Journal of Enzyme Inhibition

ISSN: 8755-5093 (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/ienz19

Fluorescence Monitoring of the Conformational Change in α_2-Macroglobulin Induced by Trypsin Under Second-Order Conditions: The Macroglobulin Acts Both as a Substrate and a Competitive Inhibitor of the Protease

Inci özer & Hilal şimşek

To cite this article: Inci özer & Hilal şimşek (2000) Fluorescence Monitoring of the Conformational Change in α_2-Macroglobulin Induced by Trypsin Under Second-Order Conditions: The Macroglobulin Acts Both as a Substrate and a Competitive Inhibitor of the Protease, Journal of Enzyme Inhibition, 15:2, 101-110, DOI: 10.1080/14756360009030344

To link to this article: https://doi.org/10.1080/14756360009030344

J. Enzyme Inhibition, 2000, Vol. 15, pp. 101–110
Reprints available directly from the publisher
Photocopying permitted by license only

FLUORESCENCE MONITORING OF THE CONFORMATIONAL CHANGE IN α_2-MACROGLOBULIN INDUCED BY TRYPSIN UNDER SECOND-ORDER CONDITIONS: THE MACROGLOBULIN ACTS BOTH AS A SUBSTRATE AND A COMPETITIVE INHIBITOR OF THE PROTEASE

INCI ÖZER* and HILAL ŞIMŞEK

Department of Biochemistry, School of Pharmacy,
Hacettepe University, 06100 Ankara, Turkey

(Received 25 April 1999)

The reaction of bovine pancreatic trypsin with human plasma α_2-macroglobulin (α_2M) was studied at 25°C, using equimolar mixtures of E and I in 50 mM potassium phosphate buffer, pH 7. The conformational change in α_2M was monitored through the increase in protein fluorescence at 320 nm (exc λ, 280 nm). At $[\alpha_2M]_0 = [E]_0 = 11.5–200$ nM, the fluorescence change data fit the integrated second-order rate equation, $(F_\infty - F_0)/(F_\infty - F_t) = 1 + k_{i,obsd}\,[\alpha_2M]_0 t$, indicating that cleavage of the bait region in α_2M was the rate-determining step.

The apparent rate constant ($k_{i,obsd}$) was found to be inversely related to reactant concentration. The kinetic behavior of the system was compatible with a model involving reversible, non-bait region binding of E to α_2M, competitively limiting the concentration of E available for bait region cleavage. The intrinsic value of k_i was $(1.7 \pm 0.24) \times 10^7\ M^{-1}\,s^{-1}$. K_p, the inhibitory constant associated with peripheral binding, was estimated to be in the submicromolar range.

The results of the present study point to a potential problem in interpreting kinetic data relating to protease-induced structural changes in macromolecular substrates. If there is non-productive binding, as in the case of trypsin and α_2M, and the reactions are monitored under pseudo first-order conditions ($[S]_0 \gg [E]_0$), an intrinsically second-order process (such as the rate-limiting bait region cleavage in α_2M) may become kinetically indistinguishable from an intrinsically first-order process (e.g. rate-limiting conformational change). Hence an excess of one component over the other should be avoided in kinetic studies addressing such systems.

Keywords: α_2-Macroglobulin; Protease-induced conformational change; Protein fluorescence

Abbreviations: α_2M, α_2-macroglobulin; BAEE, N_α-benzoyl-L-arginine ethyl ester

* Corresponding author. Fax: (90)312 311 47 77.

INTRODUCTION

α_2-Macroglobulin (α_2M) is a 725 kDa homotetrameric plasma glycoprotein, long established as a general protease inhibitor, with recent findings pointing to an additional role in the modulation of growth factor action.[1–5] Protease inhibition by α_2M is a multi-step process, sequentially involving: (a) proteolytic cleavage of a peptide bond in the "bait region" of the inhibitor which spans residues 666–706 in each subunit, (b) nucleophilic cleavage of the thiol ester bond between the side chains of cys-949 and glu-952 and (c) a gross, conformational change which culminates in the physical entrapment of up to two protease molecules within the central cavity of tetrameric α_2M.[6–8] Proteases so entrapped retain their enzymatic activity. Synthetic substrates which can diffuse into the trap freely are readily hydrolyzed;[9] inhibition applies to macromolecular substrates which are sterically excluded from the vicinity of the enzyme.

