Journal of Thermal Analysis and Calorimetry, Vol. 66 (2001) 123–132

PROTONATION CURVES OF THE D10N VARIANT OF ENDOGLUCANASE CEL45 CATALYTIC DOMAIN FROM *HUMICOLA INSOLENS*
Isothermal calorimetry and combined isoelectric focusing-electrophoresis

F. Attanasio[1], *M. Bruschi*[1], *G. Candiano*[2], *R. Ponassi*[1], *N. Castagnino*[1], *M. Schülein*[3] *and G. Rialdi*[1*]

[1]Istituto di Studi Chimico-Fisici di Macromolecole Sintetiche e Naturali, CNR, Via De Marini 6, 16149 Genova, Italy
[2]Istituto G. Gaslini, Dipt. di Nefrologia Genova, Italy
[3]Novozymes A/S, Bagsvaerd, Denmark

Abstract

Titration of ionizable groups of D10N variant catalytic domain has been performed by isothermal calorimetric technique and by combined isoelectric focusing-electrophoresis in the range of pH 3–9. pK_i^o and ΔH_i^o related to the ionizable groups have been estimated using Lindestrøm–Lang equation. Both experimental techniques are in agreement and confirm the validity of the applied theory. Slight differences in protonation enthalpies of carboxyl groups are caused by Asp10 interaction with the other part of the macromolecule.

Keywords: Cel45, protein isoelectric focusing electrophoresis, protein isothermal calorimetry, protonation heat

Introduction

Protonation heat of the Cel45 protein endoglucanase catalytic domain – formerly named EGV [1] – cloned from *Humicola insolens* fungus and produced by *Aspergillus oryzae*, has been reported in a previous work [2]. The macromolecule consists of 210 amino acids. It hydrolyzes β-1,4 links with configuration inversion at the anomeric carbon. The number of ionizable groups present in the molecular structure and exposed to the solvent, as well as ionization pK_i^o and ΔH_i^o, ionization heats of each single group, have been determined by isothermal calorimetry (ITC). Combined isoelectric focusing and electrophoresis have also been applied to confirm the electrical charges of the molecule exposed to the solvent and their pK_i^o. Both analytical procedures have given the same results [3].

* Author to whom all correspondence should be addressed.

1418–2874/2001/ $ 5.00
Akadémiai Kiadó, Budapest

In this work, the catalytic domain of this cellulase variant, D10N, where the –COOH in position 10 has been removed, has been prepared and purified according to the already reported procedure [4]. The electrical charge has been calculated according to Lindestrøm–Lang theory and compared with results obtained by ITC and IEFE.

The three-dimensional structure of Cel45 catalytic domain in native, inactive mutant (D10N), and oligosaccharide-bound forms has been determined at a resolution of 1.9 Å [4, 5].

Experimental

Protein preparation

The catalytic domain variant (D10N-core) has been produced by cloning the gene and transforming it into *Aspergillus oryzae*. A plasmid was used with the gene inserted between the gene coding for fungal amylase promoter and the gene coding for the AMG terminator from *A. niger* [6].

The protein was separated from *Aspergillus* proteins by cation exchange chromatography on the extracellular proteins. Proteins were ultrafiltrated (concentrated and washed with water) on a DOW GR81PP membrane with a 6 kD cut-off, until eluate conductivity dropped below 1000 mS cm^{-1}. The sample was applied to an S-Sepharose column with a pH 3.5 balance obtained with a 20 mM citrate buffer. The pure eluted enzyme was concentrated on an Amicon cell with DOW GR81PP membrane and then lyophilised. The lyophilised powder was dissolved in degassed 50 mM NaCl solution without buffer and then gel filtrated in a BioGel P10 column (350 mm h; 0.8 mm ř).

Protein concentration was spectrophotometrically determined by means of a Cary BIO-400 instrument (Varian, Palo Alto, CA, USA) at 280 nm using a $E_{1cm}^{1\%}$=1.846 and assuming a molecular mass of 22.855 [7]. Measured absorbance was corrected for light scattering according to Leach and Sheraga [8].

For calorimetric ITC experiments, the pH in the final solution was adjusted to 8.40±0.03 (Orion, Boston, MA, USA) using small amounts of NaOH solution.

