Characterization of Two Cold-sensitive Mutants of the β-Galactosidase from Lactobacillus delbrückii subsp. bulgaricus*

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MATERIALS AND METHODS

Plasmid Constructions—The wild-type β-galactosidase gene from L. delbrückii subsp. bulgaricus B131 (Centre International de Recherche Daniel Carasso, BSN Group, Le Plessis-Robinson, France) was cloned previously in our laboratory (Schmidt et al., 1989). An XbaI restriction site was introduced at nucleotide 441 by site-directed mutagenesis (Zoller and Smith, 1982). Then, the 3.4-kilobase pair XbaI-BamHI fragment carrying the promoterless β-galactosidase gene was ligated with a 418-base pair fragment containing the alkaline phosphatase (phoA) promoter (Oka et al., 1985) into the vector pBR322 (Boivin et al., 1977) that had been previously digested with EcoRI and BamHI. The resultant plasmid was used for mutagenesis of the gene and expression of the β-galactosidase variants.

Chemical Mutagenesis and Variant Selection—Up to 1 µg of plasmid DNA was added to 150 µl of methoxylamine hydrochloride (Aldrich), prepared according to Kadonaga and Knowles (1985), and incubated in the dark at 50 °C. Aliquots were removed every 30 min for up to 5 h, 50 µl of ethylene glycol and 20 µl of 3 sodium acetate, pH 5.5, were added, and the DNA precipitated by ethanol. The mutagenized plasmids were resuspended in 10 µl of 10 mM Tris-HCl, pH 8.0, with 1 mM EDTA and used to transform E. coli JM109 cells (lac–; Yanisch-Perron et al., 1985). Bacteria were spread on a nitrocellulose filter (Schleicher & Schuell) lying on top of a Luria-Bertani agar (Difco Laboratories) plate containing 50 µg of carbenicillin (Sigma) and grown overnight at 37 °C. Two replica filters were made and the duplicate colonies on these filters were grown as described above. One replica filter was then placed at 4 °C and after 24 h transferred to a cold agar plate containing 40 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Sigma). A duplicate filter was placed on a 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside agar plate at 37 °C. Desired mutant had near wild-type activity at 37 °C and reduced activity at 4 °C relative to wild-type as detected visually by the rate of blue color formation.

Mutation Site Determination—The mutations of two selected cold-sensitive variants, Cs2 and Cs4, were mapped to the 1.2-kilobase pair NcoI fragment by swapping wild-type and mutant gene fragments. The nucleotide base substitutions present in these two variants was determined by DNA sequencing (Sanger et al., 1977) of the entire NcoI nucleotide base pair fragment containing the alkaline phosphatase (phoA) promoter (Oka et al., 1985) into the vector pBR322 (Boivin et al., 1977) that had been previously digested with EcoRI and BamHI. The resultant plasmid was used for mutagenesis of the gene and expression of the β-galactosidase variants.

Enzyme Purification—Wild-type and variant β-galactosidases were expressed from the corresponding genes in E. coli JM109 cells and purified from cytoplasmic extract as detailed earlier (Schmidt et al., 1989). The enzymes were judged to be homogeneous as only one band was visible after SDS-PAGE analysis. All of our studies were done with these purified enzymes.

Molecular Mass Determinations—The molecular mass of the monomeric subunits of the E. coli lacZ β-galactosidase, and the subunits of the L. delbrückii subsp. bulgaricus enzyme and its variants, were determined by SDS-PAGE (Power et al., 1986). The apparent molecular mass of the native states and the urea dissociated monomers of each of these enzymes was determined by gel filtration liquid chromatography on two Superose-12 columns (Pharmacia LKB Biotechnology Inc.) connected in series. The column was equilibrated at a flow rate of 0.2 ml/min with 25 mM HEPES, pH 7.4, 0.2 mM KC1, containing 1 mM MgCl2 (native conditions), 8 M urea, and 5 mM dithiothreitol (denaturing conditions).

