Bartkiewicz, H. A. Gold, S. Altman, *Genes Dev.* **3**, 488 (1989)] in 50 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 100 mM NH_4Cl at 37°C for 2 hours. (One unit of human RNase P is defined as that amount of enzyme that cleaves 1 pmol of precursor to $tRNA^{Tyr}$ from *E. coli* in 30 min at 37°C.) For assays in subsequent rounds of selection, the amount of enzyme was reduced, and the incubation time was shortened so that less than 20% of the substrate was cleaved. Cleavage products were separated from uncleaved substrates by electrophoresis on an 8% polyacrylamide, 7 M urea gel. RNA was extracted from the gels by the crush and soak method. The purified cleavage product RNAs were reverse transcribed and amplified by PCR with SEC-1A (*8*) and SEC-1C (5′-TGGTGAGGCATGAAGG-3′) as primers with a Perkin-Elmer RNA PCR kit. The double-stranded DNA generated by PCR regained the T7 promoter sequence and the leader sequence from the sequence in the primer SEC-1A, and it was then used as a template for transcription of RNA for the next round of selection.

10. After eight cycles of selection and amplification, the resulting double-stranded DNAs were cloned into the Bluescript vector (Promega). Sixteen plasmid DNAs were sequenced by means of Sequenase 2.0 (U.S. Biochemical).
11. As a test for how well EGSs derived from the individual chimeric substrate clones can target mRNA, the EGS sequences were amplified by PCR with primers SCE-1C and SCE-1T (5′-TAATACGACTCACTATAGGCCAAACTGAGCAGAC-3′), which contains a promoter sequence for T7 RNA polymerase. RNAs were then transcribed with T7 RNA polymerase and used as substrates for human RNase P. Rates of EGS-directed cleavage of mRNA were determined in 10 μl of 50 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 100 mM NH_4Cl that contained 1 pmol (1000 cpm) of target RNA and 2.5 pmol of EGS RNA. Reaction mixtures were incubated at 37°C for various times with 0.5 μl (0.6 unit) of RNase P from HeLa cells and were then analyzed by electrophoresis in 5% polyacrylamide–7 M urea gels.
12. Partial digestion of CAT mRNA-EGS complexes with RNases T1 and T2 (Pharmacia) were performed in RNase P assay buffer (*9*). The reaction mixture contained substrate RNA labeled with ^{32}P at its 5′ terminus (2000 cpm), rat 5S RNA as carrier (at 0.2 mg/ml), and RNase T1 or RNase T2 (at three different concentrations, 2×10^{-4}, 1×10^{-3} and 5×10^{-3} units/ml). Reactions were incubated at room temperature for 5 min. The samples were analyzed by polyacrylamide gel electrophoresis in 12.5% sequencing gels. Conditions for digestion with cobra venom nuclease (Pharmacia) were as described above except that incubation was at 37°C.
13. H. Inokuchi, personal communication.
14. Y. Yuan and S. Altman, unpublished data.
15. DNA coding for EGS^{CAT} ΔAC was synthesized by PCR with $pEGS^{CAT}$ DNA (*3*) as template; and oligonucleotide EC-1ΔAC (5′-GCCAAACTGACGTCATCGACTTCG-3′) and M13 reverse primer (5′-AACAGCTATGACCATG-3′) were used as primers. The DNA generated by PCR was digested with Hind III and then inserted into pUC19 downstream from a T7 RNA polymerase promoter sequence. EGS^{CAT} ΔAC RNA was prepared by transcription in vitro after the new plasmid DNA had been linearized with Dra I. DNA that coded for EGS 19 was synthesized by PCR procedure in a manner similar to that used for the synthesis of DNA for EGS 9, that is, with oligonucleotides SCE-1C (*9*) and SCE-1I (5′-GTAATACGACTCACTATAGGCCAAACTGAGCAGACTCTAAATCTGCAAACGGAAGGTTC-3′): the newly inserted T residue in SEC-1I is underlined The DNA was transcribed in vitro with T7 RNA polymerase to give EGS 19 RNA. The EGS 19 RNA differs from EGS 9 RNA only in the additional U that restores the structure of the anticodon stem.
16. The dissociation constants of the CAT mRNA-EGS RNA complexes were determined [A. M. Pyle, J. A. McSwiggen, T. R. Cech, *Proc. Natl. Acad. Sci. U S.A.* **87**, 8187 (1990)]. A fragment of CAT mRNA, 160 nucleotides in length, was prepared by transcription with T7 RNA polymerase in the presence of [α-^{32}P]GTP. EGS RNA (10 μl) in 2× binding buffer was heated at 80°C for 4 min before it was mixed with an equal volume of 2 nM CAT mRNA fragment in water (1× binding buffer contains 50 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM NH_4Cl, 3% glycerol, 0.05% xylene cyanol). The mixtures were incubated at 37°C for 20 min and immediately separated on 5% polyacrylamide gels (9 watts). The electrophoresis buffer consisted of 36 mM tris base, 64 mM Hepes, 0.1 mM EDTA, 10 mM $MgCl_2$ (pH 7.5 without any adjustment). Quantitation of free target RNA and of the complex was performed with a Betascope (Betagen, Waltham, MA). The free energies of binding were determined from the equation $\Delta G^{\circ} = -RT\ln(1/K_d)$, where $R = 0.00198$ kcal/mol and $T = 310.15$ K.
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Heat-Inducible Degron: A Method for Constructing Temperature-Sensitive Mutants