Calorimetric titration (ITC)

Microcalorimetric experiments were carried out at 30°C by isothermal titration microcalorimeter (ITC) (Microcal, Northampton, MA, USA) [9] equipped with 250 μL microsyringe.

Protein concentration was 0.082 mM and the effective calorimetric cell volume 1.3622 mL. A $5 \cdot 10^{-3}$ mol L^{-1} HCl solution, also containing $50 \cdot 10^{-3}$ mol L^{-1} NaCl, was placed in the calorimetric syringe and 28 injections of 4–16 μL were made. The stirring device rotated at 500 rpm. Any corrections for dilution heats of the solutions, always very small, were made on observed heat effects. Ionisation enthalpy was calculated based on peak areas after drawing the baseline (Fig. 1) and using Origin software program (Microcal, Northampton, MA, USA).

Electrophoretic titration (IEFE)

The experimental procedure was performed according to Righetti *et al.* [10]. Square gel slabs (145×145×1.5 mm) containing acrylamide (5.02% T, 1.96% C) and 2.5% mass/v carrier ampholytes, pH 3-10 were prepared at 25°C. Two-dimensional separation was carried out: the first-dimensional separation was run at 10°C at constant voltage (400 V) in order to develop a pH gradient and to reach a steady state; in the second-dimensional separation, electrode gel layers were cut away, the trench was filled with the sample (200 μg of protein), and new electrode strips were overlaid onto the gel. Electrophoresis was performed perpendicular to the first dimension, turning the gel 90°, constantly at 10°C. 0.25 M electrolytes were employed, with NaOH at the cathode and H_3PO_4 at the anode. The pH gradient was measured cutting the gel into 29 slices (each 0.5 cm wide), perpendicular to the central trough and eluted with 5 mL of 10 mM KCl. The values were read with a digital pH meter. The gel with the protein pattern was stained with Coomassie Brilliant Blue R-250.

Results

The net Z charge of the macromolecule was calculated according to Lindestrøm–Lang equation (Eq. 1) [11] in a pH range between 2 and 10. It was assumed that the protein remained compact and no structural changes occurred throughout the pH range.

In analogy to the calculations reported in [2], it was also assumed that all charged groups were exposed to the solvent. They were divided in seven titrable groups.

$$\mathrm{pH}-\log\left[\frac{n_\mathrm{i}}{n_\mathrm{i}^\mathrm{o}-n_\mathrm{i}}\right]=pK_\mathrm{i}^\mathrm{o}-\left(\frac{1}{2.3RT}\right)wz \tag{1}$$

where n_i is the number of groups of ionised type i out of a total of n_i^o, calculated from aminoacid composition [2]; pK_i^o is the intrinsic pK of this group of side chains, R is the gas constant, T is temperature. The electrostatic factor w was calculated according to the Debye-Hückel theory:

$$w=\frac{e^2}{2DR_\mathrm{s}kT}\left(1-\frac{kR_\mathrm{s}}{1+ka}\right) \tag{2}$$

where e is the electron elementary charge, D the dielectric constant at 30°C, R_s=1.693 nm the protein Stokes radius, a=R_s+0.2 nm the ion exclusion radius, and k the Debye–Hückel parameter

$$k=\left(\frac{8\pi Ne^2}{1000DkT}\right)^{1/2}I^{1/2} \tag{3}$$

where N is Avogadro's number, k Boltzmann's constant.

For ITC technique, the ionic strength was 50 mole m^{-3}; for IEFE, it was estimated using the following equation according to Righetti [12]:

$$I = \frac{1}{20} C_{\text{amph}} + C_{\text{H}} \text{ (or } C_{\text{OH}}) \tag{4}$$

where $C_{\text{amph}}=3.57\cdot10^{-2}$ mol L^{-1} is the molarity of focused carrier ampholytes and C_{H} (or C_{OH}) the molarity of protons (oxydryl ions) at a given pH.

Protein protonation heat can be calculated assuming that all ionizable amino acids contribute to it with their protonation enthalpy change, depending on the extent of protonation at different pH values.

$$\Delta H^{\circ}_{\text{ion}} = \sum n_{\text{i}} \Delta H^{\circ}_{\text{i}} \tag{5}$$

where n_{i} is the number of groups of type *i* ionised out of a total of n°_{i}, and $\Delta H^{\circ}_{\text{i}}$ the value of the ionisation heat observed.

