Biochimica et Biophysica Acta, 370 (1974) 546–555

BBA 67362

ON THE MECHANISM OF THE UROKINASE-CATALYSED HYDROLYSIS OF α-*N*-ACETYL-L-LYSINE METHYL ESTER

D PETKOV and I STOINEVA

Department of Mechanism of Organic and Enzymatic Reactions and Organic Synthesis (MOEROS), Institute of Organic Chemistry, Bulgarian Academy of Sciences, Sofia 13 (Bulgaria)

(Received May 27th, 1974)

SUMMARY

The following kinetic results are consistent with an acyl enzyme mechanism of the urokinase-catalysed hydrolysis of α-*N*-acetyl-L-lysine methyl ester (Ac-Lys-OMe)

1 The $K_{m(app)}$ value ($8\,4\ 10^{-4} \pm 1\,4\ 10^{-4}$ M) for the urokinase hydrolysis of Ac-Lys-OMe is lower than the corresponding $K_{m(app)}$ values ($5\,0\ 10^{-3}$ M) for the urokinase hydrolysis of α-*N*-acetyl-L-lysine anilides

2 The rate of urokinase hydrolysis of Ac-Lys-OMe, followed by measuring the formation of α-*N*-acetyl-L-lysine in a pH-stat, decreases in the presence of the added nucleophile, methanol

3 The calculated equilibrium constant for the formation of the acyl enzyme is smaller than unity, indicating that the acyl enzyme is thermodynamically more stable than the Michaelis complex

4 The acylation rate constant ($59\,40 \pm 9\,85\ s^{-1}$) exceeds the deacylation rate constant ($11\,68 \pm 1\,47\ s^{-1}$), thus suggesting the accumulation of the acyl enzyme along the reaction pathway

5 Kinetically important basic and acidic groups with $pK_{a(app)} \approx 6\,65$ and $\approx 9\,35$, respectively, are operative in both the acylation and deacylation reactions

INTRODUCTION

A long period of time has passed since Kjeldgaard and Ploug [1] demonstrated the esterase activity of urokinase (EC 3 4 99 26), but there have been relatively few investigations on the mechanism of this enzyme action [1–3] The specific ester substrates are competitive inhibitors of the physiological function of urokinase of activating human plasminogen to plasmin [1, 4] The same active site of urokinase, therefore, catalyzes both plasminogen activation and ester hydrolysis This suggests that a study of ester hydrolysis by urokinase should provide information about the active site structure of urokinase and its mechanism of action

The investigation described in this paper was undertaken to elucidate the validity of the acyl enzyme mechanism (Bender and Kezdy [5]) in the urokinase hydrolysis of α-*N*-acetyl-L-lysine methyl ester (Ac-Lys-OMe) When kinetic evidence

Abbreviation Ac-Lys-OMe, α-*N*-acetyl-L-lysine methyl ester

for acyl enzyme formation was obtained, the effects of pH on both the acylation and deacylation steps were studied in order to identify the catalytically essential ionizable groups taking part in urokinase catalysis

MATERIALS AND METHODS

Urokinase

Three different preparations of human urokinase were used standard preparations (4800 CTA units/vial) of the Committee on Thrombolytic Agents, National Heart Institute (U S A), preparations (5000 Ploug units/vial) purchased from Koch-Light Laboratories Ltd (England) and preparations with amidase activity ranging from 1000 to 1500 CTA units/mg protein obtained in our laboratory [6] The kinetic parameters for Ac-Lys-OMe obtained using these urokinase preparations with a different degree of purification are identical within the range of experimental error This is in accordance with the findings of Sherry et al [4] that the hydrolysis of Ac-Lys-OMe is insensitive to contamination in crude urokinase preparations

The functional molarity of the urokinase solutions was determined by active site titration with *p*-nitrophenyl, *p'*-guanidinobenzoate (Petkov et al [7]) Assays of the urokinase amidase activity were performed spectrophotometrically using the chromogenic substrate α-*N*-acetyl-L-lysine *p*-nitroanilide (Petkov et al [6])

α-N-Acetyl-L-lysine methyl ester (Ac-Lys-OMe)