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A temperature-sensitive (*ts*) mutant retains the function of a gene at a low (permissive) temperature but not at a high (nonpermissive) temperature. Arg-DHFR, a dihydrofolate reductase bearing an amino-terminal (N-terminal) arginine, is long-lived in the yeast *Saccharomyces cerevisiae*, even though arginine is a destabilizing residue in the N-end rule of protein degradation. A *ts* derivative of Arg-DHFR was identified that is long-lived at 23°C but rapidly degraded by the N-end rule pathway at 37°C. Fusions of *ts* Arg-DHFR to either Ura3 or Cdc28 of *S. cerevisiae* confer *ts* phenotypes specific for these gene products. Thus, $Arg\text{-}DHFR^{ts}$ is a heat-inducible degradation signal that can be used to produce *ts* mutants without a search for *ts* mutations.

Conditional mutants make possible the analysis of physiological changes caused by inactivation of a gene or a gene product and can be used to address the function of any gene. Several types of conditional mutants and methods for producing them have been developed (*1*, *2*) since Horowitz's demonstration of the utility of *ts* mutants (*3*), but the *ts* phenotype is still the one most frequently used (*4*, *5*). One limitation of the *ts* approach is the uncertainty as to whether a given gene can be mutated to yield a *ts* product. For example, only six loci were identified after repeated searches for *ts* lethal mutations mapping to the *S. cerevisiae* chromosome I, which contains many essential genes (*5*). Another problem with conventional *ts* mutations is that they are often too leaky to be useful (*4*). We now describe a strategy for producing *ts* mutants that does not require a search for a *ts* mutation in a gene of interest. This strategy is based on a portable, heat-inducible N-degron (Fig. 1).

The N-degron is an intracellular degradation signal whose essential determinant is a "destabilizing" N-terminal residue of a protein. A set of N-degrons containing different destabilizing residues is manifested as the N-end rule, which relates the in vivo half-life of a protein to the identity of its N-terminal residue (*6*). In eukaryotes, the N-degron consists of at least two determinants: a destabilizing N-terminal residue and a specific internal Lys residue (or residues) of a substrate (*6*, *7*). The Lys residue is the site of attachment of a multiubiquitin chain (*6*–*8*). Ubiquitin is a protein whose covalent conjugation to other proteins plays a role in a number of cellular processes, primarily through routes that involve protein degradation (*6*, *8*).