Calorimetric titration curve by isothermal titration calorimetry

Figure 1 shows the heat evolved during isothermal titration of the D10N-core protein. In the experiment reported in the figure (starting pH=8.25 not shown), the starting pH was 8.40. The heat developed as a function of added HCl is shown in Fig. 2. The amount of protons, $[H^{+}]$, reacting with the ionizable groups, and the amount of free pH modifying protons in solution have been calculated from the theoretical curve. As a first approximation, protonation heat values of the single groups reported for other proteins [15–23] have been used to draw a theoretical curve. The differences from the experimental curves have been minimised by an iterative method. The calculated isoelectric point, $pI_{\text{c}}=5.76\pm0.05$, corresponds to the calculated experimental value and to the value experimentally measured by electrophocusing procedure (see below).

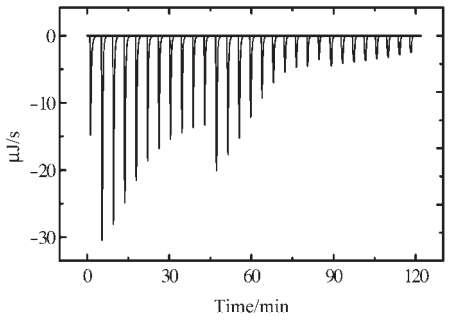

Fig. 1 Heat evolved during titration of D10N-core by isothermal calorimetry at 30°C. 29 injections, 1 of 2 μL, 10 of 4 μL, 10 of 8 μL and 8 of 16 μL, of 5 mM HCl in the presence of 50 mM of NaCl; starting pH=8.40

The sum of heat evolved by acid solution injections *vs.* the amount of added protons is reported in Fig. 2. The line represents the calculated curve after error minimisation.

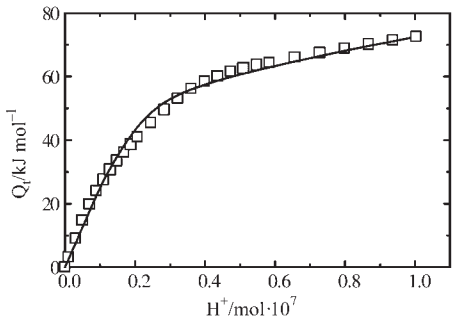

Fig. 2 Heat developed during D10N-core thermal titration depending on added HCl amount. (□) Experimental data point, (—) calculated best-fitting curve

Electrophoretic titration curve by isoelectric focusing electrophoresis

D10N-core variant has been assumed to maintain the same molecular dimensions of Cel45-core, as calculated from X-ray experiments [4]. Therefore, the frictional coefficient will be the same as calculated for Cel45-core [3]. In any case, the molecule frictional coefficient needs correction depending on the experimental conditions: i) the molecule shape must be taken into account and the equation referred to an oblate ellipsoidal structure must be applied [11, 25, 26]; ii) the gel pore size can be exactly calculated and reproducibility controlled by total acrylamide concentration T; $\%T$ (apparent gel concentration) influence must be corrected according to the procedure reported for the wild type molecule [3, 28, 29]; iii) the ion atmosphere surrounding the macromolecule must be considered and the Henry equation [27] must be applied [3]. Experimental controls have been performed to verify that the constants for the equations referred to D10N-core are the same as those reported for the wild type molecule.

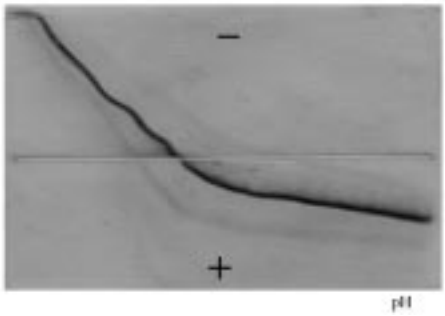

Fig. 3 Titration curve of D10N-core. Running test conditions were described in experimental electrophoresis titration

The electrophoretic titration curve, corresponding to electrophoresis across a stationary pH gradient is reported in Fig. 3. A single curve indicating the presence of a monodisperse protein has been observed. Protein electrophoretic mobility, U, was

