BOVINE LENS LEUCINE AMINOPEPTIDASE NUMBER AND STATE OF TRYPTOPHYL RESIDUES

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1. Introduction

Investigating the spectral behaviour and the enzymatic activity of the leucine aminopeptidase in presence of denaturants, a typical change in the absorption region of the tryptophyl and tyrosyl residues was observed, which was accompanied by changes in enzymatic activity [1,2]. But different values have been published for the number of tryptophyl residues of the enzyme [3,4] and no information has been available concerning the state of the chromophores. In the present paper an attempt was made to estimate the number and the arrangement of the tryptophyls in the enzyme molecule using the techniques of thermal perturbation [5–7] and solvent perturbation [7–9]. The results are, on the one hand a contribution to the elucidation of the topography of the enzyme molecule, and on the other hand an aid in the interpretation of fluorescence spectra of the enzyme.

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** *Abbreviations:* LAP Leucine aminopeptidase, EC 3.4.11.1; Tris 2-Amino-2 hydroxymethyl-propandiol-(1,3); GuHCl Guanidine hydrochloride; *N*-Ac-Tyr-OEt *N*-Acetyl-tyrosine-ethylester.

2. Materials and methods

Twice re-crystallized LAP** (Zn-enzyme) was prepared by the procedure of Hanson et al. [10] and was solved in 0.1 M Tris–HCl buffer pH 8. Enzyme concentrations were determined spectrophotometrically at 282 nm using a molar absorption coefficient of 3.2×10^5 M^{-1} cm^{-1} (calculated by means of the relation $A^{1\%}_{282\,nm} = 14.75 - 0.625 \times pH$; mol. wt = 326 000 [11,12]). *N*-Ac-Tyr-OEt (Mann Research Laboratories), L-Tryptophan (Schuchardt), methanol (Merck), polyethylene glycol 'Carbowax 300' (Merck-Schuchardt), Tris (Ferak Berlin), GuHCl (ultra pure, Schwarz/Mann) were used without further purification. The concentrations of the model compounds were determined using the molar absorption coefficients [9].

Spectral measurements were performed with a Beckman Acta CV spectrophotometer in the optical density range 0 to 0.1 using quartz cells of 1 cm path length closed with Teflon stoppers. The temperature was checked by thermistors. The base line was adjusted in the range of 340 to 240 nm at 25°C.

An appropriate concentration of the model compounds and the enzyme was chosen to get a maximum of extinction between 1 and 2 units. In distances of 2°C the thermal difference spectra were recorded. The mean value of multiple measurements was calculated.

In the range of 10–25°C a linear correlation between

$\Delta\epsilon$ and temperature was found. The ratio of $\Delta(\Delta\epsilon)/°C$ for the protein to $\Delta(\Delta\epsilon)/°C$ for the model compound was taken as the number of exposed chromophores.

Following the experimental conditions described by Herskovits et al. [9] solvent perturbation experiments were performed at 25°C ± 0.2°C in rectangular tandem cells of 0.876 cm path length (Firma Hellma). The mean error of the measurements was 2–3% in the case of model compounds L-tryptophan and *N*-Ac-Tyr-OEt, and 5% for the protein.

