Binding of a substrate analogue can induce co-operative structure in the plasmin serine-proteinase domain

Andrew J. TEUTEN,* Alan COOPER,† Richard A. G. SMITH‡ and Christopher M. DOBSON*§

*Oxford Centre for Molecular Sciences and Inorganic Chemistry Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QR,

†Department of Chemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, and

‡SmithKline Beecham Pharmaceuticals Ltd., Biosciences Research Centre, Yew Tree Bottom Road, Great Burgh, Epsom, Surrey KT18 5XQ, U.K.

Human miniplasminogen and miniplasmin were studied by n.m.r. spectroscopy and differential scanning calorimetry (d.s.c.) in order to investigate the structural properties of the serine-proteinase domain. The d.s.c. thermograms of both miniplasminogen and non-inactivated miniplasmin at pH 4.0 can be closely fitted to two transitions, at 62 ± 2 and 72 ± 2 °C, corresponding to unfolding of the kringle 5 and proteinase domains respectively. No evidence was found, under these conditions, for non-co-operative unfolding of the proteinase domain. On inactivation of miniplasmin with an affinity label, a number of additional resonances arising from residues of the proteinase

domain are observed in resolved regions of the n.m.r. spectrum. A combination of variable-temperature n.m.r. and d.s.c. has shown that part of the proteinase domain undergoes a major conformational transition on heating which is distinct from the unfolding of the remainder of the proteinase domain or the kringle 5 domain. This additional transition occurs at a temperature that depends on the nature of the affinity label present and is not observed in the absence of an inactivating agent. These results provide direct evidence for the existence of a region of the proteinase domain which, under these conditions, becomes structured only in the presence of a bound substrate.

INTRODUCTION

Plasminogen, a key enzyme in the pathway of fibrinolysis, is a multidomain glycoprotein of M_r 92000 comprising five kringle domains and a trypsin-like proteinase domain. A two-domain fragment of plasminogen, miniplasminogen, can be formed by cleaving between Val-442 and Val-443 in the kringle 4–kringle 5 linker (Sottrup-Jensen et al., 1978). Despite an M_r of 38000, the n.m.r. spectrum of miniplasminogen at pH 4 is well resolved; this has been attributed to segmental mobility between the proteinase and kringle 5 domains (Teuten et al., 1991). Activation of miniplasminogen, a zymogen, to the active enzyme miniplasmin can be catalysed by plasminogen activators in a process analogous to the activation of intact plasminogen itself. In this case, a cleavage takes place between Arg-561 and Val-562 [the plasminogen numbering is based on that given by Forsgren et al. (1987)], resulting in unmasking of the active site.

To understand the structural changes resulting from activation of the zymogen proteinases has been the goal of much research over many years, and important contributions to this have come from detailed comparisons of the X-ray crystal structures of bovine trypsin and trypsinogen, and chymotrypsin and chymotrypsinogen [Wright (1973a,b), Kraut (1977), Huber and Bode (1978) and Brünger et al. (1987) among others]. The conclusion of the studies on trypsin was that the major structural changes resulting from the proteolytic cleavages involved in the activation process were limited to a rather small portion of the molecule in the region of the active site. The 15% of the molecule that was significantly perturbed (and which also had the property of significantly lower crystallographic b-factors in the active molecule) was labelled the “activation domain” (Huber and Bode, 1978); the remaining 85% showed almost no perturbation.

In our previous paper n.m.r. studies had provided evidence for independent unfolding of the kringle and proteinase domains in miniplasminogen (Teuten et al., 1991). However, that study was not able to provide a detailed description of the unfolding of the proteinase domain. In particular, the question of whether the proteinase domain can itself unfold in two transitions, as proposed by Novokhatny et al. (1984), remained to be answered. This is an interesting question because although the known crystal structures of the digestive proteinases imply the existence of two domains and even indicate some two-fold symmetry (Dufton, 1990), they are found to unfold co-operatively in d.s.c. (Privalov, 1979). Two-step unfolding of the proteinase domain in the fibrinolytic protein urokinase has, however, been proposed (Bogusky et al., 1989). Here we present new calorimetric data on the plasminogen proteinase as a contribution to this debate. In particular, we describe how the presence of an affinity label bound to the active site appears to cause a significant alteration of the unfolding properties of the molecule.

