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Amidase Activity and Thermal Stability of Human Thrombin

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ABSTRACT

Previous studies of amidase activity of human α-thrombin have yielded variable results and the decrease of this activity as a function of time and temperature has never been quantified. As this protease is an efficient tool in biochemistry and biotechnology thanks to its extreme selectivity, amidase activity and stability of thrombin were investigated with the synthetic substrate Tos-Gly-Pro-Arg-pNa. Enzyme activity as a function of temperature showed an optimum peak at 45°C. The pH dependence of the activity showed a maximum around 9.5. The addition of NaCl promoted an increase of the activity. Stability of thrombin decreased rapidly when increasing the temperature from 25–45°C and when diluting the enyzme. The presence of glycerol and ethylene glycol promoted a small increase of thrombin half life, whereas polyethylene glycol had a more pronounced positive effect even at very low concentrations.

Index Entries: Thrombin; amidase activity; thermostability, polyethylene glycol; fusion protein; specific cleavage.

INTRODUCTION

Human α-thrombin (EC 3.4.21.5) is a serine protease that plays a central role in the blood coagulation cascade (*1,2*). Owing to its significant participation in physiological processes, this enzyme has been extensively

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studied, in particular with regard to its specificity. Like other serine proteases, thrombin exhibits distinct specificities for proteolysis and for esterolytic hydrolysis of small substrates. The special feature of thrombin lies in its highly restricted specificity toward protein substrate. This specificity is best known by its selective cleavage of two Arg-Gly bonds from among 181 Arg/Lys-Xaa bonds in fibrinogen, but thrombin also exhibits an extreme selectivity in cleaving nonfibrinogen polypeptides. The study of the selective cleavage of a number of peptides enabled an optimum synthetic cleavage site for thrombin to be defined. It has the structure of P_4-P_3-Pro-Arg-P'_1-P'_2, where P_3 and P_4 are hydrophobic amino acids and P'_1 and P'_2 are nonacidic amino acids (*3*). Therefore, thrombin falls into the category of site-specific proteases like blood coagulation factor Xa, clostripain, collagenase, and some other enzymes. These proteases find many applications in biochemistry and biotechnology, as a major tool in the sequence analysis of proteins, and in the design and synthesis of therapeutic agents (*4*). In particular these enzymes are used in recombinant DNA technology to cleave gene fusion proteins site-specifically under nondenaturing conditions. For example, thrombin was used to release the native target protein from fusion proteins, which consisted of the recombinant target protein linked to a tail allowing efficient protein purification via a specific cleavage site for thrombin (*5–8*). The efficiency of this enzymatic cleavage varied according to the particular case, ranging from 5–10% (*5*) to the major part (*7*) of released target protein. The exact reasons for the failure to cleave were not always understood, although the poor accessibility of the thrombin recognition site was often questioned. This problem was partially overcome by introducing a glycine-rich linker located immediately following the thrombin cleavage site. This linker alters the structure of the site and hence facilitates its cleavage (*8*). Another reason for the poor cleavage may be that partial denaturation of thrombin during its use affected its activity. Indeed, as a tool in research and clinical laboratories, thrombin is known for its susceptibility to decomposition by autolysis and to classic denaturation. This poor stability leads to the use of very important quantities of enzyme at room temperature to obtain an efficient cleavage. During denaturation, the different activities of thrombin decrease more or less rapidly. For example, during autolytic degradation human thrombin undergoes conversion to forms that lack clotting activity but retain similar activities toward low molecular weight substrates (*9,10*). Thermal stability of human thrombin has been studied by following both the residual proteolytic activity of the enzyme for the clotting of fibrinogen and its esterolytic activity, as a function of incubation time in the presence of different salt concentrations (*10*). However, the residual amidase activity of thrombin on synthetic peptides as a function of time has never been studied, nor the influence of organic compounds on the stability of this enzymatic activity. The objective of this study is thus to quantify rigorously the stability of human thrombin and to find methods for enhancing it. Moreover, we studied amidase

activity of thrombin using a synthetic amide substrate as a function of temperature, pH, and NaCl concentration to determine the optimal operating conditions of thrombin with this particular substrate.

