Use of amber suppressors to investigate the thermostability of Bacillus licheniformis alpha-amylase

Nathalie N. Declerck, Philippe P. Joyet, Claude Gaillardin, Jean-Michel Masson

THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 265, No. 26, Issue of September 15, pp. 15481-15488, 1990
Printed in U.S.A.

Use of Amber Suppressors to Investigate the Thermostability of *Bacillus licheniformis* α-Amylase

AMINO ACID REPLACEMENTS AT 6 HISTIDINE RESIDUES REVEAL A CRITICAL POSITION AT His-133*

(Received for publication, January 5, 1990)

Nathalie Declerck, Philippe Joyet, Claude Gaillardin, and Jean-Michel Masson‡

From the Institut National Agronomique, Laboratoire de Génétique, 78850 Thiverval-Grignon and the ‡Institut National des Sciences Appliquées, Laboratoire de Génie Biochimique et Alimentaire, Av. de Rangueil, 31077 Toulouse Cedex, France

A set of 12 *Escherichia coli* suppressor tRNAs, inserting different amino acids in response to an amber codon, has been used to create rapidly numerous protein variants of a thermostable amylase; by site-directed mutagenesis, amber mutations were first introduced into *Bacillus licheniformis* α-amylase gene at position His^{35}, His^{133}, His^{247}, His^{293}, His^{406}, or His^{450}; genes carrying one or two amber mutations were then expressed in the different suppressor strains, generating over 100 amylase variants with predicted amino acid changes that could be tested for thermostability. Within the detection limits of the assays, amino acid replacements at five histidine positions had no significant effect. In contrast, suppressed variants substituted at residue His^{133} clearly exhibited modified thermostability and could be either less stable or more stable than the wild-type amylase, depending on the amino acid inserted at this position; comparison of the variants indicates that the hydrophobicity of the substituting residue is an important but not a determinant factor of stabilization. The effect of the most stabilizing and destabilizing amino acid substitutions, His^{133} to Tyr and to Pro, respectively, were confirmed by introducing the corresponding missense mutations into the gene sequence. The advantages and limits of informational suppression in protein stability studies are discussed as well as structural features involved in the thermostability of *B. licheniformis* α-amylase.

Nonsense suppressors are mutant tRNAs mediating insertion of an amino acid in response to a termination codon introduced into a gene sequence. This informational suppression phenomenon has already proved to be a powerful tool for investigating protein structure and function by generating easily defined amino acid substitutions (see Ref. 1 for review). However, until recently this approach has been limited by the number of suppressor tRNAs and nonsense mutations that can occur naturally; for example, the genetic code allows only eight sense codons to be mutated to the amber triplet UAG in a single base change, and in *Escherichia coli,* a few species of suppressor tRNAs with a mutated anticodon have been isolated by classical genetics. With the "classical" suppressors, only serine, glutamine, tyrosine, leucine, lysine, or glycine can be specifically inserted in response to an amber codon (see Refs. 2 and 3 for review).

Recent developments in oligonucleotide synthesis and site-directed mutagenesis have made possible the *in vitro* construction of both new tRNA suppressor genes and nonsense mutations at any position of a gene sequence. By chemical synthesis, six additional efficient *E. coli* amber suppressor tRNA genes have been constructed, inserting phenylalanine, cysteine, alanine, histidine, proline, or glutamic acid, and their characterization has just been completed (4–7); to the detection limits of the assays (about 95%), these "synthetic" suppressors mediate the insertion of only one amino acid at an amber mutation site, except the glutamic acid-inserting suppressor, which inserts 10–15% glutamine as a secondary amino acid. These suppressive molecules have already been used to study the *lac* repressor (4, 7) and the mechanism of tRNA recognition by aminoacyl-tRNA synthetase (8, 9).

We report here for the first time the use of this newly extended collection of amber suppressors in protein thermostability studies. Multiple amino acid substitutions have been generated by informational suppression to study the role of specific amino acid residues in the stability of *Bacillus licheniformis* α-amylase. For its remarkable activity and stability at extreme temperature and pH, the amylase secreted by *B. licheniformis* is the liquefying enzyme most widely used in the industrial processes of starch hydrolysis (10, 11). Among the numerous other bacterial α-amylases that have been described and sequenced, those from *Bacillus amyloliquefaciens* and *Bacillus stearothermophilus* are very similar in their primary structure to that from *B. licheniformis* (12, 13) but both show lower thermostability (14, 15). By comparing the amino acid composition and sequence of these three amylases, we have noticed an excess of histidine residues in the *B. licheniformis* enzyme that could contribute to the enhancement of thermostability. Although this assessment was not confirmed by substituting some of these residues by suppression, we have identified a critical position where the nature of the inserted amino acid had dramatic consequences on thermostability.

MATERIALS AND METHODS

Genes, Plasmids Strains, and Media—The *B. licheniformis* α-amylase gene has been cloned (16) and sequenced in our laboratory. Its DNA and deduced amino acid sequence is identical to that reported by Gray *et al.* (13). For use of amber suppressors, its natural UAG termination codon has been changed by site-directed mutagenesis to a UAA codon followed by another ochre codon. For stable expression in *E. coli,* the amylase gene on a *Eco*RI-*Hin*dIII fragment was subcloned into a pJRD158 (17) low copy number derivative having a *Sal*I-*Xho*I deletion; the resulting plasmid was named pINA901.

The classical suppressors employed here are Su1(*supD*) inserting serine (2, 3); Su2-89 (*supE*), an improved glutamine-inserting sup-

* This research was supported by a grant from the Institut National de la Recherche Agronomique (to N. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