MATERIALS AND METHODS

The irreversible inhibitors N^α -*p*-tosyl-lysylchloromethane (Tos-Lys-CH₂Cl; ‘TLCK’) and valylphenylalanyl-lysylchloromethane (Val-Phe-Lys-CH₂Cl) were obtained from Sigma Chemical Co., Poole, Dorset, U.K. and Novabiochem, Nottingham, U.K., respectively. The chromogenic substrate S-2251 was obtained from KabiVitrum, Stockholm, Sweden.

Miniplasminogen was prepared as described by Teuten et al. (1991). Miniplasmin was prepared by the activation of miniplasminogen with urokinase; 50 mg of purified freeze-dried miniplasminogen were dissolved in a buffer containing 130 mM NaCl, 88 mM Trizma base and 33% glycerol at pH 7.4 and were

Abbreviations used: d.s.c., differential scanning calorimetry; Tos-Lys-CH₂Cl, N^α -*p*-tosyl-lysylchloromethane (‘TLCK’); Val-Phe-Lys-CH₂Cl, valylphenylalanyl-lysylchloromethane; T_m , midpoint temperature of unfolding transitions.

§ To whom correspondence should be sent, at the following address: Inorganic Chemistry Laboratory, University of Oxford, South Parks Rd., Oxford OX1 3QR, U.K.

incubated with 1 mol % of human two-chain urokinase (a gift of the Japan Chemical Research Co.) at room temperature with table rotation. After 2 h it was shown by electrophoresis under reducing conditions that activation was complete. Miniplasmin was irreversibly inactivated by incubation at 25 °C for 3 h with Tos-Lys-CH₂Cl or Val-Phe-Lys-CH₂Cl at 10 and 3 times molar excess respectively. That inactivation was more than 99 % complete was determined by assay with the chromogenic substrate S-2251. The sample was gel-filtered into 0.1 M ammonium bicarbonate buffer, pH 8.5, and freeze-dried. Samples for d.s.c. were dialysed against 100 mM acetic acid/sodium acetate, pH 4.0, overnight. Samples for n.m.r. were buffer-exchanged into 100 mM [2H₄]acetic acid/sodium [2H₃]acetate by repeated cycles of concentration and dilution using Amicon Centricon 10 ultrafiltration cells.

N.m.r. spectra were acquired on the Bruker AM500 spectrometer of the Oxford Centre for Molecular Sciences. Sample concentrations varied between 0.4 and 1.2 mM, and 500–3200 scans were acquired to give adequate signal-to-noise ratios. One-dimensional spectra were acquired with quadrature detection over 4K data points and spectral widths of 14.9 p.p.m. using a pulse angle of 60°. Spectra are shown resolution-enhanced using a Lorentz–Gauss transformation with parameters GB 0.2, LB-10 followed by zero filling to 8K data points. Saturation of the residual water signal was achieved by low-power irradiation during the 0.7 s relaxation delay introduced between scans.

Samples of miniplasminogen and miniplasmin for d.s.c. were diluted to 2 ml with buffer and degassed briefly before loading into the Microcal MC-2D calorimeter; the final sample concentration being normally in the range 1.5–3 mg/ml. Thermograms were recorded over the range 10–100 °C at a scan rate of 60 °C/h with dialysis buffer in the reference cell. Following a baseline correction, thermal parameters were obtained by best fitting of a minimum number of thermal transitions using unfolding models as described in the Results sections. Errors in the deconvoluted thermal parameters are estimated to be approx. ± 2 °C for transition temperatures and ± 10 % for enthalpies.

RESULTS

The d.s.c. thermogram of miniplasminogen at pH 4.0, together with a deconvolution to two independent 2-state transitions, is shown in Figure 1(a), and the data are presented in Table 1. The mid-point temperatures (T_m) of the unfolding transitions determined here are within a few degrees of those first determined by Novokhatny et al. (1984) and of the n.m.r. data reported previously (Teuten et al., 1991). The n.m.r. experiments have, in addition, shown that the transitions at approximately 61 and 70 °C are associated with the unfolding of the kringle 5 and proteinase domains respectively (Teuten et al., 1991). Assuming a non 2-state model, the calorimetric and Van't Hoff enthalpies can be calculated independently from d.s.c. data (Privalov and Potekhin, 1986; Sturtevant, 1987). The close similarity of the calorimetric and Van't Hoff enthalpies indicates that the thermal unfolding of miniplasminogen under these conditions may be described adequately by two independent co-operative unfolding transitions.