MATERIALS AND METHODS

Enzyme

Amidase activity studies were performed with pasteurized human α-thrombin, which was obtained as a solution (3.9 mg/mL and 2664 NIH U/mg) from the CNTS (Centre National de la Transfusion Sanguine, Les Ullis, France). The NIH units refer to the international human thrombin standard from the National Institute of Health (Bethesda, MD). For thermal stability studies, human α-thrombin was obtained as a dried powder from Sigma Chemical Co. (St. Louis, MO) (T 6759; 3230 NIH U/mg of protein). Lyophilyzed enzyme corresponding to 320 NIH contained in each flask was redissolved by adding 200 μL of MilliQ quality water (Millipore, Bedford, MA) and stored at −20°C. These samples could be stored in these conditions without any loss of activity. It was checked that both batches of enzyme presented the same activity and stability properties.

Assay of Thrombin Activity

Thrombin-catalyzed hydrolysis of Tos-Gly-Pro-Arg-pNA (Sigma T1637) was monitored at 400 nm on a Hewlett-Packard (Waldbronn, Germany) diode array spectrophotometer 8452, using chromogenic substrate concentration of 209 μ*M*. Stock solution of substrate was prepared by dissolving peptide *p*-nitroanilide in MilliQ water that had been adjusted to pH 4.0 with hydrochloric acid to avoid its degradation and stored frozen (*11*). The hydrolytic reactions were performed at 37°C in 50 m*M* HEPES buffer, pH 8.3, containing 0.1% PEG 6000. All assays were performed in polyethylene tubes to avoid adsorption of the enzyme at the vessel–solution interfaces (*12*).

Thermal Stability

Thrombin was incubated in polyethylene tubes at the denaturation temperature in 50 m*M* HEPES buffer, pH 8.3 containing some additives of analytical grade. At regular intervals of time, the residual activity was measured as described before. The enzyme half-life was obtained by plotting the logarithmic evolution of the residual enzyme activity (percentae of the original activity) as a function of the incubation time. Thus, the time at which 50% initial activity is reached corresponded to the half-life of the enzyme in the reaction conditions. To study the effect of additives on the thermal stability of the enzyme, the determination of the half-life for a reference solution (i.e., an enzyme solution without any additive) was performed in parallel. The results obtained were expressed in

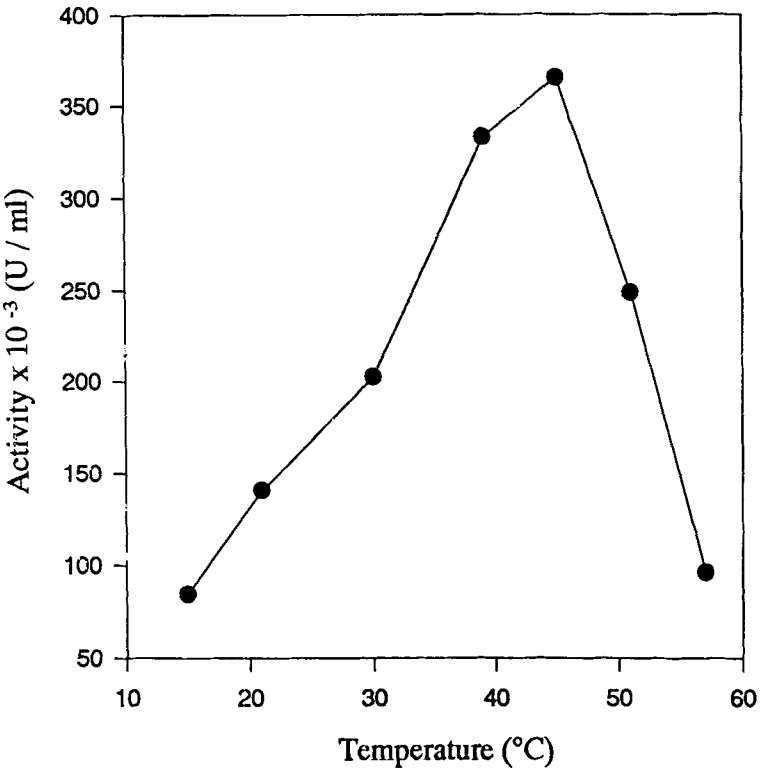

Fig. 1. Effect of temperature on thrombin activity. Activity of thrombin is followed spectrophotometrically at 400 nm using 209 μM Tos-Gly-Pro-Arg-pNa solution at 50 mM HEPES buffer, pH 8.3.