The kinetics of the reaction of α_2M with target proteases and the target specificity of the inhibitor have been studied by a variety of methods, indexed to the rate of appearance of titratable sulfhydryl groups,[6,7,10] changes in intrinsic or ligand-associated fluorescence[6,7,11,12] or changes in X-ray solution scattering behavior.[13] Additional information relating to target specificity derives from experiments on the partitioning of proteases between α_2M and α_1-proteinase inhibitor.[14,15] Second-order rate constants (k_i) reported for the chemical phase of the inhibitory process (bait region/thiol ester cleavage) range from $6 \times 10^3\,M^{-1}\,s^{-1}$ (for thrombin[7]) to $4 \times 10^7\,M^{-1}\,s^{-1}$ (for neutrophil elastase[14]). The rate constant[11,13] for the conformational rearrangement step is of the order of $1\,s^{-1}$.

This report concerns the estimation of k_i in the reaction of bovine pancreatic trypsin with α_2M, as monitored under second-order conditions, through changes in intrinsic protein fluorescence. The reaction has previously been studied in several laboratories, under pseudo first-order conditions. At $[\alpha_2M]_T = 2.5\,\mu M$ and excess benzamidine to buffer $[E]_{free}$ at 0.54–1.8 nM, kinetic analysis[6] of the appearance of titratable SH groups yielded $k_i = 2 \times 10^7\,M^{-1}\,s^{-1}$. Stopped-flow fluorimetric analysis of TNS-probed conformational changes[11] at $[\alpha_2M]_T = 10[E]_T = 2\text{–}11\,\mu M$ yielded a similar value, $1 \times 10^7\,M^{-1}\,s^{-1}$. Upon monitoring the α_2M–trypsin system in the absence of modulatory or reporter ligands and under second-order conditions ($[I]_T = [E]_T$), we have found that the observed k_i is not a constant, as expected, but an inverse function of protein concentration. The results suggest that bait region cleavage by trypsin is subject to competitive inhibition by peripheral E-binding sites on α_2M and further point to a possible source

of error in the mechanistic interpretation of kinetic data when protease action on α_2M is studied under the pseudo first-order conditions, $[I] \gg [E]$.

MATERIALS AND METHODS

Biochemicals and chromatographic matrices were purchased from Sigma Chemical Co. (USA). Stocks of *N*-tosyl-L-phenylalanine chloromethyl-ketone-treated bovine pancreatic trypsin were prepared in 2 mM HCl and active site-titrated with *p*-nitrophenyl-*p'*-guanidinobenzoate.[16] α_2M was purified from human plasma obtained from the Blood Bank of Hacettepe University Hospitals, essentially as described previously.[17]

Assay of α_2-Macroglobulin Activity

A constant aliquot of the α_2M stock solution was preincubated for 3 min at 25°C with increasing amounts of active site-titrated E in 50 mM potassium phosphate buffer, pH 7. Enzyme not entrapped in α_2M was inactivated by the addition of excess human α_1-proteinase inhibitor (prepared in this laboratory). Following further preincubation for 3 min, α_2M-associated enzymatic activity was assayed in 50 mM tris-HCl (pH 8) containing 0.5 mM BAEE as substrate. The esterase reaction was monitored spectrophotometrically at 253 nm. (One μM α_2M-associated trypsin was found to cause an increase in absorbance of 0.66 ± 0.076 units per min.) Observed activity increased linearly with added E and reached a plateau at the point where $[E]_T = [\alpha_2M]_T$. The trypsin entrapment capacity of the α_2M stock (1.5 nmol E/nmol tetrameric I) was calculated from the equivalence point observed in the titration experiment.

Kinetic Measurements

The kinetics of the reaction between trypsin and α_2M were studied fluorimetrically at 25°C in 50 mM potassium phosphate buffer, pH 7, using equimolar mixtures of active enzyme and inhibitor in the 11.5–200 nM range. (The concentration of α_2M was defined in units of trypsin entrapment capacity.) Total reaction volume was 2 ml; the reactions were initiated by the addition of 3–35 μl enzyme by means of a lambda pipette. The change in intrinsic protein fluorescence intensity was monitored using a Shimadzu RF-5301 PC spectrofluorimeter equipped with a magnetically stirred, thermostatted cuvette compartment. The instrument was operated in the "quantitative"

mode to avoid continuous irradiation of the samples. Readings were taken every 6–15 s. Wavelengths and slit widths were: excitation, 280 and 1.5 nm; emission, 320 and 10 mm. The emission λ corresponded to the peak of the fluorescence difference spectrum of trypsin-treated *vs* native α_2M.