The n.m.r. spectrum of miniplasminogen consists of resonances from the kringle 5 and proteinase domains. By comparison with the spectrum of the isolated kringle 5 domain (Thewes et al., 1987), the kringle 5 resonances are seen to be in closely similar chemical-shift positions in the two molecules (Teuten et al., 1991). Those resonances which are not present in the spectrum of the isolated kringle 5 domain can be assigned, by elimination, to

the proteinase domain. The n.m.r. spectra of miniplasminogen and its activated form, miniplasmin, are shown in Figure 2 and can be seen to be closely similar. Neither the kringle 5 resonances nor the proteinase resonances appear to be significantly perturbed as a result of the activation process. A d.s.c. analysis was performed on non-inactivated miniplasmin, and the thermogram is shown in Figure 1(b). Data resulting from a deconvolution are given in Table 2, and the calorimetric parameters of this molecule are seen to resemble closely those of miniplasminogen.

Figure 2 also shows the effect on the spectrum of miniplasmin of inactivating the molecule with the affinity label Tos-Lys-CH₂Cl or Val-Phe-Lys-CH₂Cl. These reagents (their structures are illustrated in Figure 3) are known to alkylate the active-site

Figure 1 D.s.c. thermograms of (a) miniplasminogen and (b) non-inactivated miniplasmin in 100 mM sodium acetate/acetic acid at pH 4.0 showing a deconvolution to a model assuming two 2-state transitions

Solid line, experimental data; dotted lines, deconvoluted transitions; broken line, summation of deconvoluted transitions showing the overall fit. C_p is heat capacity.

Table 1 Thermodynamic parameters determined from deconvolution of the miniplasminogen d.s.c. thermogram assuming independent 2-state and non-2-state models

Model	Independent 2-state		Non-2-state	
T_m (°C)	61	74	61	74
ΔH (calorimetric) (kJ · mol ⁻¹)	345	261	335	259
ΔH (Van't Hoff) (kJ · mol ⁻¹)	—	—	357	269

Figure 2 Aromatic and upfield parts of the 500 MHz ^1H n.m.r. spectra at 27 °C of (A) miniplasminogen, (B) non-inactivated miniplasmin, (C) Tos-Lys- CH_2Cl -inactivated miniplasmin and (D) Val-Phe-Lys- CH_2Cl -inactivated miniplasmin at pH 4.0

Table 2 Thermodynamic parameters determined from deconvolution of the non-inactivated miniplasmin d.s.c. thermogram assuming independent 2-state and non-2-state models

Model	Independent 2-state		Non-2-state	
T_m (°C)	61	74	61	74
ΔH (calorimetric) ($\text{kJ} \cdot \text{mol}^{-1}$)	319	247	307	249
ΔH (Van't Hoff) ($\text{kJ} \cdot \text{mol}^{-1}$)	—	—	333	249

Figure 3 Structures of Val-Phe-Lys- CH_2Cl and Tos-Lys- CH_2Cl

histidine residue specifically (Shaw et al., 1965). In neither spectrum of the inactivated miniplasmins is there significant perturbation of the kringle 5 resonances from their positions in the spectra of miniplasminogen and miniplasmin. This is consistent with the kringle and proteinase domains interacting only weakly (Teuten et al., 1991). Several resonances are, however,

Figure 4 Temperature-dependence of the 500 MHz ^1H n.m.r. spectrum of Tos-Phe- CH_2Cl -inactivated miniplasmin in 100 mM sodium $[\text{}^2\text{H}_3]\text{acetate}/[\text{}^2\text{H}_4]\text{acetic acid}$ at pH 4.0

perturbed by the binding of affinity labels, and are attributed to residues in the proteinase domain. In particular, in the molecules where an affinity label is present, a number of additional resonances are observable in resolved regions of the spectrum and are particularly clearly visible in the region where methyl-group resonances are normally found. As the spectra of miniplasminogen and non-inactivated miniplasmin are very similar, the strong implication of this observation is that these spectral changes arise from perturbations to the proteinase domain structure, associated with the presence of an inactivating agent.