α-*N*-Acetyl-L-lysine methyl ester hydrochloride was obtained as a thick oil by the method of Irving and Gutman [8] The oil was solidified after drying in vacuo over P_2O_5 and triturating under dry ether The amorphous product had a melting range (115–125 °C) and specific rotation ($[\alpha]_D^{25} = -20°$ (c1, H_2O)) as reported [4] The amount of KOH used for the pH-static titration of α-*N*-acetyl-L-lysine released in the complete urokinase hydrolysis of Ac-Lys-OMe corresponded within experimental error to the amount of Ac-Lys-OMe subjected to hydrolysis

Kinetic runs

The hydrolysis of Ac-Lys-OMe was followed by automatic end-point titration with 5 0 10^{-3} M KOH of the α-*N*-acetyl-L-lysine liberated by the action of urokinase at a constant pH using a Radiometer pH-stat assembly (TTT11/PHM26 titrator + SBU1a syringe burette assembly + SRBR2c titrigraph) Ac-Lys-OMe was reacted with urokinase under turnover conditions in the following manner A 0 3-ml aliquot of 1 M KCl and an appropriate volume of distilled water were allowed to equilibrate at 37 ± 0 1 °C for 10 min in the temperature controlled pH-stat reaction vessel An aliquot of Ac-Lys-OMe stock solution (3 0 10^{-2} M in water) was added to the thermostated solution to give the final substrate concentration (total volume 3 ml) and non-enzymatic hydrolysis was followed for 3 min 0 05 ml of the stock urokinase solution (5000 CTA units/ml in 0 5 M NaCl), previously titrated with *p*-nitrophenyl, *p'*-guanidinobenzoate, was then added to the reaction mixture and the urokinase-catalysed hydrolysis was followed up to 25% completion

In the experiments with added nucleophilic agent, an appropriate volume of distilled water was replaced by pure methanol and the reaction was followed at pH 7 6 —the pH optimum [4]

Evaluation of the apparent and individual rate and equilibrium constants

The corrected values of the initial rates for non-enzymatic hydrolysis were used for the Lineweaver–Burk plots The Michaelis–Menten parameters k_{cat} and $K_{m(app)}$ and their standard deviations were determined by the least-square treatment of these plots

The individual rate and equilibrium constants were calculated as described by Berezin et al [9] According this this method, the reciprocal of the true Michaelis–Menten constant K_s is equal to the abscissa of the common intersection point of the Lineweaver–Burk plots corresponding to various nucleophilic agent concentrations With K_s known, the all individual rate constants, associated with an acyl enzyme mechanism of action can be calculated from the simplified parts of the Eqns 3 and 4 and the unsimplified part of Eqn 3

RESULTS

Kinetics in the presence of an added nucleophile, methanol

Stability test To determine the effect of methanol on the stability of urokinase, the stability test of Seydoux and Yon [10] was used In the presence of methanol (2 46 M, the largest methanol concentration used in the experiments) equal volumes of 3 0 10^{-2} M Ac-Lys-OMe were introduced at regular intervals of time in the

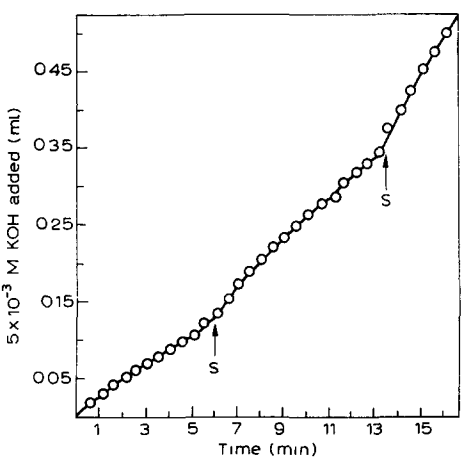

Fig 1 Test for the urokinase stability in the presence of methanol The arrows indicate the introduction of 0 078 ml of the Ac-Lys-OMe stock solution (3 0 10^{-2} M) in the reaction mixture The initial rates are 39 12, 37 16 and 42 38 μM min^{-1} Experimental conditions pH 7 6, [methanol] = 2 46 M, [S] = 7 8 10^{-4} M, [E] = 2 20 10^{-7} M, 0 1 M KCl, 37 ± 0 1 °C

reaction mixture (Fig 1) The initial rates obtained were identical within experimental error, indicating that no changes occurred in the urokinase activity during a kinetic run (5 min)

The effect of added nucleophile, methanol Double-reciprocal plots of initial rates in the absence and presence of 0 82 and 2 46 M methanol gave straight lines (Fig 2) intersecting in a common point in the upper left-hand quadrant The common

