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A THERMAL INVESTIGATION OF THE STABILITY OF CRYSTALLINE CROSS-LINKED CARBOXYPEPTIDASE A

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ABSTRACT

Crystals of carboxypeptidase A were cross-linked with glutaraldehyde, and samples of the enzyme and apoenzyme were examined by thermogravimetric, differential thermal and thermomechanical analyses. Further samples were heat-treated under vacuum for various lengths of time, and examined by thermal analysis. Enzymatic activities were measured using the substrate carbobenzoxy-glycyl-L-phenylalanine. It proved possible to put forward explanations for all the phenomena observed, and in particular to identify the peak obtained due to loss of water from the zinc ion situated at the active site of the enzyme. The great stability of the cross-linked crystals, which had previously been commented on, was confirmed and the importance of water as a stabilising factor was indicated.

INTRODUCTION

Carboxypeptidase is an enzyme whose structure has been extensively studied. Early work showed that the enzyme occurred in four forms according to the mode of isolation and the differences were found to be centred in the composition of the N-terminal section of the protein chain[1]. Later, extensive structural studies[2] finally resulted in the complete elucidation of the tertiary structure and the amino acid sequence of the single protein chain[3]. Further work[4] has provided more evidence for the cystine bridge first reported in the X-ray analysis[2] and experiments with model systems[5] led to inferences in conformity with conclusions drawn earlier[2] regarding the binding of zinc in the active site of the enzyme. The structure has been reviewed by Lipscomb[6], and the relative importance of various forces as factors in determining the secondary and tertiary structure of the protein have been discussed[7].

An examination of the properties of carboxypeptidase A crystals also has been carried out[8]. In general, properties were found to be broadly similar to those of solutions of the enzyme but significant differences did occur[9–11]. The crystals were cross-linked with glutaraldehyde in order to render them insoluble and to

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eliminate the problem of fragmentation, the uncross-linked crystals being both soluble and fragile[8]. Extensive investigations indicated that glutaraldehyde did not alter the structure of the molecules in the crystals[8,10] in any significant way. Glutaraldehyde has been used extensively for stabilising protein crystals and the nature of the reagent and the cross-linking reaction have been discussed in detail[12].

The work reported in this paper represents some of the results of a further investigation into the properties of crystalline carboxypeptidase A. The investigation was prompted by the assertion of various authors in different disciplines that ion-exchangers should be good models for proteins and living cells[13–15]. Techniques that had been used successfully to elucidate information on zeolite ion-exchangers[16] were applied (after appropriate modifications) to cross-linked crystalline carboxypeptidase A.

EXPERIMENTAL

Materials

Carboxypeptidase A was purchased from Calbiochem, Lot No. 900846. It was supplied as an aqueous suspension of crystals that had been purified by five recrystallisations. Toluene had been added as a preservative. The enzyme was purified further by another recrystallisation using a dialysis method similar to that described by Quiocho[17]. The substrate carbobenzoxy-glycyl-L-phenylalanine was purchased from Sigma Chemicals.

Cross-linking

The dialysis bag containing the crystals was opened and placed in a test-tube. The crystals were allowed to settle to the bottom of the bag and the supernatant removed. 40 ml of 1% glutaraldehyde in 0.02M sodium veronal buffer (pH 7.5) were added, the dialysis bag tied up again, and the tube stoppered. The tube was rotated along its longitudinal axis for one hour at room temperature. The crystals, now yellow in colour, were washed into a centrifuge tube using the buffer solution. After centrifugation, the glutaraldehyde solution was removed and the crystals washed four times with the veronal buffer. The crystals were cross-linked immediately after dialysis to minimise fragmentation.

Contamination

To minimise contamination by adventitious metal ions either from reagents or from glass vessels, the cleaning methods and purification procedures using dithizone in carbon tetrachloride of Coleman and Vallee[18] were observed carefully. All solutions were stored in plastic bottles.

Enzymatic activity

The method of measurement of the enzymatic activity of the crystals described by Quiocho[17] was not suited to the experimental requirements of this work. Peptidase activity was measured using the substrate carbobenzoxy-glycyl-L-phenylalanine. The

assay solution used was metal-extracted 0.01M substrate in the veronal buffer with 1M sodium chloride. The details of the experimental method are described elsewhere[19]. For the work described in this paper 5 mg (±0.05 mg) of the enzyme crystals were agitated with 1 ml of the substrate solution at 25°C. Enzymatic activity was expressed in terms of micromoles of phenylalanine released into solution per minute.

