Effect of temperature on the association step in thrombin–fibrinogen interaction

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Kinetics of fibrinopeptide A release by human α -thrombin at low fibrinogen concentration allowed us to measure the specificity constant, i.e. $k_{\text{cat.}}/K_m$, for the interaction between the enzyme and human fibrinogen. A study of the dependence of the ratio $k_{\text{cat.}}/K_m$ upon the viscosity of the medium revealed that fibrinogen acts as a 'sticky' substrate, or, in other words, as a substrate that dissociates from the Michaelis complex with a rate comparable with that for acylation of the active site. These experiments allowed us also to compute for the first time the second-order rate constant for thrombin–fibrinogen association. A study of the temperature-dependence of the association rate, carried out over the temperature range spanning from 10 °C to 37 °C (pH 7.50; 10.15) permitted the estimation of the enthalpy and entropy of

activation, $\Delta H^\ddagger$ and $\Delta S^\ddagger$, which were found to be equal to $5.69 \pm 0.77 \text{ kJ} \cdot \text{mol}^{-1}$ and $-80.25 \pm 1.79 \text{ kJ} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ respectively. In addition, the values of K_m for thrombin–fibrinogen reaction were measured at different solution viscosities in order to derive the equilibrium dissociation constant, K_s , of this interaction. These experiments showed that the K_s values for thrombin–fibrinogen binding was equal to $1.8 \mu\text{M}$ at 25 °C. Altogether these results indicated that fibrinogen, though interacting with both the catalytic pocket and the fibrinogen recognition site on the thrombin molecule, dissociates from Michaelis complex with a rate comparable with that shown by amide substrates, which interact only with the catalytic site.

INTRODUCTION

Thrombin–fibrinogen interaction is a fundamental reaction in blood clotting process, since it produces fibrin monomers whose aggregation leads to formation of insoluble fibrin fibres [1]. The first step in thrombin–fibrinogen interaction is the binding of α -thrombin to fibrinogen, followed by hydrolysis at the *N*-terminal α -chains of fibrinopeptide A. Release of fibrinopeptide B at the *N*-terminal β -chain occurs upon release of fibrinopeptide A and is strongly affected by aggregation of $\alpha\text{B}\beta$ -fibrin monomers [2,3].

Although the steady-state parameters for hydrolysis of $\text{A}\alpha$ -chain have been well characterized by many workers [3–7], biochemical and biophysical aspects concerning the pre-catalytic steps were only partially investigated.

A method based on viscosity perturbation has been recently exploited to dissect the kinetic scheme of amidase activity of human α -thrombin toward synthetic peptides [8,9]. This strategy allowed us to compute the individual rate constants for the multiple steps over the global catalytic cycle in human α -thrombin. On the basis of these findings, an extensive investigation on the individual steps pertaining to thrombin–fibrinogen interaction demanded an immediate attention. In the present study we begin this investigation by using a viscometric method to explore the encounter step in thrombin–fibrinogen interaction. The second-order rate constant for thrombin–fibrinogen reaction was directly measured for the first time and studied as a function of temperature. This experimental strategy permitted estimation of the thermodynamic activation parameters for the encounter step in thrombin–fibrinogen association.

MATERIALS AND METHODS

Data analysis

Thrombin–fibrinogen interaction was analysed by the widely

accepted scheme of serine–proteinase activity [10]

where E is thrombin, F is fibrinogen, EF and EP are the enzyme–fibrinogen and enzyme–product intermediates, whereas k_{+1} , k_{-1} , k_2 and k_3 are the rate constants for fibrinogen association and dissociation, acylation and deacylation reactions respectively.

All the constants but k_{+1} are first-order, the latter pertaining to a second-order kinetic process. It is known that solution viscosity slows down all reactions involving diffusion [11] and thus it should be expected that all the rate constants but k_2 (which refers to an intramolecular reaction) in eqn. (1) are inversely proportional to solution viscosity. This was recently demonstrated for human α -thrombin amidase activity by using several synthetic substrates [8,9]. According to this model, apparent $k_{\text{cat.}}$ and K_m , measured as a function of relative viscosity, $\eta_{\text{rel.}}$, are given by [8,9]

$$k_{\text{cat.}} = \frac{k_2 k_3}{(k_2 \eta_{\text{rel.}} + k_3)} \quad (2)$$

$$K_m = \frac{k_3 (k_{-1} + k_2 \eta_{\text{rel.}})}{k_{+1} (k_2 \eta_{\text{rel.}} + k_3)} \quad (3)$$