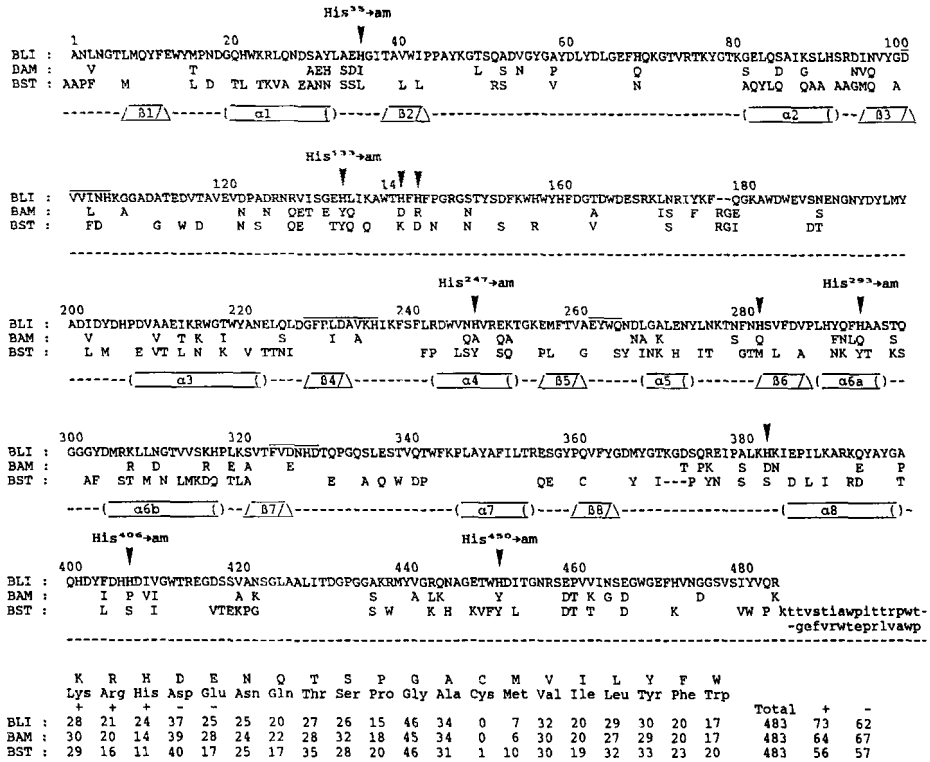

FIG. 1. **Comparison of amino acid sequence and composition of *B. licheniformis* (*BLI*), *B. amyloliquefaciens* (*BAM*), and *B. stearothermophilus* (*BST*) α-amylases and predicted location of some secondary structures.** The sequences of the mature amylases are aligned according to Yuuki *et al.* (12) with revised sequences (13) corresponding for *B. licheniformis* to the amylase gene sequence determined in the laboratory. *Dashes* in the amino acid sequences denote the deletion of the corresponding residues. *Vertical arrows* indicate the histidine residues specific to the *B. licheniformis* sequence including the six amber mutation sites. *Overlined* stretches correspond to the four highly conserved regions observed among α-amylases of various origins (34). *Cylindrical* and *triangular* forms symbolize the putative α-helices and β-sheets forming a $(\beta/\alpha)_8$ barrel structural motif as found in the two eucaryotic α-amylases of known structure (30, 31); they have been positioned here by combining the predictions made by two different groups (32, 33) and our personal analysis (N. Declerck, P. Joyet, C. Gaillardin, and J.-M. Masson, unpublished results). The amino acid composition of the three amylases for 483 aligned residues are given at the bottom of the figure as well as the total number of positively (+) or negatively (−) ionizable residues.

pressor (18); Su3 (*supF*) inserting tyrosine (2, 3); Su5 (*supG*), a lysine-inserting ochre suppressor which can also recognize UAG codons (2, 3) having improved efficiency in a strain containing the *uar*-1 mutation (5, 19); Su6 (*supP*) inserting leucine (2, 3); SuUAG (*GlyU*), a glycine-inserting suppressor obtained by *in vivo* genetic manipulation (20). The *E. coli* strains harboring the natural suppressors have been described (7, 19, 20, 21).

Synthetic suppressors are named for the tRNAs encoded by the synthetic genes provided by J. Miller's laboratory at UCLA; Phe-$tRNA_{CUA}$, Cys-$tRNA_{CUA}$, Ala-$tRNA_{CUA}$, HisA-$tRNA_{CUA}$, GluA-$tRNA_{CUA}$, and ProH-$tRNA_{CUA}$ insert phenylalanine, cysteine, alanine, histidine, glutamic acid, and proline, respectively. To provide compatibility with any plasmid having a ColE1 replication origin, the *Hin*dIII-*Pvu*II fragments from pGFIB-1 (4) carrying the synthetic tRNA genes downstream from a synthetic promoter (22) have been recombined with the *Hin*dIII-*Hin*cII fragments from pACYC-184 (23) having P15A replication origin and Tn9 chloramphenicol resistance gene. Six pCT2 derivative plasmids, each carrying a different synthetic gene were constructed by this mean and introduced into the *E. coli* XAC-1 (4) strains carrying the amber mutated amylase genes.

To detect amylase activity of the *E. coli* transformants, cells were plated on Luria broth agarose containing 0.5% soluble starch (Merck) and, after overnight growth, exposed for a few minutes to iodine vapors. For amylase production, all strains were grown in 2 × tryptone-yeast liquid medium supplemented with thiamine (0.001%), arginine (50 μg/ml), methionine (25 μg/ml), and ampicillin (100 μg/ml); chloramphenicol (30 μg/ml) was added when needed.

Site-directed Mutagenesis—The *Eco*RI-*Hin*dIII fragment from pINA901 carrying the complete amylase gene was inserted in Bluescript™ KS-M13⁺ phagemid. Initial experiments indicated that this vector was very unstable in *E. coli*. A promoterless derivative was thus constructed by deleting a *Pst*I fragment upstream from the gene sequence coding for mature amylase. Single-stranded phagemid DNA was generated using M13K07 helper phage, and templates were prepared for site-directed mutagenesis (24). All oligonucleotides were made with a Biosearch Cyclone™ DNA Synthesizer. Amber mutations were introduced one at a time following the procedure of Carter *et al.* (24) except that the extension-ligation step was performed at 37 °C for 90 min and followed by a first transformation in strain BMH71-18*mutL* (25). After overnight culture of the pooled transformants, double-stranded phagemid DNA was extracted and diluted several times to transform HB2151 (24) competent cells. Transformants were then screened by colony hybridization with the mutagenic oligonucleotides as probe and single-stranded DNA was prepared from positive clones for nucleotide sequencing by the dideoxy chain termination method (26) using Sequenase™. The restriction fragments carrying the amber mutations were then introduced at the sites of their wild-type counterparts in pINA901.

To replace the amber codon at position His^{133} by a sense codon restoring amylase activity, the DNA template prepared for sequencing the H133am mutation was re-used. The previous procedure was employed for site-directed mutagenesis except that the colony hybridization step was avoided; after the first transformation in BMH71-18*mutL* and extraction of phagemid DNA, the restriction fragment carrying the mutation site was directly introduced in place of the amber mutated gene fragment in pINA901-H133am. Transformants of HB2151 were then plated on starch medium to detect clones with restored amylase activity. The missense mutation carried by the revertants was confirmed by sequencing plasmid DNA (27).