The thermal unfolding of Tos-Lys- CH_2Cl -inactivated miniplasmin was followed by recording n.m.r. spectra as a function of temperature; these spectra are shown in Figure 4. Resolved resonances assigned to the kringle domain, labelled K in the Figure, disappear simultaneously with a T_m of approx. 63 °C; this is therefore the transition temperature for co-operative unfolding of this domain. As this is within experimental error of the temperature of this transition in miniplasminogen, see above, it can thus be concluded first that the thermal unfolding properties of the kringle 5 domain are not significantly affected by the activation process. The behaviour of the resonances assigned to the proteinase domain, however, makes it clear that this domain does not unfold in a single step. One set of resonances disappears with a characteristic temperature of approximately 44 °C, whereas a second set of resonances disappears at a substantially higher temperature, with a characteristic temperature of approx. 70 °C. Several resonances in the upfield region corresponding to these two patterns of behaviour are marked P1 and P2 respectively in Figure 4. The higher-temperature spectra in Figure

Figure 5 D.s.c. thermograms of Tos-Lys-CH₂Cl-inactivated miniplasmin in 100 mM sodium acetate/acetic acid at pH 4.0 showing deconvolution to models assuming (a) two independent 2-state transitions or (b) two non-2-state transitions

Solid line, experimental data; dotted lines, deconvoluted transitions; broken line, summation of deconvoluted transitions showing the overall fit.

Table 3 Thermodynamic parameters determined from deconvolution of the Tos-Lys-CH₂Cl-inactivated miniplasmin d.s.c. thermogram assuming independent 2-state and non-2-state models

Model	Independent 2-state			Non-2-state		
T_m ($^{\circ}\text{C}$)	51	62	75	50	61	74
ΔH (calorimetric) ($\text{kJ} \cdot \text{mol}^{-1}$)	197	328	242	155	300	286
ΔH (Van't Hoff) ($\text{kJ} \cdot \text{mol}^{-1}$)	—	—	—	297	385	221

4 containing the second set of resonances (including those labelled P2) are closely similar both to the high-temperature spectra observed in the unfolding of miniplasminogen (see Teuten et al., 1991), and to the spectra of non-inactivated miniplasmin (Figure 2). The thermal transition occurring at approx. 70 $^{\circ}\text{C}$ can therefore be attributed to the co-operative unfolding of the major part of the proteinase domain. The resonances disappearing at the low-temperature transition (~ 44 $^{\circ}\text{C}$) are, by contrast, those observable in miniplasmin only as a result of binding of Tos-Lys-CH₂Cl (see Figure 2). This transition therefore appears to arise from a reversal of the conformational change induced in this region of the protein by the binding of substrate.

Figure 6 Upfield region of the 500 MHz ^1H n.m.r. spectrum of Tos-Lys-CH₂Cl-inactivated miniplasmin at pH 4.0 (A) freshly dissolved and (B) after 15 h at 37 $^{\circ}\text{C}$; (C) shows the same region of the spectrum of miniplasminogen for comparison

The signal marked * is an impurity.

A sample of Tos-Lys-CH₂Cl-inactivated miniplasmin prepared in the same way as for the n.m.r. experiment above was studied by d.s.c. The appearance of the resultant thermogram, which is shown in Figure 5, indicates the presence of an unfolding transition additional to those observed for miniplasmin itself and occurring close to the temperature at which the conformational change induced by Tos-Lys-CH₂Cl was observed to be reversed in the n.m.r. spectra. Deconvolution of the thermogram yields three transitions with T_m values at 50, 61 and 74 $^{\circ}\text{C}$ respectively. In this instance, however, only a model assuming three independent non-2-state transitions gave a close fit to the experimental data; data for this simulation are presented in Table 3 and Figure 5(b). The poorer-fitting model, assuming three independent 2-state transitions, is shown in Figure 5(a) for

Figure 7 Temperature-dependence of the 500 MHz ^1H n.m.r. spectrum of Val-Phe-Lys- CH_2Cl -inactivated miniplasmin in 100 mM sodium $[\text{H}_3]\text{acetate}/[\text{H}_4]\text{acetic acid}$ at pH 4.0

comparison. The part of the thermogram describing the two higher-temperature transitions can be closely fitted using 2-state transitions. These transitions strongly resemble those in the d.s.c. unfolding of miniplasminogen and miniplasmin (Figure 1), both in terms of unfolding temperature and enthalpy. The low-temperature transition is not well-fitted, however, because the area and shape of the transition do not correlate in the manner required for a 2-state model. For this reason, in a fit to non-2-state transitions, the Van't Hoff enthalpy for the low-temperature transition is much higher than the calorimetric enthalpy, and this implies that either an irreversible process is taking place, or that there exists higher co-operativity. Exothermic irreversible processes have been shown to result in a distortion or sharpening of the d.s.c. transition peak that would inevitably yield an over-estimate of the Van't Hoff enthalpy (Galisteo et al., 1991). That the lowest-temperature transition is in fact irreversible is apparent from observations of the refolding of the protein by n.m.r. On cooling the sample to 27 from 80 °C, the n.m.r. spectrum (results not shown) shows a restoration of resonances from the kringle domain and those resonances associated with the high-temperature proteinase transition; thus refolding of these parts of the molecule has occurred. The resonances associated with the region of the substrate-bound proteinase domain which unfolds at the lower temperature are not, however, restored to the

