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Thermostabilization of carboxymethylcellulase from Aspergillus niger by carboxyl group modification

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The carboxyl groups of purified carboxymethylcellulase (CMCase) from Aspergillus niger NIAB280 were modified by 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC) in the presence of glycinamide for 15 min (GAM15) and glycinamide plus cellobiose for 75 min (GAM75). The half-lives of GAM15 at different temperatures were significantly enhanced whereas those of GAM75 were reduced as compared with the native CMCase. The activation energies of denaturation of native, GAM15 and GAM75 were 40, 35 and 59 kJ mol^{-1} respectively. Native CMCase and GAM15 showed no compensation effect, whereas native and GAM75 gave temperature of compensation of 44°C. Gibb's free energy of activation for denaturation (ΔG^*) of GAM15 was increased as compared with native CMCase. Surprisingly the entropies (ΔS^*) of activation for denaturation were negative for native and GAM75 and decreased further for GAM15 between the temperature range of 45 to 65°C. A possible explanation for the thermal inactivation of native and increased thermal stability of GAM15 is also discussed.

Introduction

Carboxymethylcellulase (endo-β-1,4-glucanase, EC 3.2.1.4) cleaves β-linked bonds within cellulose molecule. It is highly desirable that the thermal stability of this industrially important enzyme be increased so that the hydrolysis of cellulose could be carried out at higher temperatures (Godfrey and West, 1996). Previously we have shown that non-covalently attached polysaccharides impart stability to CMCase from *Aspergillus niger* (Siddiqui *et al.*, 1996). There are reports of thermostabilization of enzymes by chemical modification. Acetylation of amino groups of horseraddish peroxidase (Miland *et al.*, 1996) and α-amylase from *Bacillus subtilis* (Urabe *et al.*, 1973) increased their stability toward heat. Charge reversal by chemical modification of carboxyl groups of glucoamylase from *Aspergillus niger* also increased its thermostability (Munch and Tritsch, 1990). However, there have been hardly any attempt to increase the thermal stability of CMCases by chemical modification of its carboxyl groups.

In this paper we report the effect of charge neutralization of purified CMCase from *Aspergillus niger* NIAB280 on its thermostability. We have also determined, for the first time, the thermodynamic parameters for denaturation of native and modified CMCases in order to get an insight into the mechanism of thermal unfolding pathway.

Materials and methods

CMCase used in this study was purified to homogeneity level (unpublished results).

CMCase assay

All CMCase assays were performed as described earlier (Siddiqui *et al.*, 1996) with the modification that assay solution contained 1.5% (w/v) carboxymethylcellulose-Na salt (CMC) as the substrate whose pH was adjusted to 5.2 with 2-[N-morpholino]ethanesulfonic acid.

Glycinamide modified CMCase for 15 min (GAM15)

Carboxyl groups of purified CMCase from *A. niger* were activated by 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC) in the presence of glycinamide as a nucleophile (Hoare and Koshland, 1967). Glycinamide (1 M) was added to 5 ml (11 U/ml) CMCase solution and the pH was adjusted to 5.5 with 12 M NaOH. The reaction was initiated by adding 0.05 g (50 mM) EDC. After 15 min the reaction was quenched by adding 5 ml 0.5 M sodium acetate buffer, pH 5.5. The modified CMCase was exhaustively dialyzed against 20 mM sodium acetate, pH 5.5, to remove reagents.

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Glycinamide modified CMCase for 75 min (GAM75)

As above except that the reaction mixture also contained 50 mM cellobiose as the competitive inhibitor of CMCase in order to protect the active-site carboxyls from modification and the reaction time was extended to 75 min.

Thermostability

Thermal inactivation of native and modified CMCases were determined by incubating the enzyme solution in 20 mM sodium acetate, pH 5.5 buffer. Aliquots were withdrawn at different time intervals, cooled on ice before assaying for residual CMCase activity at 40°C. This procedure was repeated at five temperatures. The data were fitted to first-order plots (Figs 1a,b,c). These semi-logarithmic plots were slightly concurved due to the presence of heterogeneous population of modified enzyme.

Activation energy for denaturation (E_a)

The first-order rate constants for denaturation (k_d) of native and modified CMCases at different temperatures were determined as described above under thermostability. These rate-constants (k_d) for each species were plotted (Fig. 1d) and analysed as described by Montes *et al.*(1995); and Munch and Tritsch (1990). The thermodynamic data were calculated by rearranging the Eyring absolute rate equation (Eyring and Stearn, 1939) as given below:

$$k_d = (K_B T/h)\ e^{(-\Delta H^*/RT)} \cdot e^{(\Delta S^*/R)} \qquad (1)$$

where h (Planck constant) = 6.63×10^{-34} Js and K_B (Boltzman constant, [R/N]) = 1.38×10^{-23} J K^{-1} where N (Avogadros no.) = 6.02×10^{-23} mol^{-1}.

$$\Delta H^* \text{ (enthalpy of activation)} = E_a - RT \qquad (2)$$

where R (gas constant) = 8.314 J K^{-1} mol^{-1}.

