EFFECT OF METHYLATION OF THE HISTIDINE RESIDUE IN THE ACTIVE SITE OF α-CHYMOTRYPSIN ON THE CONFORMATIONAL STABILITY OF THE ENZYME*

V.K. ANTONOV and T.I. VOROTYNTSEVA

Shemyakin Institute for Chemistry of Natural Products, USSR Academy of Sciences, Moscow, USSR

Received 10 April 1972

1. Introduction

X-Ray data have shown [1, 2] that sorption of a quasi-substrate, *N*-formyl-L-tryptophan, on the active site of α-chymotrypsin does not perturb the enzyme conformation, whereas acylation of the active site noticeably affects the positions of several of its constituent atoms. Acylation of serine-195 is accompanied by disruption of the "charge relay system" [3]. One may therefore assume that this system plays an important role in maintaining the native conformation of the active site.

In this paper, we present evidence in favour of the fact that the charge relay system is a conformation stabilizing factor not only of the active site but of the entire chymotrypsin globular molecule. This follows from comparison of the rates of urea denaturation of the native and N^{ϵ}-histidine-57 methylated α-chymotrypsins.

2. Materials and methods

α-Chymotrypsin was a crystalline preparation from the Olaina factory with 70% content of active enzyme, by titration of the active site. The enzyme was purified by CM-Sephadex chromatography and methylated by treatment with methyl *p*-nitrobenzene sulphonate [4]. The modified protein solution was passed through a Sephadex G-25 column, the eluate was concentrated with dry Sephadex G-50 [5]. The concentration of N^{ϵ}-methylhistidine-57 α-chymotrypsin was measured spectrophotometrically at 282 nm. Urea of "chemically pure" grade was used without further purification. Optical rotatory dispersion measurements were made on a Cary-60-spectropolarimeter in a 2 ml cell (width 0.1 cm). Spectrophotometric studies were done with a Specord instrument in a 3 ml cell (width 1 cm). Stock solutions of 8.2 M and 12 M urea and of 1.07×10^{-3} M proflavine bisulphate (ϵ_{444} $3.3 \pm 0.1 \times 10^4$) were prepared in a 0.02 M Tris-HCl buffer, pH 7.6. pH was measured with a LPU-0.1 pH-meter. Proflavine solution, 0.1 ml, was added to the cell containing protein (9.3×10^{-5} M) and urea solutions. The initial absorption of the protein–proflavine complex (465 nm) was found by extrapolating the kinetic curve to zero time. Spectropolarimetric experiments were performed with 9.25×10^{-5} M protein solutions. From the observed α_{233} values was subtracted the rotation of the denatured protein, i.e. the value of α_{233} after denaturation of chymotrypsin or its methylated derivative for 30 min in 7.8 M urea solution. Absence of autolysis was shown in a blank experiment with chymotrypsin in 3–7 M urea solutions (up to 2 hr) by the ninhydrin method. CD-spectra were recorded on a Cary-60 instrument (fitted with a Cary 6001 accessory) at 25°, pH 4.8. Concentrations of the methylated and native enzymes were 1.1 mg/ml. The cell was 0.01 cm thick (at 180–250 nm), or 1 cm thick (at 250–350 nm).

3. Results and discussion

Earlier we have shown [6] that chymotrypsin methylated at the N^{ϵ}-atom of histidine-57, although practically catalytically inactive, completely retains its

* The paper is dedicated to Prof. A.E. Braunstein on his 70th anniversary.

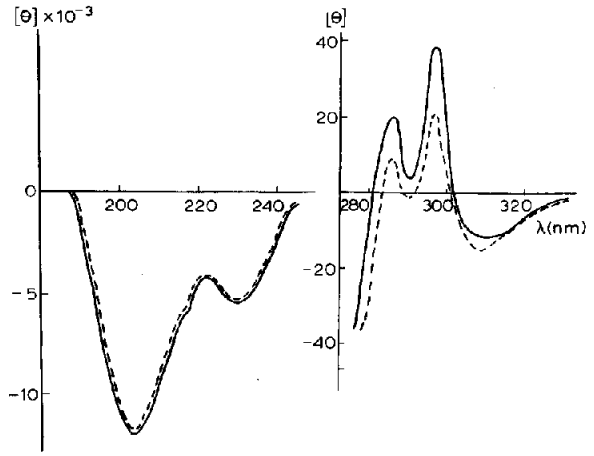

Fig. 1. CD Spectra of native (——) and methylated (- - - -) chymotrypsins.

Table 1
Denaturation rate constants for native and methylated chymotrypsins.

Urea concentration (M)	$k \cdot 10^2$, min^{-1}	
	Native	Methylated
1.8		0.5
2.7		0.7
4.5	0.5	2.0
4.95	0.7	2.3
5.12	8.9	17.5
5.65	14.5	26.7
6.15	20.4	42.3
7.5	83.0	83.0

substrate (ethyl *N*-acetyl-L-tyrosinate) or competitive inhibitor (proflavine) binding ability and without change in the binding stoichiometry*. The ORD and CD curves of the native and modified chymotrypsins (fig. 1), showed considerable similarity in the conformations of both proteins. Some differences between the CD curves refer mainly to the aromatic chromophores region, being practically zero for the far UV-region.