spectrum, indicating that this transition is not reversible. It has been observed that, after the sample has been standing at 37 °C, the resonances which are resolved only in the spectrum of Tos-Lys- CH_2Cl -inactivated miniplasmin are reduced in intensity; this reduction was approx. 75% after 15 h at pH 4.0 (Figure 6). Furthermore, a thermogram of a sample treated in this way closely resembles that of miniplasminogen.

A sample of the Val-Phe-Lys- CH_2Cl -inactivated miniplasmin has been studied by n.m.r. in an experiment analogous to that described for the Tos-Lys- CH_2Cl -inactivated sample described above. The temperature-dependence of the spectrum of Val-Phe-Lys- CH_2Cl -inactivated miniplasmin is shown in Figure 7, and again three transitions are observed. The kringle and proteinase transitions at ~ 62 and ~ 70 °C are clearly evident in the spectra, but in this case the upfield resonances, which are characteristic of the inactivated miniplasmin, disappear only at temperatures above approx. 60 °C. This greater stability is reflected in the fact that the n.m.r. spectrum of this sample, unlike that of Tos-Lys- CH_2Cl -inactivated miniplasmin, was unchanged for many hours at 37 °C; further, this 60 °C transition was found to be reversible provided the sample was not heated above about 70 °C. D.s.c. of a Val-Phe-Lys- CH_2Cl -inactivated sample of miniplasmin (results not shown) indicates the existence of a poorly resolved additional transition in the 60–64 °C region, entirely consistent with the n.m.r. data.

DISCUSSION

Studies on miniplasminogen at pH 4.0 in the presence of 100 mM acetic acid/sodium acetate by both d.s.c. and n.m.r. have shown that the unfolding of miniplasminogen is best described by two transitions, at approx. 62 ± 2 and 72 ± 2 °C, associated with the unfolding of the kringle 5 and the proteinase domain respectively. No evidence has been found for a third transition, and thus it is concluded that the proteinase domain unfolds co-operatively under these conditions. Activation of miniplasminogen to miniplasmin results in little perturbation of the n.m.r. spectrum at pH 4, and the calorimetric properties of the two molecules have been determined by d.s.c. to be the same to within 10%. Both the activation of miniplasminogen to miniplasmin and its inactivation with Tos-Lys- CH_2Cl have no significant effect on either the denaturation temperature or the unfolding enthalpy of the kringle 5 domain.

Inactivation of miniplasmin with either of the affinity labels Val-Phe-Lys- CH_2Cl or Tos-Lys- CH_2Cl causes the appearance of a set of additional resonances, clearly observable in resolved regions of the spectrum of the protein. It is likely that these are resonances that were previously unresolved in the spectrum of the protein, that have been shifted as a result of a conformational transition on binding or from ring-current shifts induced by the presence of the aromatic ring of the ligand; it is, however, possible that one or more of the resonances could arise from the protons of the ligand itself. The part of the molecule giving rise to these resonances in inactivated miniplasmin has been found to unfold on heating in a manner that is independent of the unfolding of both the kringle domain and the remainder of the proteinase domain. In the n.m.r. spectrum the upfield-shifted resonances from the region of the proteinase domain are observed to disappear uniformly in this lower-temperature transition without perturbation of resonances from the remainder of the proteinase domain. This implies that the different regions of the proteinase domain have structural autonomy. That the value for ΔH for this process is comparable in magnitude with that for unfolding transitions within the remainder of the molecule suggests that the process being observed is the co-operative

