A Fluorimetric Study of the Role of Calcium Ions in the Stability of Thermolysin

By ANGELO FONTANA, CLAUDIO VITA, ENRICO BOCCU' and
FRANCESCO M. VERONESE

*Institute of Organic Chemistry (Centro di Studio sui Biopolimeri, C.N.R.) and
Pharmaceutical Chemistry (Centro di Studio sulla Chimica del Farmaco e dei Prodotti
Biologicamente Attivi, C.N.R.), University of Padova, Via Marzolo 1, 35100 Padova, Italy*

(Received 4 January 1977)

1. Fluorimetric techniques were used to characterize the environment of tryptophan residues in thermolysin and apo-thermolysin. The apo-thermolysin was obtained by dissolving the enzyme in the presence of 10mM-EDTA, which removed the functional Zn^{2+} ion and the four Ca^{2+} ions/molecule from the enzyme. 2. At 25°C in aqueous solution the fluorescence-emission spectrum of the native holoenzyme, on excitation at 290nm, was essentially characteristic of tryptophan, with an emission maximum at 333nm. The emission maximum of the apoenzyme is red-shifted to 338nm and the relative intensity of fluorescence is decreased by 10%, both effects indicating some unfolding of the protein molecule, with the indole groups being transferred to a more hydrophilic environment. 3. Fluorescence quenching studies using KI, *N*'-methylnicotinamide hydrochloride and acrylamide indicated a more open structure in the apoenzyme, with the tryptophan residues located in a negatively charged environment. 4. The thermal properties of the apoenzyme, as monitored by fluorescence-emission measurements, are dramatically changed with respect to the native holoenzyme. In fact, whereas the native enzyme is heat-stable up to about 80°C, for the apoenzyme a thermal transition is observed near 48°C. The apoenzyme is also unstable to the action of unfolding agents such as urea and guanidinium chloride, much as for other globular proteins from mesophilic organisms. 5. The functional Zn^{2+} ion does not contribute noticeably to the stability of thermolysin. 6. It is concluded that a major role in the structural stability of thermolysin is played by the Ca^{2+} ions, which have a bridging function within this disulphide-free protein molecule.

Thermolysin binds one functionally essential Zn^{2+} ion at its active site. It also binds four Ca^{2+} ions, which do not participate in catalysis but stabilize the enzyme against heat denaturation (Endo, 1962; Feder *et al.*, 1971; Drucker & Borchers, 1971). The Zn^{2+} -free thermolysin in a Ca^{2+} -containing buffer retains its thermal stability completely, indicating that the Zn^{2+} ion does not contribute noticeably to the heat-stability of the enzyme (Holmquist & Vallee, 1974; Dahlquist *et al.*, 1976; Voordouw *et al.*, 1976). There are no disulphide bonds in the enzyme, and the role of Ca^{2+} was suggested by X-ray crystallography to be the linking together of different parts of the protein molecule, with a resulting increase in heat-stability (Colman *et al.*, 1972; Matthews *et al.*, 1974).

In a previous paper (Fontana *et al.*, 1976) we have reported preliminary results on the heat-stability of thermolysin dissolved in 10mM-EDTA, which leads to complete inhibition on removal of both Zn^{2+} and Ca^{2+} ions from the enzyme (Mori-hara & Tsuzuki, 1966; Matsubara, 1970; Matsubara & Feder, 1971). On the basis of circular-dichroism measurements in the far-u.v., the chelating agent had a dramatic

effect on the conformational stability of thermolysin, since the EDTA-treated enzyme unfolds near 50°C. Dahlquist *et al.* (1976) reported similar studies on the effect of EDTA on thermolysin. Conformational transitions of the protein molecule were monitored by determining the changes in u.v. absorbance at 286nm on heating.

The fluorescence spectra of non-conjugated proteins are essentially determined by the tyrosine and tryptophan residues, of which thermolysin contains 28 and 3 residues per molecule respectively (Titani *et al.*, 1972). On irradiation of a protein at 290nm most of the absorption of light is due to the tryptophan residues only, and the resultant emission spectrum is due mainly to these residues (Brand & Witholt, 1967). The sensitivity of protein fluorescence to the micro-environments surrounding the emitting tryptophan residues allows fluorescence to be used as a sensitive tool to monitor conformational properties and transitions of protein molecules. Sometimes fluorescence can reveal structural changes in a protein that are too subtle to be observed by other techniques. In the present study fluorescence-emission spectro-

scopy and fluorescence quenching by perturbants (Lehrer, 1971; Eftink & Ghiron, 1976) have been used to investigate both the environment and the exposure of tryptophan residues in the native enzyme and in the apoprotein and to monitor the unfolding of protein structure by the action of heat and denaturing agents.