the form of protective effect (or stabilizing factor), as defined by the ratio of thrombin half-life in the presence of additives to the half-life of thrombin without any additives.

RESULTS AND DISCUSSION

Amidase Activity Studies

Specific activity of the enzyme preparation used was equal to 161.5 U/mg (0.63 U/mL), where 1 U is defined as the quantity of enzyme that hydrolyzes 1 μmol of substrate/min using the standard conditions of assay described in the previous section.

Effect of Temperature on the Enzyme Activity

The initial reaction rate of thrombin was determined at various temperatures. The results obtained showed an optimum value at 45°C (Fig. 1). The rapid decrease of activity above this temperature can be attributed to enzyme denaturation. These results permitted the activation energy required for the catalyzed reaction to be obtained. It was equal to 8.8 kcal/mol, which falls within the normal range for enzyme catalysis.

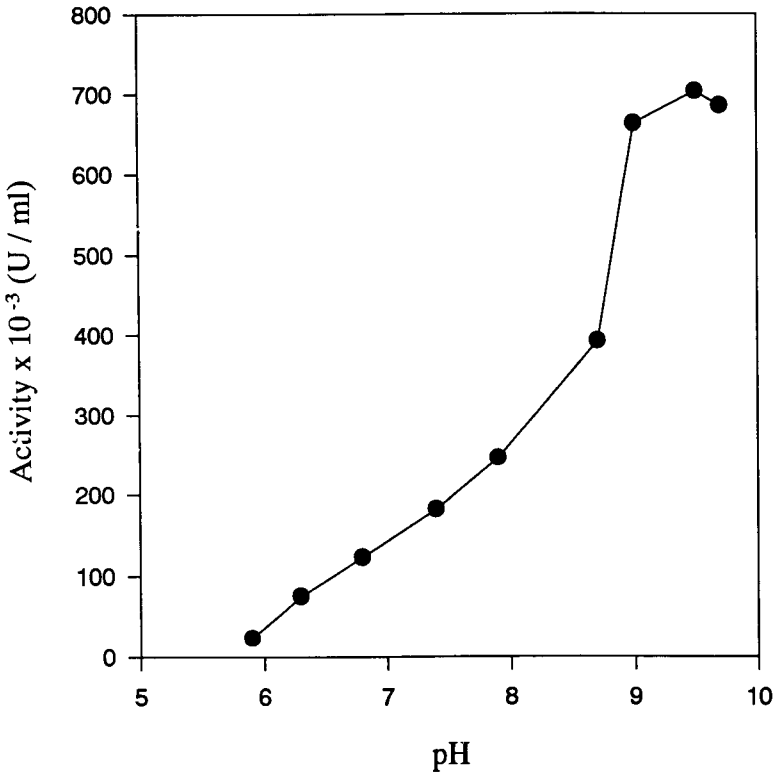

Fig. 2. Effect of pH on enzyme activity. Activity of thrombin is measured at 37°C using 209 μM Tos-Gly-Pro-Arg-pNa solution in the triple buffer containing 25 m*M* BisTris, 25 m*M* Tris, and 25 m*M* CHES adjusted to the desired pH with HCl.

Effect of pH on Thrombin Activity

The pH dependence of thrombin amidase activity was studied in the pH range 6–10 with a triple buffer containing 25 m*M* BisTris (pKa = 6.5 at 25°C), 25 m*M* Tris (pKa = 8.0 at 25°C), and 25 m*M* CHES (pKa = 9.5 at 25°C), to cover the entire pH range without changing the buffer (*13*). The pH dependence of thrombin activity is shown in Fig. 2. The activity presents a maximum around 9.5.