α-Amylase Recovery, Assay, and Immunoassay—α-Amylase was recovered from the periplasm of the cells after 24-h growth in liquid medium at 37 °C. Periplasmic extracts from 3-ml cultures were pre-

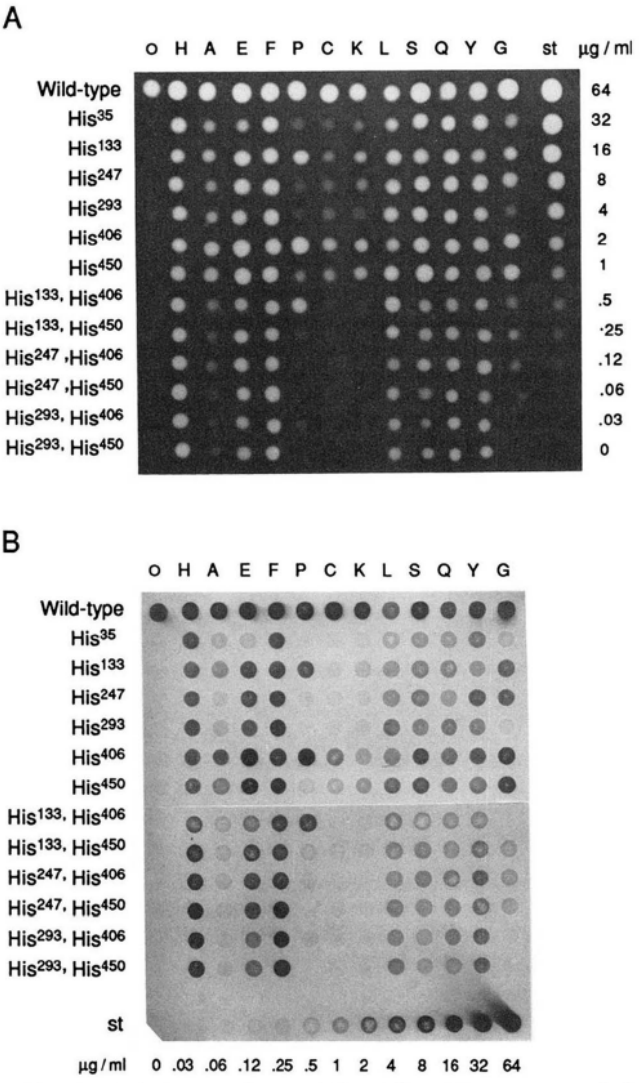

FIG. 2. **Relative amylase activity (*A*) and immunoreactivity (*B*) of the periplasmic extracts prepared from XAC-1 and the 12 *E. coli* suppressor strains (*columns*) expressing the wild type amylase gene or one of the 12 amber mutated genes (*lines*).** The predicted amino acids inserted by the suppressor carried by the strains are indicated with the one letter code recalled in Fig. 1; *0* means no suppressor (strain XAC-1). *St* corresponds to a standard range made with commercial *B. licheniformis* α-amylase (Sigma) at the indicated concentrations (micrograms/ml). *A*, starch hydrolysis halos made by 3 µl of periplasmic extracts and amylase range spotted on 0.8% agarose plates containing 0.5% soluble starch, 50 mM sodium acetate buffer, pH 6, 2 mM $CaCl_2$ and exposed to iodine vapors after 1-h incubation at 45 °C. *B*, dot immunoassay of *B. licheniformis* α-amylase antigens in the same protein samples 16-fold diluted in appropriate buffer, spotted and stained on nitrocellulose filters as described under "Materials and Methods." Missing sample: double mutant H133am+H406am/Gly-inserting suppressor.

pared by osmotic shock (28) with 1 ml of ice-cold 20 mM sodium acetate buffer, pH 6, 0.1 mM $CaCl_2$ and aliquoted in microplaques to be stored at −80 °C until use. Kinetic determination of α-amylase activity was performed on the extracts using a *p*-nitrophenyl maltoheptasoid derivative as substrate purchased from BioMérieux, France; the method adapted for rapid assay in microplaques will be described elsewhere. For dot-blotting experiments, 40 µl of diluted protein samples were spotted on nitrocellulose filters as described (29). α-Amylase was revealed using rabbit antiserum directed against purified *B. licheniformis* α-amylase (Sigma) and an alkaline phosphatase-linked probe (Promega).

Test of Thermostability—20 µl of the periplasmic extracts were diluted in 80 µl 50 mM sodium acetate buffer, pH 5.5, 0.025 mM $CaCl_2$ (0.04 mM final concentration) in 2 × 8 microwell modules (Nunc). Modules were then almost completely immersed in a water bath at different temperatures and removed after 10-min incubation. 100 µl of ice-cold R1 × 4 buffer (200 mM MOPS,[1] pH 7.15, 200 mM NaCl, 8 mM $CaCl_2$) was rapidly added to each well. After at least 10 min of cooling, 40 µl of incubated and nonincubated samples were assayed at room temperature to measure residual amylase activity.

RESULTS

Introduction of Amber Mutations—The amino acid sequence and composition of *B. licheniformis* (*BLI*), *B. amyloliquefaciens* (*BAM*), and *B. stearothermophilus* (*BST*) α-amylases are compared in Fig. 1. Also represented are the secondary structures predicted to form the $(\beta/\alpha)_8$ barrel structural motif common to all α-amylases (25–28). Comparison of the three homologous amylases reveals no relevant differences but an excess of histidine residues in the *B. licheniformis* enzyme. Ten of these are found exclusively in the *B. licheniformis* sequence and are indicated in Fig. 1. These histidine residues are changed in the two other less thermostable bacteria amylases by either hydrophobic or hydrophilic residues, although 4 of them (His^{133}, His^{247}, His^{293}, and His^{450}) are replaced by tyrosine in the *B. stearothermophilus* sequence, 2 of these (His^{133} and His^{450}) being also replaced by Tyr in the *B. amyloliquefaciens* sequence. We have decided to substitute by suppression these 4 histidine residues specific to the *B. licheniformis* sequence and two others, His^{35}, replaced by Ile and Leu, and His^{406}, replaced by Pro and Ser in the *B. amyloliquefaciens* and *B. stearothermophilus* sequences, respectively.

Amber mutations were introduced at the corresponding sites in the *B. licheniformis* α-amylase gene by oligonucleotide-directed mutagenesis. In initial attempts, we used a high expression amylase vector providing a way to screen directly for mutant clones having lost amylase activity on starch plates. However, this high copy number vector was found to be very unstable in *E. coli* and the amylase minus clones isolated after the site-directed mutagenesis procedure had a rearranged rather than an amber mutated gene. Such a deleterious effect related to the high expression of the *Bacillus* amylase gene in *E. coli* had already been observed (35). Therefore, a nonexpressing vector was preferred for mutagenesis. Mutants were screened by colony hybridization and the mutated fragments were subcloned into pINA901, a low copy number plasmid allowing amylase gene expression at a moderate but nondetrimental level in *E. coli*.