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β-Galactosidase (EC 3.2.1.23), hydrolizes β-galactosyl linkages, such as the β(1,4)-linkage found in the milk sugar lactose. The enzyme is produced by a variety of organisms (for a review, see Wallenfels and Weil (1972)), but only the β-galactosidase from Escherichia coli has been extensively characterized. This enzyme is reported to be a tetramer of four identical subunits of 110,000 Da (Zipser, 1963). Enzymatic catalysis proceeds through a galactosyl-enzyme intermediate (Fink and Angelides, 1975). Transgalactosylation can also occur by the enzyme is reported to be a tetramer of four identical subunits of 110,000 Da (Zipser, 1963). Enzymatic catalysis proceeds through a galactosyl-enzyme intermediate (Fink and Angelides, 1975).
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were taken and quenched with an equal volume of 50 mNPG substrate and saturating magnesium concentration.

The total protein was determined by the BCA protein determination method (Smith et al., 1985), using bovine serum albumin as the standard. Specific activity was determined from the initial rate at 25 °C in the presence of 50 mM NPG substrate and saturating magnesium concentration.

Progress curve determinations were performed as described previously (Estell et al., 1985), using a thermostated HP spectrophotometer. Appearance of o-nitrophenol was followed at 400 nm for low substrate concentrations (ε = 9609 ν⁻¹) and 460 nm for high substrate concentrations (ε = 4311 ν⁻¹). NPG at 0.35–0.47 mM was used for the high substrate concentration, and 0.068–0.073 mM for the low substrate concentration. Replicate assays were run over the temperature range of 11–45 °C.

Determination of Magnesium Binding Constants—Samples of purified wild-type and P429S mutant enzymes were dialyzed overnight at 4 °C versus 150 volumes of 25 mM Bis-Tris, 200 mM NaOAc, 10 mM EDTA, pH 7.1, and then twice versus 100 volumes of 25 mM Bis-Tris, 200 mM NaOAc, 1 mM EDTA, pH 7.1. Magnesium sulfate and EDTA were used to fix the magnesium concentrations in the individual reactions (Portzehl et al., 1964) between 0.03 μM and 16 μM, pH 7.1. Two assumptions were made in the determination of free magnesium: 1) only the forms of the ligand with three and four negative charges will bind magnesium at pH 7.1 (Portzehl et al., 1964); and 2) the affinity of EDTA for magnesium does not change substantially over the temperature range used in these experiments. The assays were carried out in replicates of 4–5 in the presence of at least 5 different substrate concentrations below Kₜₐₕ and 5 above. Assays were performed from 5 °C (necessitating the use of He gas to prevent condensation on the cuvette walls) to 45 °C. The enzyme was added to pre-equilibrated buffer, and allowed to further equilibrate at the appropriate temperature for 20 min. Substrate was added and the reaction quantitated as described above.

The initial rate determinations were averaged and fit to a two-state binding model (Lippincott, 1965). This assumption assumes one rate (Rₑₛ)ₜₐₕ in the presence of substrate (S₀) for the enzyme with metal bound, (E₂Mₜₐₕ):

\[ Rₑₛ = E₂MₜₐₕS₀/(Kₑₛ + S₀) \]  

and one rate (Rₑ) for the enzyme with no metal bound, (E₂):

\[ Rₑ = E₂S₀/(Kₑ + S₀) \]  

with the measured rate (ν) being derived as the sum of these two rates:

\[ ν = ([L + M/K₉ₜₐₕ]E₂S₀/(Kₑₛ + S₀)I + \)  

where: kₑ = kₑₛ for the rate with no metal bound; Kₑ = Kₑₛ for the rate with no metal bound; Kₑₛ = kₑₛ for the rate with metal bound; M = magnesium concentration; Kₑₛ = dissociation constant for magnesium binding; Eₚ = total enzyme concentration.