In the following studies, thermal stability was measured at pH 8.3, as thrombin stability is relatively high at this pH value. Moreover, previous studies (*13*) have shown that the addition of NaCl in the medium displaced the maximum of activity to lower pH values (with NaCl 1*M*, the maximum is around 8.5) and we were interested in knowing the stability of thrombin in the presence of NaCl for further studies.

Effect of NaCl on Thrombin Activity

Activity was measured in the presence of different NaCl concentrations. Activity increased by a factor 2 with NaCl concentration between 0–500 m*M* and increased more slowly above this value (Fig. 3). Enhancement of thrombin activity in the presence of NaCl has been reported with

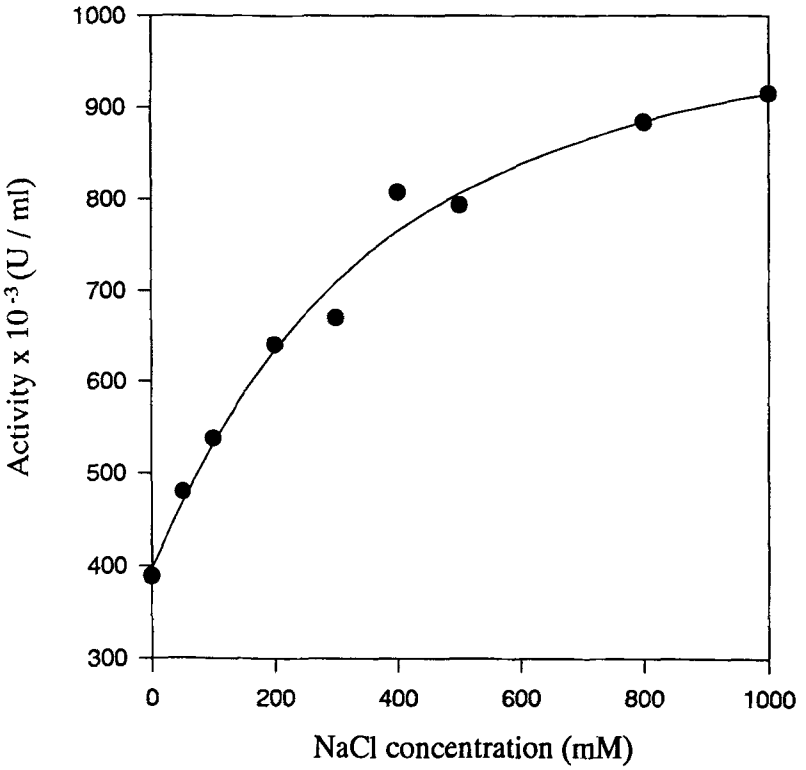

Fig. 3. Effect of NaCl concentration on thrombin activity. Activity of thrombin is measured at 37°C and pH 8.3 with experimental conditions as defined in Fig. 1.

some synthetic substrates. The cation Na^+ would bind to thrombin, causing a conformational change in the active site that increases the reactivity of functional groups (*14*). Nevertheless, inhibitory effects of the sodium ions on the amidase and esterase activities of thrombin have been observed with other types of synthetic substrates (*15*). Consequently, the effect of NaCl on enzyme activity depends on the substrate used. As an example, addition of NaCl has been reported both to increase and decrease thrombin digestion of fusion proteins depending on the type of fusion (*16*). In this study, we have quantified the increase of thrombin activity with the particular substrate Tos-Gly-Pro-Arg-pNa over a large range of NaCl concentration, which had never been done before.

Thermal Stability Studies

Effect of Temperature on Thrombin Stability

To minimize the influence of unknown additives on the denaturation rates determined for thrombin, a preparation of enzyme with a high degree of purity was used. Moreover, the thrombin concentration used in this experiment corresponded to the commercial preparation diluted 500 times, i.e., approx 3 NIH U/mL.