At $[\alpha_2M]_0 = [E]_0 = 57$ nM, the reaction was also studied in the presence of 0.13–1.5 mM BAEE as competitive inhibitor. Flourescence intensities were recorded in the initial time interval where < 10% of BAEE was hydrolyzed (and at $t = \infty$, to allow for the slight fluorescence quenching caused by the BAEE/benzoyl arginine couple).

Data Analysis

Depending on whether or not BAEE was present, the data were analyzed basically according to either Equation (1) or (2) applicable to second-order reactions when reactant concentrations are equal. Modifications were introduced, as called for by the system under study (see below).

$$\frac{F_\infty - F_0}{F_\infty - F_t} = 1 + [\alpha_2M]_0 k_i t \tag{1}$$

$$\frac{F_\infty - F_0}{F_\infty - F_t} = 1 + [\alpha_2M]_0 \{K_{BAEE}/(K_{BAEE} + [BAEE])\} k_i t \tag{2a}$$

$$\frac{1}{k_{i,obsd}} = \frac{1}{k_i} + \frac{[BAEE]}{k_i K_{BAEE}} \tag{2b}$$

RESULTS

The time course for the fractional change in fluorescence intensity in the reaction of equimolar mixtures of α_2M and trypsin (11.5 and 115 nM active protein) is shown in Figure 1. Lacking rapid mixing equipment, the reactions could only be monitored beyond the first half-life ($t_{experimental} = t_{real} - (4 \pm 1)$ s), so that F_0 refers to the sum of the fluorescence signals of α_2M and E in isolation rather than an experimentally observed parameter. Nevertheless, within the period of observation, the progress curves were found to be much less sensitive to initial reactant concentrations than expected from a simple second-order reaction mechanism. Accordingly, while secondary plots of the data according to Equation (1) were linear (Figure 2),

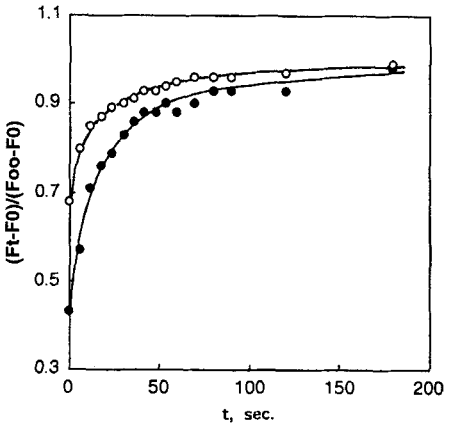


FIGURE 1 Time course for the change in protein fluorescence in the reaction of trypsin with α_2M. $[\alpha_2M]_0 = [E]_0 = 11.5$ (●) and 115 (○) nM. (Concentrations refer to the active dimeric unit of α_2M and to catalytically active trypsin.)

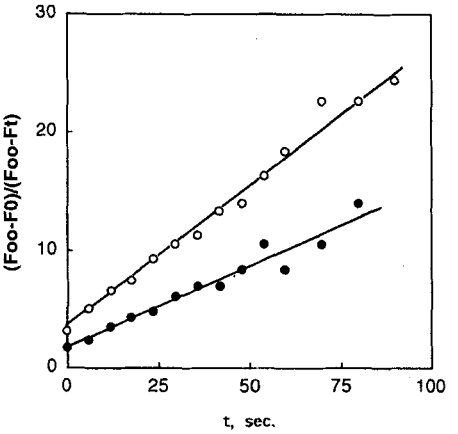


FIGURE 2 Second-order plots of the fluorescence change in the reaction of E with α_2M. Data and symbols as in Figure 1.

k_i as estimated from the slopes of such plots was found to be an "apparent" constant ($k_{i,obsd}$) and to vary inversely with protein concentration (Figure 3). Values ranged from $(1.2 \pm 0.2) \times 10^7\ M^{-1}\ s^{-1}$ at $[\alpha_2M]_0 = [E]_0 = 11.5$ nM to $(1.5 \pm 0.13) \times 10^6\ M^{-1}\ s^{-1}$ at $[\alpha_2M]_0 = [E]_0 = 200$ nM. Upon extrapolation of the data to infinite dilution, k_i was found to be $(1.7 \pm 0.24) \times 10^7\ M^{-1}\ s^{-1}$.