Lactose Hydrolysis—The hydrolysis of lactose at 10 mg/ml in 10 mM sodium succinate, pH 6.7, in the presence and absence of 20 mM MgSO₄, was measured using high performance liquid chromatography methods. Identical reactions were first preincubated at 9 and 30 °C; purified wild-type, C62, or C64 mutant enzyme was then added and the reaction allowed to proceed for 1–24 h. At appropriate time intervals, samples were taken and quenched with an equal volume of 20 mM H₂SO₄. The sugar profile of the quenched samples was stable over 48–96 h. The samples were chromatographed using an IBM LC/9533 liquid chromatograph equipped with a LC9506 SE automated sampler and system 9000 computer. The samples were injected onto a 300 x 7.8-mm Bio-Rad Aminex HPX-87H column preceded by a Cation H⁺ guard column. The column was run isocratically in 10 mM H₂SO₄ at 0.6 ml/min and 60 °C. Peaks were detected by monitoring refractive index. This system allowed the baseline resolution of allolactose, lactose, glucose, galactose, and succinate. Succinate served as an internal standard in each experiment. Standard injections of lactose, glucose, galactose, and succinate were reproducible to within 5%.

RESULTS

Generation and Identification of Variants—Methoxylamine mutagenesis of the β-galactosidase gene from L. delbrückii subsp. bulgaricus resulted in the generation of a variety of cold-sensitive mutants. The nucleotide sequence alterations discovered in two of these mutants, C62 and C64, were introduced in the wild-type gene by oligonucleotide site-directed mutagenesis to make the cold-sensitive variants L317F and P429S, respectively. The mutant C62 appeared to be more cold-sensitive on plate screens than the corresponding variant L317F due to a suppressor mutation that reduced the expression of the enzyme in E. coli (data not shown). All characterization of the variants reported here was performed on protein isolated from the site-directed mutants L317F and P429S.

Molecular Mass—The molecular mass of denatured L. delbrückii subsp. bulgaricus β-galactosidase subunits from both the wild-type and the mutants, L317F and P429S, appear on SDS-PAGE to be approximately 110,000 Da. This is in agreement with the estimated molecular mass inferred from the DNA sequence (Schmidt et al., 1989). However, their native molecular mass, as measured by gel filtration on Superose-12 columns, appears closer to 220,000 Da (Fig. 1A). The elution profile does not change with the addition of 5 mM EDTA to the buffer. This molecular mass as well as the absence of heterogeneity in the N-terminal sequence (Schmidt et al., 1989) suggests that the enzyme exists as a dimer of two identical subunits. This contrasts with the tetrameric structure of the E. coli lacZ β-galactosidase. The possibility that the L. delbrückii subsp. bulgaricus enzyme is a monomer in its native form but chromatographs aberrantly during gel filtration was eliminated by further studies in the presence of 8 mM urea. Work on the E. coli enzyme has shown that treatment with 8 mM urea causes the tetramer to dissociate into four identical monomers. This has been shown with both native gels and sedimentation studies (Zipser, 1963). Gel filtration, in the presence of 8 mM urea using the E. coli enzyme as an internal control, shows clearly that the urea-treated L. delbrückii subsp. bulgaricus enzyme runs identically to the E. coli monomer (Fig. 1B), suggesting that the native form of the enzyme is indeed a dimer made up of identical subunits.

Specific Activity—The NPG hydrolysis specific activities of the wild-type, P429S, and L317F enzymes were determined to be 850, 760 (90% of wild-type), and 570 units/mg (67% of wild-type) of protein, respectively. In comparison, the E. coli enzyme had a specific activity of 690 units/mg (81% of the L. delbrückii subsp. bulgaricus wild-type enzyme).

The specific activities of the enzymes did not change significantly during storage through the duration of this study. There was also essentially no differences in the specific activities measured from different preparations. Thus, it is unlikely that the lower specific activities of the mutants represents the presence of a constant fraction of inactive enzyme.