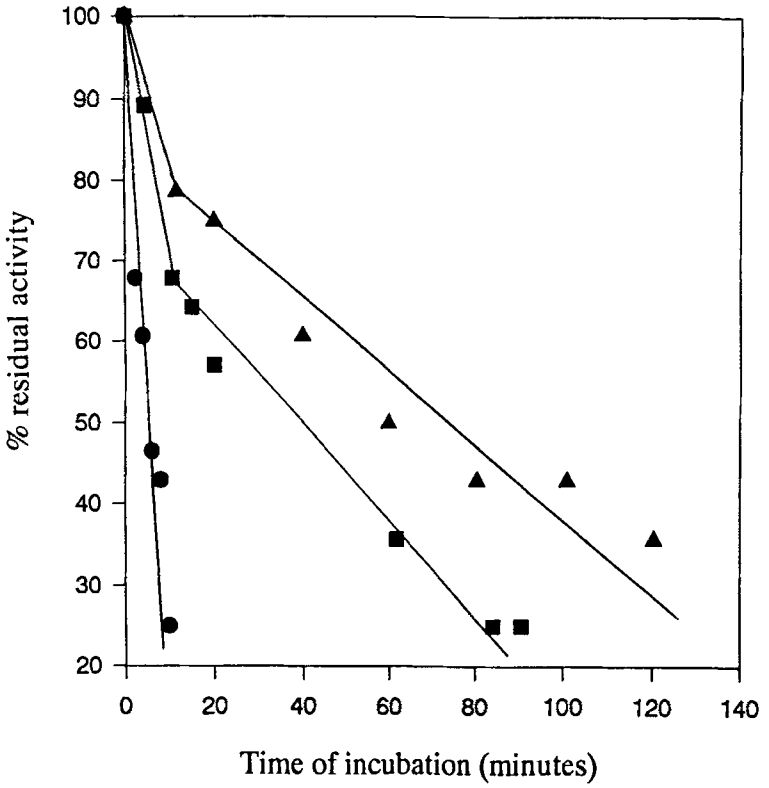

Fig. 4. Effect of temperature on thrombin half-life. Residual activity of thrombin is measured at 37°C and pH 8.3 with experimental conditions as defined in Fig. 1. ●, 45°C; ■, 37°C; ▲, 25°C.

Figure 4 shows the evolution of the residual activity as a function of the incubation time at different temperatures. Thrombin stability decreased very rapidly with an increase of temperature. Half-lives obtained at 25, 37, and 45°C were equal to 60, 35, and 6 min, respectively. These results show that pure thrombin exhibits stability problems at relatively low temperatures that correspond to the standard operating conditions for cleaving fusion proteins (*7*).

In log (residual activity) vs time plots at 25 and 37°C two-slope curves are observed, instead of the linear behavior corresponding to single-step deactivation. At first, rapid loss in activity takes place, followed by a second slower stage. Such behavior is often observed, as enzyme deactivation follows complex mechanisms, including several steps. From this curve pattern it can be assumed either that thrombin denatures by a two irreversible first-order steps deactivation process, or that different forms of thrombin (α, β, or γ) that differ in both specific activity and stability are present in the initial preparation (*17*).

The denaturation temperature chosen for the following stability studies was 45°C, since the half-life obtained at this temperature (6 min) was short enough for sufficient precision and to allow the experiments to be carried out rather quickly.

Table 1
Effect of Concentration of Thrombin on Its Half-Life at 45°C

Thrombin concentration, U/mL	Half-life, min
3	6
7.5	7
15	10
30	13

Effect of Thrombin Concentration on Its Thermal Stability

Table 1 gives the half-life of thrombin at 45°C in terms of its concentration. These were obtained by diluting the commercial preparation with 50 m*M* HEPES buffer pH 8.3. Half-life of thrombin increases with its concentration ranging from 6 min at 3 NIH U/mL to 13 min at 30 NIH U/mL. This result dismisses autolysis as the major cause of activity loss. Indeed, autolytic degradation would increase with thrombin concentration, since thrombin is both the enzyme responsible and the protein substrate. It is well known that enzymes that are not susceptible to autolysis are more stable at high concentration in aqueous solution, owing to the presence of impurities in the preparation of enzyme that ensure a better thermostability or owing to the self-stabilizing effect of any soluble protein (*18*).