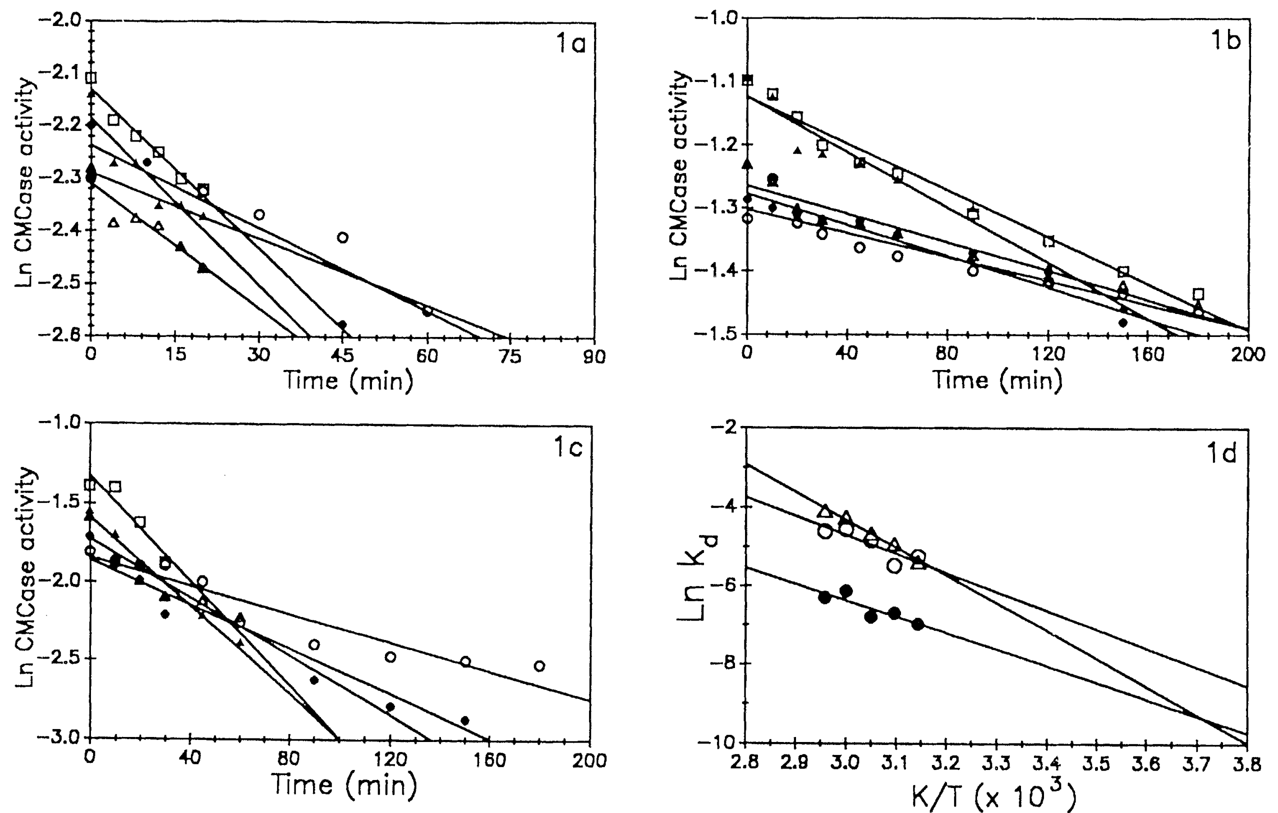

Figure 1a First-order plots of the effect of thermal denaturation on native CMCase. Samples were incubated at 45°C (open circle), 50°C (closed circle), 55°C (open triangle), 60°C (closed triangle) and 65°C (open square). **1b**: first-order plots of the effect of thermal denaturation on GAM15. **1c**: first-order plots of the effect of thermal denaturation on GAM75. **1d**: Arrhenius plot for the determination of activation energy (E_a) of denaturation of native CMCase (open circle), GAM15 (closed circle) and GAM75 (open triangle). The data regarding the first-order rate constants for native, GAM15 and GAM75 CMCases were taken from figures 1a, 1b and 1c respectively. The temperature corresponding to the point of intersection of native and GAM75 CMCases is called isokinetic temperature or temperature of compensation (T_c).