Experimental

Materials

EDTA (Fluka A.G., Buchs, Switzerland), tetraethylenepentamine (Eastman Kodak Co., Rochester, NY, U.S.A.), thioglycolic acid (Fluka), *N*-methyl-nicotinamide hydrochloride (Sigma Chemical Co., St. Louis, MO, U.S.A.), acrylamide (Fluka) and *N*-acetyltryptophanamide (Fluka) were used without further purification. Urea (C. Erba, Milan, Italy) was recrystallized from 95% (v/v) ethanol and only fresh solutions of the reagent were used. Guanidinium chloride (Fluka) was recrystallized twice from benzene and once from 95% (v/v) methanol. All other materials were obtained from commercial sources and were used without purification.

Thermolysin was obtained from Daiwa Kasei K.K. (Osaka, Japan) as a freeze-dried, three-times-recrystallized material containing 30% (w/v) calcium acetate and was further purified by gel filtration on a Sephadex G-75 column (Titani *et al.*, 1972). The commercial enzyme was found to contain about 10% of autodigestion products. The protein concentration was determined spectrophotometrically by using $A_{280\text{nm}}^{1\%} = 15.2$ (Voordouw & Roche, 1974), which corresponds to a molar extinction (ϵ) of 52400 for a mol.wt. of 34600 (Titani *et al.*, 1972). Occasionally, protein solutions were clarified by centrifugation or by passing through a Millipore filter (0.45 μm pore size).

Methods

Fluorescence measurements were carried out with a Hitachi-Perkin-Elmer model MPF-2A spectrofluorimeter equipped with a thermostatically controlled cell holder. All fluorescence measurements were made by using an excitation wavelength of 290nm. The absorbance of samples examined never exceeded 0.1. Fluorescence-emission quenching by KI (Lehrer, 1971), *N*-methyl-nicotinamide hydrochloride (Holmes & Robbins, 1974) and acrylamide (Eftink & Ghiron, 1976) was measured as described in the literature.

'Melting' experiments were carried out by continuous recording of the fluorescence-emission intensity, while the temperature inside the cell was increased at a constant rate, 1–5°C/min, by circulating water through the cuvette holder with a Haake

thermostat. The temperature of the sample was directly monitored by a thermistor probe attached through the cap of the cell. The derivative was calculated at each temperature and a plot of $-d(F/F_0)/dT$ versus T constructed (where F_0 is fluorescence-emission intensity at room temperature). Maximum values of the derivative plots were taken as the temperature of 'melting' (T_m).

Results

Effect of EDTA on thermolysin

EDTA has been found to be a potent inhibitor of thermolysin, competing with the active site for the Zn^{2+} ion and removing Ca^{2+} ions from the protein (Mori-hara & Tsuzuki, 1966; Feder *et al.*, 1971; Dahlquist *et al.*, 1976). The physicochemical measurements reported here were made in the presence of 10mM-EDTA at a protein concentration of 1–3 μM and performed immediately after preparation of the enzyme solutions. It was found important to dissolve the enzyme directly in the EDTA-containing buffer, as subsequent addition of EDTA to the enzyme solution resulted in some autolysis. We refer below to this

Fig. 1. Fluorescence-emission spectra of thermolysin and apo-thermolysin

The protein was dissolved at a concentration of 0.035mg/ml in 20mM-Tris/HCl buffer, pH7.0, containing 10mM- CaCl_2 (—) or 10mM-EDTA (---). The wavelength of excitation was 290nm and spectra were recorded at 25°C.

Fig. 2. Stern-Volmer plots for the quenching of tryptophan fluorescence by KI (a), *N'*-methylnicotinamide hydrochloride (b) and acrylamide (c)

The wavelength of excitation was 290nm and spectra were recorded at 25°C. Relative intensities were measured at 350nm for *N*-acetyltryptophanamide (Δ) dissolved in 20mM-Tris/HCl buffer, pH7.0, at 333 nm for thermolysin ($\bullet$), in the same buffer containing 10mM- CaCl_2 and at 338 nm for apo-thermolysin ($\circ$) in buffer with 10mM-EDTA. The protein concentration in each case was 0.09 mg/ml.

