ACETOACETYLATION OF RIBONUCLEASE A

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Blocking groups which can be removed easily represent a useful tool for the elucidation of primary structure and for structure-reactivity correlations in proteins.

To date, acetic anhydride ([1]) has been used as an irreversible reagent for amino-groups, whereas carbon disulfide ([2]), methyl acetamidate or benzimidate ([3,4]) and the trifluoroacetyl group ([5]) have been used as reversible reagents.

We have studied the acetoacetyl group, which was formerly proposed as a protective agent in peptide synthesis ([6]), as a new blocking group for chemical modification of proteins. Ribonuclease A (Fluka AG 4 x crystallized), purified according to Hirs, Stein and Moore ([7]) has been used as a standard protein. Acetoacetylation was carried out with freshly distilled diketene *, in a molar ratio suited to yield complete acetoacetylation of the free amino-groups, on the basis of the lysine content. The pH was maintained at a constant value of 8.5 by addition of standard alkali. The reaction was followed with a pH meter (Beckman Model G.S.) until no further uptake of alkali occured. The progress of the reaction was measured also by following the increasing reactivity of the products with ferric chloride and the decreasing reactivity with ninhydrin, after gel filtration and lyophilization. These results are shown as a function of the molar

* Diketene is a commercially available product of T.Schuchardt, GMBH & Co. München.

ratio between diketene and protein in Figure 1. It is evident that diketene acetoacetylates other groups besides the eleven amino-groups (10 lysine residues) present in the RNase molecule. This is not surprising if one takes into account the acylating action of diketene on other susceptible groups. Optimum acetoacetylation was observed when diketene was used in a ten-fold molar excess with respect to the $-NH_2$ groups in the protein. The extent of amino-group protection was measured by means of dinitrofluoro benzene reaction and amino-acid analysis after acid hydrolysis of the protein.

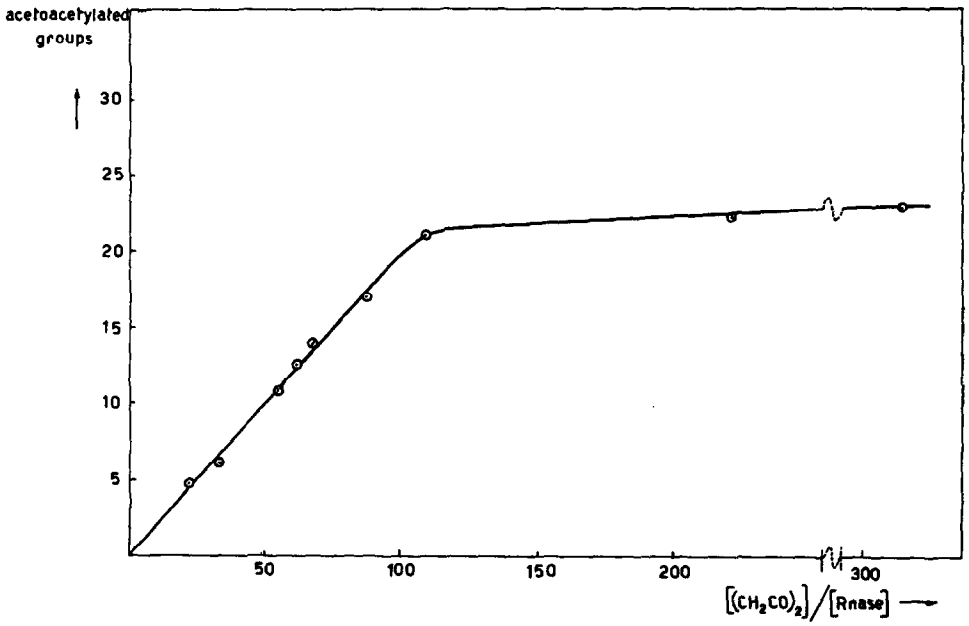

Fig. 1 – Acetoacetylation of RNase A at 25 ° C. The molar ratio diketene / protein is expressed on the basis of the free amino-groups present in the molecule; the pH was maintained at 8.5 by addition of alkali.

The acetoacetylation causes a progressive inactivation of the protein. Figure 2 shows, in fact, that while three groups could be blocked without appreciable loss of activity a total of 17 modified groups (including all the free $-NH_2$) made the enzyme completely inactive.