We constructed a thermolabile protein that becomes a substrate of the N-end rule pathway only at a temperature high enough to result in at least partial unfolding of the protein. This unfolding activates a previously cryptic N-degron in the protein by

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increasing exposure of its destabilizing N-terminal residue, by increasing mobilities of its internal Lys residues, or because of both effects at once. Since proteolysis by the N-end rule pathway is highly processive (6), one can make any protein of interest conditionally short-lived by expressing it as a fusion to the thus engineered thermolabile protein, with the latter serving as a portable, heat-inducible N-degron (Fig. 1).

Arg-DHFR, a variant of the 21-kD mouse DHFR in which the wild-type N-terminal Val is replaced by Arg, is long-lived in the yeast *S. cerevisiae* [half-life ($t_{1/2}$) > 6 hours at 30°C], even though Arg (unlike Val) is a destabilizing residue in the N-end rule (7). We searched for a *ts* allele of Arg-DHFR whose cryptic N-degron would be activated at 37°C but not at 23°C. A plasmid (pPW17R) was constructed that expressed Ub-Arg-DHFR-ha-Ura3 in *S. cerevisiae* (9). The ubiquitin (Ub) moiety of this fusion protein was required for production of the desired residue, such as Arg, at the N-terminus of the DHFR moiety. Ubiquitin fusions are rapidly cleaved in vivo after the last residue of ubiquitin, making possible the production of otherwise identical proteins bearing different N-terminal residues (6) (Fig. 1). The "ha" epitope allowed immunoprecipitation of the Arg-DHFR-ha-Ura3 fusion with a monoclonal antibody to ha (anti-ha) (9). The *S. cerevisiae* Ura3 moiety made possible selections for or against the fusion's presence in cells (Fig. 2A), while also serving as a test protein (Fig. 1).

We carried out a screen for derivatives of pPW17R that could confer onto Ura$^-$ cells a *ts* Ura$^+$ phenotype whose *ts* aspect required the N-end rule pathway (9, 10). This screen yielded two mutant plasmids with the desired properties: at 23°C, these plasmids conferred a Ura$^+$ phenotype, whereas at 37°C they conferred a Ura$^-$ phenotype in [*UBR1 ura3*] cells but a Ura$^+$ phenotype in congenic [*ubr1Δ ura3*] cells (Figs. 1 and 2A). The [*ubr1Δ ura3*] strain lacked the N-end rule pathway because it lacked N-recognin (encoded by *UBR1*), the recognition component of the pathway (6, 11, 12). The relevant change in both plasmids was a single missense mutation that replaced Pro with Leu at position 66 in the DHFR moiety of Ub-Arg-DHFR-ha-Ura3, yielding Ub-Arg-DHFR^{ts}-ha-Ura3 (10). The Pro^{66} region of DHFR connects its αII helix to the βC strand (13).

We then used Arg-DHFR^{ts} to produce a *ts* version of the *S. cerevisiae* Cdc28 protein kinase—an essential component of the cell cycle oscillator (14). The chromosomal *CDC28* gene was replaced with a gene that expressed Ub-Arg-DHFR^{ts}-ha-Cdc28 (15). The resulting *S. cerevisiae* strain was compared to the wild-type (*CDC28*) strain YPH500. Whereas the wild-type strain grew at both 23°C and 37°C, a representative strain expressing Ub-Arg-DHFR^{ts}-ha-Cdc28 (instead of the wild-type Cdc28) grew at 23°C but was inviable at 37°C (Fig. 2B). The morphology of these cells was examined after a temperature upshift in liquid culture. After 2 hours at 37°C, cells that expressed Ub-Arg-DHFR^{ts}-ha-Cdc28 became larger but lacked buds (G_1 phase morphology); however, by 4 hours at 37°C, many of these cells developed abnormal (elongated) buds and arrested in this configuration, which is similar to the arrest phenotype observed with some of the conventional *ts* alleles of *CDC28* (14). This Cdc28-mediated *ts* lethal phenotype required the presence of the N-end rule pathway, inasmuch as *ubr1Δ* cells that expressed Ub-Arg-DHFR^{ts}-ha-Cdc28 grew at both 23°C and 37°C (Fig. 2B), and remained morphologically normal at 37°C.