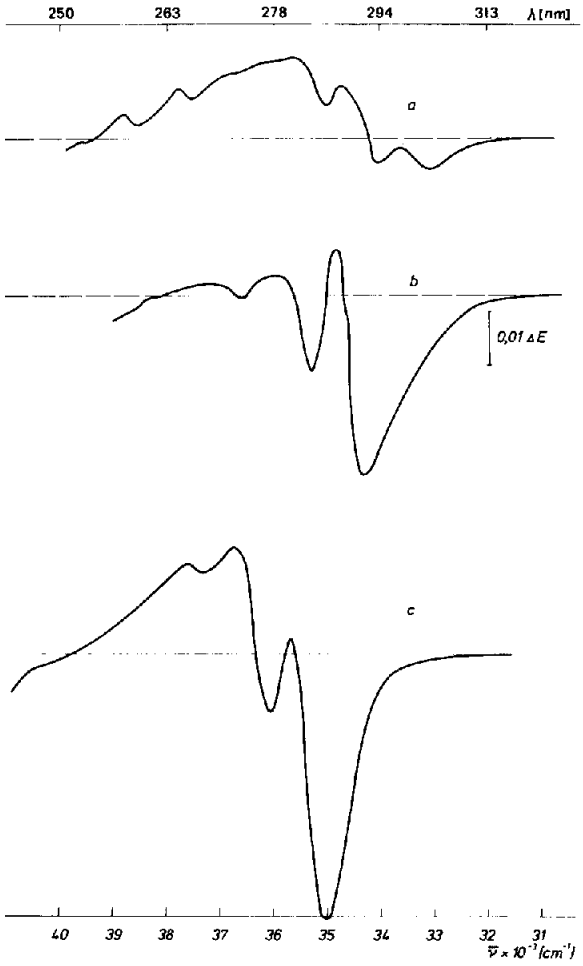

Fig.1. Thermal perturbation difference spectra. (a) LAP (6.6×10^{-6} M); (b) L-tryptophan (2.4×10^{-4} M); (c) *N*-Ac-Tyr-OEt (9.7×10^{-4} M) in 0.1 M Tris/HCl pH 8.0. The reference cell was kept at 25°C while the other cell was brought to 10°C.

3. Results and discussion

3.1. *Thermal perturbation*

The thermal perturbation difference spectrum of leucine aminopeptidase with 2 negative extrema at 300 nm and 293 nm, resp., and a broad extrema at 287 nm is shown in fig.1a. This difference spectrum resembles extensively that of tryptophan (cf. fig.1b). The plot $\Delta\epsilon$ versus temperature gives a straight line indicating that no conformational changes take place in the studied range of 10°C to 25°C (fig.2). This result is in correspondence with the established temperature stability of the enzyme [10]. The results of the thermal perturbation are listed in table 1. The slope line resulted in 7.7 (at 300 nm) and 8.3 (at 293 nm) exposed tryptophyl residues in the native protein. In the same manner the number of 41.5 exposed tryptophyls per mole enzyme was obtained for GuHCl-

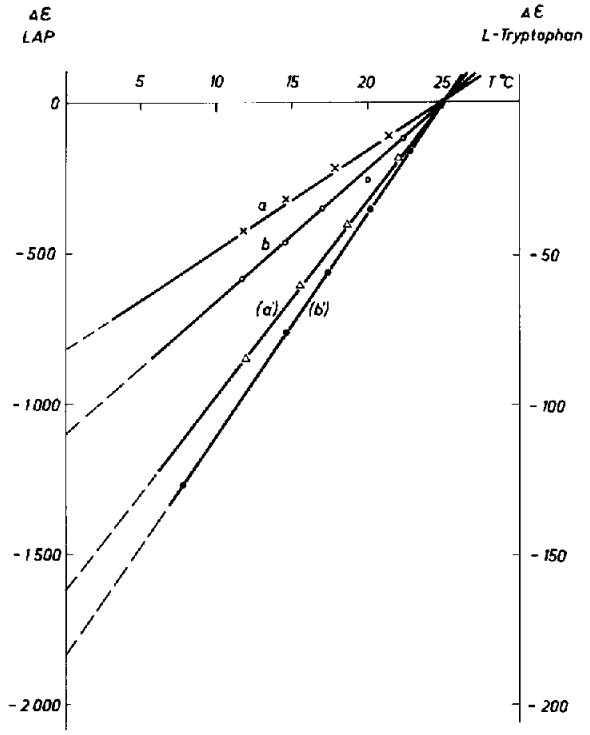

Fig.2. Changes of the molar absorption coefficient as function of the sample temperature. (a) LAP measured at 300 nm; (a') LAP at 293 nm; (b) L-tryptophan at 300 nm; (b') L-tryptophan at 293 nm. Concentrations and buffer as described in fig.1.

Table 1
Thermal perturbation spectral data of L-tryptophan and LAP

Substance	Medium	λ (nm)	Slope Δ(Δε)/°C	Δ(Δε)/°C LAP / Δ(Δε)/°C Tryptophan
L-Trypto-phan	0.1 M Tris/HCl pH = 8.0	300	4.4	
		293	7.4	
LAP	0.1 M Tris/HCl pH = 8.0	300	34	7.7
		293	62	8.3
L-Trypto-phan	0.1 M Tris/HCl 6 M GuHCl pH = 8.0	300	3.2	
LAP	0.1 M Tris/HCl 6 M GuHCl pH = 8.0	300	132	41.5

treated enzyme. This number corresponds well with the value found by oxidation with *N*-bromosuccinimide in urea solution [1].