EDTA-treated enzyme as apo-thermolysin. It should also be mentioned that apo-thermolysin, a Zn^{2+} -free low- Ca^{2+} protein prepared by the procedure described by Feder *et al.* (1971), by using tetraethylenepentamine (Voordouw & Roche, 1974) as Zn^{2+} -chelating agent, showed the same fluorescence-emission-spectrum characteristics and heat-stability as those observed with the EDTA-treated enzyme (cf. below).

Fluorescence measurements

Thermolysin in 10mM- CaCl_2 /20mM-Tris/HCl buffer, pH7.0, on excitation at 290nm shows an emission maximum at 333 nm (Fig. 1), which is blue-shifted by about 20nm from that observed with free tryptophan or structureless tryptophan peptides in aqueous solution (Brand & Witholt, 1967). This is commonly seen with proteins and is considered to indicate a hydrophobic environment for the tryptophan residues. pH has no effect on the emission properties of thermolysin in the pH range 5–9. Out-

side this pH range a red shift of emission wavelength and a decrease in relative intensity occurs.

The fluorescence characteristics of the apoenzyme are quite different in both emission intensity and maximum wavelength of emission from those of the native enzyme. As shown in Fig. 1, thermolysin in 10mM-EDTA/20mM-Tris/HCl buffer, pH7.0, shows a red shift in the maximum from 333 to 338nm, and about 10% decrease in relative intensity, both effects indicating that some unfolding of the protein molecule occurs in the presence of EDTA, with the indole groups being transferred to a more hydrophilic environment (Brand & Witholt, 1967).

Fluorescence-quenching studies

In the present study, KI (Lehrer, 1971), *N'*-methylnicotinamide hydrochloride (Holmes & Robbins, 1974) and acrylamide (Eftink & Ghiron, 1976) were used as quenchers of tryptophan fluorescence. Plots of $(F_0/F) - 1$ against quencher concentration (Stern &

Fig. 3. Relative emission of fluorescence measured as a function of temperature

The results are reported as $(F/F_0) \times 100$ as a function of temperature, where F_0 is the fluorescence-emission intensity at room temperature. The excitation wavelength was 290 nm. (a) Thermolysin dissolved in 20 mM-Tris/HCl buffer, pH 7.0, containing 10 mM- CaCl_2 , at a protein concentration of 0.03 mg/ml (●); mixture of tyrosine and tryptophan (molar ratio 28:3) in the same buffer (○). The relative intensity of emission was measured at 333 nm with thermolysin and at 350 nm with the amino acid mixture. (b) Thermolysin dissolved in 20 mM-Tris/HCl buffer, pH 7.0, containing 10 μM - (●) or 1 mM- (○) EDTA. The protein concentration was 0.035 mg/ml.

Volmer, 1919) are shown in Fig. 2. With KI, the effectiveness of quenching decreases in the order *N*-acetyltryptophan > apo-thermolysin > thermolysin. The native enzyme contains tryptophan residues, which are, in general, shielded from quencher. The enhancement of exposure in the apoenzyme is consistent with a conformational transition to a more open form. With the positively charged *N'*-methyl-nicotinamide hydrochloride as the quencher (Fig. 2b) the order of quenching is apo-thermolysin > thermolysin > *N*-acetyltryptophanamide. The fact that a higher quenching effect for *N'*-methylnicotinamide hydrochloride is obtained with the apoenzyme than with the model compound indicates that the tryptophan residues in apo-thermolysin are located in a negatively charged environment. In fact, since a protein is a polyelectrolyte, the quenching effect of charged ionic quenchers would be influenced by electrostatic effects (Lehrer, 1971).

Acrylamide has been shown to be an excellent uncharged quenching probe that is very discriminating in sensing the degree of exposure of tryptophan

residues in proteins. This molecule does not share the shortcomings of ionic quenchers and allows an estimation of the exposure of residues in a purely collisional process (Eftink & Ghiron, 1976). The results obtained with acrylamide (Fig. 2c) parallel those obtained with KI as quencher and indicate enhancement of exposure of tryptophan residues in the apoenzyme compared with in the native holoenzyme.