Determination of Kinetic Constants—In order to examine the enzymatic activity as a function of temperature, Kₑₜₐₕ and Vₚₑₜₐₕ determinations were done by progress curve analysis for both the two mutant enzymes and the wild-type enzyme from 11 to 45 °C at 2 mM Mg²⁺. The Kₑₜₐₕ for the wild-type enzyme was determined to be 0.365 mM for NPG and remained constant over the temperature range examined. This value was further...
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FIG. 1. Determination of molecular mass by Superose-12 gel filtration. Purified β-galactosidases from E. coli (○) and L. delbrückii subsp. bulgaricus (+) were fractionated as described under "Materials and Methods" in the absence (A) or presence (B) of 8 m urea. Fractions were assayed by activity using NPG as a substrate (A), or by monitoring the absorbance at 280 nm (B). As comparison, in the absence of urea, apofemin (440,000 Da) and P-amylase (200,000 Da) elute at fractions 50 and 80, respectively. The variant, P429S, chromatographs identically to the wild-type L. delbrückii subsp. bulgaricus enzyme (data not shown).

verified by initial rate determinations of $K_m$ and $V_{max}$ at 25 °C. The reported $K_m$ (0.12 mM) for NPG of the E. coli β-galactosidase (Cuppers et al., 1990; Martinez-Bilbao et al., 1991) is thus approximately three times lower than that for the L. delbrückii subsp. bulgaricus wild-type enzyme. The $K_m$ values for NPG of the L317F and P429S mutants were 0.371 mM (102% of wild-type) and 0.479 mM (130% of wild-type), respectively, and both remained constant over the temperature range studied. Linear Arrhenius plots of $\ln(V_{max}/E_T)$ versus reciprocal temperature (K$^{-1}$) can be generated from the data (Segal, 1975) for the wild-type and L317F enzymes (Fig. 2). As shown, the slope of this line ($-E_a/R$) is essentially equivalent for both of these enzymes; however, $V_{max}/E_T$ for wild-type is ~1.7 times greater than $V_{max}/E_T$ for the L317F enzyme at all temperatures investigated.

In contrast to L317F, the values of $V_{max}/E_T$ and the slope of the Arrhenius plot for the P429S variant essentially matches that of the wild-type at temperatures greater than 20 °C, suggesting that the enzymes are similar at these elevated, permissive temperatures. However, the Arrhenius plot for P429S does not appear to be linear and curves downward at lower temperatures (Fig. 3).

Lactose Hydrolysis—Enzymatic hydrolysis of lactose was performed in order to correlate the synthetic substrate data (NPG) to the natural substrate lactose. The reaction profiles of the wild-type enzyme and the two mutants were compared at identical enzyme concentrations in the presence or absence of 20 mM MgSO$_4$ at 9, 33, and 41 °C. The results were plotted as reaction time versus the area of the eluted substrate, products, or succinate control peaks. A reaction profile of the hydrolysis of
lactose by the wild-type enzyme is shown in Fig. 4 (42 °C, no Mg$^{2+}$). The appearance of allo-lactose over time demonstrates that the *L. delbrückii* subsp. *bulgaricus* enzyme carries out transgalactosylation like the *E. coli* β-galactosidase (Fig. 4).

In the absence of magnesium, at 42 °C, the initial reaction rates are similar, although a slightly decreased rate is seen for the P429S variant (compare the initial slopes in Fig. 5). At 9 °C, the wild-type and L317F initial rates are essentially identical while very little lactose hydrolysis is seen for the mutant. This demonstrates that the mutant P429S is also cold-sensitive for the hydrolysis of its natural substrate, lactose, in the absence of magnesium. In the presence of 20 mM Mg$^{2+}$, the initial rates for all three enzymes are approximately the same and similar to the initial rates observed for wild-type and L317F in the absence of Mg$^{2+}$ (Fig. 5). For the P429S enzyme, Mg$^{2+}$ appears to suppress its low temperature lability suggesting that its cold sensitivity is a function of Mg$^{2+}$ binding to the enzyme.

**Magnesium Binding Constants**—The discovery that Mg$^{2+}$ could suppress the cold lability of P429S led to the determination of the magnesium ion binding constants for the wild-type and P429S enzymes as a function of temperature. The $K_D$ for wild-type β-galactosidase remains constant at 1.0 μM from 11 to 37 °C, at which point it increases rapidly (above 55 °C the loss of activity becomes irreversible). For comparison, the $K_D$ for Mg$^{2+}$ of *E. coli* β-galactosidase has been reported to be 0.15 μM (Edwards et al., 1990b) and 2.0 μM (Edwards et al., 1990a).