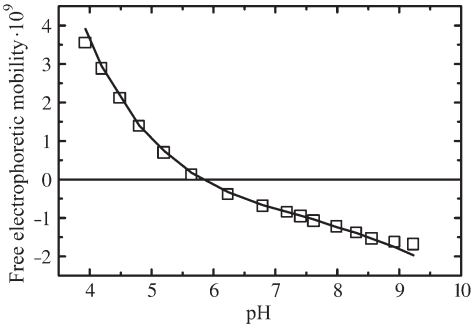

Fig. 4 Electrophoretic mobility, *U*, of D10N-core as a function of pH increase. (□) experimental data points; (—) best fitting curve according Eq. (6) using the best estimate of n_i, pK_i^o in the Table 1

measured as a function of monotonic pH increase (Fig. 4). Electrophoretic mobility values are related to the net macromolecule charge, *Z*, calculated from Eq. 1, according to the following equation:

$$U = \frac{v}{E} = \frac{Ze}{f} [\mathrm{m}^2\ \mathrm{V}^{-1}\ \mathrm{s}^{-1}] \tag{6}$$

where *U* is the electrophoretic mobility, *v* is the migration velocity and *f* the frictional coefficient after corrections as reported, *e* is the electron elementary charge. The best estimate of experimental n_i, pK_i^o, *w* and *f* for D10N-core was obtained by fitting electrophoretic titration values to Eq. (6) (Table 1). A graphical representation of *w*=0.181±0.002 for different ionizable groups is reported in Fig. 5 according to Eq. (1), where the intercept is the experimental value of pK_i for the single groups and *w* is calculated from the slopes of the lines.

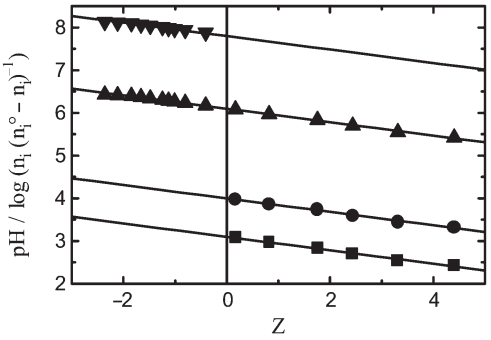

Fig. 5 Graphic representation of Eq. (1) for titrable groups, where pK_i's can be calculated from intercepts and *w* from slopes. (▪) α-COOH, *w*=0.181±0.002, *pK*=3.096±0.005, *R*=0.998; (•) R-COOH, *w*=0.181±0.002, *pK*=3.997±0.005, *R*=0.998; (▲) Imidazole, *w*=0.181±0.002, *pK*=6.097±0.005, *R*=0.998; (▼) ε-amino, *w*=0.180±0.005, *pK*=7.797±0.002, *R*=0.998

Table 1 Ionization parameters of the catalytic domain of Cel45-core and D10N variant calculated from Lindestrøm–Lang, ITC and IEFE techniques

	Cel45-core[a]						D10N-core					
	IEFE			ITC			IEFE			ITC		
Group	N° theor.	N° exp.	pK_i	N° exp.	pK_i	$\Delta H°$/ kJ mol^{-1}	N° theor.	N° exp.	pK_i	N° exp.	pK_i	$\Delta H°$/ kJ mol^{-1}
α-COOH	1	1	3.5±0.2	1	3.13±0.01	5.0±0.4	1	1	3.10 ±0.20	1	3.10±0.02	4.2±0.4
R-COOH	21	21	3.80±0.02	21	3.87±0.01	4.68 ±0.04	20	20	4.00±0.02	20	3.87±0.01	4.18±0.04
Imidazole	1	1	6.98±0.01	1	6.90±0.02	28.42±0.04	1	1	6.10±0.01	1	6.15±0.02	28.55±0.04
α-amino	1	1	7.80±0.02	1	7.90±0.01	39.7±0.4	1	1	7.80±0.02	1	7.90±0.01	37.6±0.4
Phenol	5	5	10.2±0.3	5	10.10±0.01	25.5±0.4	5	5	10.10±0.4	5	10.10±0.01	25.5±0.4
α-amino	8	8	10.4±0.3	8	10.50±0.05	25.6±0.4	8	8	10.50±0.4	8	10.50±0.05	25.6±0.4
Guanidyl	11	11	>12	11	12.6±0.1	58±4	11	11	>13	11	12.6±0.1	54±4
pI			5.12±0.01		5.14±0.05				5.74 ±0.01		5.76±0.05	
w[b]			0.170±0.002		0.097±0.002				0.181±0.002		0.064±0.003	

[a]Values reported in [2, 3]
[b]w values are not referred at the same temperature; IEFE: 10°C, ITC: 30°C

pH values corresponding to zero mobility, indicating zero charge on the macromolecule, are in agreement with calculated values, Eq. (1), as well as with calorimetric values (Table 1).