Effect of temperature

The temperature-dependence of the fluorescence intensity of thermolysin dissolved in 10 mM- CaCl_2 /20 mM-Tris/HCl buffer, pH 7.0, and of a mixture of tryptophan and tyrosine in the same molar ratio (3:28) as in the enzyme molecule (Titani *et al.*, 1972) is shown in Fig. 3(a). The intensity of fluorescence of the amino acid mixture decreased in a monotonic way as the temperature was increased, owing to thermal quenching (Gally & Edelman, 1962; Steiner & Edelhoch, 1962; Brand & Witholt, 1967). The intensity-versus-temperature profile obtained with thermolysin was similar to that observed with the amino acid mixture in the temperature range (20–85°C) investigated. In both cases, the decrease in quantum yield is not accompanied by any red shift of the fluorescence maximum. On the other hand a red shift was observed when the protein was held at 85°C. These results indicate that no structural alteration occurs when the native holoenzyme is heated in the presence of 10 mM- CaCl_2 up to about 80°C.

When the enzyme was heated in 10 μM -EDTA in Tris/HCl buffer, the intensity of the fluorescence-versus-temperature profile clearly showed the occurrence of a transition in the temperature range 65–70°C (Fig. 3b). At higher concentrations (0.1–10 mM) of EDTA this transition was shifted to 45–50°C. The structural disorganization produced on heating in the presence of EDTA caused a red shift of the emission maximum near 350 nm.

'Melting' studies of apo-thermolysin were also performed by continuous recording at a fixed wavelength of the intensity of fluorescence while the temperature inside the cell was continuously increased at a constant rate. The results were expressed by plotting the relative intensity of fluorescence versus temperature. The corresponding first-derivative plots from 'melting' experiments carried out at 10 μM –10 mM-EDTA are presented in Fig. 4. Assigning the 'melting' point (T_m) to the temperature corresponding to the centre of the peak in the derivative plot, a T_m of $48 \pm 0.5^\circ\text{C}$ was obtained at 10–0.1 mM- and $70 \pm 0.5^\circ\text{C}$ at 10 μM -EDTA. Possible variations in T_m of the apoenzyme were investigated on heating at three different heating rates, i.e. 1, 2 and $5^\circ\text{C}/\text{min}$. The deviation in T_m observed was found to fall within the usual experimental error in the 'melting' experiment.

The role of Zn^{2+} on the conformational stability of thermolysin was negligible. 'Melting'-profile determinations, obtained from fluorescence measurements of thermolysin in 10mM- CaCl_2 in the presence of the zinc-specific chelating agent tetraethylenepentamine

Fig. 4. Derivative curves of fluorescence emission intensity as a function of temperature of thermolysin in the presence of EDTA

The protein was dissolved (0.035mg/ml) in 20mM-Tris/HCl buffer, pH7.0, containing 10 μM - (----), 0.1 mM- (.....) and 10 mM- (—) EDTA. The derivative was calculated from continuous tracings of fluorescence intensity with increasing temperature. The excitation wavelength was 290nm and emission intensity was recorded at 338nm. The ordinate represents $-\text{d}(F/F_0)/\text{d}T \times 100$, where F_0 is the intensity of emission measured at room temperature.

(5mM) (Voordouw & Roche, 1974), indicated that the structural stability of the Zn^{2+} -free enzyme was the same as that of the native enzyme. This result is in accord with previous work (Holmquist & Vallee, 1974; Voordouw *et al.*, 1976) dealing with thermal stability of the Zn^{2+} -free enzyme determined by measuring residual activity of the heated enzyme. Similarly, other metal-binding agents such as 2-mercaptoethanol, thioglycolic acid and imidazole, known to form complexes with Zn^{2+} and to inhibit thermolysin (Sillen & Martell, 1964; Latt *et al.*, 1969; Holmquist & Vallee, 1974), did not have any significant effect on the T_m of the enzyme.

Effect of denaturing agents

The effects of increasing concentrations of urea and guanidinium chloride on the fluorescence characteristics of apo-thermolysin are shown in Fig. 5. The progressive unfolding of the protein molecule is clearly indicated by the gradual decrease in fluorescence intensity and red shift of emission maximum on increasing the perturbant concentration. At 8M-urea or 4M-guanidinium chloride the apoenzyme shows a fluorescence maximum near 350nm, typical of structureless tryptophan-containing peptides in aqueous solution (Brand & Witholt, 1967). Relatively low concentrations of urea or guanidinium chloride at neutral pH are sufficient to cause elimination of the non-covalent interactions responsible for the globular structure of apo-thermolysin. A midpoint transition can be evaluated at about 5M-urea or 1.2M-guanidinium chloride.