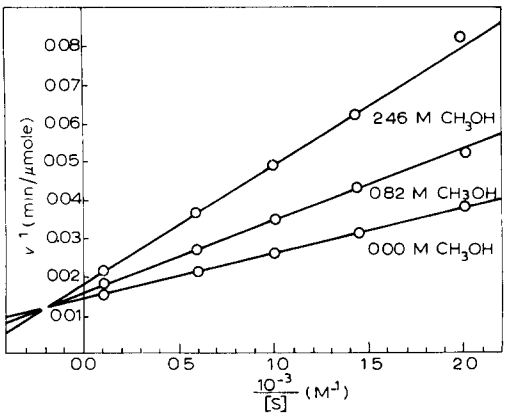

Fig 2 Lineweaver–Burk plots for the urokinase hydrolysis of Ac-Lys-OMe at different methanol concentrations Experimental conditions pH 7 6, [E] = 1 17 10^{-7} M, 0 1 M KCl, 37 ± 0 1 °C

intersection point is at a sufficient distance from the $1/v$ axis This indicates that methanol does not act as a competitive inhibitor of the urokinase-catalysed hydrolysis of Ac-Lys-OMe

The $1/v$ against methanol concentration plots at a number of constant substrate concentrations also gave straight lines, which intersect at a common point in the upper left-hand quadrant (Fig 3) with abscissa [methanol] = −1 65 ± 0 1 M

Steady-state kinetic data

The apparent rate and equilibrium constants and the individual rate and equilibrium constants associated with an acylenzyme mechanism of urokinase-catalysed hydrolysis of Ac-Lys-OMe are summarized in Table I The results are quoted ± S D

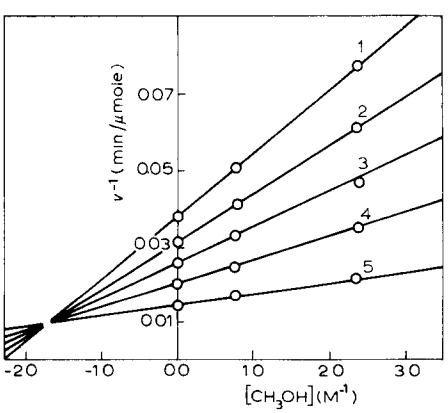

Fig 3 Plots of $1/v$ against methanol concentration at different Ac-Lys-OMe concentrations 1, 5 0 10^{-4} M, 2, 7 0 10^{-4} M, 3, 1 0 10^{-3} M, 4, 2 0 10^{-3} M and 5, 1 0 10^{-2} M Experimental conditions pH 7 6, [E] = 1 17 10^{-7} M, 0 1 M KCl, 37 ± 0 1 °C

TABLE I

STEADY-STATE KINETIC DATA FOR THE UROKINASE-CATALYSED HYDROLYSIS OF Ac-Lys-OMe

pH 7 6, 0 1 M KCl, 37 ± 0 1 °C The results are quoted ± S D

k_{cat} (s^{-1})	k_2 (s^{-1})	k_3 (s^{-1})	k^1_{-2} (M^{-1} s^{-1})	k_2/k_3	$K_{m(app)}$ (mM)	K_s (mM)	k_2/K_s (s^{-1} mM^{-1})	K_{EA} (M^{-1})
10 01 ± 1 25		11 68 ± 1 47		5 10 ± 0 28		5 0 ± 0 6		0 116 ± 0 005
	59 40 ± 9 85		6 88 ± 0 86		0 84 ± 0 14		11 80 ± 0 54	

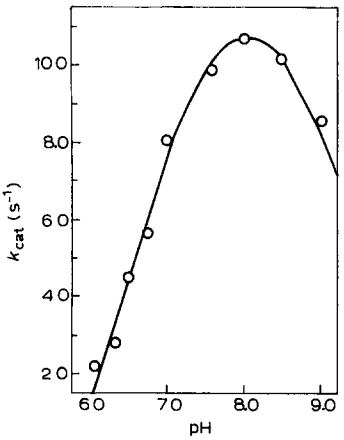

Fig 4 The pH profile of k_{cat} of the urokinase hydrolysis of Ac-Lys-OMe The circles are the experimental data, the solid curve is the theoretical one, calculated from Eqn 9 for $K_{ea} = 10^{-6.6}$ and $K'_{ea} = 10^{-9.3}$ Experimental conditions [E] = 1 56 10^{-7} M, 0 1 M KCl, 37 ± 0 1 °C