Eqns. (2) and (3) provide rigorous expressions for $k_{\text{cat.}}$ and K_m as a function of relative viscosity without any assumption on the relative magnitude of k_2 and k_3 . From eqns. (2) and (3) the ratio $K_m/k_{\text{cat.}}$ as a function of $\eta_{\text{rel.}}$ is given by:

Abbreviations used: FpA, fibrinopeptide A; FpAP, fibrinopeptide 3-phosphate; FpB, fibrinopeptide B; FpAY, des-Ala-fibrinopeptide A; PEG, poly(ethylene glycol).

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$$\frac{K_m}{k_{cat}} = \frac{\eta_{rel.}}{k_{+1}} + \frac{k_{-1}}{k_{+1}k_2} \quad (4)$$

Application of equation 4 should permit one to estimate the value of k_{+1} , the association rate constant for thrombin–fibrinogen interaction, and the ratio k_2/k_{-1} , which measures the ‘stickiness’ of fibrinogen [12]. We define the ‘stickiness’ of a substrate as the particular tendency of a substrate to dissociate from the Michaelis complex with a rate comparable with that of the catalytic reaction.

In the present study fibrinogen concentration was kept below 0.5 μ M, i.e. below the K_m values of thrombin interaction with fibrinogen A α -chain [7,13]. Under these conditions the Michaelis equation for fibrinopeptide A (FpA) release reduces to:

$$\frac{d[FpA]}{dt} = \frac{k_{cat}e_0[A\alpha]}{K_m} \quad (5)$$

and thrombin-catalysed steps are first-order with respect to the concentrations of enzyme (e_0) and A α -chain $[A\alpha]$. Integration of eqn. (5) gives:

$$[FpA]_t = [A\alpha]_0 (1 - \exp^{-k_1 t}) \quad (6)$$

where $[FpA]_t$ is the concentration of fibrinopeptide A at the time t and k_1 is a pseudo-first-order rate constant equal to the product of thrombin concentration and the specificity constant (k_{cat}/K_m) for thrombin–A α -chain interaction [2]. Eqn. (6) was used in the present study to compute the ratio K_m/k_{cat} under different solution viscosities. These values were then fitted to eqn. (4) to derive the values of k_{+1} and k_2/k_{-1} , as described above. This procedure has been recently exploited for the analysis of data pertaining to interaction of human α -thrombin with several synthetic amide substrates [8,9,14].

The thermodynamic activation parameters for the encounter step in thrombin–fibrinogen interaction may be computed by using the activated-complex theory for which the Eyring equation applies [15,16]

$$k_{+1} = \frac{k_b T}{h} \exp^{-\frac{\Delta G^\ddagger}{RT}} \quad (7)$$

where k_{+1} is the second-order rate constant for thrombin–fibrinogen encounter, k is Boltzmann’s constant, h is Planck’s constant, T the absolute temperature, R the gas constant and where:

$$\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger \quad (8)$$

with $\Delta G^\ddagger$, $\Delta H^\ddagger$ and $\Delta S^\ddagger$ being the free energy, enthalpy and entropy of activation respectively. Rearrangement of eqns. (7) and (8) provides the relationship:

$$\log \frac{k_{+1}}{T} = -\frac{\Delta H^\ddagger}{2.3RT} + \log \frac{k}{h} + \frac{\Delta S^\ddagger}{2.3R} \quad (9)$$

Thus by measuring k_{+1} as a function of temperature and plotting $\log k_{+1}/T$ against $1/T$ the values of the enthalpy and entropy of activation can be calculated.

Reagents

Human prothrombin, *Echis carinatus* (carpet viper) venom, gelatin- and lysine-agarose (4%), fibrinopeptide A, *p*-nitrophenyl guanidinobenzoate hydrochloride, Hepes and sucrose were purchased from Sigma Chemical Co. (St. Louis, MO,

U.S.A.). S-2238 (D-Phe-Pip-Arg *p*-nitroaniline) and human fibrinogen (grade L) was from Kabi Vitrum (Stockholm, Sweden). All other reagents were of the best quality commercially available.