Six mutated genes were thus created as described under "Materials and Methods," carrying one of the following amber mutations: H35am, H133am, H247am, H293am, H406am, or H450am at residue His^{35}, His^{133}, His^{247}, His^{293}, His^{406}, or His^{450}, respectively. In mutant H133am, we introduced simultaneously a silent mutation at position 134; the CTA triplet was replaced by a CTG triplet both coding for leucine, assuming that the presence of the original rare codon following the amber mutation could alter the suppression efficiency (21).

Genes with two amber mutations were also constructed by *in vitro* recombination between appropriate restriction fragments carrying single mutation; only six genes were made with the following double mutations: H133am+H406am, H133am+H450am, H247am+H406am, H247am+H450am, H293am+H406am, or H293am+H450am.

Suppression—pINA901 carrying the wild-type amylase gene or one of the 12 mutated genes constructed above was introduced in strain XAC-1 and in the six *E. coli* strains harboring a classical amber suppressor gene. The 13 XAC-1

[1] The abbreviation used is: MOPS, 4-morpholinepropanesulfonic acid.

TABLE I

Efficiency at restoring amylase activity by suppression of the amber mutations

Amylase activity was assayed as described under "Materials and Methods" in duplicate on two series of periplasmic extracts except for the variants which where not tested for thermostability (single extraction). Average values are given in percentage of the wild-type amylase activity produced in the respective suppressor strains. Values over 30% are underlined.

Amber mutation sites	Amino acid inserted by suppression											
	His	Ala	Glu	Phe	Pro	Cys	Lys	Leu	Ser	Gln	Tyr	Gly
							%					
Wild-type[a]	100	100	100	100	100	100	100	100	100	100	100	100
His^{35}	40	7	6	34	3	1	12	23	17	35	27	4
His^{133}	39	11	27	31	24	3	25	46	24	41	19	8
His^{247}	36	4	21	36	1	2	12	36	25	32	41	10
His^{293}	36	4	20	33	1	2	7	35	16	39	25	1
His^{406}	40	22	55	30	60	12	35	41	34	49	32	16
His^{450}	35	17	35	44	5	9	33	61	33	52	33	18
His^{133}, His^{406}	18	4	16	19	16	1	4	32	8	16	12	1
His^{133}, His^{450}	21	2	18	22	1	<1	4	28	12	14	17	2
His^{247}, His^{406}	27	3	17	14	1	<1	4	18	13	19	21	2
His^{247}, His^{450}	34	1	16	22	<1	<1	1	19	10	18	17	1
His^{293}, His^{406}	32	1	15	20	1	1	4	22	7	22	16	<1
His^{293}, His^{450}	23	1	14	15	<1	<1	3	22	6	15	19	<1

[a] Amylase activity produced by expression of the wild-type amylase gene varied from 3 to 40 μg/ml depending on the suppressor strains genetic background.

TABLE II

Residual activity of the wild-type amylase and different variants produced by the suppressing strains after 10-min incubation at 80 °C, pH 5.5, and 0.04 mM $CaCl_2$

Thermoinactivation experiments were carried out on periplasmic extracts and residual activities were measured as described under "Materials and Methods." Presented here are the average values calculated from at least two independent tests on different enzymatic extracts. Values over 40% or under 20% are underlined. Amylase variants produced at a too low level were not tested (missing values).

Amber mutation sites	Amino acid inserted by suppression											
	His	Ala	Glu	Phe	Pro	Cys	Lys	Leu	Ser	Gln	Tyr	Gly
							%					
Wild-type[a]	32	31	32	36	33	31	33	30	34	31	35	30
His^{35}	30	35	26	31			37	35	38	39	38	35
His^{133}	29	14	38	46	5		40	45	19	43	61	13
His^{247}	30	21	35	31			25	34	31	29	39	26
His^{293}	28	30	26	30			31	31	33	32	36	
His^{406}	28	30	31	32	30	31	38	33	36	39	33	33
His^{450}	25	36	24	32		32	31	28	29	31	29	32
His^{133}, His^{406}	27	19	37	47	5		42	49	17	44	58	
His^{133}, His^{450}	23		35	45			30	44	17	41	56	
His^{247}, His^{406}	30		35	35					31	30	38	
His^{247}, His^{450}	35		39	36				35	31	40	37	
His^{293}, His^{406}	33		31	36				32	27	37	39	
His^{293}, His^{450}	30		30	34				30	33	33	31	

transformants were then transformed with one of the six pCT2 derivates carrying a synthetic suppressor gene. Fig. 2, *A* and *B*, visualizes the relative amylase activity and immunoreactivity of the periplasmic extracts prepared from the different *E. coli* transformants. Since cell growth and protein recovery varied depending on the genetic background, amylase yields should be compared only for genes expressed in the same suppressor strain (columns in Fig. 2).

It can first be noted that when the mutated genes were expressed in absence of suppressor, no activity nor immunoreactivity could be detected indicating that none of the truncated peptides they encode were stable and/or exported to the periplasm of the cells. No immunoreacting material could be detected in total cell extracts (data not shown), although it cannot be ruled out that inactive forms could be produced with unrecognizable epitopes for the polyclonal antibodies prepared against the wild-type amylase.

In the presence of any suppressor, all single mutants produced active enzymes in sufficient amount to be detected in the periplasm. Some amylase variants have been separated by gel electrophoresis and all presented the same molecular weight as the wild-type amylase (not shown). Complete and functional polypeptide chains were thus synthesized by suppression at every amber codon introduced into the amylase gene. However, the yield of suppressed amylase appeared to be very variable, depending on both the nature of the suppressor tRNA and the amber mutation site. In the double mutants, these effects were cumulated so that enzyme recovery was sometimes under the detection threshold in Fig. 2.

To estimate the efficiency of the suppressors in each case, amylase activity was measured in the periplasmic extracts of the variants and compared to the level of amylase synthesized from the wild-type gene in the same suppressor strains (Table I). Natural suppressors inserting leucine or glutamine and synthetic suppressors inserting phenylalanine or histidine were found to act efficiently at any amber codon whereas all mutations were poorly suppressed by the Cys- or Gly-inserting suppressors. In contrast, suppression mediated by Pro- or

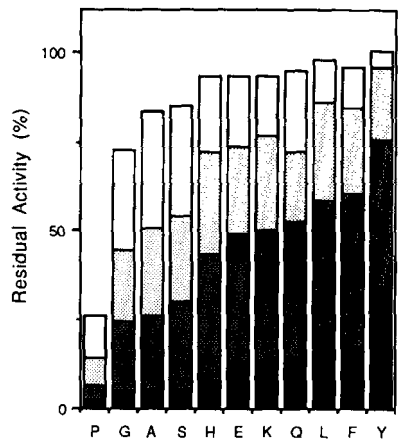

FIG. 3. **Thermostability of 11 amylase variants having different amino acids inserted by suppression at position 133.** *Superimposed bars* represent residual amylase activity retained by each variant after 10-min incubation at 68 (□), 74 (□), 79 (▩), or 86 (■) °C. Written below in one letter code are the predicted amino acids introduced in place of the His^{133} residue. Experiments were carried out under the conditions described in the legend to Table II, at pH 5.5 and 0.04 mM $CaCl_2$.

