Circular Dichroism of Acetoacetylated Proteins

The use of diketene as a protecting group in peptide synthesis and as reagent for selective and reversible modification of proteins in studies of primary structure and of structure-function relationship has been described elsewhere[1-3]. It has been demonstrated that acetoacetylation causes a progressive inactivation of the enzyme up to complete loss of the enzymatic activity[1,2]. It is not possible, at this point, to establish whether the effect of the modification of the enzymatic activity is the indirect result of a conformational change or of the blocking of groups which participate directly in binding the substrate or in catalytic function.

In this paper the conformational changes of bovine pancreatic ribonuclease (RNase A) and white-egg lysozyme after acetoacetylation with different amounts of diketene were studied by means of the circular dichroism technique.

Native RNase A and lysozyme were subjected to diketene treatment; the extent of the acetoacetylation and the residual enzymatic activity were estimated according to the procedure described by MARZOTTO[1,2].

Two modified RNases were prepared by using 1:55 and 1:330 molar ratios respectively between protein and diketene. The former modified enzyme showed 10–11 acetoacetylated groups and a 35–40% residual activity, the latter contained 16–17 acetoacetyl groups and was completely inactive.

The acetoacetyl lysozyme, prepared by using a 1:330 molar ratio between protein and diketene, contained 11–12 modified groups and showed a 15–20% residual activity.

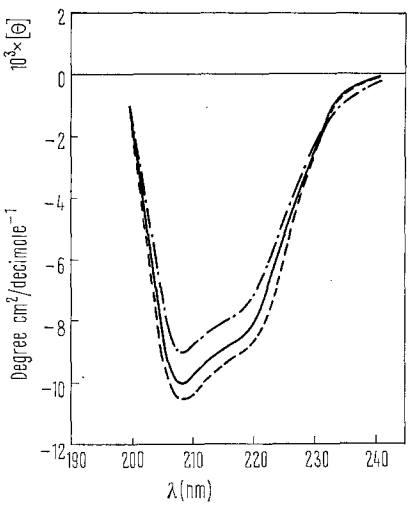
Figure 1, a shows the circular dichroism (CD) behaviour in the far ultraviolet of aqueous solutions of the 2 acetoacetylated RNases (1:55 and 1:330 molar ratios) compared with the one of native RNase A. In any case the minimum of ellipticity $[\theta]$ at 222 nm, typical of the n-π^* peptide transition of the α-helix and the large dichroic band centred at 208 nm, associated with the π-π^* α-helical peptide transition[4,5], are present. The magnitude of these dichroic bands is not too far from those of native RNase. The diminution observed even for the more extensively acetoacetylated RNase can be evaluated within a 20% $[\theta]$ value.

It therefore appears that diketene treatment induces very little conformational change of RNase.

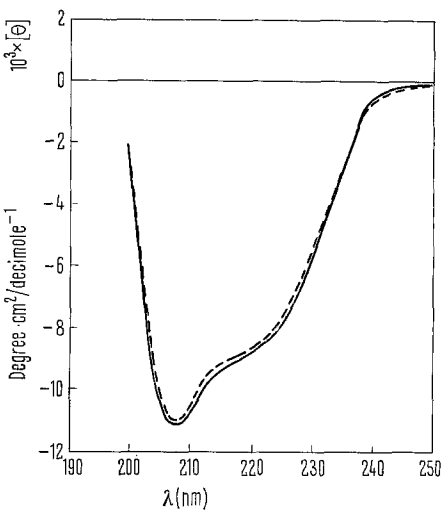
These conclusions are confirmed by the CD spectra of native lysozyme and lysozyme acetoacetylated with protein diketene 1:330 molar ratio. Figure 1, b shows the circular dichroic behaviour in the far ultraviolet of aqueous solutions of the native and modified enzyme. In this case, even with an extensively modified lysozyme, no noticeable difference is detectable between the 2 dichroic curves. Both the bands at 222 nm and at 208 nm of the acetoacetylated lysozyme show a $[\theta]$ value very close to that of native lysozyme.

The present results allow us to conclude that no appreciable conformational changes of protein molecules occur after diketene treatment, thus playing a negligible role in the loss of enzymatic activity. This supports the view that the modification of enzymatic activity has to be attributed to the selective blocking of one or more amino groups involved in the binding or in the catalytic function of an enzyme.

Furthermore these data emphasize the advantageous use of the acetoacetyl group as a selective and reversible blocking agent in the complete protection of the free



a) Circular dichroism in the far ultraviolet of aqueous solutions of native RNase A (– – –); 1:55 acetoacetylated RNase A (——) and 1:330 acetoacetylated RNase A (–·–·–) by a Roussel-Jouan dichrograph II at 25 °C. The concentrations used were 1 mg/ml. Cylindrical quartz cells were used with 0.05 and 0.01 cm optical path. The sensitivity was kept at 1.10^{-5}. The symbol $[\theta]$ represents the mean residue molecular ellipticity expressed in degree cm²/decimole^{-1}.



b) Circular dichroism spectra in the far ultraviolet of aqueous solutions of native lysozyme (– – –) and 1:330 acetoacetylated lysozyme (——). The experimental conditions are the same as reported in Figure a.