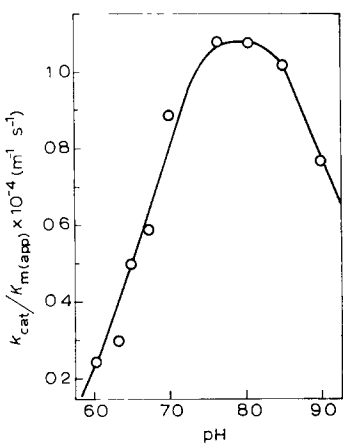

Fig 5 The pH profile of the ratio $k_{cat}/K_{m(app)}$ of the urokinase hydrolysis of Ac-Lys-OMe The circles are the experimental data, the solid line is the theoretical one, calculated from Eqn 10 for $K_e = 10^{-6.7}$ and $K'_e = 10^{-9.4}$ Experimental conditions [E] = 1 56 10^{-7} M, 0 1 M KCl, 37 ± 0 1 °C

pH dependence of k_{cat} and $k_{cat}/K_{m(app)}$

The pH profile of the catalytic constant k_{cat} and the $k_{cat}/K_{m(app)}$ ratio are represented in Fig 4 and Fig 5 The circles are the experimental data The solid lines are the theoretical lines calculated from Eqns 9 and 10 using for k_3 and k_2/K_s the values in Table I and $K_e = 10^{-6.7}$, $K_{ea} = 10^{-6.6}$, $K'_e = 10^{-9.4}$ and $K'_{ea} = 10^{-9.3}$ These values gave the closest fit (determined by visual comparison) between the theoretical and experimental pH profiles

DISCUSSION

The saturation kinetics, observed by Sherry et al [4], are consistent with an equilibrium formation of a noncovalent complex (Michaelis complex) between uro-

kinase and Ac-Lys-OMe followed by an intra-complex reaction to produce the free enzyme and the products of the reaction (Michaelis–Menten kinetics)

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P_1 + P_2 \tag{1}$$

The mechanism of the intra-complex reaction being the rate-limiting step in the reaction pathway 1 is the subject of further consideration

The urokinase esterase and activator activities have been shown [11] to be irreversibly inhibited by diisopropylfluorophosphate (DFP) This finding is indicative of the operation of a serine hydroxymethyl group in the urokinase active site, which is uniquely blocked by an irreversible (covalent) interaction with DFP resulting in the formation of a stable acyl enzyme (EA) The successive acylation and deacylation of such a uniquely reactive serine hydroxyl constitutes the most important feature of the mechanism of ester and amide hydrolysis catalysed by the serine proteases (Bender and Kezdy [5])

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k'_{-2}\,[P_1]}{\overset{k_2}{\rightleftharpoons}} EA + P_1 \xrightarrow{k'_3\,[H_2O]} E + P_2 \tag{2}$$

The steady-state derivation gives the following simple connection between the kinetic parameters of the kinetic schemes 1 and 2

$$k_{cat} = k_2 \times \frac{k_3}{k_2 + k_{-2} + k_3} = k_2 \times \frac{k_3}{k_2 + k_3} \text{ if } [P_1] \to 0 \tag{3}$$

$$K_{m(app)} = \frac{k_{-1}k_{-2} + k_{-1}k_3 + k_2k_3}{(k_2 + k_{-2} + k_3)k_1} = K_s \times \frac{k_3}{k_2 + k_3} \text{ if } [P_1] \to 0 \text{ and } k_{-1} \gg k_2 \tag{4}$$

in which K_s is the equilibrium binding constant (the true Michaelis–Menten constant), $k_{-2} = k'_{-2}\,[P_1]$ and $k_3 = k'_3\,[H_2O]$