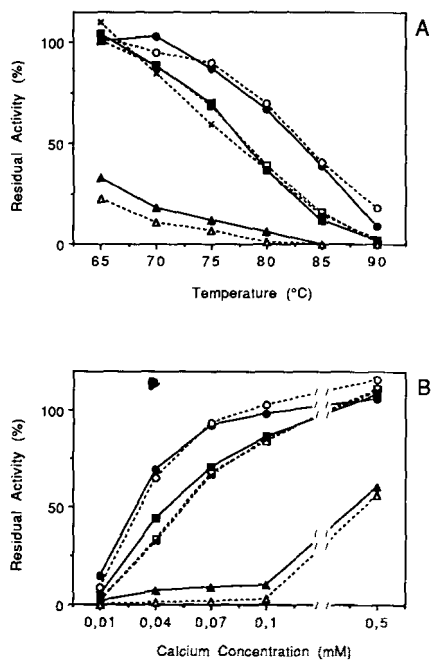

FIG. 4. **Thermostability of the H133Y and H133P variants obtained by suppression or reversion and compared at increasing temperatures (*A*) or calcium concentrations (*B*).** *A*, residual activity measured in the periplasmic extracts after 10-min incubation at pH 5.5, 0.04 mM $CaCl_2$ and increasing temperatures. *B*, same as *A* at pH 5.5, 80 °C, and increasing calcium concentrations. The *curves* in *solid lines* are for the wild-type and mutant amylases produced in strain XAC-1, whereas the *dotted ones* are for the enzymes produced in the tyrosine- or proline-inserting suppressor strains. ■, wild-type amylase produced in strain XAC-1 expressing the wild-type gene; □ and ×, wild-type amylase produced in Tyr-tRNA and Pro-tRNA suppressor strains, respectively, expressing the wild-type gene; ●, amylase variant having tyrosine inserted at position 133, produced in strain XAC-1 expressing the H133Y revertant gene; ○, same as ●, produced in Tyr-tRNA suppressor strain expressing the H133am amber mutant gene; ▲, amylase variant having proline inserted at position 133, produced in strain XAC-1 expressing the H133P revertant gene; △, same as ▲, produced in Pro-tRNA suppressor strain expressing the H133am amber mutant gene.

Lys-inserting suppressor was very dependent on the mutation site. Except for Pro-$tRNA_{CUA}$, the amber sites at position 406 or 450 provided usually the best reading "context" with all suppressor tRNAs; earlier work has already shown the importance of surrounding sequences on the suppression of nonsense codons (1, 21).

When two amber mutations had to be suppressed in the same gene (double mutants), the suppression efficiencies should be the product of those obtained for the two respective single mutations. For unknown reasons, this was not always the case here, and the values observed for the double mutants were often different from what could be expected from the values obtained for the single mutants in Table I.

It can however be argued that very low efficiencies of suppression actually reflect misacylation or a basal level of nonspecific suppression due either to pCT2 or to other differences in genetic background. We therefore did not investigate further proteins made in less than 4% of the wild-type levels.

Thermostability of the Variants—To facilitate the detection of both thermolabile and thermostable forms among the numerous amylase variants, we have determined the incubation conditions leading to around 50% heat inactivation of the wild-type amylase. Unfortunately for such experiments, *B. licheniformis* α-amylase is a very thermostable enzyme and it was crucial to find experimental conditions compatible with rapid test in microplaques. In the procedure we finally retained, the final concentration of calcium, a well-known amylase stabilizer (31, 36), was lowered to 0.04 mM so that residual activity of the wild-type enzyme after 10-min incubation at 75 and 80 °C was about 70 and 30%, respectively.

The thermostability of the amylase variants produced in sufficient amount was tested under these conditions. Table II presents residual activities measured after incubation at 80 °C. It was important to assess two points before interpreting the values obtained for the variants. First, in spite of some variations inherent to the test procedure, similar values were observed for the enzyme produced by expression of the wild-type gene in the different strains (first line); this meant that it was reasonable to perform the test on non-purified proteins even if recovered in different amount from cells of various genetic background. Second, the amylase recovered from the transformants expressing the mutant genes in the presence of the histidine-inserting suppressor (first column) displayed the same thermostability as the wild-type enzyme; this suggested that a protein identical to the wild-type amylase was re-generated with the synthetic His-$tRNA_{CUA}$ suppressor mediating the insertion of the original His residue at the amber sites. The range of responses observed with these controls reflects variations due both to the test procedure and the suppression method. Although this fluctuation is limited, it obviously prevents detection of minor modifications of enzyme parameters.

Due to this limitation, for interpreting the data in Table II residual activity values ranging from 20 to 40% were considered as being not significantly different from those obtained with the wild-type enzymes. Within these detection limits, we concluded that most of the amylase variants exhibited a thermoinactivation similar to the wild-type amylase. Substitution of either one or two histidine residues at position His^{35}, His^{247}, His^{293}, His^{406}, or His^{450} had no detectable effect on the thermostability of the variants in the conditions used here.

Some variants having modified thermostability were clearly identified from Table II; all were substituted at position 133. At this position, either thermolabile or more thermostable forms of the *B. licheniformis* α-amylase were generated depending on the amino acid inserted in place of the original

His residue. The most stabilizing and destabilizing substitutions were by tyrosine and proline, respectively, but almost any amino acid replacement tested increased or decreased the thermostability of the resulting protein compared to the wild-type enzyme. Similar effects were observed for the variants produced by suppression of double mutants having additional amino acid substitutions at either position His^{406} or His^{450}. This confirmed the effects of amino acid replacements at position 133 and the apparent neutrality of the substitutions at positions 406 and 450.

To assess the relative effect of multiple amino acid substitutions at the unique His^{133} residue, the thermostability test was performed at different temperatures on the 11 amylase variants obtained by suppression (Fig. 3). Based on these results, we propose the following order of stabilizing replacements: Pro < Gly < Ala < Ser < His (wild type) < Glu ≤ Lys ≤ Gln < Leu ≤ Phe < Tyr.

Reversion of the H133am Mutation—To confirm that the effects revealed in the thermostability test were due to the predicted amino acid substitutions, the corresponding missense mutations were introduced into the gene sequence. This was done only for the most and the least stabilizing substitutions at position His^{133}, *i.e.* by tyrosine and proline, respectively.