Similar to the observed behavior of the *E. coli* enzyme, exhaustively chelated (dialysis versus 10 mM EDTA) *L. delbrückii* subsp. *bulgaricus* β-galactosidase still retains a low but measurable 1–5% activity (data not shown). This agrees with the $k_{cat}/K_m$ value at 25 °C for *E. coli* β-galactosidase in the absence of Mg$^{2+}$ which is 2% of its value in 1 mM Mg$^{2+}$ (Cupples et al., 1990).

In direct contrast to wild-type, the $K_D$ for Mg$^{2+}$ of the P429S...
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![Graph showing the reaction profile for the hydrolysis of lactose by the L. delbrückii subsp. bulgaricus β-galactosidase in the absence of Mg²⁺ at 42 °C. The normalized peak areas for the substrate lactose (■), products galactose (●), glucose (x), and allolactose (○); as well as the internal succinate standard (△) are shown as a function of time.](image1)

FIG. 4. The reaction profile for the hydrolysis of lactose by the L. delbrückii subsp. bulgaricus β-galactosidase in the absence of Mg²⁺ at 42 °C. The normalized peak areas for the substrate lactose (■); products galactose (●), glucose (x), and allolactose (○); as well as the internal succinate standard (△) are shown as a function of time.

![Graph showing the dependence of lactose hydrolysis on temperature and Mg²⁺ concentration for wild-type and variant β-galactosidases. The disappearance of lactose in the absence of Mg²⁺ (solid lines) is shown at 41 °C for wild-type (■), and variants L317F (○) and P429S (△). The same reactions performed at 9 °C are denoted by filled symbols. For comparison, the hydrolysis of lactose by variant L317F in 20 mM MgSO₄ (broken lines) at 41 °C (○) and 9 °C (□) is included. Lactose hydrolysis at 20 mM Mg²⁺ by the wild-type and P429S enzymes are essentially identical to the L317F data (not shown).](image2)

FIG. 5. The dependence of lactose hydrolysis on temperature and Mg²⁺ concentration for wild-type and variant β-galactosidases. The disappearance of lactose in the absence of Mg²⁺ (solid lines) is shown at 41 °C for wild-type (■), and variants L317F (○) and P429S (△). The same reactions performed at 9 °C are denoted by filled symbols. For comparison, the hydrolysis of lactose by variant L317F in 20 mM MgSO₄ (broken lines) at 41 °C (○) and 9 °C (□) is included. Lactose hydrolysis at 20 mM Mg²⁺ by the wild-type and P429S enzymes are essentially identical to the L317F data (not shown).

mutant was not constant. At 5 °C, the $K_D$ was 3.0 mM, a 3000-fold increase over the wild-type value. The $K_D$ decreased with an increase of temperature, reaching a minimum of 0.19 mM at 40 °C, but still almost 200 times the $K_D$ of the wild-type enzyme. A van't Hoff plot of ln($K_D$) versus reciprocal temperature ($K^{-1}$) is shown in Fig. 6. This clearly shows the contrast between the varying $K_D$ of the P429S and the constant $K_D$ of the wild-type enzyme. Both plots give good linear fits up until about 40 °C where there is an abrupt change so that $K_D$ now increases with temperature. This is most likely caused by the onset of the thermal denaturation of the protein. In addition, P429S appears more heat labile than wild-type, rapidly inactivating at temperatures above 48 °C at all Mg²⁺ concentrations tested (data not shown).

**DISCUSSION**

In this study, we have generated and characterized two apparent cold-sensitive variants of the β-galactosidase enzyme from L. delbrückii subsp. bulgaricus. There are a variety of conditions which could result in cold sensitivity of an enzyme: 1) a cold-dependent change in the reaction mechanism; 2) low temperature denaturation of the tertiary or quaternary struc-
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Assuming that the cold sensitivity of P429S is entirely due to metal dependence in the range of interest (Fig. 6). It is interesting that where the enzyme with no metal bound is essentially inactive. The dissociation of an essential cofactor. In addition, in our initial screens, variants with reduced protein expression could also appear as being cold-sensitive.