Effect of Glycerol and Ethylene Glycol on Thrombin Thermal Stability

The protective effect of glycerol and ethylene glycol at concentration up to 2*M* were studied at 45°C. As shown in Fig. 5, these effects are very weak with ethylene glycol reaching the values of 2 in a 1*M* solution. However, glycerol at 2*M* concentration significantly increases thrombin stability. For different enzymes the intensity of the stabilizing factor of polyols is very variable. The protective effect is not an absolute effect, but it depends on the nature of the enzyme studied and the degree of its interaction with the additive (*19*).

Effect of PEG on Thrombin Thermal Stability

The influence of PEG (polyethylene glycol) addition on thrombin stability was determined using PEG 6000 (average mol wt 6000). PEG 6000 presented a protective effect that increased with its concentration (Table 2). Very low concentrations were sufficient to significantly stabilize thrombin. Indeed, the thrombin half-life was multiplied by a factor 6.5 in the presence of 1 g/L of this additive. The protective effect remained equal to approx the same value up to 100 g/L and increased again for

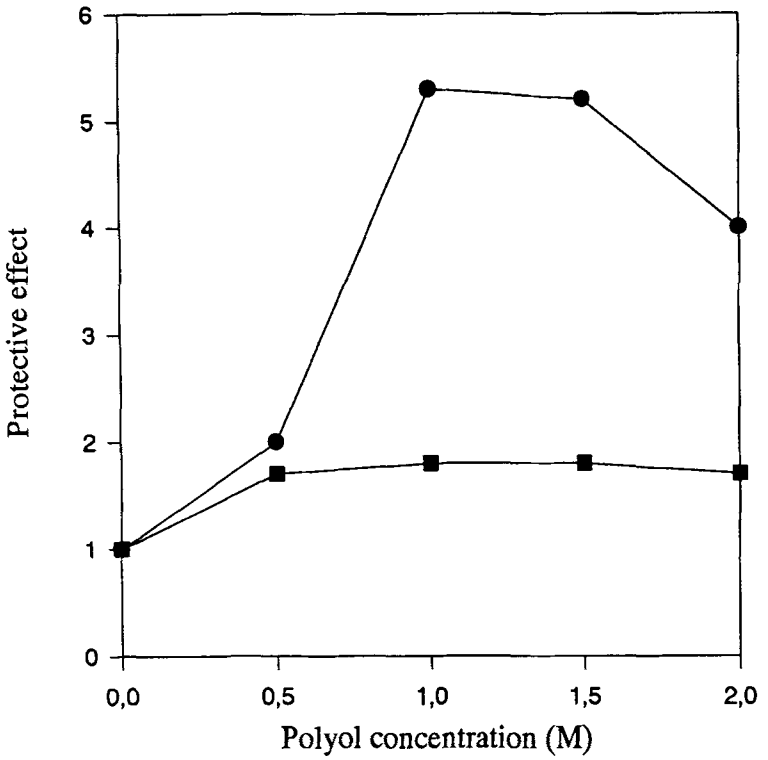

Fig. 5. Effect of glycerol (●) and polyethylene glycol (■) on thrombin stability. Protective effect is defined as the ratio of thrombin half-life with additive to thrombin half-life without additive. Denaturation is performed at 45°C and residual activity of thrombin is measured at 37°C and pH 8.3 with experimental conditions as defined in Fig. 1.

Table 2
Effect of Concentration of PEG 6000
on Its Protective Effect at 45°C

PEG 6000 concentration, g/L	Protective effect
0.025	2.5
0.05	3.5
1	6.5
5	6.5
50	7.5
100	8
150	10
200	15

higher PEG concentrations. Thrombin stability increased slightly with PEG molecular weight at a concentration of 1 g/L ranging from 5.5 with PEG 4000 to 9.5 with PEG 20,000.