To eliminate the possibility that the observed dependence of $k_{i,obsd}$ on protein concentration was an experimental artifact arising from the technical difficulties of monitoring a fast reaction with conventional equipment,

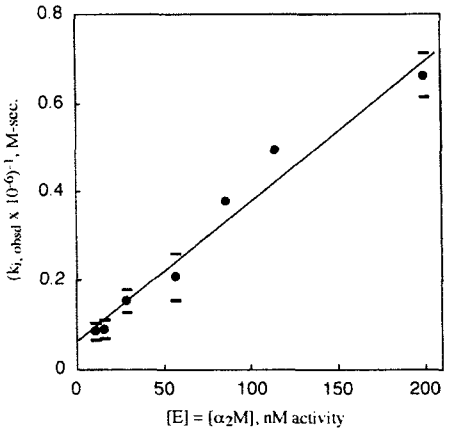


FIGURE 3 Dependence of the apparent second-order rate constant for the reaction between E and α_2M on protein concentration. Each point is the average of at least two determinations; error bars have been included where SD $\geq \pm 10\%$.

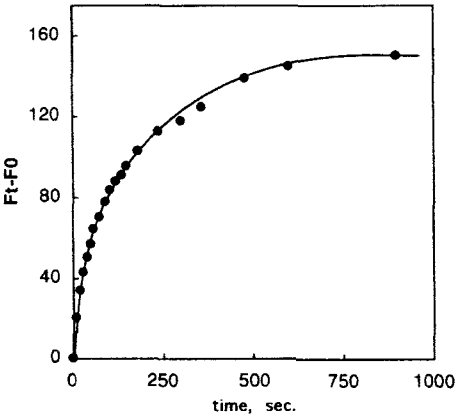


FIGURE 4 Time course for the change in protein fluorescence in the BAEE-inhibited reaction of trypsin with α_2M. $[\alpha_2M]_0 = [E]_0 = 57$ nM; $[BAEE]_0 = 0.5$ mM.

the system was also studied in the presence of varying concentrations of BAEE (and $[\alpha_2M]_0 = [E]_0 = 57$ nM). A typical course for the BAEE-inhibited reaction is shown in Figure 4. As required by Equation (2b), $1/k_{i,obsd}$ was linearly related to [BAEE] (Figure 5). The ordinate intercept yielded a limiting value, $k_{i,obsd} = (4.6 \pm 0.55) \times 10^6\,M^{-1}\,s^{-1}$, in close agreement with the experimental value obtained in the absence of BAEE ($(4.8 \pm 0.48) \times 10^6\,M^{-1}\,s^{-1}$) and validating the data in Figures 1–3.

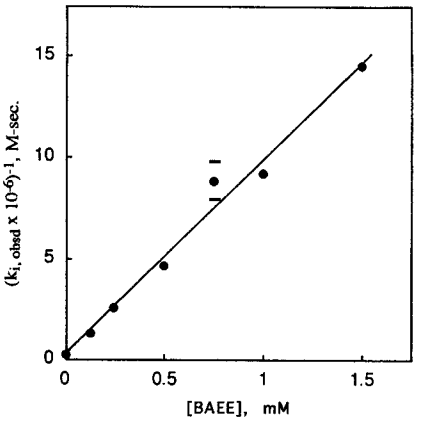
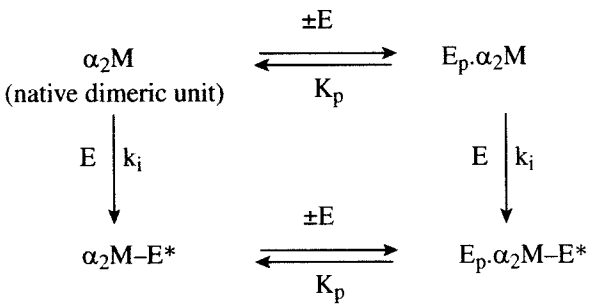


FIGURE 5 Dependence of the apparent second-order rate constant for the reaction between E and α_2M on $[BAEE]_0$. $[\alpha_2M]_0 = [E]_0 = 57$ nM. Each point is the average of at least two determinations; error bars have been included where SD $\geq \pm 10\%$.

DISCUSSION

The inverse relationship between $k_{i,obsd}$ and protein concentration in the reaction of trypsin with α_2M could arise from: (a) reversible self-associations of E and/or α_2M to yield species with inferior activity, (b) inhibition due to binding of inactive trypsin to native α_2M, thereby rendering it resistant to bait region cleavage, (c) competitive inhibition of E by α_2M. The first possibility was dismissed, because the second-order plots for the reactions (Figure 2) were linear, with no evidence for the time-dependent acceleration that should be observed as the presumptive association–dissociation equilibrium is shifted towards the right. Self-association of E was also ruled out by the constancy of specific esteratic activity in the range, $[E] = 0-700$ nM. Alternative (b) was deemed unlikely, since the level of inactive protein in the trypsin preparation (*ca.* 30%) could not account for the 10-fold decrease in $k_{i,obsd}$, even if quantitative binding were invoked. Hence further deliberations were focused on alternative (c), i.e. competitive inhibition of trypsin-catalyzed bait region cleavage in α_2M by α_2M itself.