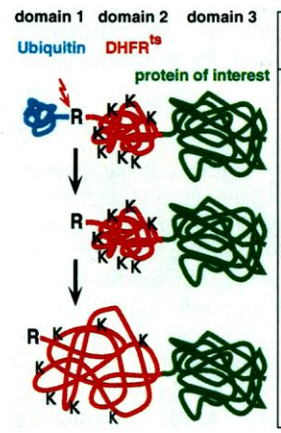
Pulse-chase experiments confirmed that Arg-DHFR^{ts}-ha-Cdc28 was long-lived at 23°C but short-lived at 37°C ($t_{1/2}$ < 10 min) (Fig. 3). The onset of metabolic instability of Arg-DHFR^{ts}-ha-Cdc28 after the temperature upshift was extremely rapid (Fig. 3). As could be expected from the results of phenotypic analysis (Fig. 2B), Arg-DHFR^{ts}-ha-Cdc28 was long-lived at both temperatures in *ubr1Δ* cells that lacked the N-end rule pathway (Fig. 3).

We conclude that Arg-DHFR^{ts} can be used as a portable, heat-inducible N-degron to produce *ts* mutants of a new class, called *td* (temperature-inducible degron). Features of the *td* technique that should make it useful in a variety of settings include the following.

1) The *td* method does not require an extensive, often unsuccessful search for a *ts* mutation in a gene of interest.

2) If the protein of interest can tolerate an N-terminal extension, the corresponding *td* fusion is likely to be functionally unperturbed at permissive temperature, as appears to be the case with Ura3 and Cdc28 (Fig. 1). By contrast, low activity at permissive temperature is a common problem with conventionally derived *ts* proteins (4) and is also expected to be a complication with proteins expressed from genes whose nonsense mutations are suppressed by a conditional suppressor tRNA (2).

3) A frequent problem with conditional phenotypes is the "phenotypic lag" that may occur between the imposition of nonpermissive conditions and the emergence of a relevant null phenotype. The *td* method eliminates or reduces this problem, because the heat-induced activation of the conditional N-degron results in rapid disappearance of a *td* protein (Fig. 3). In an earlier



T (°C)	Half-life in *S. cerevisiae*		Phenotypes with Ura3 as domain 3		Phenotypes with Cdc28 as domain 3	
	UBR1	*ubr1Δ*	*UBR1*	*ubr1Δ*	*UBR1*	*ubr1Δ*
23°C	deubiquitination (cotranslational)					
23°C	long	long	Ura$^+$	Ura$^+$	growth	growth
37°C	short	long	Ura$^-$	Ura$^+$	arrest	growth