ΔG^* (free energy of activation)
$= -RT \cdot \ln[(k_d \cdot h)/(K_B \cdot T)]$ (3)

Equation 3 is derived by rearranging eq. (1).

ΔS^* (entropy of activation) $= (\Delta H^* - \Delta G^*)/T$ (4)

Results and discussion

Thermal denaturation of enzymes is a two step process as shown earlier (Montes *et al.*, 1995)

where N is the native enzyme, U is the unfolded inactive enzyme which could be reversibly refolded upon cooling and I is the inactivated enzyme formed after prolonged exposure to heat and therefore cannot be recovered upon cooling (Zale and Klibanov, 1986). The thermal denaturation of enzymes results in the breakage of non-covalent linkages including hydrophobic interactions with concomitant increase in the enthalpy of activation (ΔH^*). The opening up of the enzyme structure is accompanied by increase in the disorder or entropy of activation (ΔS^*) (Vieille and Zeikus, 1996) but contrary to this we found that native CMCase from *A. niger* has negative ΔS^* and positive ΔH^* between the temperature range of 45 and 65°C (Table 1). The denaturation of many proteins like chicken egg albumin (Eyring and Stearn, 1939) and lactoglobulin at low temperatures and in the presence of urea (Nelson and Hummel, 1962) show negative ΔS^* because water ordering increases in the vicinity of non-polar amino acids which are exposed during unfolding (Privalov and Gill, 1988). This ordering of water around hydrophobic residues is disrupted at higher temperatures; therefore, this could not be the reason for negative ΔS^* in case of native CMCase. The unfolding of hemoglobin in acid at 45°C also shows negative ΔS^* of –155 J mol^{-1} K^{-1} in the presence of acid (Eyring and Stearn, 1939), whereas the thermal denaturation of metmyoglobin at 40°C and high pressure gave ΔS^* of –84 J mol^{-1} K^{-1} (Zipp and Kauzmann, 1973). Eyring and Stearn (1939) have rated different denaturants in increasing values of ΔS^* with urea and acid denaturation producing minimum ΔS^* and salt and alcohol maximum ΔS^*. In case of α-amylase (apo-enzyme) from *Bacillus licheniformis*, thermal denaturation at even greater temperature of 80°C gave ΔS^* of –150 J mol^{-1} K^{-1}, ΔG^* of 103 kJ/mol and ΔH^* of 50.5 kJ mol^{-1} whereas surprisingly the same enzyme in the presence of Ca^{++} gave positive ΔS^* (Violet and Meunier, 1989). The values of all three thermodynamic parameters for native CMCase are given in Table 1. Apart from *A. niger* CMCase, this is the only other enzyme with negative ΔS^* at such a high temperature. At low temperatures, some enzymes such as chymotrypsinogen (Brandts, 1964) and *Staphylococcus* nuclease (Griko *et al.*, 1988) show cold denaturation phenomenon which means that below a certain critical temperature, called the temperature of maximum stability (T_{max}), both ΔS^* and ΔH^* have negative

Table 1 Thermodynamic and kinetic parameters for thermal inactivation of native CMCase from A. niger

T (K)	k_d (sec^{-1})	$t_{1/2}$ (min)	ΔH^* (kJ mol^{-1})	ΔG^* (kJ mol^{-1})	ΔS^* (J mol^{-1}K^{-1})
318	8.67×10^{-5}	133	37.26	102.78	–206
323	6.92×10^{-5}	167	37.22	105.04	–210
328	1.31×10^{-4}	88	37.18	104.97	–207
333	1.75×10^{-4}	66	37.14	105.80	–206
338	1.67×10^{-4}	69	37.10	107.57	–209

E_a (activation energy of denaturation) = 40 kJ mol^{-1} is calculated from Fig. 1d.

Table 2 Thermodynamic and kinetic parameters for thermal inactivation of GAM15 from A. niger

T (K)	k_d (min^{-1})	$t_{1/2}$ (min)	ΔH^* (kJ mol^{-1})	ΔG^* (kJ mol^{-1})	ΔS^* (J mol^{-1} K^{-1})
318	9.30×10^{-4}	745	32.14	96.50	–202
323	1.23×10^{-3}	564	32.09	97.31	–202
328	1.12×10^{-3}	619	32.05	99.12	–204
333	2.18×10^{-3}	318	32.01	98.82	–200
338	1.84×10^{-3}	376	31.97	100.83	–204

E_a (activation energy of denaturation) = 34.78 kJ mol^{-1} is calculated from Fig. 1d.

values. According to Brandts (1964), high hydrophobic content in the interior of the protein will raise T_{max} as in the case of thermal denaturation of metmyoglobin which was 25°C (Zipp and Kauzmann, 1973) as compared to 10°C in case of chymotrypsinogen. A possible explanation is as follows. *The polar interactions are weakened whereas hydrophobic ones are strenghtened with increasing temperatures. That could be the reason for negative* ΔS^* *as the resistance of the enzyme to unfolding due to stronger hydrophobic interactions overcomes the tendency of the enzyme to fall apart due to weakened polar interactions at high temperatures*. On the contrary, we found positive ΔS^* for β-glucosidase from *A. niger* under identical conditions of thermal denaturation (unpublished results).