unfolding of a significant region of the proteinase domain. The temperature of the additional transition was found to vary depending on the affinity label used, the larger Val-Phe-Lys-CH₂Cl giving rise to greater stability than Tos-Lys-CH₂Cl. Unlike the other two transitions, this third unfolding transition in Tos-Lys-CH₂Cl-inactivated miniplasmin is not fully reversible, and a calorimetric deconvolution reveals that it cannot be modelled by a simple 2-state process. When the conformational change takes place at elevated temperature, it is unlikely that the affinity label itself can be cleaved, and it is presumed that the structural changes in this part of the domain are not always able to reverse on cooling. These observations suggest that the region of the molecule is not stable at high temperature or after prolonged exposure to elevated temperature; it is probable that, for this reason, the temperature found for the midpoint of this transition is slightly lower in the n.m.r. unfolding experiment (which takes much longer) than in the d.s.c. experiment. It appears that the Val-Phe-Lys-CH₂Cl affinity label causes a greater stabilization of its environment than Tos-Lys-CH₂Cl, resulting in unfolding of a region of the active site taking place at a significantly higher temperature.

The results described here reveal a phenomenon whereby a part of the proteinase domain of plasmin appears to be unfolded at pH 4.0 in the absence of a bound ligand. The binding of the substrate analogues then induces formation of structure which is locally co-operative, although independent of the remainder of the proteinase structure. Thus, in the presence of the substrate analogue, the proteinase domain as a whole appears under these conditions to unfold in a distinctly non-co-operative manner. The nature of the additional structure induced in the plasmin proteinase domain by the covalently bound ligand could in principle be identified if specific assignments in the n.m.r. spectrum of the protein were available. At present this is not the case; indeed to make such assignments in a rigorous manner would be a formidable undertaking for a protein of this size. The results presented here, however, allow speculation as to the nature of the additional structure. As stabilization of this

structure requires the presence of substrate and not simply activation of the protein by cleavage of the Arg⁵⁶¹-Val⁵⁶² peptide bond, the structured region is very likely to be in the vicinity of the active site of the serine proteinase. It may well, therefore, correspond to an 'activation region', of the type identified in crystallographic studies of trypsin and trypsinogen, which is disordered in the zymogen but ordered in the active enzyme (Huber and Bode, 1978). Whether or not this is the origin of the phenomenon observed here requires further study, but the present results show clearly that significant structural changes may be induced in a local region of a serine-proteinase domain as a consequence of activation and substrate binding.

This is a contribution from the Oxford Centre for Molecular Sciences, which is funded by the Science and Engineering Research Council (SERC) and the Medical Research Council. The biological microcalorimetry facilities at the University of Glasgow are supported by the SERC.

REFERENCES

- Bogusky, M. J., Dobson, C. M. and Smith, R. A. G. (1989) *Biochemistry* **28**, 6728–6735
- Brünger, A. T., Huber, R. and Karplus, M. (1987) *Biochemistry* **26**, 5153–5162
- Dufton, M. J. (1990) *FEBS Lett.* **271**, 9–13
- Forsgren, M., Raden, B., Israelsson, M., Larsson, K. and Heden, L.-O. (1987) *FEBS Lett.* **213**, 254–260
- Galisteo, M. L., Mateo, P. L. and Sanchez-Ruiz, J. M. (1991) *Biochemistry* **30**, 2061–2066
- Huber, R. and Bode, W. (1978) *Acc. Chem. Res.* **11**, 114–122
- Kraut, J. (1977) *Annu. Rev. Biochem.* **4**, 331–358
- Novokhatny, V. V., Kudinov, S. A. and Privalov, P. L. (1984) *J. Mol. Biol.* **179**, 215–232
- Privalov, P. L. (1979) *Adv. Prot. Chem.* **33**, 167–241
- Privalov, P. L. and Potekhin, S. A. (1986) *Meth. Enzymol.* **131**, 4–51
- Shaw, E., Mares-Guia, M. and Cohen, W. (1965) *Biochemistry* **4**, 2219–2224
- Sottrup-Jensen, L., Claeys, H., Zajdel, M., Petersen, T. E. and Magnusson, S. (1978) *Prog. Chem. Fibrinolysis Thrombolysis* **3**, 191–209
- Sturtevant, J. M. (1987) *Annu. Rev. Phys. Chem.* **38**, 463–488
- Teuten, A. J., Smith, R. A. G. and Dobson, C. M. (1991) *FEBS Lett.* **278**, 17–22
- Thewes, T., Ramesh, V., Simplaceanu, E. L. and Llinás, M. (1987) *Biochim. Biophys. Acta* **912**, 254–269
- Wright, H. T. (1973a) *J. Mol. Biol.* **79**, 1–11
- Wright, H. T. (1973b) *J. Mol. Biol.* **79**, 13–23