Fig. 1. The *td* method. A fusion protein on the left contains an N-terminal ubiquitin (Ub) moiety (blue), a *ts* dihydrofolate reductase (DHFR^{ts}) moiety (red), with a destabilizing residue such as Arg (R) at the Ub-DHFR junction, and a test protein moiety (green) at the C-terminus of the fusion. In the present work, the test proteins were Ura3 and Cdc28 of *S. cerevisiae*. Some of the Lys (K) residues of DHFR^{ts} are indicated as well. Expression of this fusion in a eukaryote such as the yeast *S. cerevisiae* results in rapid cleavage at the Ub-DHFR junction and the exposure of a destabilizing Arg (R) residue at the N-terminus of a deubiquitinated fusion (6). At permissive temperature (23°C), the N-degron of the Arg-DHFR^{ts} moiety is inactive, apparently because none of the Lys residues in the folded DHFR^{ts} can serve as an efficient ubiquitination site (6). However, at nonpermissive temperature (37°C), a conformational destabilization of Arg-DHFR^{ts} results in at least some of its lysines becoming available as ubiquitination sites of the previously cryptic N-degron. The processive degradation of the fusion by the N-end rule pathway then ensues, greatly reducing its level in the cell. In the examples shown, the yeast Ura3 (orotidine-5′-phosphate decarboxylase) as the C-terminal moiety of the fusion resulted in Ura$^+$ cells at 23°C but in Ura$^-$ cells at 37°C. Similarly, when the essential kinase Cdc28 was expressed as an Arg-DHFR^{ts}-Cdc28 fusion, cells grew at 23°C but not at 37°C. With either Arg-DHFR^{ts}-Ura3 or Arg-DHFR^{ts}-Cdc28, the absence of the N-end rule pathway (in *ubr1Δ* cells) precluded these conditional phenotypes at 37°C. Thus, Arg-DHFR^{ts} can be used as a portable, heat-inducible N-degron that yields *ts* mutants of a new class, called *td* (temperature-inducible degron).

application of the N-end rule to the problem of phenotypic lag, Park *et al.* (*16*) fused a constitutive N-degron to a protein expressed from a regulatable promoter. Aside from being a non-*ts* technique, this otherwise useful method (*16*) is constrained by the necessity of using a heterologous promoter and by the constitutively short half-life of a target protein, whose levels may therefore be suboptimal under permissive conditions. (Although the test genes of the present work were also expressed from a heterologous promoter, this limitation is not intrinsic to the *td* technique.)

4) One advantage of the *td* method is the possibility of using two sets of experimental conditions: a *td* protein-expressing strain at permissive versus nonpermissive temperature or, alternatively, the same strain versus a congenic strain lacking the N-end rule pathway, with both strains at nonpermissive temperature. This powerful internal control, provided in the *td* technique by two alternative sets of permissive and nonpermissive conditions, is unavailable with conventional *ts* mutants.

At present, the *td* method is confined to proteins of the cytosol and the nucleus—compartments where the N-end rule pathway is known to operate (*6*). However, the *td* concept should also be applicable to degrons in other compartments. Cytosolic degradation signals distinct from N-degrons and residing in either DHFR or other carriers should be feasible as well and may prove superior for certain applications. We recently found that the heat induction of the Arg-DHFR^{ts} N-degron is inhibited in the presence of methotrexate, a DHFR-specific, tightly binding substrate analog (*17*). The resulting possibility of controlling a degron with agents other than temperature can be used to construct new classes of conditional mutants.