Purification of α -thrombin and fibrinogen

Purification of human α -thrombin was obtained by means of prothrombin activation by ecarin, and cation-exchange h.p.l.c., as previously reported [8]. Active-site titration, performed as detailed elsewhere [17], showed that this preparation was 96% active. Fibrinogen preparation was purified from contaminating plasmin(ogen) and fibronectin by affinity chromatography on lysine- and gelatine-agarose. Fibrinogen concentration was calculated by using an ϵ_{280} value of 1.506 ml·mg⁻¹·cm⁻¹ [18].

Experiments on the kinetics of fibrinopeptide A release

Fibrinopeptide release was studied at low fibrinogen concentration (0.3–0.5 μ M) in order to compute the ratio k_{cat}/K_m as described under ‘Data analysis’. Typically 500 μ l of human α -thrombin (final concn. 0.2 nM) was added to 1 ml of fibrinogen under solution conditions of 10 mM Hepes, 0.15 M NaCl, 0.1% poly(ethylene glycol) (PEG), pH 7.50 at 10, 15, 25 and 37 °C. The pH values of solutions was always adjusted at 25 °C by using a Δ pH/ Δ T coefficient of $-0.0131 (\pm 0.0002)$. This coefficient was calculated by using a Beckman Φ -60 pH-meter and a HETO (Allerød, Denmark) DT 622/1 thermostat, equipped with a CB 13-25E cryostatic bath. The above reported coefficient was in excellent agreement with the value at pH 7.50 reported in the literature [19]. At ten appropriate time intervals, ranging from 1 to 60 min, hydrolysis reaction was stopped by addition of 0.3 M HClO₄ (final concn.). The precipitated protein was then centrifuged down at 12000 rev./min (r_{av} 4 cm) for 5 min and the supernatants (1 ml) were injected into the h.p.l.c. apparatus. These experiments were performed by using a ‘matrix’ strategy, whereby the release of fibrinopeptide A at ten time intervals was studied as a function of seven solution viscosities which were changed by adding sucrose to buffer solutions. This procedure reduced the error arising from preparation of different thrombin solutions for each data set collected at a given medium viscosity. Six sucrose concentrations were employed, starting from 0.84 M and scaling by a factor of 1.25. The seventh concentration was equal to zero and used as the reference. Relative viscosity was measured by a rotating-cylinder Brookfield LVT thermostatically controlled viscometer (Stoughton, MA, U.S.A.). Viscosity was measured by using ten sucrose concentrations spanning from 0.1 M to 1 M under the solution conditions of 10 mM Hepes, 0.15 M NaCl/0.1% PEG, pH 7.50 at 10, 15, 25 and 37 °C. Under all solution conditions measurements were taken in duplicate. The value of the relative viscosity at any sucrose concentration was computed as:

$$\eta_{rel.} = 1 + \alpha Suc + \beta Suc^2 \quad (10)$$

where α and β are constants and Suc is sucrose concentration. The values of α and β were found to change as a function of temperature. At 10, 15, 25 and 37 °C the values of α were 0.60, 0.59, 0.54 and 0.53 respectively. At the same temperatures the values of β were 1.75, 1.65, 1.49 and 1.27 respectively.

Separation and quantification of the various fibrinopeptides were obtained on a Perkin–Elmer model LC10 h.p.l.c. instrument equipped with an LC-85B spectrophotometer, by using an Applied Biosystems model OD-224 Speri-5 RP18 (5 μ m) 220 mm $\times$ 4.6 mm column. The elution buffers were: (A) 20 mM sodium phosphate/5% (v/v) acetonitrile, pH 5.80; (B) 50% (v/v) acetonitrile in buffer (A). Elutions were carried out by

using a linear gradient (0–40% B) in 18 min. This gradient allowed for a sharp resolution of all fibrinopeptides, i.e. FpA, fibrinopeptide A3-phosphate (FpAP), des-Ala-fibrinopeptide A (FpAY), fibrinopeptide B (FpB) and des-Arg-FpB. The areas under chromatographic peaks were measured and converted into concentration by a calibration curve prepared with standard samples of FpA. Although in these experiments the kinetics of FpB release could also be monitored, we did not analyse these data because of the uncertainty of the effect of sucrose on fibrin aggregation, which is known to strongly affect fibrinopeptide B release [2,3].