The $K_{m(app)}$ values for the urokinase hydrolysis of Ac-Lys-OMe, obtained by Sherry et al [4] (7 7 10^{-4} M) and in this work (8 4 $10^{-4} \pm 1$ 4 10^{-4} M) are lower than the $K_{m(app)}$ values obtained for the urokinase hydrolysis of α-*N*-acetyl-L-lysine anilides (5 0 10^{-3} M) (Petkov et al [7]) These findings are consistent with the interpretation of the urokinase ester and anilide hydrolysis in terms of different rate-limiting steps in the stepwise mechanism 2 Actually, when the overall rate is controlled by the acylation step (anilide hydrolysis [7]), i e $k_2 \ll k_3$, $K_{m(app)} = K_s$ When deacylation is the rate-limiting step (ester hydrolysis, this work), i e $k_3 \ll k_2$, $K_{m(app)} = K_s\ k_3/k_2$ Therefore, the $K_{m(app)}$ value for Ac-Lys-OMe should be k_3/k_2 times smaller than the corresponding $K_{m(app)}$ values for α-*N*-acetyl-L-lysine anilides, which is found

Indirect evidence for the validity of the acyl enzyme mechanism for the urokinase hydrolysis of Ac-Lys-OMe may be obtained by studying the kinetics of this reaction in the presence of the added nucleophile [12], methanol In this case the reaction between water and the acylenzyme yielding the free enzyme and α-*N*-acetyl-L-lysine (P_2) should be superimposed on the reaction of methanol (P_1) with the acyl

enzyme yielding the free enzyme and Ac-Lys-OMe (Eqn 2) Due to this competition between nucleophiles, water and methanol for the acyl enzyme, the rates observed by registering P_2 (pH-stat) should be smaller than those observed in the absence of methanol This is found (Fig 2) The stability test (Fig 1) shows that the observed decreases in the rate is a result only of the nucleophilic competition and any denaturation effects and conformational changes, induced by variation of the dielectric constant of the medium, should be excluded from the interpretation of the results

In the presence of methanol, even under initial rate conditions, $[P_1] \neq 0$ and the unsimplified Eqns 3 and 4 for k_{cat} and $K_{m(app)}$ are valid Using the simplified and unsimplified parts of Eqn 3, the abscissa of the straight-lines intersection point in Fig 3 can be easily shown to be equal to $-k_3/k'_{-2}$ The good agreement between the calculated (1 70 M) by the use of k_3 and k'_{-2} values from Table I and the determined (1 65 M) from the Fig 3 ratio k_3/k'_{-2} supports the interpretation of the results in terms of the reaction mechanism 2

As can be seen from Table I, $k_3 < k_2$ This suggests that deacylation is the rate-limiting step in the acyl enzyme mechanism of urokinase hydrolysis of Ac-Lys-OMe From the application of the steady-state approximation to EA it follows that $[EA] = k_2/k_3$ [ES] and, therefore, the acyl enzyme accumulates to steady-state concentrations Moreover, as the nucleophile used is the same as P_1, the equilibrium constant

$$K_{EA} = \frac{k'_{-2}}{k_2} \tag{5}$$

of the formation of the acyl enzyme can also be evaluated It is smaller than unity (Table I), indicating that the acyl enzyme is thermodynamically more stable than the Michaelis complex ES

If the urokinase hydrolysis of Ac-Lys-OMe is formulated according to the acylenzyme mechanism 2, the reaction between this ionizable substrate and urokinase at different pH values may be represented in the following manner

$$\begin{array}{ccccccc} EH & & & ESH & & EAH & \\ \Downarrow K_e & & & \Downarrow K_{es} & & \Downarrow K_{ea} & \\ E & + S & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & ES & \xrightarrow{k_2} & EA & + P_1 \xrightarrow{k_3} E + P_2 \\ \Downarrow K'_e & \Downarrow K'_s & & \Downarrow K'_{es} & & \Downarrow K'_{ea} & \\ E^- & S^- & & ES^- & & EA^- & \end{array} \tag{6}$$

where the K values are the corresponding acid dissociation constants of reagents and intermediates Assuming that only ES, EA and the protonated form of Ac-Lys-OMe are productive in the enzyme reaction, the catalytic rate constant k_{cat} and the ratio $k_{cat}/K_{m(app)}$ should be dependent upon the H^+ concentration in the reaction medium This is exactly what is observed (Figs 4 and 5)