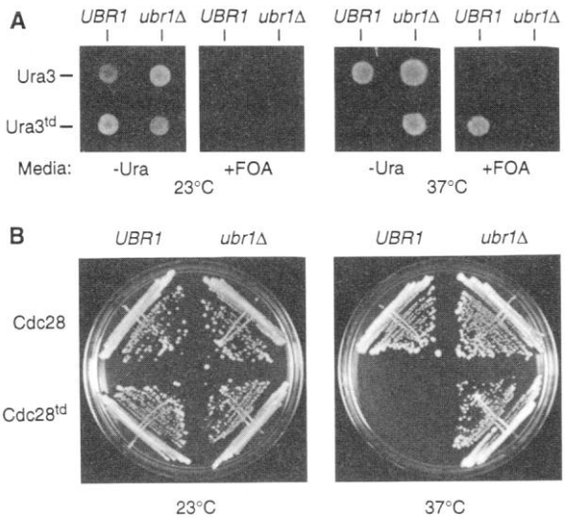


Fig. 2. The *td* mutants. (**A**) Ura3 as a test protein. *Saccharomyces cerevisiae* YPH500 (*MATα ura3 his3 UBR1*) (*18*) and the congenic strain JD15 (*ubr1Δ ura3*) were transformed with pPW17R, expressing Ub-Arg-DHFR-ha-Ura3 (denoted as Ura3), or with pPW43R, expressing Ub-Arg-DHFR^{ts}-ha-Ura3 (denoted as Ura3^{td}) (*10*). Individual colonies of plasmid-carrying cells were dispersed in water, and 5 μl of each suspension, containing approximately equal amounts of cells, was spotted either on SD(-Ura, -His) plates, which allowed the growth of Ura$^+$ but not of Ura$^-$ cells, or on SD(FOA, Ura, -His) plates, which allowed the growth of Ura$^-$ but not of Ura$^+$ cells (*10*). The plates were incubated at either 23°C or 37°C, as indicated, and photographed 3 to 5 days later, depending on the growth temperature and media composition. (**B**) Cdc28 as a test protein. YPH500 (*18*) was transformed with the linearized plasmid pPW66R, yielding the strain PWY1, which expressed Ub-Arg-DHFR^{ts}-ha-Cdc28 (denoted as Cdc28^{td}) instead of the wild-type Cdc28 (*15*). PWY1 was crossed to JD55 [*MATa ura3 ubr1Δ::HIS3*]. The resulting diploid was sporulated, and haploid *UBR1* and *ubr1Δ* segregants (*11, 12*) that expressed either wild-type Cdc28 or Cdc28^{td} were isolated; in these segregants, *HIS3* and *URA3* marked, respectively, *ubr1Δ* and the Cdc28^{td}-encoding allele of *CDC28*. These strains (their relevant genotypes are indicated around the plates) were streaked in sectors on YPD plates containing 0.1 mM $CuSO_4$, and incubated at 23°C or 37°C, as indicated. The plates were photographed after 4 or 3 days of growth at 23°C or 37°C, respectively.

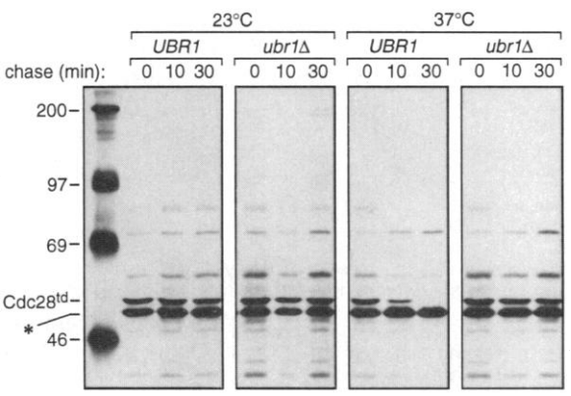


Fig. 3. Pulse-chase analysis of Cdc28^{td}. Exponential cultures of either *UBR1* or *ubr1Δ S. cerevisiae* that expressed Arg-DHFR^{ts}-ha-Cdc28, denoted as Cdc28^{td} (see the legend to Fig. 2B and (*15*)], were labeled with ^{35}S-methionine for 5 min at 23°C, followed by a chase at 23°C or 37°C for 0, 10, and 30 min, extraction, immunoprecipitation with anti-ha, and SDS–polyacrylamide gel electrophoresis analysis (*11, 12, 19*). Durations of chase, temperatures, and the relevant genotypes are indicated above the lanes. The band representing Cdc28^{td} and molecular size markers (in the leftmost lane) are also indicated (the latter in kilodaltons). The asterisk denotes an unrelated *S. cerevisiae* protein that cross-reacted with anti-ha; this protein (*19*) was present in control immunoprecipitates from cells lacking Arg-DHFR^{ts}-ha-Cdc28.