Enzymes could be made more thermostable by either stabilizing the native form by putting non-covalent bonds including hydrogen bonds, salt-bridges and hydrophobic-interactions or by decreasing the entropy of unfolding (Daniel, 1996; Matthews *et al.*, 1987). These total stabilizing interactions will give an average protein a ΔG of approx. 1000 kJ mol^{-1} but in reality ΔG is only about 40–50 kJ mol^{-1} because the destabilizing force of entropy is decreased to counter-balance it. The neutralization of 1–5 carboxyls in case of GAM15 (unpublished results) resulted in a 5.5 fold increase in half-life and 5 kJ mol^{-1} increase in ΔG^* as compared with native CMCase at 65°C (Tables 1 and 2). The interesting feature is that both ΔH^* and ΔS^* of GAM15 are decreased as compared with native CMCase (Tables 1 and 2). The thermostabilization of enzymes is mostly accompanied by decrease in ΔS^* and ΔH^* as in case of carboxyl group modified-glucoamylase from *A. niger* (Munch and Tritsch, 1990), acetylated α-amylase (Urabe *et al.*, 1973) and protein-engineered T4 lysozyme (Matthews *et al.*, 1987). Further decrease in ΔS^* of GAM15 as compared to native CMCase (Tables 1 and 2) could be due to elimination of repulsion between negatively charged carboxyl groups thus decreasing the flexibility of an external loop (Clark and Gurd, 1967; Matthew, 1993; Vieille and Zeikus, 1996) thereby stabilizing GAM15. It has been shown that changes on the surface of a protein could lead to thermostabilization (Querol and Parrilla, 1987). The neutralization of excessive negative or positive charges on the surface of enzymes by chemical modification have been shown to increase the thermostability of horse-raddish peroxidase (Miland *et al.*, 1996) and α-amylase (Urabe *et al.*, 1973). Conversely, it has been shown that deamidation at high temperatures leads to inactivation of *Bacillus licheniformis* α-amylase because of increased flexibility due to repulsion of carboxyl groups (Tomazic and Klibanov, 1988). In case of GAM75, the neutralization of 6–10 carboxyls resulted in slightly decreased thermostability; therefore, both ΔH^* and ΔS^* have been increased compared with native CMCase (Tables 1 and 3). This clearly means that further neutralization of negative charge leads to more conformational changes which decreased the thermostability due to less compact structure of GAM75.

The Arrhenius plots of native CMCase and GAM75 met at 44°C (Fig. 1d). This temperature is called the temperature of compensation (T_c) or isokinetic temperature and is defined by Barnes (1969) as related reactions in which the mechanism of thermal unfolding or nature of transition state is identical. No effect is seen at T_c and opposite effects will be observed on either side of the isokinetic temperature which is found to be between −10°C and 100°C (Urabe *et al.*, 1973). On the contrary, the Arrhenius plots of native CMCase and thermostable GAM15 are almost parallel (Fig. 1d), which imply a different transition state for the unfolding of enzymes.

Conclusion

We have shown that apart from α-amylase from *B. licheniformis* (Violet and Meunier, 1989), native and modified CMCases have also transition states of negative entropy at high temperatures. We have also shown that GAM15 is more thermostable than native CMCase which is mainly due to further decrease in ΔS^*. It was also found that the mechanism of unfolding of native and GAM15 was different whereas that of native and GAM75 was same.

Table 3 Thermodynamic and kinetic parameters for thermal inactivation of GAM75 from A. niger

T (K)	k_d (min^{-1})	$t_{1/2}$ (min)	ΔH^* (kJ mol^{-1})	ΔG^* (kJ mol^{-1})	ΔS^* (J mol^{-1} K^{-1})
318	4.53×10^{-3}	153	56.06	92.31	−114
323	7.12×10^{-3}	97	56.02	92.60	−113
328	9.36×10^{-3}	74	55.98	93.33	−114
333	14.1×10^{-3}	49	55.94	93.66	−113
338	16.7×10^{-3}	41	55.90	94.62	−115

E_a (activation energy of denaturation) = 58.7 kJ mol^{-1} is calculated from fig. 1d.

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