Reversion of the amber mutation was facilitated by the fact that revertants with restored amylase activity could be easily screened on starch plates. By site-directed mutagenesis, the TAG sequence at position 133 of the amber mutated gene was replaced by the TAC or CCG triplets coding for tyrosine and proline, respectively. The silent mutation at the Leu^{134} site was also reverted so that the DNA sequence of the two newly mutated genes were identical to that of the wild-type gene except for the H133Y or H133P mutation.

The thermostability of the enzymes produced by these mutants was compared with that of the wild-type amylase and of their counterparts produced by suppression. Residual activities were measured after incubation at increasing temperatures or calcium concentrations. Central curves in Fig. 4, *A* and *B*, are those obtained with the wild-type gene expressed in the three *E. coli* strains used in this experiment. As expected, they are well aligned and often overlap. Above and below are the curves obtained with the amylase variants having tyrosine and proline in place of His^{133}, respectively, introduced by either informational suppression or genetic reversion.

The two important conclusions that can be made by comparing these curves are 1) the variants obtained here by genetic reversion behave in the same way as the substituted molecules obtained previously by suppression and 2) insertion of tyrosine or proline in place of His^{133}, respectively, increases or decreases very significantly the thermostability of *B. licheniformis* α-amylase, at least at low calcium concentrations. At 0.04 mM $CaCl_2$ (Fig. 4*A*), 50% inactivation occurred at around 83 °C for the H133Y variant, approximately 5 °C higher than for the wild-type enzyme and below 65 °C for the H133P variant.

DISCUSSION

Informational Suppression in Protein Thermostability Studies—The aim of this work was first to know whether the informational suppression system would be a powerful tool to investigate the stability properties of a thermophilic α-amylase. The obvious advantage of this approach is that it enabled to create rapidly numerous variants of the protein substituted at defined positions with all the possible amino acid exchanges permitted by the set of amber suppressors. Making multiple substitutions at a unique position of a protein has already proved fruitful in understanding the molecular basis of protein stability (37–42). However, no general rules have yet been established to predict which sites should be targeted and how substituted amino acids modify the thermostability of a protein even when its three dimensional structure is known (which is not the case for *B. licheniformis* α-amylase). Moreover, rather than particular amino acid replacements, many small changes distributed throughout the molecule may be responsible for thermal stability as suggested by comparing the amino acid sequence of thermophilic proteins with that of their mesophilic counterparts (43). Since the number of different substitutions that may be envisioned can exponentially increase, it is crucial to have rapid and efficient methods of investigation.

Degenerated oligonucleotide-directed mutagenesis or cassette mutagenesis have usually been employed to saturate targeted sites with all possible amino acid replacements (38, 40, 44). However, the use of amber suppressors is a rather simpler method since the production of 12 different proteins with known substitutions requires a single site-directed mutagenesis and DNA sequencing. Furthermore, introduction of a nonsense mutation into an enzyme gene sequence abolishes usually an enzymatic activity that can be subsequently restored by informational suppression or genetic reversion. Therefore, mutant screening can be greatly facilitated by monitoring *in vivo* deactivation or reactivation of a catalytic function rather than modification of a particular protein property. Although efficient methods for genetic screening of mutations modifying protein thermostability have been described (45–47), these are hardly applicable in case of extremely thermostable proteins such as *B. licheniformis* α-amylase, whereas it is very easy to visualize in cells the loss or gain of amylase activity.

But the collection of efficient and specific amber suppressor tRNAs is still incomplete, although at least an arginine-inserting suppressor could now be added to the set used in this work (8). Because of gross misacylation of certain suppressor tRNA species, seven amino acids cannot be inserted (at this time) by suppression of an amber mutation. Among them, some as aspartic acid or asparagine would be of particular interest in protein stability studies since these residues can play a critical role in enzyme thermoinactivation processes (48).

In early works where suppressor tRNAs have been employed, plus or minus responses were already full of information on protein structure and function (1, 7, 49). In contrast, investigation on protein properties such as thermostability requires quantitative analysis and sufficient amount of suppressed proteins to make reliable serial tests. With the mutated amylase genes constructed here, the efficiency at restoring amylase activity by suppression rarely exceeded 50% and it was often under 5%, limiting the number of amylase variants that could be tested for thermostability. Suppression efficiency and enzyme recovery can depend on many factors including the nucleotide sequence in the vicinity of the UAG codon and the nature of the suppressor tRNAs; the suppressibility of an amber mutation is thus difficult to predict.

The other main restrictive aspect of this system concerns the specificity of the suppression phenomenom. Although all the suppressors used in this work (except $Glu\text{-}tRNA_{CUA}$) are supposed to insert only one amino acid in response to a UAG codon (6), it cannot be ruled out that some aspecifically substituted molecules are synthetized that can interfere with the observations or even be responsible for some slight effects. Therefore, interpretation of the data had to be restricted for

variants exhibiting clearly modified stability compared to the wild-type enzyme. Conversely, the apparent neutrality of some presumed amino acid substitutions are not necessarily due to their lack of influence but to the lack of precision of the assays. Here too, the background of possible misacylation presents a potential problem, especially in cases where suppression efficiencies are low. Nevertheless, we think that this method is quite appropriate for a rapid survey of mutant proteins with various amino acid exchanges in order to detect quickly those of interest before undertaking extensive site-directed mutagenesis for definitive analysis.

The best confirmation that the predicted amino acid inserted by suppression is responsible for the observed protein modification is to generate directly the substitution at the gene level. Two direct substitutions were made in the amylase gene at position 133, changing the original histidine codon for a tyrosine or proline codon. In both cases the enzymes encoded by the missense mutant genes behaved in the same way as the enzymes obtained by suppression of the amber mutation by Tyr-tRNA$_{CUA}$ or Pro-tRNA$_{CUA}$. Hence, the use of informational suppression did allow to reveal real effects on protein stability attributable to the expected amino acid replacements.

Structural Features Involved in the Stability of B. licheniformis α-Amylase—To test the informational suppression system, we decided to substitute several amino acid residues of the same nature, distributed all along the *B. licheniformis* amylase sequence. The choice for histidine residues was dictated by the facts that: (i) histidine is the only amino acid for which such a discrepancy is observed in the primary structure of the three homologous bacterial amylases; histidine is among the uncommon amino acids found in proteins, and its unusual high frequency in *B. licheniformis* α-amylase could be not fortuitous; (ii) these extra histidines introduce an excess of 11 positively charged residues compared with the total number of negatively charged residues, whereas in the two other less thermostable amylases and in globular proteins in general, amino acid charges are usually well balanced; (iii) at the time this study was started, little was known on the thermoinactivation process of these enzymes and no convincing structural features had been proposed as being responsible for their differences in thermostability; (iv) His-tRNA$_{CUA}$ is an efficient synthetic suppressor providing the possibility to regenerate the wild-type enzyme by suppression, an important control in such experiments; and (v) histidine is a rather big amino acid with a moderately charged aromatic group, which should thus be easily exchangeable by many other amino acids, including bulky ones as tyrosine or phenylalanine.