As observed with acetylated proteins ([1]), the acetoacetyl groups bound to residues other than $-NH_2$ can be removed successfully by means of 0.2 M $NaHCO_3$-Na_2CO_3 buffer at pH 9.5 for eleven hours at 25° C. With such a procedure all the acetoacetylated NH_2-residues were untouched, since the

protein did not give a ninhydrin reaction, and no ε-dinitrophenyl lysine residues were detectable after dinitrophenylation. The enzymatic activity was not restored at the end of the alkali treatment.

The complete removal of the acetoacetyl groups from acetoacetylated Ribonuclease A (Aa RNase A) was performed at pH 7 and 25°C in an aqueous solution of hydroxylamine hydrochloride in a three-fold molar excess with respect to the total number of modified groups. The reaction was carried out for about twelve hours, based on previous experiments with some acetoacetylated amino-acids. The complete release of the protected groups was shown both with ninhydrin and ferric chloride. The activity was fully restored, as shown in Figure 2.

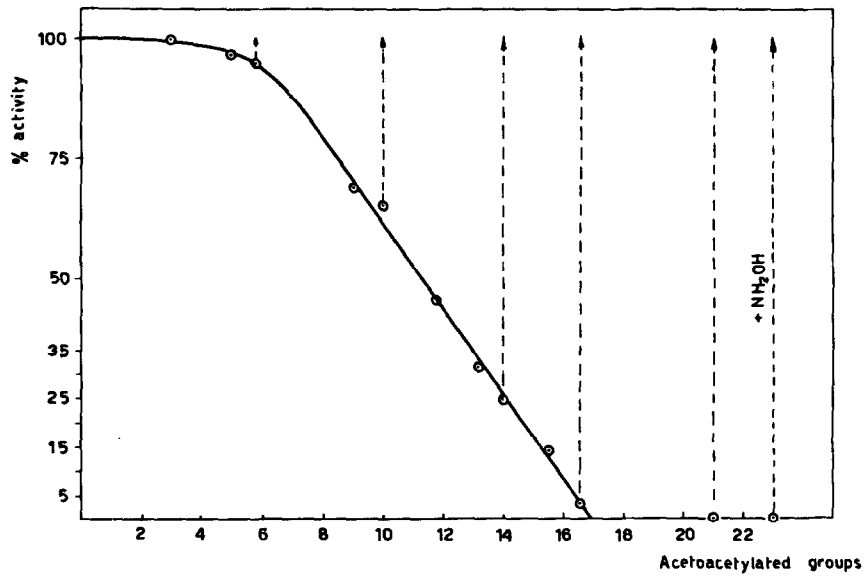

Fig. 2 – Enzymatic activity of the acetoacetylated RNase A. The arrows indicate reactivation of the modified protein following the removal of acetoacetyl groups.

The enzymic activity of the products has been checked by, using yeast Ribonucleic acid (RNA) (Schwartz Bioresearch Inc.) as substrate according to the Kunitz procedure ([8]).

The electrophoretic and chromatographic patterns of native, modified and deacetoacetylated RNase A (DAaRNase) are shown in the Figures 3 and 4 respectively. No difference is detectable between native RNase A and DAa-

-RNase A. The similarity of the native and DAa-RNase A is supported further by the U.V. spectra.

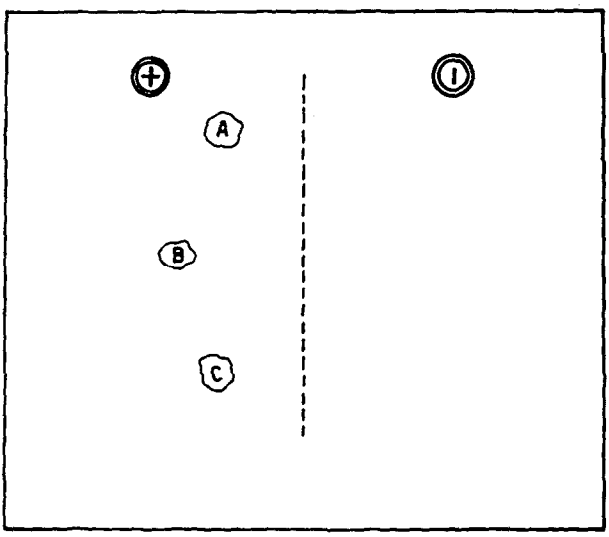

Fig. 3 – Paper electrophoresis on Whatman N° 1 18 V/cm, 2.5 hours, 25% acetic acid pH 1.9. A) native RNase A; B) fully AaRNase A; C) DAaRNase A.