Zinc-free carboxypeptidase A (apocarboxypeptidase A)

This was prepared by a modification of the method described by Vallee et al.[20], using a metal-extracted solution of 0.025M citrate buffer in 0.5M sodium chloride (pH 3.5). Details of the method are described elsewhere[19]. A residual activity was detected in the enzyme after extraction. This residual activity had been previously noted[20] and was about 10% of the original activity.

Thermal analyses

Samples of the crystals of the enzyme, the apoenzyme, and enzyme that had been subjected to heat-treatments of different degrees of severity, were examined by thermogravimetry (TG) and differential thermal analysis (DTA). In addition, a thermomechanical analysis (TMA) of a sample of the crystalline enzyme was carried out. All analyses were under an atmosphere of nitrogen, and conditions were as recorded in Table 1. The instruments used were the DuPont 950 Thermobalance, 900 Differential Thermal and the 942 Thermo-Mechanical Analysers.

Heat-treatments of the enzyme

Aliquots (5 ±0.05 mg) of the enzyme crystals were tared into three sintered assay tubes[19] (labelled X, Y, Z). The following procedure was then adopted:

(a) the peptidase activity of the enzyme crystals in tubes X, Y, Z were determined;

(b) the tubes X, Y, Z were fitted into a vacuum manifold and heat-treated at 95°C under a vacuum of 10 Nm^{-2} for four hours in a heating mantle. After this period of time had elapsed, the manifold was removed from the mantle, the tubes cooled and the vacuum gently released to atmospheric pressure. Metal-free water was washed through the crystals for one hour, then the peptidase activity of the crystals determined;

(c) tube X was put aside. Tubes Y and Z were heat-treated at 95°C under vacuum for four hours as before. The peptidase activity of all three tubes was determined;

(d) tube Y was also put aside. Tube Z was heat-treated as before for a further four hours. The peptidase activity of all three tubes was determined;

(e) the three tubes containing the enzyme samples that had now undergone heat-treatments of differing severities were allowed to stand for 72 hours in the presence of water, then once more enzymatically assayed. Further assays were carried out on tubes Y and Z 24 hours later, and finally on all three tubes after a further 72 hours had elapsed.

Approximately 24 hours elapsed between each heat-treatment. Samples of X, Y and Z were examined by DTA under the conditions noted in Table 1. Prior to the examinations the crystals, which had been thoroughly wetted with metal-free water after the heat-treatments, were dried at room temperature under a vacuum of 10 Nm^{-2} in a desiccator for 24 hours.

TABLE 1
CONDITIONS OF THERMAL ANALYSIS

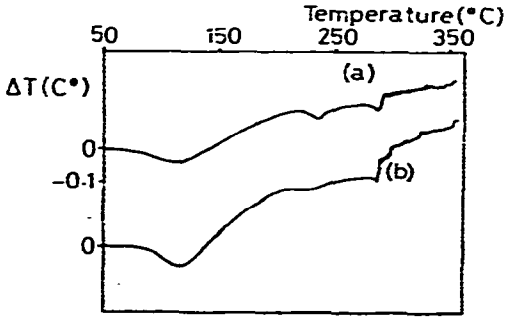
Sample	*Technique*	*Temperature range (°C)*	*Heating rate (°C/min)*	*Other comments*
Enzyme crystals	TG	20–500	20	
	DTA	20–400	10	reference material: glass beads sample depth: 3 mm ΔT: 0.2 °C/25 min
	TMA	20–450	10	expansion probe wt.: 2 g sample depth: 5 mm
Apoenzyme crystals	TG	20–500	20	
	DTA	20–400	10	conditions as above
Heat-treated enzyme crystals	TG	20–450	10	
	DTA	20–400	10	conditions as above

In addition a large sample of crystals was heat-treated at 95 °C under a vacuum of 10 Nm^{-2} for 16 hours. After treating with metal-free water, and drying under vacuum as with samples X, Y, Z, these crystals were examined by DTA and TG. (The purpose of wetting the heat-treated crystals before examining them by thermal analysis was to eliminate any effects arising from a readily reversible dehydration from the DTA and TG traces.)

RESULTS

The DTA, TG and TMA curves obtained for the cross-linked carboxypeptidase A and apocarboxypeptidase A crystals are shown in Figs. 1–3. In the TG curves, two weight losses were observed; the first was over the range 50 to 100 °C (ΔW ca. −2%), and a large one occurred between 190 and 500 °C (ΔW ca. −67%). The weight losses were almost the same for both the enzyme and apoenzyme. The DTA curves showed three phenomena. Firstly, after an initial fall, a diffuse low exotherm appeared at about 125 °C and reached a maximum at approximately 200 °C. It is arguable that this phenomenon might be better described in terms of a large endotherm peaking at ca. 110 °C, but an examination of Fig. 5, where the DTA curve of the enzyme is compared with those of the heat-treated samples, seems to rule this out. Secondly, a

small sharp endotherm was observed in the enzyme trace at a temperature of 231 °C; this peak was hardly visible in that of the apoenzyme. Finally, both the enzyme and apoenzyme showed a sharp endotherm at 281 °C.