From our results, one of the mutants studied, L317F, has a slightly reduced activity compared to wild-type on the synthetic substrate NPG at all temperatures tested. Thus, although L317F appears to be somewhat cold-sensitive on plate screens (data not shown), it must really be considered an “activity” mutant and not truly “cold-sensitive” (cold-sensitive meaning having approximately wild-type activity at ambient temperature and reduced activity at lower temperatures). By these criteria, another mutant, P429S, is cold-sensitive on both the synthetic substrate NPG as well as lactose, its natural substrate. Our work demonstrates that this cold sensitivity correlates to a change in the affinity of the mutant enzyme for essential magnesium ions. In fact, the cold temperature effect can be totally suppressed by the addition of high concentrations of magnesium ions in vitro. The reversible nature of this change, and the absence of a temperature-sensitive $K_m$ for NPG, suggests that the P429S enzyme does not undergo a large structural alteration or denaturation at these temperatures. Assuming that the cold sensitivity of P429S is entirely due to the dissociation of Mg$^{2+}$, and that the activity of the enzyme without bound Mg$^{2+}$ is negligible, then $V_{max}$ as a function of temperature ($T$) can be derived from Equation 3 above:

$$V_{max}(T) = E_0 k_{cat} [M][M + K_D(T)]$$

where $K_D(T)$ can be calculated from the linear fit of the van’t Hoff plot shown in Fig. 6. The theoretical curve determined from Equation 4 fits the data generated at 2 mM Mg$^{2+}$ quite well as shown in Fig. 3. Thus, the cold sensitivity of P429S can be explained by a reduction in the affinity of this variant for Mg$^{2+}$ where the enzyme with no metal bound is essentially inactive. The wild-type enzyme, unlike the P429S variant, is not cold sensitive since its $K_D$ for Mg$^{2+}$ has no strong temperature dependence in the range of interest (Fig. 6). It is interesting that the $K_D$ for Mg$^{2+}$ of the wild-type E. coli β-galactosidase has been reported to decrease with increasing temperature similar to that of P429S (Edwards et al., 1990b). Thus, one might expect the wild-type E. coli enzyme to be cold sensitive as well. Of course, in this case, much lower Mg$^{2+}$ concentrations would be required to observe the effect (the $K_D$ values for the E. coli and P429S β-galactosidases are 0.15 and 530 μM, respectively, at 25°C). The zero slope of the van’t Hoff plot for the wild-type β-galactosidase suggests that the binding of magnesium ions to the enzyme is driven by entropy. It seems likely, that upon the binding of Mg$^{2+}$, a significant number of water molecules would be released from the solvated magnesium ions and from the Mg$^{2+}$ binding pocket on the protein causing the entropy change necessary as a driving force. Binding of magnesium ions to the P429S variant requires added energy in addition to the entropy change as reflected in the positive slope of the van’t Hoff plot. Thus, the P429S mutation might skew the configuration of the binding site reducing the affinity of this variant for Mg$^{2+}$ (the $K_D$ for P429S is over 500 times larger than wild-type at 25°C).

The P429S mutation is not in a highly conserved region but in a short segment (<40 amino acids) that connects highly conserved Regions III and IV (Schmidt et al., 1989). It is possible that one or both of these regions is part of a Mg$^{2+}$ binding site. In fact, Edwards et al. (1990a) have found that substitutions in Region IV at one of the putative active site residues (E461) in the E. coli β-galactosidase can significantly decrease the affinity of the protein for Mg$^{2+}$ (a 1500-fold increase in $K_D$ for the E461H variant at 25°C). The mutagenesis/selection procedure outlined here allows the identification of potentially different cold-sensitive phenotypes. Mutants P429S and L317F represent two of these cases. In both variants, dramatic effects result from what is apparently the small localized change introduced by a single amino acid substitution. Cold denaturation seems unlikely as neither aggregation nor precipitation of either variant at any temperature is observed, even at very high ionic strengths. The decrease of activity at low temperatures is fully reversible and time independent. As with the E. coli enzyme, magnesium ions are not required for the quaternary structure of the protein at ambient temperatures as evidenced by the gel filtration studies run in 5 mM EDTA (data not shown). Also, importantly, the $K_m$ values for both the variants remain constant over the full temperature range examined. We conclude that L317F is crippled.
catalytically (specifically in its $V_{\text{max}}$) while the cold sensitivity of P429S is due to an increase in the essentially inactive "Mg$^{2+}$-free" enzyme concentration as the temperature is lowered.