Actually, the protein was found to accommodate well amino acid replacements at any of the six mutation sites tested here. Indeed, the specific activity of the amylase variants obtained by suppression did not seem significantly modified, since a good correlation was observed between the size of the hydrolysis halos made by the periplasmic extracts spotted on starch agarose plates (Fig. 2*A*) and the amount of protein revealed by immunodetection (Fig. 2*B*). Inspection of numerous proteins and their variants generated in nature and laboratories has already shown that proteins can sustain many changes in their primary structure, even important ones, provided that the structural integrity of the core is preserved (40, 41, 50). Indeed, protein sequence comparison of α-amylases (32–34, 51) suggests that these histidine residues are located outside the highly conserved regions comprising the active site as well as outside the predicted β sheet regions forming the β-barrel motif buried in the interior of the protein structure (Fig. 1).

Not only does the protein tolerate the modifications inflicted here, it also was not significantly affected in its thermostability, irrespective of the amino acid inserted in place of a histidine residue at five out of six positions. Within the detection limits of the assays, all the substitutions generated at position His^{35}, His^{247}, His^{293}, His^{406}, or His^{450} were found to be neutral. Even when two of them were substituted simultaneously or when the inserted amino acid corresponded to that found in *B. amyloliquefaciens* or *B. stearothermophilus* amylase, no stabilizing or destabilizing effect was observed. We concluded that these extra ionizable residues might play a minor role, if any, in the stabilization of *B. licheniformis* α-amylase compared with the two homologous enzymes. This conclusion is in agreement with recent studies attempting to identify the structural elements responsible for the extra thermostability exhibited by the *B. licheniformis* enzyme. Tomazic and Klibanov (14, 15) have elucidated the molecular mechanisms of irreversible thermoinactivation of *B. amyloliquefaciens* and *B. stearothermophilus* amylases (14) and showed that the rate of the main inactivating process (monomolecular conformational scrambling) is lowered in the *B. licheniformis* enzyme thanks to additional electrostatic interactions involving a few specific lysine residues (15). Recently, Suzuki *et al.* (52) have elegantly showed by constructing chimeric genes of *B. amyloliquefaciens* and *B. licheniformis* α-amylases that a deletion (Arg^{176}-Gly^{177}) and, to a lesser extent, two substitutions (Ala for Lys^{269} and Asp for Asn^{266}) were responsible for the enhancement of stability against irreversible thermal inactivation (52). Although the biochemical approach and the genetical approach identified different structural elements, none suggested that some histidine residues were implicated in the increase of thermostability.

In contrast, substitutions at residue 133 were found to either decrease or increase the thermostability of the variants. They are now increasing examples demonstrating that single amino acid changes can have dramatic effects on protein stability (37, 38, 41, 45–47, 53–58). Increased thermostability has been obtained either by genetic screen (45–47, 53) or rational design (54–58), although the mechanisms through which stabilization is achieved are not always understood. In cases where the role of individual residues has been highlighted by making multiple amino acid substitutions (37–41), it has been found several times that changes in thermostability were directly related to the hydrophobicity of the substituting residue (37, 39, 41). Numerous hydrophobicity scales have been proposed, but those based on the solubility of amino acids in organic solvents have provided the best correlation with the observed stabilizing effects of amino acid replacements (41). For some amino acids inserted with the suppressors set, the classical scale assessed in a water-ethanol transfer system (59) assigns the following hydrophobicity order: Ser < Gly < Ala = His < Leu < Tyr < Phe; this is not far from the order Gly < Ala < Ser < His < Leu ≤ Phe < Tyr relative to their stabilizing effect at position 133 as determined here. This suggests that some hydrophobic interactions might be implicated in protein stabilization at this position. However, Ser and Tyr, the two amino acids from the above series having a hydroxyl group in their side chain, introduce more stability than what could be expected from simple linear correlation with hydrophobicity. Furthermore, variants substituted with either Lys, Glu, or Gln appear more thermostable than the wild-type enzyme, indicating that factors other than hydrophobicity contribute to protein stability. Finally, the fact that insertion of proline at this position drastically alters thermostability suggests that the propensity of some amino acids at forming and stabilizing secondary structures must also be considered. Work is in progress to understand better the effects observed here by

generating other amino acid replacements at this position, testing the variants in various conditions, and attempting to predict the structural organization of the region surrounding residue 133.

Surprisingly, the most stabilizing substitution introduced here at position 133 *i.e.* His → Tyr, had already been performed by nature in the two homologous *Bacillus* amylases, but both are less thermostable than the wild-type *B. licheniformis* enzyme. This is in fact consistent with the previous findings reported here, indicating that the extra thermostability of the latter relative to the former relies on structural features other than histidine residues. However, the singular effects observed at the His^{133} site are not in agreement with the assessment made by Tomazic and Klibanov (15) that irreversible thermoinactivation of *B. licheniformis* α-amylase is brought about by deamidation of Asn/Gln residues and thus approaches the upper limit of enzyme thermostability.

Although further characterization of the different variants constructed in this work is required, the present results obtained thanks to a rapid investigation method and stability test show that even the thermostability of an extremely thermostable enzyme is not maximized and can be enhanced in laboratories.

Acknowledgments—We thank L. M. Houdebine for preparing the antibodies against α-amylase as well as J. Garnier and J. M. Levin for fruitful discussion.