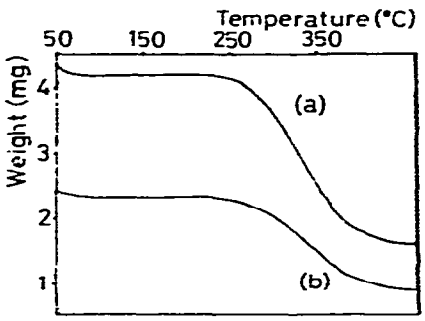


Fig. 1 (left). DTA curves of (a) carboxypeptidase A and (b) apocarboxypeptidase A crystals.

Fig. 2 (right). TG curves of (a) carboxypeptidase A and (b) apocarboxypeptidase A crystals.

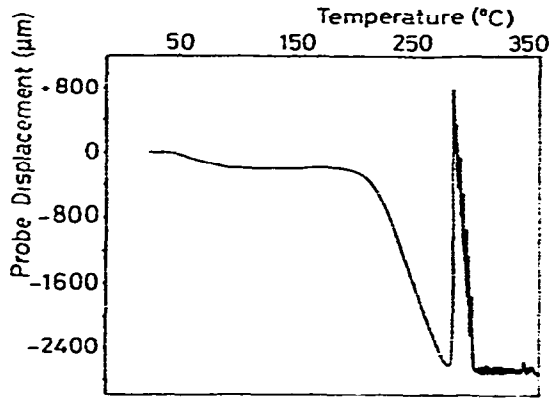


Fig. 3. TMA curve of carboxypeptidase A crystals.

The TMA curves showed two volume changes which corresponded to the weight losses observed by TG. The first volume change (ΔV ca. −4%) was observed from 25→100 °C and the second (ΔV ca. −50%) occurred from 200→280 °C, reached a maximum at 290 °C, and then fell back to the volume reading observed at 280 °C. At higher temperatures, the trace oscillated strongly but stayed at the same probe displacement reading.

Examination of the samples after thermal analysis indicated charring, and the crystals appeared to have melted.

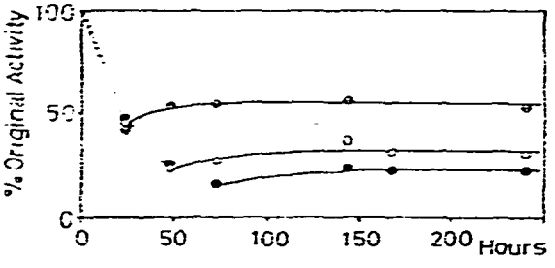
The effect of heat-treating the carboxypeptidase A crystals for various lengths of time on the peptidase activity of the enzyme is shown in Table 2 and Fig. 4. The peptidase activity decayed with each heat-treatment, and each sample, on being allowed to stand at room temperature in the presence of water, showed a small but

distinct recovery of activity over approximately four days. Thereafter, the activity remained constant.

TABLE 2
EFFECT OF HEAT ON PEPTIDASE ACTIVITY OF CRYSTALS OF CARBOXYPEPTIDASE A

Assay time	*Tube X*		*Tube Y*		*Tube Z*	
	Activity	*% Original activity*	*Activity*	*% Original activity*	*Activity*	*% Original activity*
Zero	0.46	100	0.55	100	0.57	100
1st denaturation	0.19	41.3	0.25	45.5	0.27	47.3
2nd denaturation	0.25	54.4	0.13	23.6	0.14	24.6
3rd denaturation	0.25	54.5	0.15	27.3	0.10	17.5
72 hours later	0.26	56.5	0.21	38.2	0.14	24.6
24 hours later	no assay	no assay	0.18	32.7	0.13	22.8
72 hours later	0.24	52.2	0.17	31.0	0.13	22.8

The sample of enzyme that was heat-treated for 16 hours (and examined by TG as well as DTA) showed a final peptidase activity of 20% of original activity, which was in accord with the data shown in Table 2 and Fig. 4.