REFERENCES

Bolivar, F., Rodriguez, R. L., Green, P. J., Betlach, M. C., Heyneker, H. L., Boyer, H. W., Croiss, J. H., and Falkow, S. (1977) *Gene (Amst.)* 2, 95–113

Brandts, J. F. (1964) *J. Am. Chem. Soc.* 86, 4291–4301

Cupples, C. G., Miller, J. H., and Huber, R. E. (1990) *J. Biol. Chem.* 266, 5512–5518

Edwards, R. A., Cupples, C. G., and Huber, R. E. (1990a) *Biochem. Biophys. Res. Commun.* 171, 35–37

Edwards, R. A., Jacobson, A. L., and Huber, R. E. (1990b) *Biochemistry* 29, 11001–11008

Estell, D. A., Graycar, T. P., and Wells, J. A. (1985) *J. Biol. Chem.* 260, 6518–6521

Fink, A., and Angelides, K. (1975) *Biochem. Biophys. Res. Commun.* 62, 701–706

Huber, R. E., Kurr, G., and Wallenfels, K. (1976) *Biochemistry* 15, 1994–2001

Kadonaga, J. T., and Knowles, J. R. (1985) *Nucleic Acids Res.* 13, 1733–1745

Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math.* 11, 481–484

Martinez-Bilbao, M., Holdsworth, R. E., Edwards, L. A., and Huber, R. E. (1991) *J. Biol. Chem.* 266, 4979–4986

Oka, T., Sakamoto, S., Miyoshi, K., Fuwa, T., Yoda, K., and Yamasaki, M. (1985) *Proc. Natl. Acad. Sci. U. S. A.* 82, 7212–7216

Portzehl, H., Caldwell, P. C., and Roegg, J. C. (1964) *Biochim. Biophys. Acta* 79, 581–591

Power, S. D., Adams, R. M., and Wells, J. A. (1986) *Proc. Natl. Acad. Sci. U. S. A.* 83, 3096–3100

Privalov, P. L., Grik, Y. V., and Venyaminov, S. Y. (1986) *J. Mol. Biol.* 190, 487–498

Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* 74, 5463–5467

Schmidt, B. F., Adams, R. A., Requadt, C., Power, S., and Mainzer, S. E. (1989) *J. Bacteriol.* 171, 635–635

Segel, I. H. (1976) *Enzyme Kinetics*, pp. 931–934, John Wiley and Sons, Inc., New York

Smith, P. K., Krohn, R. I., Hermansen, G. T., Mallia, A. L., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Geake, N. M., Olsen, B. J., and Klenk, D. C. (1985) *Anal. Biochem.* 150, 76–85

Strom, R., Attardi, G., Forsei, S., Turini, P., Celada, F., and Antonini, E. (1971) *Eur. J. Biochem.* 23, 119–124

Tenu, J., Virinette, O. M., and Yon, J. (1972) *Eur. J. Biochem.* 23, 119–124

Ullmann, A., and Monod, J. (1959) *Biochem. Biophys. Res. Commun.* 5, 85–89

Wallenfels, K., and Weil, R. (1972) *The Enzymes*, Vol. VIII, pp. 617–663, Academic Press, New York

Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) *Gene (Amst.)* 33, 103–119

Zipser, D. (1963) *J. Mol. Biol.* 7, 113–121

Zoller, M. J., and Smith, M. (1982) *Nucleic Acids Res.* 10, 6487–6500