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amino-groups present in peptides and proteins, since even a very large excess of diketene is unable to induce appreciable conformational changes of a protein.

Riassunto. Sono state analizzate le variazioni conformazionali indotte nella ribonucleasi pancreatica di bue (RNase A) e nel lisozima del bianco di uovo dopo acetoacetilazione con differenti quantità di dichetene. La modifica dell'attività enzimatica, susseguente al trattamento con dichetene, non è attribuibile a variazioni conformazionali delle proteine considerate ma al bloccaggio selettivo di uno o piú gruppi amminici coinvolti nell'attività catalitica o nel «binding» col substrato.

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Molecular Alterations of Human Fibrinogen by Ultrasonic Frequencies

Although the fact that gaseous cavitation produced by ultrasonic frequencies has been used to disrupt plant and animal cells[1,2], and was shown to alter the physicobiochemical characteristics of human serum[3–5], little is known about its effects on the biological activities of purified proteins. After ultrasonication of human serum, SEARCY and BERQUIST[3] observed the disappearance of heat precipitable fibrinogen with no fibrin formed by thrombin.

The present report describes the formation by brief period of ultrasonication of non-clottable with an anticoagulant activity protein from highly purified 95% clottable human fibrinogen. Sedimentation data from the analytical ultracentrifuge, electrophoretic mobility on polyacrylamide gel and chromatographic behavior, indicated no noticeable changes between the native human fibrinogen and the 2 min ultrasonicated fibrinogen. The ultrasonicated fibrinogen is shown to act as an anticoagulant and as an inhibitor of thrombin activity.

Materials and methods. Human fibrinogen, Pentex lot 44 purified by series of salt precipitation and adsorption on tricalcium phosphate[6] to obtain a 95% clottable protein, sedimenting as a homogeneous single peak at a velocity of 7.35 S.

A 1% solution in physiological saline of this fibrinogen was freshly prepared and exposed to ultrasonic vibration, using Raytheon Sonic Oscillator Model DF 101, 250 W and 10 kc with an output current of 0.78 A and 60 cycles. The temperature of the solution was kept at 0–4 °C during ultrasonication. Aliquots were withdrawn after 2, 5, 15, 30 and 60 min of ultrasonication. All the aliquots were immediately freeze-dried in vacuum. Sedimentation experiments were carried out on a Spinco Model L analytical Ultracentrifuge equipped with a Schlieren system.

DEAE-cellulose of 100–200 mesh with 0.70 mEq nitrogen/g was first equilibrated in 0.005*M* phosphate-0.040*M* *tris*, of pH 8.6, then packed into a column of 35 × 2.2 cm. 300 mg of purified human fibrinogen or its 2 or 5 min ultrasonicate were dissolved in 5 ml of the phosphate-*tris* buffer and equilibrated at 4 °C against the buffer, then applied onto the DEAE-cellulose column. Elution was carried out with a gradient pH using the same buffer starting at pH 8.6 and gradually decreasing to pH 4.1 with a flow rate of 75 ml/h and 15 ml fractions were collected. Optical densities of the fractions were measured at 280 nm against distilled water as a blank in a model DU Beckman Spectrophotometer.

Disc electrophoresis was carried out on columns of polyacrylamide gel[7] containing 0.15*M* e-aminocaproic acid[8].

The clottability of the ultrasonicated fibrinogen was compared with that of fibrinogen. Solutions (2 mg/ml) of fibrinogen before and after 2, 5, 15, 30 and 60 min ultrasonicates were prepared and incubated at 36 °C. 1 ml aliquots of each were removed at various times, 1 drop of stock thrombin solution was added. The time of the appearance of a clot, or fibrin strands were noted. Thrombin clotting time[9] was determined by adding

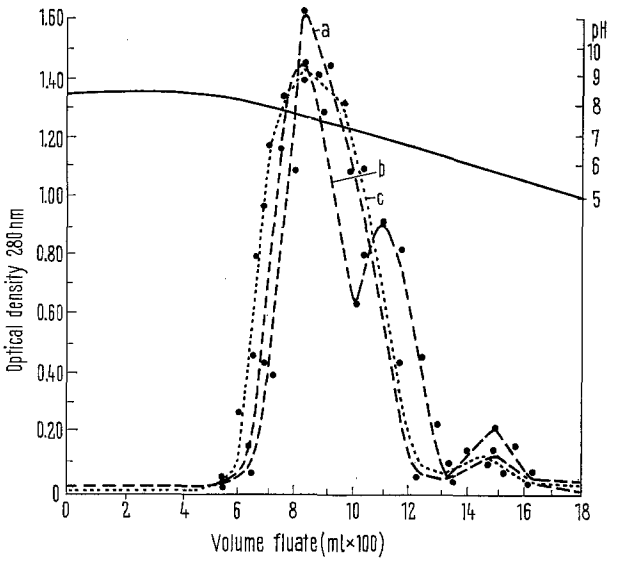

Fig. 1. DEAE-cellulose chromatography of human fibrinogen and its ultrasonicate. Elution patterns of purified human fibrinogen (a) ——— and its 60 min ultrasonicate (b) --------- and its 2 min ultrasonicate (c) ---------- from DEAE-cellulose column.

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