A working model, which takes the α_2M half-molecule (M_r 360 000) as the functional unit in protease entrapment, is outlined in Scheme 1. Further assumptions, inspired by the simplicity of the observed kinetics, were: (a) the half-molecules are noncooperative, (b) each half-molecule has n ($\gg 1$) peripheral (nonbait region) trypsin-binding sites which are independent and have similar affinities ($1/K_p$) for E. Hence, at any time t,



SCHEME 1 α_2M–E*, conformationally altered α_2M with entrapped enzyme; E_p, enzyme bound to a peripheral site on α_2M or α_2M–E*.

$[E_p{\cdot}\alpha_2M]_t + [E_p{\cdot}\alpha_2M{-}E^*]_t \ll [\alpha_2M]_t + [\alpha_2M{-}E^*]_t \approx [\alpha_2M]_0$, and the concentration of enzyme available for reaction with α_2M is given by $[E]_{free,t} = K_p[E]_{total,t}/(K_p + n[\alpha_2M]_0)$, (c) K_p is insensitive to the conformational state of α_2M and, (*d*) the rate constant for bait region cleavage is independent of the state of occupancy of the peripheral sites.

Under these conditions the E-catalyzed conformational change in α_2M will conform to Equations (3a) and 3(b):

$$\frac{F_\infty - F_0}{F_\infty - F_t} = 1 + [\alpha_2M]_0\{K_p/(K_p + n[\alpha_2M]_0)\}k_i t \tag{3a}$$

$$\frac{1}{k_{i,obsd}} = \frac{1}{k_i} + \frac{n[\alpha_2M]_0}{k_i K_p}. \tag{3b}$$

Using Equation (3b), the slope of the straight line in Figure 3 and $k_i = 1.7 \times 10^7\,M^{-1}\,s^{-1}$, K_p/n was calculated to be 24 nM (corrected to refer to $[\alpha_2M]_0$ in units of nM *protein*. The actual value obtained from Figure 3 was 18 nM). The data at hand cannot give independent estimates for K_p and n. However, since n must ≈ 10 for the approximation, $[\alpha_2M]_t + [\alpha_2M{-}E^*]_t \gg [E_p{\cdot}\alpha_2M]_t + [E_p{\cdot}\alpha_2M{-}E^*]_t$ to be valid, the lower limit for K_p is likely to be in the 10^2 nM range.

The parallellism between the admittedly over-simplified Scheme 1 and how the trypsin–α_2M system actually operates may be disputable. The biological significance of the proposed peripheral binding may also be challenged. Although a tight binding interaction between α_2M and chymotrypsin has previously been noted within a chromatographic context,[8] such binding does not appear to be common to all proteases acting on α_2M.

(The rate constant for the reaction of plasmin with α_2M, for instance, has been reported to be invariant in the [I] = [E] = 50–500 nM range).[6] On the other hand, the results presented here should be of technical interest in future studies on other protease–α_2M couples. The specific point to which we would like to draw attention is the following. When experiments monitoring chemical or conformational changes are conducted under pseudo first-order conditions with $[\alpha_2M]_0 \gg [E]_0$, a system conforming to Scheme I will be governed by Equation (4) or (5) depending on the relative magnitudes of $n[\alpha_2M]_0$ and K_p. In the range where Equation (5) applies, the reaction will appear intrinsically first-order, and the observed rate constant may erroneously be taken to reflect a rate limiting unimolecular rearrangement in bait

$$\text{Rate} = \frac{k_i K_p}{K_p + n[\alpha_2M]_0} [\alpha_2M]_{t\approx 0}[E]_t \tag{4}$$

if $n[\alpha_2M]_0 \gg K_p$,

$$\text{Rate} = \frac{k_i K_p}{n} [E]_t \tag{5}$$

region-cleaved α_2M, rather than the bimolecular cleavage process itself.