From the reaction scheme 6 and the application of the steady-state approximation, the equations for the pH dependence of k_{cat} and $K_{m(app)}$ follow

$$k_{cat} = k_2 \frac{k_3}{k_2 f^-_{ea} + k_3 f^-_{es}} \tag{7}$$

$$K_{m(app)} = K_s \frac{k_3}{k_2 f^-_{es} + k_3 f^-_{es}} \frac{1}{f^-_e f^-_s} \tag{8}$$

in which f_e^-, f_s^-, f_{es}^- and f_{ea}^- are the Michaelis pH functions [13] of the enzyme, substrate, ES and EA, respectively As the experiments with added nucleophile reveal, the $k_3 \ll k_2$, Eqn 7 may be simplified to

$$k_{cat} = \frac{k_3}{f_{ea}^-} = \frac{k_3}{1 + \frac{[H^+]}{K_e} + \frac{K'_{ea}}{[H^+]}} \tag{9}$$

and a plot of k_{cat} vs pH should yield the acid dissociation constants K_{ea} and K'_{ea} of the acyl enzyme

Upon division of Eqn 7 by Eqn 8 one obtains

$$\frac{k_{cat}}{K_{m(app)}} = \frac{k_2/K_s}{f_e^- f_s^-} = \frac{k_2/K_s}{\left(1 + \frac{[H^+]}{K_e} + \frac{K'_e}{[H^+]}\right)\left(1 + \frac{K'_s}{[H^+]}\right)} \tag{10}$$

The acid dissociation constant K'_s of lysine ε-NH^+_3 is about $10^{-10\,5}$ (Alberty [14]) With the use of this value for K'_s, when the reaction within the region of pH 6 0–9 0 is considered, the last multiplier in the denominator of the right-hand side of Eqn 10 tends to 1 Therefore, a plot of $k_{cat}/K_{m(app)}$ vs pH should yield the dissociation constants K_e and K'_e of the kinetically important groups in the active site of the free enzyme

The good fit between the experimental pH profiles of k_{cat} and $k_{cat}/K_{m(app)}$ and the theoretical curves calculated from Eqns 9 and 10 using for k_3 and k_2/K_s the values in Table I and $K_e = 10^{-6\,7}$, $K_{ea} = 10^{-6\,6}$, $K'_e = 10^{-9\,4}$ and $K'_{ea} = 10^{-9\,3}$ (Figs 4 and 5) suggests that a kinetically important basic group with an apparent pK 6 6–6 7 and an acidic group with an apparent pK 9 3–9 4 are operative in both the acylation and deacylation reactions

The ionizable group of pK_a around neutrality is usually identified [14] as the imidazole group of a histidine moiety or the terminal α-ammonium group As it has been found by Landmann and Markward [11], the urokinase esterase activity is not affected by incubation with α-*N*-tosyl-L-lysine chlormethyl ketone, which having the backbone of the specific substrates should alkylate the active site histidine side chain Since this is not the case, this indicates that a terminal α-ammonium group controls the catalytic properties of urokinase There still remains an explanation of the pH dependence due to a histidine imidazole participation in the urokinase active site This follows from the fact that is not proper to exclude the participation of a functional group in the active site of the enzyme on the basis of the negative results of a single modification experiment

REFERENCES

1 Kjeldgaard, N O and Ploug, J (1957) Biochim Biophys Acta 24, 283–289
2 Lorand, L and Condit, E V (1965) Biochemistry 4, 265–270
3 Walton, P L (1966) Biochim Biophys Acta 132, 104–114
4 Sherry, S , Alkjaersig, N and Fletcher, A (1964) J Lab Clin Med 64, 144–153
5 Bender, M L and Kezdy, F J (1965) Annu Rev Biochem 34, 49–76
6 Petkov, D , Christova, E and Karadjova, M (1973) Thromb Diathes Haemorr 29, 276–285

7 Petkov, D , Christova, E , Pojarlieff, I and Stambolieva, N , Eur J Biochem , in the press
8 Irving, C C and Gutman, J R (1959) J Org Chem 24, 1979–1983
9 Berezin, I V , Kazanskaya, N F and Klyosov, A A (1971) FEBS Lett 15, 121–124
10 Seydoux, F and Yon, J (1967) Eur J Biochem 3, 42–56
11 Landmann, H and Markwardt, F (1970) Experientia 26, 145–147
12 Bender, M L , Clement, Gunter, C R and Kezdy, F J (1964) J Am Chem Soc 86, 3697–3703
13 Dixon, M and Webb, E C (1964) Enzymes, 2nd edn, pp 108–116, Longmans Green, London
14 Alberty, R A (1953) in The Proteins (Neurath, H and Baily, K , eds) Vol 28, p 467, Academic Press, New York