Discussion

Figure 6 indicates the electrical charge of both macromolecules calculated by Lindestrøm–Lang Eq. (1) assuming that all theoretical postulates are valid: the protein remains compact and impenetrable to the solvent; all protonable groups are exposed to the solvent; no structural changes occur throughout the pH range. The points in triangles and circles represent experimental data: the results are in agreement with both techniques. The straight line indicates the best fitting parameters with experimental values obtained by the two techniques with the D10N variant. The dotted line refers to the calculations and experimental values reported in previous works [2, 3] with Cel45-catalytic domain.

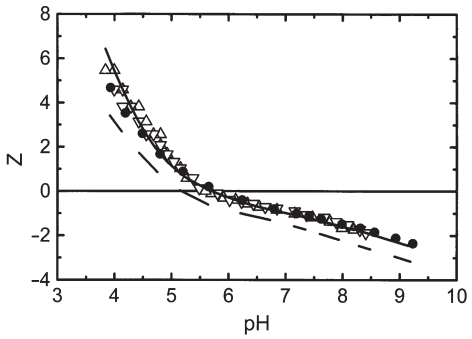

Fig. 6 Titration curve of D10N catalytic domain calculated from electrophoretic migration (Fig. 5) and from isothermal calorimetry (Fig. 2) at different pH values. The solid line represents the best fitting of calculated values obtained from Eq. (1) using the ionization parameters reported in Table 1 for D10N-core; the dashed line represents the calculated and experimental values for Cel45-core. Experimental values: (Δ) starting pH=8.40 and (∇) starting pH=8.25, calorimetric values from two different runs; (•) IEFE values

The structural difference between the two proteins – wild type and its variant – is determined by the removal of one negative charge from –COOH in position 10. The agreement between the calculated curves according to Lindestrøm–Lang theory in both experimental procedures indicates that the analytical method is also valid for the mutated protein. Similarly to the wild type protein, there is agreement in the whole range of investigated pH, therefore the protein appears stable. Actually, the *w* value is constant and depends on macromolecule shape and conformation [11].

Furthermore, ITC technique measures the heat developed during titration, according Eq. (5). It must be pointed out that the enthalpic contribution during protonation of ionizable groups is in the order of 2–4 kJ mol^{-1} for carboxyl groups and 40 kJ mol^{-1} for amino groups, as reported for model compounds [13, 14] or standard proteins [15–23]. In

the case of an unfolding transition, the heat measured will be the sum of all protonation heat values plus the heat due to unfolding. This last term is remarkably higher and easy to detect, as reported by [20]. From this point of view, ITC is more sensitive than IEFE. The agreement between the experimental curve and the calculated one confirms that no unfolding process takes place during the titration experiment.

Neither experimental technique is able to distinguish the values of the single ionizable groups, if their properties are not significantly different [19, 24], i.e. their values are calculated as the average of all them. In this case, assuming that the two molecules – wild type and D10N variant – have similar conformations, the single carboxyl group of the aspartic 10 can be studied by subtraction of the two curves. Its pK_i cannot be differentiated, but the enthalpic contribution is different by ΔH= 14.68 kJ mol^{-1}. The enthalpic contribution calculated for this D10 carboxyl group is too high to be considered as protonation heat only. Some other interactions must be taken into account. The molecular structure calculated by X-ray measurements [4] on the wild type and the D10N variant suggest different interactions of this aspartic amino acid, in particular with the Pro125–Asp165 region. This molecular region can be considered as an independent molecular region with few interactions with the remaining molecule which has a compact barrel structure. Asp10 also interacts with two water molecules. A slight structural change referred to the variant molecule is reported by Davies [4].