Fig. 5. Effect of urea and guanidinium chloride on the fluorescence-emission properties of apo-thermolysin

The protein was dissolved (0.03 mg/ml) in urea (a) or guanidinium chloride (b) solutions of increasing concentrations in 20mM-Tris/HCl buffer, pH7.0, containing 10mM-EDTA. The excitation wavelength was 290nm and data were taken from spectra recorded at room temperature after 5 min of the preparation of the solutions. ○, Relative fluorescence intensity; ●, maximum wavelength of emission.

Discussion

The present fluorimetric study has provided evidence that the environment around tryptophan residues is affected by the change from the native holoenzyme to the apoenzyme, as shown by the red shift of the maximum wavelength of the intrinsic protein fluorescence in apo-thermolysin and also by the decrease in fluorescence intensity. These alterations in the fluorescence characteristics can be accounted for by a conformational change with an increase in polarity around the emitting tryptophan residues, related to the transfer of these residues from the interior to the more hydrophilic exterior of the protein molecule (Brand & Witholt, 1967). This interpretation is consistent also with the fluorescence-quenching experiments (Lehrer, 1971). With KI and acrylamide as quenchers, indication has been obtained of a more unfolded structure in the apoprotein with respect to the native holoenzyme, since quenching was in the order *N*-acetyltryptophanamide > apo-thermolysin > native holoenzyme. This is the order that would be postulated by considering decreased steric hindrance and rates of diffusion in the more open form of the apoprotein compared with the native enzyme.

The fact that with the positively charged *N*'-methyl-nicotinamide hydrochloride as quencher the effectiveness of quenching is practically the same in thermolysin and the model compound, and both are less than in apo-thermolysin, indicates that in this case charge effects play an important role in the quenching effect (Lehrer, 1971). *N*'-Methylnicotinamide hydrochloride exhibits a much more efficient quenching effect with the apoprotein, implying that tryptophan residues are located in a more negatively charged environment. This can be explained by the fact that Ca^{2+} ions are bound to the protein essentially through the carboxylate groups of aspartic and glutamic residues, and the removal of these ions by EDTA would leave more anionic groups in the vicinity of tryptophan residues. In particular, tryptophan-186 is flanked in the protein molecule by aspartate-185 and glutamate-187, both residues being involved in the binding of Ca^{2+} (Colman *et al.*, 1972; Weaver *et al.*, 1976).

The heat-stability of apo-thermolysin is remarkably less than that of the native enzyme. The apoprotein has been found to denature near 48°C by 'melting'-profile experiments using fluorescence-emission measurements (Fig. 4). The observed T_m of apo-thermolysin is in agreement with that previously determined by using circular dichroism in the far-u.v. (Fontana *et al.*, 1976) or difference absorption measurements at 286 nm (Dahlquist *et al.*, 1976).

When native thermolysin was heated in the presence of 10 μM -EDTA a T_m near 70°C was observed. It has been previously demonstrated that

two of the four Ca^{2+} ions dissociate more easily from the polypeptide chain when the concentration of external Ca^{2+} is 1 mM–1 μM (Voordouw & Roche, 1974). Weaver *et al.* (1976) reported the successive removal of Ca^{2+} from the holoenzyme in the solid state by soaking the crystals in EDTA solution and also found that two of the four ions were removed more easily. The conclusions reported by the above authors were at variance with respect to the less tightly bound Ca^{2+} ions. Without going into the question of which sites are involved in the dissociation, from our work we can conclude that not all four Ca^{2+} ions contribute similarly to the stabilization of thermolysin, since a low- Ca^{2+} thermolysin, as obtained by dissolving the holoenzyme in the presence of 10 μM -EDTA, appears to be rather heat-stable, being denatured near 70°C (Fig. 4).

Previously, the conformation of native thermolysin in the presence of Ca^{2+} ions has been found to be unaffected when dissolved in 8M-urea or 6M-guanidinium hydrochloride at room temperature (Ohta, 1967; Fontana *et al.*, 1976). On the other hand, the apoprotein is immediately denatured by these agents at high concentration, as indicated by fluorescence measurements. Determination of the transition curve of the apoprotein by using increasing concentrations of denaturant revealed that unfolding occurs in a range of urea or guanidinium chloride concentrations quite similar to that found with most globular proteins from mesophilic organisms (Tanford, 1968, 1970). This would imply that the removal of Ca^{2+} ions from the protein molecule would determine the loss of stability not only towards heat, but also to common protein denaturants.