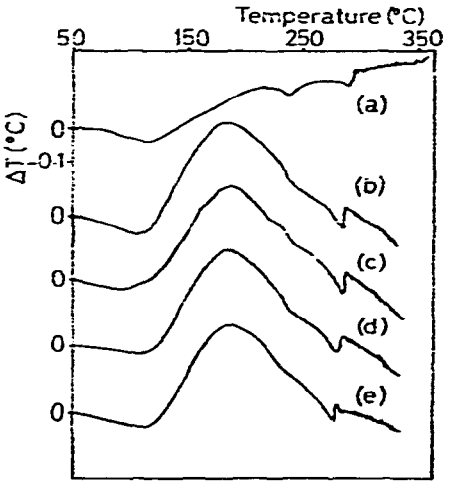


Fig. 4. Effect of heat-treatments on the peptidase activity of the enzyme crystals. The dashed lines represent a heat-treatment stage.

Fig. 5. Effect of heat-treatments on the DTA curves of the enzyme crystals: (a) no heat-treatment, (b) 4 hours heat-treatment, (c) 8 hours heat-treatment, (d) 12 hours heat-treatment, and (e) 16 hours heat-treatment.

A comparison of the DTA of the heat-treated samples with that of the undamaged enzyme is interesting (Fig. 5). The broad diffuse exotherm observed in both

the enzyme and apoenzyme samples became much more prominent, even after four hours heat-treatment. The exotherm peak appeared to be 180 °C. Further heat-treatment did not markedly increase the size of this exotherm. The small sharp endotherm observed at 231 °C in the native carboxypeptidase A gradually disappeared, being first replaced by two small peaks at 225 and 240 °C, these also having disappeared after 16 hours at 95 °C under vacuum (Fig. 5). The sharp endotherm at 281 °C showed little change, although there was some suggestion of it occurring at progressively lower temperatures as the samples were further heat-treated.

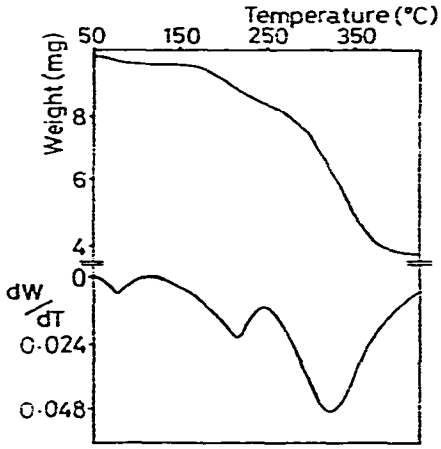


Fig. 6. TG curve of enzyme crystals that had been heat-treated for 16 hours; comparison to the differential thermogravimetric plot.

The TG curve of the enzyme heat-treated for 16 hours revealed that the damaged enzyme showed a more complex behaviour. A differential thermogravimetric plot was computed (Fig. 6) and exhibited three peaks at approximately 80, 215 and 325 °C. (The weight losses were for these peaks, $\Delta W - 2$, -12 and -55% respectively.)

DISCUSSION

An examination of the DTA curves obtained from the enzyme and apoenzyme show one significant difference, namely, that the sharp endotherm observed at 231 °C in the metalloenzyme was hardly visible in the apoenzyme (Fig. 1). Since the enzyme and apoenzyme differ only by the presence of zinc at the active site in the former, it would seem reasonable to attribute this peak to some phenomenon connected with the zinc. As it is known that one of the ligands on the zinc in carboxypeptidase A is water[2], it would appear that this peak was due to the removal of water bound to zinc. It is noteworthy that the temperature at which this peak occurred was almost identical to that observed in the DTA curve of zinc-2.62Y zeolite[16], which was also ascribed to water bound to zinc.

Both the enzyme and apoenzyme DTA showed a diffuse exothermic peak maximising around 200 °C. At about the same temperature the TMA curve (Fig. 3)

showed that the crystal volume began to decrease sharply and, at a slightly higher temperature (ca. 230 °C), the TG curves of the enzyme and apoenzyme displayed the commencement of a substantial weight loss (Fig. 2). It is therefore postulated that the diffuse DTA exotherm observed in both the enzyme and apoenzyme was due to conformational changes in the protein structure (i.e. denaturation) which involved the destruction of the tertiary structure, and released substantial amounts of structuring water. (The weight losses observed were too large to be due to water alone, and must have involved some decomposition of the protein.) Further support for this hypothesis is provided by the DTA and TG curves of the heat-treated enzyme, discussed below.

The small weight loss observed on the TG curves between 50 and 100 °C (Fig. 2), and the small decrease in volume observed on the TMA over the same temperature range, can be attributed to loss of small amounts of loosely bound water.