Measurement of K_m for thrombin–fibrinogen interaction

The K_m value for thrombin–fibrinogen interaction was measured by the strategy of competitive inhibition of S-2238 hydrolysis, as recently detailed [13,21]. In these experiments seven curves of S-2238 hydrolysis were collected, using seven substrate concentrations scaled by a factor of 2 and ranging from 0.5 to 40 μ M. One curve was taken in the absence of fibrinogen and six other curves were collected in the presence of fibrinogen concentrations scaled by a factor of 1.5. The highest fibrinogen concentration was approximately half of the experimental value of K_m . Corrections for light-scattering due to fibrin aggregation were made as detailed elsewhere [13]. A total of 49 experimental points were fitted to the following equation:

$$v = e_t \frac{k_{\text{cat}}[S]}{K_m(1 + [F])'K_m + [S]} \quad (11)$$

where e_t is thrombin concentration, k_{cat} is the catalytic constant for S-2238 hydrolysis, sK_m and $'K_m$ are the Michaelis constants for thrombin–S-2238 and thrombin–fibrinogen interaction, and $[S]$ and $[F]$ are the substrate and fibrinogen concentration respectively. The values of $'K_m$ measured at 0, 0.2, 0.4, 0.6, 0.8 and 1 M sucrose were then fitted to a rearranged form of eqn. (3):

$$K_m = K_s \frac{(1 + \alpha\eta_{\text{rel}})}{(1 + \beta\eta_{\text{rel}})} \quad (12)$$

where α is equal to k_2/k_{-1} , β is k_2/k_3 and K_s is the equilibrium dissociation constant for thrombin–fibrinogen interaction.

All parameters contained in eqns. (6), (11) and (12), along with their S.E.M. values at the cut-off of 1 S.D. (68%) were resolved with good accuracy by a non-linear least-squares method using the Marquardt algorithm [20]. In all linear regressions (eqns. 4 and 9) points were weighted according to the inverse of their variances.

RESULTS

The experimental and theoretical strategy employed in the present study allowed us to estimate, for the first time, the value of the second-order rate constant for thrombin–fibrinogen association. Figure 1 showed two progress curves for FpA release obtained at different solution viscosities. It is evident that the solution viscosity greatly slows down the apparent pseudo-first-order rate constant for FpA release. At low substrate concentration this rate constant, as demonstrated above, is equal to k_{cat}/K_m times the concentration of thrombin. It was thus possible to estimate the second-order rate constant, k_{+1} , and the ratio k_2/k_{-1} for

thrombin–fibrinogen association by measuring k_{cat}/K_m at different solution viscosities and fitting these values to eqn. (4), as shown by Figure 2 for data obtained at 37 °C.

It was previously demonstrated that the viscogenic agent sucrose does not specifically perturb the interaction of thrombin with a macromolecular ligand, such as hirudin [8], so that one

Figure 1 Progress curves for the release of FpA at 0 M sucrose ($\eta_{\text{rel}} = 1$, $\square$) and 0.84 M sucrose ($\eta_{\text{rel}} = 2.50$, $\blacksquare$) obtained by using 0.2 nM human α -thrombin and 0.3 μ M fibrinogen under the experimental conditions reported in the text at 37 °C

For the sake of clarity, only two curves are shown. Continuous lines were drawn according to eqn. (6) with the best-fit parameter values: $[A\alpha] = 368.7 \pm 5.4$ nM and $k = 0.265 \pm 0.015$ min $^{-1}$ for the curve at $\eta_{\text{rel}} = 1$ and $k = 0.155 \pm 0.005$ min $^{-1}$ for the curve at $\eta_{\text{rel}} = 2.50$.

Figure 2 Effect of η_{rel} on k_{cat}/K_m for FpA release under the experimental conditions reported in the text at 37 °C

The straight line is drawn according to eqn. (4) with the best-fit parameter values as listed in Table 1.

Table 1 Effect of temperature on the association rate constant and the ratio k_2/k_{-1} of thrombin–fibrinogen interaction

Temperature (°C)	$10^{-7} \times k_{+1}$ (M $^{-1}$ · s $^{-1}$)	k_2/k_{-1}
10	3.33 ± 0.36	1.46 ± 0.62
15	3.50 ± 0.29	0.96 ± 0.23
25	4.06 ± 0.27	1.10 ± 0.24
37	4.85 ± 0.37	0.81 ± 0.18

Figure 3 Effect of temperature on the second-order rate constant for thrombin–fibrinogen association step

The straight line is drawn according to eqn. (9) with the best-fit parameter values: $\Delta H^\ddagger = 5.69 \pm 0.77 \text{ kJ} \cdot \text{mol}^{-1}$; $\Delta S^\ddagger = -80.25 \pm 1.79 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$. The S.E. of the fit is $\sigma = 0.01$ (log units).