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9. The *CEN6, HIS3*-based plasmid pPW17R, which expressed Ub-Arg-DHFR-ha-Ura3 from the *S. cerevisiae* P_{CUP1} promoter, was constructed in the background of pRS313 (*18*). Briefly, a ~0.4-kb fragment from pJDC22-2 (*12*) that contained P_{CUP1} was ligated to a separately constructed fragment encoding Ub-Arg-DHFR-ha-Ura3. The DHFR moiety of Ub-Arg-DHFR-ha-Ura3 contained the sequence His-Gly-Ser-Gly-Ile-Met between Arg at the Ub-DHFR junction and Val, the wild-type N-terminal residue of DHFR (*7*). The DHFR moiety was followed by a 14-residue, ha epitope–containing sequence (*19*). The Ura3 moiety of Ub-Arg-DHFR-ha-Ura3 was actually a fusion of the last 91 residues of *S. cerevisiae* His4 to residue 6 of the Ura3 protein [E. Alani and N. Kleckner, *Genetics* **117**, 5 (1987)]. Purified pPW17R was mutagenized with hydroxylamine [S. Busby, M. Irani, B. de Crombrugghe, *J. Mol. Biol.* **154**, 197 (1982)]. The resulting DNA was used to transform the Ura$^-$ (*pyrF*) *Escherichia coli* MC1066 [M. J. Casadaban, A. Martinez-Ariaz, S. K. Shapira, J. Chow, *Methods Enzymol.* **100**, 293 (1983)] to ampicillin (amp) resistance, with selection on Luria broth–amp plates at 37°C. Transformants were replica-plated

onto M9 plates containing amp, Trp, and Leu, and lacking uracil. The yeast *URA3* gene complements the Ura$^-$ phenotype of *E. coli pyrF* mutants [M. Rose, P. Grisafi, D. Botstein, *Gene* **29**, 113 (1984)]. This *E. coli* screen eliminated mutant plasmids that did not express a functional Ura3 moiety of Ub-Arg-DHFR-ha-Ura3 at 37°C. However, those (potentially relevant) plasmids that expressed a mutant DHFR moiety were expected to pass this test. [*E. coli* lacks the ubiquitin system (*6*). The N-terminal ubiquitin moiety of Ub-Arg-DHFR-ha-Ura3 was therefore retained in *E. coli*, precluding the formation of an N-degron.]

10. Plasmids that passed the *E. coli* screen (*9*) were introduced into *S. cerevisiae* YPH500 (*his3 ura3*) (*18*), with transformants selected at 23°C on SD(-Ura) plates containing 0.1 mM $CuSO_4$. The colonies were replica-plated onto SD plates (*20*) lacking His (to select for plasmid retention) and containing 5-fluoroorotic acid (FOA) and uracil [J. D. Boeke, F. Lacroute, G. R. Fink, *Mol. Gen. Genet.* **197**, 345 (1984)]. The FOA plates were incubated at 37°C to select against cells (carrying pPW17R plasmids) that could yield a functional Ura3 at 37°C. (FOA kills Ura3$^+$ cells.) After several rounds of the FOA-mediated selection against cells that were Ura$^+$ at 37°C, we verified the *ts* Ura$^+$ phenotype of surviving cell clones by replica-plating them onto SD(-Ura, -His) plates at 37°C. Plasmids from cells that passed these screens were introduced into the YPH500-derived strain JD15 [*ubr1-Δ1::LEU2 ura3*, produced identically to *ubr1Δ* strains in (*11, 12*)], with transformants selected on SD(-Ura) plates at 37°C. This step narrowed the selection to plasmids whose ability to confer the *ts* Ura$^+$ phenotype required the presence of the N-end rule pathway (*6, 11, 12*). In both of the plasmids thus obtained, the *ts* lesion was found to be a single missense mutation in the region encoding DHFR (see text). The mutation was identified by first determining, in restriction fragment-swapping tests, that the relevant alteration resided within the DHFR moiety of Ub-Arg-DHFR-ha-Ura3, and then by sequencing the DHFR-coding regions in the initial (pPW17R) and mutant plasmids using the chain termination method (*20*). The final construct, termed pPW43R, was produced from the unmutagenized pPW17R by replacement of its Eco RI fragment encoding Ub-Arg-DHFR-ha-Ura3 with the otherwise identical fragment from one of the above plasmids encoding Ub-Arg-DHFR^{ts}-ha-Ura3.
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15. The plasmid pPW66R was constructed in the background of the *URA3*-based integration vector pRS306 (*18*). Briefly, a DNA fragment encoding Ub-Arg-DHFR^{ts}-ha (*10*) was ligated to a fragment [produced using polymerase chain reaction and *S. cerevisiae* genomic DNA (*20*)] that encompassed the first 284 nucleotides of the *CDC28* open reading frame (ORF) (*14*). The resulting fragment, encoding Ub-Arg-DHFR^{ts}-ha-Cdc28$_{1-95}$, was positioned downstream from the P_{CUP1} promoter in pRS306, yielding pPW66R. This plasmid was linearized at the Msc I site (nucleotide 92 in the *CDC28* ORF) and transformed into *S. cerevisiae* YPH500 (*18*). In the resulting Ura$^+$ integrants, homologous recombinations [R. Rothstein, *Methods Enzymol.* **194**, 281 (1991)] between the *CDC28* region in pPW66R and *CDC28* in chromosome II (*14*) resulted in the integration of pPW66R and formation of an ORF encoding Ub-Arg-DHFR^{ts}-ha-Cdc28 (which contained the full-length CDC28$_{1-299}$ moiety), in addition to a nearby sequence encoding Cdc28$_{1-95}$. This truncated allele of *CDC28* was neither functional nor dominant negative.
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Open "Back Door" in a Molecular Dynamics Simulation of Acetylcholinesterase