It remains to postulate an explanation for the small sharp peak observed at 281 °C in the DTA curves of the enzyme and apoenzyme. A very large peak was observed at this temperature on the TMA of this enzyme. Since samples of the protein after thermal analysis were found to have charred and melted, it would seem reasonable to attribute this peak to the "melting point" of the decomposing protein. The very large positive volume change observed on the TMA curve would suggest rapid degassing and decomposition as the crystal melted. The rapid oscillations of the pen immediately after this peak are consistent with a continuing degassing of the new phase.

TABLE 3

COMPARISON OF TG ANALYSES OF DIFFERENT ENZYME SAMPLES

Sample	*Temperature, °C*	*ΔW %*	*Total ΔW %*
Enzyme	50–100	−2	
	190–500	−67	−69
Apoenzyme	30–100	−3	
	190–500	−64	−67
Denatured enzyme	50–100	−2	
	150–250	−12	
	250–500	−55	−69

The distinctive differences between the DTA curves of the native and heat-treated enzyme crystals enumerated in the previous section may be explained as follows. Before thermal analyses were carried out the enzyme crystals were thoroughly wetted and then dried, under vacuum in a desiccator, at room temperature. Thus any readily reversible dehydration that had occurred during heat-treatment would not be recorded. The gradual disappearance of the peak at 231 °C in the heat-treated samples would therefore suggest the occurrence of some change in the vicinity of

the active site, rendering the zinc unavailable for coordination with water. It is known[6] that, when a substrate binds in the active site of carboxypeptidase A, several conformational changes in the region of the active site occur including: the movement of the guanidinium group of Arg-145 through 200 pm, the movement away from the zinc atom of the carboxyl group of Glu-270 by about 200 pm, and, most striking of all, the movement of the phenolic-OH of Tyr-248 by 1.2 nm to within 300 pm of the –NH group of the scissile peptide bond[2,6]. Since the enzyme is active in the crystalline state, it follows that the "fixing" of the molecules in the crystal lattice does not prevent conformational changes in the region of the active site; it is therefore reasonable to suggest that heat-treatment created conformational changes in the region of the active site which rendered the zinc unavailable for coordination with water or a substrate (since the substrate replaces the water molecule when binding to the zinc ion[2]). The small endothermic peaks observed at 225° and 240°C during intermediate stages of the heat-treatments could be a further indication of this conformational change, together with the concomitant loss in peptidase activity.

The change observed in the exothermic DTA peak at 180°C on heat-treatment may be interpreted in the light of the TG curves of the native and heat-treated enzyme. The overall percentage weight losses observed in the different samples were practically identical and are compared for convenience in Table 3. This would suggest that the water content of the heat-treated protein was broadly similar to that of undamaged samples, a conclusion in agreement with other studies[21]. (The total weight loss observed in all three cases, of course, was not due only to water.) However the behaviour of the heat-treated sample was different to that of the native enzyme, in that the major weight loss occurred in two parts beginning at a lower temperature than that observed in the native enzyme. The differential thermogravimetric trace (Fig. 6) revealed that this first major weight loss coincided quite closely with the large exothermic peak at 180°C observed in the DTA analyses of heat-treated samples. These observations can be rationalised as follows. In the heat-treated enzyme crystals the heat-treatment caused minor conformational changes in the protein structure, which resulted in the water being less strongly bound. On thermal analysis the water was lost rapidly and rapid break-down of the tertiary structure of the protein occurred (hence the large exothermic peak observed in the DTA and the weight loss observed in the TGA over the same temperature range). With the native enzyme, on thermal analysis, water was lost at a slower rate at a comparable temperature to that at which decomposition occurred. Thus the exotherm due to break-down of the tertiary protein structure was small (being partially masked by an endotherm resulting from relatively strongly bound water being lost) and only one major weight loss was observed. It follows from this hypothesis that the second major weight loss, between 250 and 500°C in the TG curve of the denatured enzyme, was due to decomposition. This conclusion is confirmed by the large volume decrease observed, from 200 to 280°C on the TMA curve of the native enzyme, which could only have been due to a collapse of structure and decomposition. The volume decrease and weight loss observed are very similar (−50% and −55% respectively).

Although Quiocho[10] has previously commented on the stability of cross-linked carboxypeptidase A crystals at room temperature, bearing in mind the relative severity of the heat-treatments described here, the retention of peptidase activity by the crystals was high being still ½ the original value after 16 hours at 95°C. The thermal analyses indicated that the loss of peptidase activity that did occur was due to conformational changes in the environment of the active site rather than to any denaturation of the tertiary structure. The resistance of the tertiary structure to breakdown is certainly greatly enhanced by the glutaraldehyde cross-linking reagent[8] but the differences observed in the thermal analyses of the native and heat-treated enzyme samples indicate the important part water plays in the structural stability of the carboxypeptidase A molecule even in the crystalline phase.

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