The following may be a case in point. TNS-probed conformational changes in the system, $[\alpha_2M]_0 = 10$ [trypsin]$_0 = 2$–11 μM, occur in two apparently first-order phases.[11] The rate constant for the first phase is a function of $[\alpha_2M]_0$. Accordingly, this phase ($k = 1 \times 10^7\ M^{-1}\ s^{-1}$) has been taken to reflect the bait region/thiol ester cleavage step. The second phase, on the other hand, appears to be inherently first-order, since the observed rate constant ($1.4\ s^{-1}$) is independent of inhibitor concentration. Hence it has been assigned to the final conformational change leading to the formation of α_2M–E*. The results of the present study bring up the alternative possibility that the first phase in question may have been related to the peripheral binding of E to α_2M (or some other process observable only in the TNS-probed system) and that the second phase may in fact have been the one associated with bait region/thiol ester cleavage. Significantly, substitution of $k_i = 1.7 \times 10^7\ M^{-1}\ s^{-1}$ and $K_p/n = 24$ nM into Equation (5) yields k (pseudo first-order) $= 0.4\ s^{-1}$, which lies close to the value of $1.4\ s^{-1}$ quoted above.[11] A further point which suggests that $k \approx 1\ s^{-1}$ may reflect a process other than a molecular rearrangement is the magnitude of the rate constant for conformational change obtained at 5°C and $[E] \gg [\alpha_2M]$ ($k \approx 0.9\ s^{-1}$).[13] The similarity of the first-order rate constants, despite the 20-degree difference

in temperature supports the idea that the rate-limiting process at the higher temperature must be bait region cleavage rather than a conformational change.

The problem of kinetic ambiguity presented here may apply also to data relating to protease-induced structural changes in other macromolecular substrates. If there is nonproductive binding, as in the case of trypsin and α_2M, and the reactions are monitored under pseudo first-order conditions ($[S]_0 \gg [E]_0$), an intrinsically second-order process (such as the rate-limiting bait region cleavage in α_2M) may become kinetically indistinguishable from an intrinsically first-order process (e.g. rate-limiting conformational change). Hence an excess of one component over the other should be avoided in initial studies addressing such systems.

Acknowledgment

This study was supported by a grant (TBAG-1395) from the Scientific and Technical Research Council of Turkey.

References

[1] L. Sottrup-Jensen (1989) *J. Biol. Chem.*, **264**, 11 539–11 542.
[2] S. Soker, C.M. Svahn and G. Neufeld (1993) *J. Biol. Chem.*, **268**, 7685–7691.
[3] B.B. Wolf and S.L. Gonias (1994) *Biochemistry*, **33**, 11 270–11 277.
[4] J.C. Bonner and A.R. Osornio-Vargas (1995) *J. Biol. Chem.*, **270**, 16 236–16 242.
[5] H. Gavish, I. Bab, A. Tartakovsky, M. Chorev, N. Mansur, Z. Greenberg, M. Namdar-Attar and A. Muhlrad (1997) *Biochemistry*, **36**, 14 883–14 888.
[6] U. Christensen and L. Sottrup-Jensen (1984) *Biochemistry*, **23**, 6619–6626.
[7] J.P. Steiner, P. Bhattacharya and D.K. Strickland (1985) *Biochemistry*, **24**, 2993–3001.
[8] P. Gettins, B.C. Crews and L.W. Cunningham (1989) *Biochemisry*, **28**, 5613–5618.
[9] J.W. Mehl, W. O'Connell and J. DeGroot (1964) *Science*, **145**, 821–823.
[10] R.D. Feinman, A.I. Yuan, S.R. Windwer and D. Wang (1985) *Biochem. J.*, **231**, 417–423.
[11] D.K. Strickland, L.-J. Larsson, D.E. Neuenschwander and I. Björk (1991) *Biochemistry*, **30**, 2797–2803.
[12] V.F. Favaudon, M. Tourbez, F. Pochon, R. Mareix and H. Tourbez (1987) *Eur. J. Biochem.* **165**, 31–37.
[13] H. Arakawa, T. Urisaka, H. Tsuruta, Y. Amemiya, H. Kihara and A. Ikai (1994) *FEBS Lett.*, **337**, 171–174.
[14] G.D. Virca and J. Travis (1984) *J. Biol. Chem.*, **259**, 8870–8874.
[15] P. Laurent and J.G. Bieth (1989) *Biochim. Biophys. Acta*, **994**, 285–288.
[16] T. Chase and E. Shaw (1969) *Biochemistry*, **8**, 2212–2224.
[17] T. Kurecki, L.F. Kress and M. Laskowski (1979) *Anal. Biochem.*, **99**, 415–420.