The denaturation velocity for both proteins was studied at various urea concentrations at 25°. The initial rate of denaturation was measured by following intensity changes in the trough of the ORD curve at 233 nm. In separate experiments the concentration change of the protein–proflavine complex was measured at 465 nm [8], reflecting the change in the protein binding capacity (fig. 2).

Table 1 summarizes the denaturation rate constants (calculated according to first-order kinetics) of chymotrypsin and its *N*-methylated derivative, for varying urea concentrations.

The data demonstrate that N^{ϵ}-methylhistidine-α-chymotrypsin is denaturated faster than the native enzyme. This is evident from fig. 3, which presents the concentrations of folded proteins *vs*. urea concentration after 15 min incubation. Under these conditions, α-chymotrypsin is 50% unfolded at 5.45 M urea concentrations, whereas N^{ϵ}-methylhistidine-57-α-chymotrypsin is unfolded to the same extent at urea concentrations as low as 4.65 M. The spectropolarimetric and dye binding data fall on the same curve for both the native and methylated protein indicating that both local and general conformational stabilities remain at the same level for the two different proteins (cf. [8]).

In summary it may be said that the charge relay system in chymotrypsin plays an important role not only in catalysis as had been shown earlier [3], but also in the conformational stability of the enzyme molecule. This gives a better understanding of the functioning of the enzyme. Thus on formation of the Michaelis complex it is the conformational rigidity of the enzyme that is the decisive factor, providing for the "key-to-lock" arrangement of the substrate and the active site. At the stage of acyl-enzyme formation (as well as at histidine-57 methylation), the charge relay system is destroyed, augmenting conformational lability of the enzyme. In order to cleave the acyl grouping the participation of a new nucleophile, water, is necessary. This can occur only if the active site undergoes a conformational perturbation [2] which apparently occurs as the result of decrease in the conformational rigidity of the entire molecule.

* Independently the same observation was made by Henderson [7].

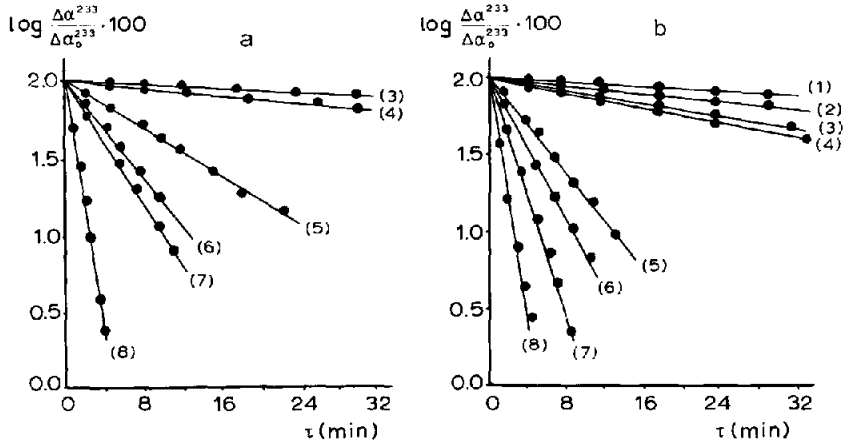

Fig. 2. Semilogarithmic kinetic plots of α-chymotrypsin (a) and N^{ϵ}-methylhistidine-57-α-chymotrypsin (b) at urea concentrations (M): (1) 1.8, (2) 2.7, (3) 4.5, (4) 4.95, (5) 5.12, (6) 5.65, (7) 6.15, (8) 7.5; concentrations of protein 6.4–9.25 × 10^{-5} M.

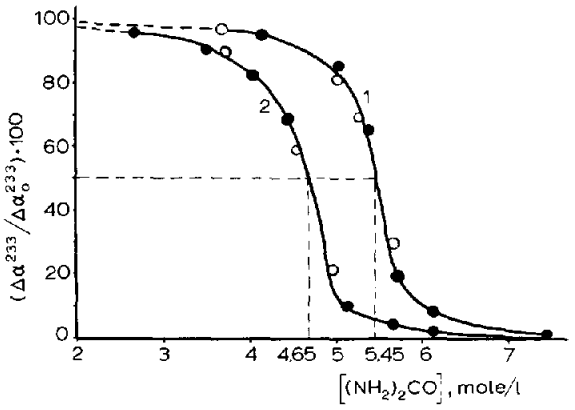

Fig. 3. Denatured protein fraction *vs.* urea concentration after 15 min incubation; (1), native chymotrypsin, (2), methylated chymotrypsin. The data were obtained by proflavine (o—o—o) and ORD (•—•—•) techniques.

References

[1] T.A. Steitz, R. Henderson and D.M. Blow, J. Mol. Biol. 46 (1969) 337.
[2] R. Henderson, J. Mol. Biol. 54 (1970) 341.
[3] D.M. Blow, J.J. Birktoff and B.S. Hartley, Nature 221 (1969) 337.
[4] Y. Nakagawa and M.L. Bender, Biochemistry 9 (1970) 259.
[5] J.M. Dechary, A.C.F. Mason and W.B. Carney, Anal. Biochem. 30 (1969) 142.
[6] V.K. Antonov and T.I. Vorotyntzeva, Dokl. Akad. Nauk SSSR 202 (1972) 201.
[7] R. Henderson, Biochem. J. 124 (1971) 13.
[8] T.I. Vorotyntzeva and V.K. Antonov, Molekul. Biol. 6 (1972) 63.