 0.01$] but no differences between groups and no interaction of group and item type ($P > 0.1$). Figure 2B shows overall performance on the classification task [percent correct; $t(20) = 1.45$, $P > 0.10$] together with the results for a second study-test sequence, scheduled an average of 1 to 2 months later, in which subjects attempted to recognize patterns that had appeared 5 min earlier (*15*). The subject groups differed in their ability to recognize the particular items that had been presented [$t(20) = 3.3$, $P < 0.01$]. There was also a significant interaction between the performance of the two groups on the classification and recognition tests [$F(1, 20) = 5.5$, $P < 0.05$].

Veterans Affairs Medical Center, San Diego, CA 92161, and Departments of Psychiatry and Neurosciences, University of California, San Diego, CA 92093.