Polyethylene glycol has been shown to significantly stabilize some enzymes such as glucose oxidase (*20*), but has also been shown to reduce the thermostability of some proteins by interacting with their denatured form (*21*). The stabilizing effect induced by PEG molecules at low PEG concentration may be attributed to a possible molecular interaction between PEG and thrombin. At high PEG concentration it may arise from a number of factors including mechanical restraint of conformational changes and a reinforcement of water molecule organization (*22*).

CONCLUSION

The information collected from our studies of the amidase activity and thermostability of thrombin will help in using this enzyme more efficiently, especially in cleaving fusion proteins. In particular, these studies provide an accurate quantification of the residual amidase activity as a function of incubation time at different temperatures, of enzyme dilutions, and in the presence of different additives. According to our results, the major cause of amidase activity loss would not be autolysis but probably conformational changes. In this context the use of soluble additives is particularly attractive to stabilize the native structure of thrombin and hence prolong its useful life. We have shown that addition of PEG, even at very low concentration, significantly stabilizes the enzyme.

REFERENCES

1. Fenton, J. W., II (1981), *Ann. NY Acad. Sci.* **370,** 468–495.
2. Shuman, M. A. (1986), *Ann. NY Acad. Sci.* **485,** 228–239.
3. Chang, J. Y. (1985), *Eur. J. Biochem.* **151,** 217–224.
4. Chang, J. Y., Alkan, S. S., Hilschmann, N., and Braun, D. G. (1985), *Eur. J. Biochem.* **151,** 225–230.
5. Knott, J. A., Sullivan, C. A., and Weston, A. (1988), *Eur. J. Biochem.* **174,** 405–410.
6. Smith, D. B. and Johnson, K. S. (1988), *Gene* **67,** 31–40.
7. Gearing, D. P., Nicola, N. A., Metcalf, D., Foote, S., Willson, T. A., Gough, N. M., et al. (1989), *Biotechnology* **7,** 1157–1161.
8. Guan, K. L. and Dixon, J. E. (1991), *Anal. Biochem.* **192,** 262–267.
9. Fenton, J. W., II, Fasco, M. J., and Stackrow, A. B. (1977), *J. Biol. Chem.* **252,** 3587–3598.
10. Landis, B. H., Koehler, K. A., and Fenton, J. W. (1981), *J. Biol. Chem.* **256,** 4604–4610.

11. Lottenberg, R., Christensen, U., Jackson, C. M., and Coleman, P. L. (1981), *Meth. Enzymol.* **80,** 341–361.
12. Baughman, D. J. (1970), *Meth. Enzymol.* **19,** 145–157.
13. Di Cera, E., De Cristofaro, R., Albright, D. J., and Fenton, J. W. (1991), *Biochemistry* **30,** 7913–7924.
14. Orthner, C. L. and Kosow, D. P. (1980), *Arch. Biochem. Biophys.* **202,** 63–75.
15. Workman, E. F. and Lundblad, R. L. (1978), *Arch. Biochem. Biophys.* **185,** 544–548.
16. Haun, R. S. and Moss, J. (1992), *Gene* **112,** 37–43.
17. Greco, G., Jr. and Gianfreda, L. (1984), *Biotechnol. Lett.* **6,** 693–698.
18. Wiseman, A. (1978), in *Topics in Enzyme and Fermentation Biotechnology,* vol. 2, Wiseman, A., ed., Ellis Horwood, Chichester, pp. 280–303.
19. Graber, M. and Combes, D. (1989), *Enzyme Microb. Technol.* **11,** 673–677.
20. Ye, W. N., Combes, D., and Monsan, P. (1988), *Enzyme Microb. Technol.* **10,** 498–502.
21. Lee, L. L. and Lee, J. C. (1987), *Biochemistry* **26,** 7813–7819.
22. Combes, D. and Monsan, P. (1984), *Ann. NY Acad. Sci.* **434,** 48–60.