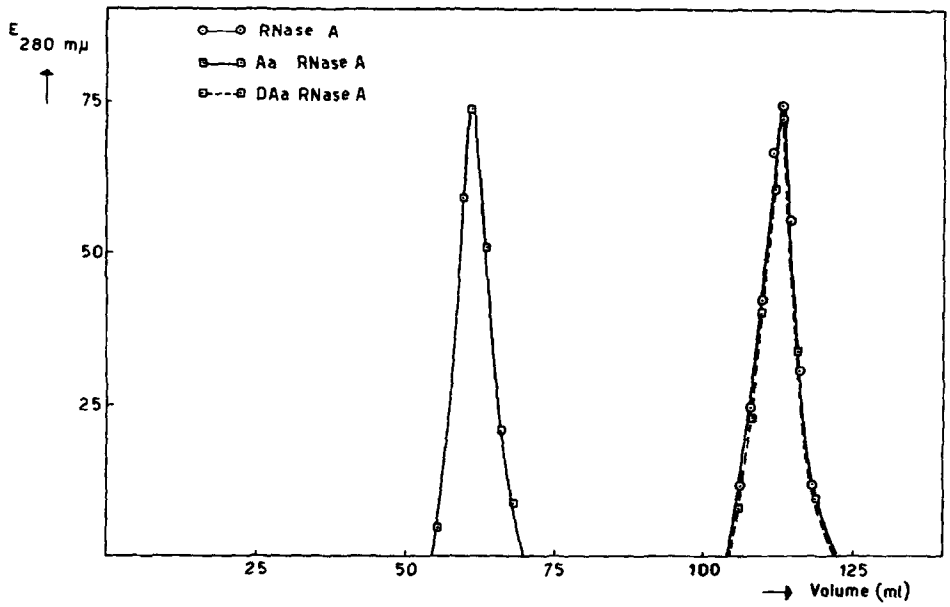

Fig. 4 – Chromatography of RNase A, AaRNase A and DAaRNase A on CG 50 Amberlite 200–400 mesh (1.8 x 90 cm.); eluent 0.2 M phosphate buffer pH 6.5.

We can recall at this point that acetic anhydride ([7]) is a non-reversible masking reagent; carbon disulfide in turn is a reversible protector which does not block all the $-NH_2$ groups ([2]), and this is true also for methyl acetamidate and benzymidate ([3]). Trifluoro-acetylation ([5]) has been employed for reversible modification of RNase, but the recovered material was devoid of enzymatic activity: this was attributed to incorrectly paired half-cystine residues.

Bearing in mind the above facts,the acetoacetyl groups may offer some advantages as a reversible protector since it requires milder conditions and yields a fully active protein after deblocking, therefore it is appropriate for reversible modification of native proteins. Moreover acetoacetylation may provide a useful procedure for investigating the primary structure of proteins. In fact it is known that in the acylated proteins, the lysine bonds not the arginine ones are resistant to hydrolysis by trypsin. Further studies concerning such an application are in progress.

REFERENCES

(1) Brown, R.K., Dutieux, J., Delaney, R., Leikhim, E. and Clark, B.J., Ann. N.Y. Acad. Sci., 81, 524 (1959).

(2) Merigan, T.C., Dreyer, W.J., and Berger, A., Bioch. Biph. Acta, 62, 122 (1962).

(3) Hunter, M.J. and Ludwig, M.L., J. Am. Chem. Soc., 84, 3491 (1962).

(4) Ludwig, M.L., and Byrne, R., J. Am. Chem. Soc., 84, 4160 (1962).

(5) Goldberger, R.F., and Anfinsen, C.B., Biochemistry, 1, 401 (1962),

(6) D'Angeli, F., Filira, F., and Scoffone, E., Tetrahedron Letters, 10, 605 (1965).

(7) Hirs, C.W.A., Stein, W., and Moore, S., J. Biol. Chem., 200, 493 (1953).

(8) Kunitz, M., J. Biol. Chem., 164, 563 (1946).