Figure 4 Results of kinetic experimental values of K_m for thrombin–fibrinogen interaction measured as a function of relative viscosity at 25 °C under the experimental conditions reported in the text

The continuous line was drawn according to eqn. (12) with the best-fit parameter values: $K_s = 1.82 \pm 0.62 \text{ } \mu\text{M}$; $k_2/k_{-1} = 1.79 \pm 1.13$; and $k_2/k_3 = 0.121 \pm 0.1$. The S.E. of the fit (σ) was 1.17×10^{-7} .

could reasonably rule out aspecific effects of sucrose also for thrombin–fibrinogen interaction.

The estimates of k_{+1} changed from 3 to $4.5 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ over the temperature range spanning from 10 to 37 °C. The value of k_{+1} was found to be directly proportional to temperature over the range 10–37 °C. The values of k_{+1} and k_2/k_{-1} as a function of temperature are listed in Table 1. Application of eqn. (9) allowed one to split the free energy of activation for the association step into its enthalpic and entropic contribution, as shown in Figure 3. The activation enthalpy for thrombin–fibrinogen association was found to be equal to $5.69 \pm 0.77 \text{ kJ} \cdot \text{mol}^{-1}$, whereas the activation entropy was estimated to be $-80.25 \pm 1.79 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$. The resulting activation free energy is equal to $30.6 \pm 3.5 \text{ kJ} \cdot \text{mol}^{-1}$ at 37 °C.

Figure 4 shows the dependence of K_m on the relative viscosity of the medium at 25 °C along with the theoretical line drawn according to eqn. (12). All parameters involved in eqn. (12) were resolved with acceptable accuracy. The estimate of k_2/k_{-1} , i.e. 1.79 ± 1.13 , was in a good agreement with the value derived from experiments on FpA release and computed by eqn. (4). The

values of the ratio k_2/k_3 showed that the acylation reaction is rate-limiting under the experimental conditions of the study at 25 °C. The value of the equilibrium dissociation constant for fibrinogen binding to thrombin was found to be $1.82 \pm 0.62 \text{ } \mu\text{M}$, in reasonable agreement with a recent report [9].

DISCUSSION

The results obtained in the present study along with the information gained by previous investigations on both thrombin–fibrinogen and thrombin–hirudin interactions could contribute to shed light on the energetic factors which drive the interaction and regulate the kinetic mechanisms of fibrinogen hydrolysis by human α -thrombin.

It is noteworthy that the association rate for thrombin–fibrinogen association is roughly equal to the value estimated for the association of thrombin with synthetic amide substrates [8,9], which bind to the catalytic site alone. On the other hand, the value of k_{+1} for thrombin–fibrinogen interaction is approximately one order of magnitude lower than the one for thrombin–hirudin association [22]. Previous studies showed that the rate constant of hirudin association is in the range 10^8 – $10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$, whereas the dissociation equilibrium constant is in the picomolar range under experimental conditions comparable with those employed for the present study [22,23]. Since both fibrinogen and hirudin ‘bridge-bind’ to the catalytic site and to the ‘fibrinogen-recognition site’ on the thrombin molecule, the difference in the energetics of these interactions demands a reasonable explanation.

The present study, along with a recent report [21], showed that the equilibrium dissociation constant for thrombin–fibrinogen interaction is in the micromolar range. This result implies that the six orders of magnitude that differentiate the equilibrium constants of hirudin and fibrinogen binding to α -thrombin seem to be not fully justified by the measured difference between the association rates. This means that a large difference of the dissociation rate constant, i.e. k_{-1} in eqn. (1), should mostly account for the affinity change between hirudin and fibrinogen. It is noteworthy that a similar difference of the dissociation rate in the interaction of the basic pancreatic trypsin inhibitor with trypsin and thrombin was previously demonstrated to be the major factor that scales down the affinity of thrombin for that inhibitor [24]. Hence it is most likely that, in the thrombin–fibrinogen adduct, a structural and/or conformational factor could lead to a destabilization of the complex. This effect could be drastically reduced in the thrombin–hirudin complex and thus the affinity greatly increased. This hypothesis, which has been recently proposed on the basis of thermodynamic arguments [21], seems to be validated by the present findings.