REFERENCES

1. Glass, R. E., Nene, V., and Hunter, M. G. (1982) *Biochem. J.* **203,** 1–13
2. Eggertsson, G., and Soll, D. (1988) *Microbiol. Rev.* **52,** 354–374
3. Gorini, L. (1970) *Annu. Rev. Genet.* **4,** 107–134
4. Normanly, J., Masson, J.-M., Kleina, L. G., Abelson, J., and Miller, J. H. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 6548–6552
5. Kleina, L. G., Masson, J.-M., Normanly, J., Abelson, J., and Miller, J. H. (1990) *J. Mol. Biol.* **213,** 705–717
6. Normanly, J., Kleina, L. G., Abelson, J., and Miller, J. H. (1990) *J. Mol. Biol.* **213,** 719–726
7. Kleina, L. G., and Miller, J. H. (1990) *J. Mol. Biol.* **212,** 295–318
8. McClain, W. H., and Foss, K. (1988) *Science* **241,** 1804–1807
9. Hou, Y.-M., and Shimmel, P. (1988) *Nature* **333,** 140–145
10. Saito, N. (1973) *Arch. Biochem. Biophys.* **155,** 290–298
11. Kindle, K. L. (1983) *Appl. Biochem. Biotech.* **8,** 153–170
12. Yuuki, T., Nomura, T., Tezuka, H., Tsuboi, A., Yamagata, H., Tsukagoshi, N., and Udaka, S. (1985) *J. Biochem. (Tokyo)* **98,** 1147–1156
13. Gray, G. L., Mainzer, S. E., Rey, M. W., Lamsa, M. H., Kindle, K. L., Carmona, C., and Requadt, C. (1986) *J. Bacteriol.* **166,** 635–643
14. Tomazic, S. J., and Klibanov, A. M. (1988) *J. Biol. Chem.* **263,** 3086–3091
15. Tomazic, S. J., and Klibanov, A. M. (1988) *J. Biol. Chem.* **263,** 3092–3096
16. Joyet, P., Guerineau, M., and Heslot, H. (1984) *FEMS Microbiol. Lett.* **21,** 353–358
17. Davison, J., Heusterpreute, M., Merchez, M., and Brunel, F. (1984) *Gene (Amst.)* **28,** 311–318
18. Bradley, D., Park, J. V., and Soll, L. (1981) *J. Bacteriol.* **145,** 704–712
19. Ryden, S. M., and Isaksson, L. A. (1984) *Mol. & Gen. Genet.* **193,** 38–45
20. Murgola, E. J. (1985) *Annu. Rev. Genet.* **19,** 57–80
21. Miller, J. H., and Albertini, A. M. (1983) *J. Mol. Biol.* **164,** 59–71
22. Masson, J.-M., and Miller, J. H. (1986) *Gene (Amst.)* **47,** 179–183
23. Chang, A. C. Y., and Cohen, S. N. (1978) *J. Bacteriol.* **134,** 1141–1156
24. Carter, P., Bedouelle, H., and Winter, G. (1985) *Nucleic Acids Res.* **13,** 4431–4443
25. Kramer, B., Kramer, W., and Fritz, H.-J. (1984) *Cell* **38,** 879–887
26. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5463–5467
27. Zhang, H., Scholl, R., Browse, J., and Somerville, C. (1988) *Nucleic Acids Res.* **16,** 1220
28. Neu, H. C., and Heppel, L. A. (1965) *J. Biol. Chem.* **240,** 3685–3692
29. Hawkes, R., Niday, E., and Gordon, J. (1982) *Anal. Biochem.* **119,** 142–147
30. Matsuura, Y., Kusunoki, M., Harada, W., and Kakudo, M. (1984) *J. Biochem. (Tokyo)* **95,** 697–702
31. Buisson, G., Duée, E., Haser, R., and Payan, F. (1987) *EMBO J.* **6,** 3909–3916
32. MacGregor, E. A. (1988) *J. Protein Chem.* **7,** 399–415
33. Raimbaud, E., Buleon, A., Perez, S., and Henrissat, B. (1989) *Int. J. Biol. Macromol.* **11,** 217–225
34. Nakajima, R., Imanaka, T., and Aiba, S. (1986) *Appl. Microbiol. Biotechnol.* **23,** 355–360
35. Willemot, K., and Cornelis, P. (1983) *J. Gen. Microbiol.* **129,** 311–319
36. Vallee, B. L., Stein, E. A., Sumerwell, W. N., and Fischer, E. H. (1959) *J. Biol. Chem.* **234,** 2901–2905
37. Yutani, K., Ogasahara, K., Tsujita, T., and Sugino, Y. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 4441–4444
38. Alber, T., Dao-pin, S., Wilson, K., Wozniak, J. A., Cook, S. P., and Matthews, B. W. (1987) *Nature* **330,** 41–46
39. Matsumura, M., Yahanda, S., Yasumura, S., Yutani, K., and Aiba, S. (1988) *Eur. J. Biochem.* **171,** 715–720
40. Alber, T. A., Bell, J. A., Dao-pin, S., Nicholson, H., Wozniak, J. A., Cook, S., and Matthews, B. W. (1988) *Science* **239,** 631–635
41. Matsumura, M., Becktel, W. J., and Matthews, B. W. (1988) *Nature* **334,** 406–410
42. Matsumura, M., Wozniak, J. A., Dao-pin, S., and Matthews, B. W. (1989) *J. Biol. Chem.* **264,** 16059–16066
43. Argos, P., Rossmann, M. G., Grau, U. M., Zuber, H., Frank, G., and Tratschin, J. D. (1979) *Biochemistry* **18,** 5698–5703
44. Wells, J. A., Vasser, M., and Powers, D. B. (1985) *Gene (Amst.)* **34,** 315–323
45. Matsumura, M., and Aiba, S. (1985) *J. Biol. Chem.* **260,** 15298–15303
46. Alber, T., and Wozniak, J. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 747–750
47. Bryan, P. N., Rollence, M. L., Pantoliano, M. W., Wood, J., Finzel, B. C., Gilliland, G., Howard, A. J., and Poulos, T. L. (1986) *Proteins: Struct. Funct. Genet.* **1,** 326–334
48. Ahern, T. J., and Klibanov, A. M. (1985) *Science* **228,** 1280–1284
49. Rennell, D., and Poteete, A. R. (1989) *Genetics* **123,** 431–440
50. Luger, K., Hommel, U., Herold, M., Hofsteenge, J., and Kirschner, K. (1989) *Science* **243,** 206–210
51. Holm, L., Koivula, A. K., Lehtovaara, P. M., Hemminki, A., and Knowles, J. K. C. (1990) *Protein Eng.* **3,** 181–191
52. Suzuki, Y., Ito, N., Yuuki, T., Yamagata, H., and Udaka, S. (1989) *J. Biol. Chem.* **264,** 18933–18938
53. Das, G., Hickey, D. R., McLendon, D., McLendon, G., and Sherman, F. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 496–499
54. Perry, L. J., and Wetzel, R. (1984) *Science* **226,** 555–557
55. Imanaka, T., Shibazaki, M., and Takagi, M. (1986) *Nature* **324,** 695–697
56. Hecht, M. H., Sturtevant, J. M., and Sauer, R. T. (1986) *Proteins: Struct. Funct. Genet.* **1,** 43–46
57. Ahern, T. J., Casal, J. I., Petsko, G. A., and Klibanov, A. M. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 675–679
58. Nicholson, H., Becktel, W. J., and Matthews, B. W. (1988) *Nature* **336,** 651–656
59. Nozaki, Y., and Tanford, C. (1971) *J. Biol. Chem.* **246,** 2211–2217