3.2. *Solvent perturbation*

The solvent perturbation spectra (cf. fig.3) show 2 negative extrema at 292 nm and 285 nm, resp., which are chiefly cuased by tryptophan residues. Only the tryptophan residues were considered in the interpretation, since the error in the spectrophotometrical determinations of tyrosine is too large due to the unfavourable ratio of the aromatic amino acids in the enzyme protein (48 tyrosine and 120 phenylalanine residues [12]).

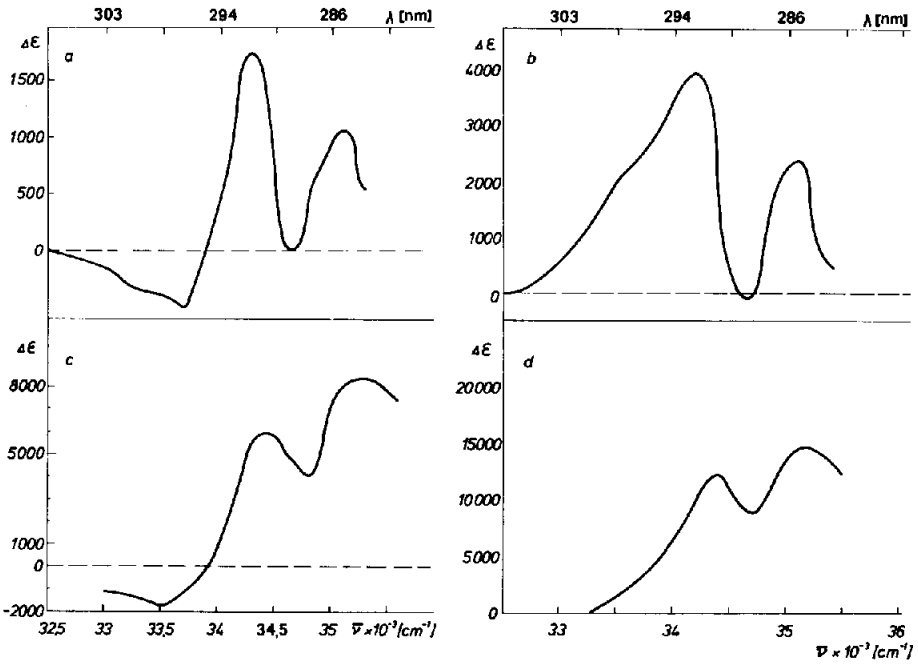

Fig.3. Solvent perturbation spectra of LAP at pH 8.0. (a) + 20% (v/v) methanol; (b) + 20% (v/v) 'Carbowax 300'; (c) in 6 M GuHcl + 20% (v/v) methanol; (d) in 6 M GuHCl + 20% (v/v) 'Carbowax 300'.

Table 2
Number of exposed tryptophyl residues of LAP

Medium	Perturbant	λ (nm)	Moles of exposed residues per mole enzyme
Buffer[a]	Methanol 20% (v/v)	292	7.5
Buffer[a]	'Carbowax 300' 20% (v/v)	292	7.6
Buffer[a]	Methanol	300	41.5
+ 6 M GuHCl	20% (v/v)	291	42.0
Buffer[a]	'Carbowax 300'	300	31.5
+ 6 M GuHCl	20% (v/v)	291	30.5

[a] 0.04 M Tris/HCl pH 8.0; temperature 25°C

The results of the measurements with methanol and 'Carbowax 300' listed in table 2 do not show significant differences in the number of exposed tryptophyls despite the different diameter of the used perturbant molecule (2.8 Å and 9.2 Å, resp.). The detected number of 7.5 exposed tryptophyls agrees well with the results observed during the oxidation of the enzyme with *N*-bromosuccinimide [1]. 6 to 8 residues could be modified without loss of activity. A higher degree of modification caused a decrease of enzymatic activity.

In 6 M GuHCl the absorption extrema at 292 and 285 nm, resp., shift to shorter wavelength. Using the perturbant methanol a number of 41.5 exposed tryptophyl residues was found.

With the perturbant 'Carbowax 300' the number of exposed residues is lower indicating that not all residues are accessible to the perturbant under the chosen experimental conditions.

(During the preparation of this paper the value of 40 tryptophyl residues per mole enzyme was determined using the method of Edelhoch [13]. Lasch, J., personal communication.)

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