The study of the temperature dependence of k_{+1} for thrombin–fibrinogen binding showed that the formation of the activated complex is an entropy-driven process. Although quantitative interpretations of activation entropy are rather uncertain [16], it is widely accepted that chemical processes which have negative activation entropies in the range spanning from -30 to $-80 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ are bimolecular reactions with orientation and steric requirements involving also the solvent molecules [15,16].

The ratio k_2/k_{-1} indicated that, under experimental conditions of the present study, fibrinogen acts as a ‘sticky’ substrate, the rate of acylation being comparable with the dissociation rate. It is, however, noteworthy that the values of k_2/k_{-1} are inversely proportional to temperature, as shown by values listed in Table 1. This means that the activation enthalpy for the dissociation of fibrinogen from the Michaelis complex is significantly higher

than that one pertaining to the acylation of the serine oxide residue in the active site of α -thrombin.

In conclusion, the experimental strategy employed in the present study seems to be a powerful tool with which to approach the biochemical and biophysical aspects underlying thrombin–fibrinogen interaction, opening also the way to a rigorous analysis of the functional properties of many congenital fibrinogen variants.

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REFERENCES

- 1 Doolittle, R. F. (1973) *Adv. Protein Chem.* **27**, 1–109
- 2 Lewis, S. D., Shields, P. P. and Shafer, S. (1985) *J. Biol. Chem.* **260**, 10192–10199
- 3 Mihalyi, E. (1988) *Biochemistry* **27**, 976–982
- 4 Martinelli, R. A. and Scheraga, H. A. (1980) *Biochemistry* **19**, 2343–2350
- 5 Higgins, D. L., Lewis, S. D. and Shafer, J. A. (1983) *J. Biol. Chem.* **258**, 9276–9282
- 6 Schmitz, T., Rothe, M. and Dodt, J. (1991) *Eur. J. Biochem.* **195**, 251–256
- 7 Hofsteenge, J., Taguchi, H. and Stone, S. R. (1986) *Biochem. J.* **237**, 243–251
- 8 De Cristofaro, R., Rocca, B., Bizzi, B. and Landolfi, R. (1993) *Biochem. J.* **289**, 475–480
- 9 Wells, C. M. and Di Cera, E. (1992) *Biochemistry* **31**, 11721–11730
- 10 Fersht, A. (1985) *Enzyme Structure and Mechanism*, Freeman, New York
- 11 Caldin, E. F. (1964) *Fast Reaction in Solution*, Wiley, New York
- 12 Cleland, W. W. (1986) *Investigation of Rates and Mechanisms of Reactions, Part I* (Bernasconi, C. F., ed.), Wiley, New York
- 13 De Cristofaro, R. and Di Cera, E. (1992) *Biochemistry* **31**, 257–265
- 14 Stone, S. R., Betz, A. and Hofsteenge, J. (1991) *Biochemistry* **30**, 9841–9848
- 15 Laidler, K. J. (1965) *Chemical Kinetics*, McGraw-Hill, New York
- 16 Jencks, W. P. (1987) *Catalysis in Chemistry and Enzymology*, Dover, New York
- 17 Di Cera, E., De Cristofaro, R., Albright, D. J. and Fenton, J. W., II (1991) *Biochemistry* **30**, 7913–7924
- 18 Mihalyi, E. (1968) *Biochemistry* **7**, 208–223
- 19 Blanchard, J. S. (1984) *Methods Enzymol.* **104**, 404–414
- 20 Bard, Y. (1974) *Nonlinear Parameter Estimation*, Academic Press, New York
- 21 Hopfner, K. P. and Di Cera, E. (1992) *Biochemistry* **31**, 11567–11571
- 22 Stone, S. R. and Hofsteenge, J. (1986) *Biochemistry* **25**, 4622–4628
- 23 De Cristofaro, R., Fenton, J. W., II and Di Cera, E. (1992) *J. Mol. Biol.* **226**, 263–269
- 24 Ascenzi, P., Coletta, M., Amiconi, G., De Cristofaro, R., Bolognesi, M., Guarneri, M. and Menegatti, E. (1988) *Biochim. Biophys. Acta* **956**, 156–161

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