From this remark, D10N variant conformation can be assumed to be very similar to the wild type molecule: shape, frictional coefficient, *w*, stability etc. are not modified. On the other hand, the assumption of a 'slight structural change' [4] is confirmed by protonation heat differences of Asp10 carboxyl group.

* * *

This research was supported by the European Commission, Biotechnology Programme, BIO4-97-2303.

References

1 B. Henrissat, T. T. Teeri and R. A. J. Warren, FEBS, Lett., 425 (1998) 352.
2 R. Galletto, F. Attanasio, E. Dossi, M. Schülein and G. Rialdi, Thermochim. Acta, 321 (1998) 17.
3 F. Attanasio, M. Bruschi, G. Candiano, R. Galletto, L. Musante, M. Schülein and G. Rialdi, Electrophoresis, 20 (1999) 1403.
4 G. J. Davies, S. P. Tolley, B. Henrissat, C. Hjort and M. Schülein, Biochemistry, 34 (1995) 16210.
5 G. J. Davies, G. G. Dodson, R. E. Hubbard, S. P. Tolley, Z. Dauter, K. S. Wilson, C. Hjort, J. M. Mikkelsen, G. Rasmussen and M. Schülein, Nature, 365 (1993) 362.
6 T. Christensen, H. Wöldike, E. Boel, S. B. Mortensen, K. Hjortshoj, L. Thim and M. T. Hansen, Biotechnology, 4 (1988) 1419.
7 M. Schülein, Biochemical J., 57 (1997) 71.
8 S. J. Leach and H. A. Scheraga, J. Am. Chem. Soc., 82 (1960) 4790.

9 T. Wiseman, S. Williston, J. F. Brandts and L. Lin, Anal. Biochemistry, 179 (1989) 131.
10 P. G. Righetti, R. Krishnamoorthy, E. Giannazza and D. Labie, J. Chromatogr., 166 (1978) 455.
11 C. Tanford, Physical Chemistry of Macromolecules, J. Wiley & Son, New York 1961, p. 317.
12 P. G. Righetti, Isoelectric Focusing: Theory, Methodology and Applications, Elsevier Biomedical Press, Amsterdam 1983, p. 1.
13 J. T. Edsall and J. Wyman, Biophysical Chemistry, Academic, New York 1958, p. 447.
14 C. Tanford, Advan. Protein Chem., 17 (1962) 69.
15 J. Hermans Jr. and G. Rialdi, Biochemistry, 4 (1965) 1277.
16 G. C. Kresheck and H. A. Sheraga, J. Am. Chem. Soc., 88 (1966) 4588.
17 M. A. Marini and C. J. Martin, Met. Enzymol., 27 (1973) 590.
18 C. J. Martin and M. A. Marini, Anal. Chem., 8 (1979) 221.
19 C. Bjurulf, Eur. J. Biochem., 30 (1972) 33.
20 D. D. Shiao and J. M. Sturtevant, Biopolymers, 15 (1976) 1201.
21 C. Tanford and M. L. Wagner, J. Am. Chem. Soc., 76 (1954) 3331.
22 R. M. Izatt and J. J. Christiansen, Vol. 1, Handbook of Biochemistry and Molecular Biology-Physical Chemical Data, CRC Press Inc., Cleveland, USA 1976, p. 151.
23 R. Biltonen, A. T. Schwartz and I. Wadso, Biochemistry, 10 (1971) 3417.
24 C. R. Cantor and P. R. Schimmel, Vol. 1, Biophysical Chemistry, W. H. Freeman, Co., San Francisco, USA 1980, p. 50.
25 C. R. Cantor and P. R. Schimmel, Vol. 2, Biophysical Chemistry, W. H. Freeman, Co., San Francisco, USA 1980, p. 539.
26 K. E. Van Holden, Physical Biochemistry, L. Hager, F. Wold (Eds.), Prentice-Hall, Englewood Cliffs, NJ 1971, p. 122.
27 D. C. Henry, Proc. Roy. Soc. A, 133 (1931) 106.
28 G. M. Rothe, Adv. Electrophor., 4 (1991) 251.
29 G. M. Rothe, Electrophoresis of Enzymes Laboratory Methods, G. M. Rothe (Eds), Springer-Verlag, Berlin 1994, p.71.