These results, together with those previously reported by us (Fontana *et al.*, 1976) and others (Dahlquist *et al.*, 1976; Voordouw & Roche, 1975), indicate that Ca^{2+} ions play an important role in maintaining the integrity of the conformation and conformational stability towards heat and protein denaturants of thermolysin. That the stabilizing effect of Ca^{2+} is much more pronounced with thermolysin than it is with several other enzyme molecules [Matthews *et al.* (1974) and references cited therein] is still not clearly understood at the molecular level. A detailed comparative structural study between thermolysin and the heat-labile neutral proteinase from *Bacillus subtilis* (Levy *et al.*, 1975; Pangburn *et al.*, 1976) would probably shed light on their differences in thermal stability.

We thank Mr. M. Zambonin, Mr. F. Miozzo, Mr. S. Fioretto Da Rin and Mrs. R. Largajolli for valuable technical assistance.

References

- Brand, L. & Witholt, B. (1967) *Methods Enzymol.* **11**, 776–855

- Colman, P. M., Jansonius, J. H. & Matthews, B. W. (1972) *J. Mol. Biol.* **70**, 701-724
- Dahlquist, F. M., Long, J. W. & Bigbee, W. L. (1976) *Biochemistry* **5**, 1103-1111
- Drucker, H. & Borchers, S. L. (1971) *Arch. Biochem. Biophys.* **147**, 242-248
- Eftink, M. R. & Ghiron, C. A. (1976) *Biochemistry* **15**, 672-680
- Endo, S. (1962) *J. Ferment. Technol.* **40**, 346-353
- Feder, J., Garrett, L. R. & Wildi, B. S. (1971) *Biochemistry* **10**, 4552-4556
- Fontana, A., Boccu', E. & Veronese, F. M. (1976) *Experientia Suppl.* **26**, 55-59
- Gally, J. A. & Edelman, G. H. (1962) *Biochim. Biophys. Acta* **60**, 499-509
- Holmes, L. G. & Robbins, F. M. (1974) *Photochem. Photobiol.* **19**, 361-366
- Holmquist, B. & Vallee, B. L. (1974) *J. Biol. Chem.* **249**, 4601-4607
- Latt, S. A., Holmquist, B. & Vallee, B. L. (1969) *Biochem. Biophys. Res. Commun.* **37**, 333-339
- Lehrer, S. S. (1971) *Biochemistry* **10**, 3254-3263
- Levy, P. L., Pangburn, M. K., Burstein, Y., Ericsson, L. H., Neurath, H. & Walsh, K. A. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 4341-4354
- Matsubara, H. (1970) *Methods Enzymol.* **19**, 642-652
- Matsubara, H. & Feder, J. (1971) *Enzymes 3rd Ed.* **3**, 721-730
- Matthews, B. W., Weaver, L. H. & Kester, W. R. (1974) *J. Biol. Chem.* **241**, 8030-8044
- Moriwara, K. & Tsuzuki, H. (1966) *Biochim. Biophys. Acta* **118**, 215-218
- Ohta, Y. (1967) *J. Biol. Chem.* **242**, 509-515
- Pangburn, M. K., Levy, P. L., Walsh, J. A. & Neurath, H. (1976) *Experientia Suppl.* **26**, 19-30
- Sillen, L. G. & Martell, A. E. (1964) *Stability Constants for Metal-Ion Complexes: Chem. Soc. Spec. Publ. No. 17*, 635
- Steiner, R. F. & Edelhoch, H. (1962) *Nature (London)* **193**, 375-376
- Stern, O. & Volmer, M. (1919) *Phys. Z.* **20**, 183-190
- Tanford, C. (1968) *Adv. Protein Chem.* **23**, 121-282
- Tanford, C. (1970) *Adv. Protein Chem.* **24**, 1-95
- Titani, K., Hermanson, M. A., Ericsson, L. H., Walsh, K. A. & Neurath, H. (1972) *Biochemistry* **11**, 2427-2435
- Voordouw, G. & Roche, R. S. (1974) *Biochemistry* **13**, 5017-5021
- Voordouw, G. & Roche, R. S. (1975) *Biochemistry* **14**, 4667-4673
- Voordouw, G., Milo, C. & Roche, R. S. (1976) *Biochemistry* **15**, 3716-3723
- Weaver, L. H., Kester, W. R., Ten Eyck, L. F. & Matthews, B. W. (1976) *Experientia Suppl.* **26**, 31-39