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The enzyme acetylcholinesterase generates a strong electrostatic field that can attract the cationic substrate acetylcholine to the active site. However, the long and narrow active site gorge seems inconsistent with the enzyme's high catalytic rate. A molecular dynamics simulation of acetylcholinesterase in water reveals the transient opening of a short channel, large enough to pass a water molecule, through a thin wall of the active site near tryptophan-84. This simulation suggests that substrate, products, or solvent could move through this "back door," in addition to the entrance revealed by the crystallographic structure. Electrostatic calculations show a strong field at the back door, oriented to attract the substrate and the reaction product choline and to repel the other reaction product, acetate. Analysis of the open back door conformation suggests a mutation that could seal the back door and thus test the hypothesis that thermal motion of this enzyme may open multiple routes of access to its active site.

The enzyme acetylcholinesterase (AChE) terminates signaling at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine (ACh). The crystal structure of AChE (*1*) raises questions regarding substrate entry and product release. First, the active site gorge is very deep and appears too narrow to admit ACh (*1, 2*). However, the demonstration that quaternary amines enter the active site of crystallized AChE (*3*) proves that the protein is sufficiently flexible to admit substrate by some route. Second, the inward electrostatic field at the gorge, which is likely to accelerate penetration of the positively charged substrate (*4, 5*), would seem to impede the exit of the product choline from the mouth of the active site. A thin wall near the base of the active site, at residues Met^{83} and Trp^{84}, could offer an alternative route for the escape of products (*4*). This back door might also provide a vent for water molecules during the passage of substrate, products, or both through the narrow gorge. We have studied these issues by performing a molecular dynamics (MD) simulation of AChE and analyzing the resulting time series of protein conformations with regard to active site accessibility.

Unobserved atoms were added to the *Torpedo californica* AChE dimer (*1*), as described in (*5*), using the program Quanta (Molecular Simulations, Waltham, Massachusetts). Protonation states at neutral pH were assigned on the basis of atomic solvent accessibilities, of salt-bridging and hydrogen-bonding opportunities, and of the putative catalytic mechanism, which requires the active site His^{440} to be neutral (*6*). The model enzyme was immersed in water, and the classical laws of motion solved numerically with weak coupling to a heat bath at 300 K to generate 119 ps of analyzable conformations (*7*).

We searched for conformations in which the active site had any aperture large enough to pass a water molecule, generating for each conformation a Lee and Richards (*8*) solvent-accessible dot surface with a 1.4 Å probe. The molecular

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