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THE FOLDING OF PANCREATIC ELASTASE : INDEPENDENT DOMAIN REFOLDING AND INTER-DOMAIN INTERACTION

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SUMMARY

The role of domains in the refolding of elastase, a two domain protein, was investigated. It has been demonstrated that fragment 126-245, corresponding to one of the two domains, is able to refold independently. Moreover, the in vitro complementation of the two domains lead to a molecule having the overall conformation of the native protein and only a weak but significant activity.

INTRODUCTION

The existence of domains in the structural organization of many globular proteins (1) and the implication of this general feature in the mechanism of folding was emphasized by Wetlaufer (2) and since by several authors (3,4,5,6). The existence of domains in proteins suggest that some parts of a continuous polypeptide chain are able to fold independently from separate nucleation centers. Once domains formed, their association may induce (or not) structural rearrangements crucial for the expression of biological activity. However, until now, the available experimental data on this aspect of protein folding does not allow any generalization (7,8,9).

We have investigated this problem with elastase, a small two domain protein (M.W. 25K) from the family of serine proteases. As evidenced by X-ray's diffraction studies, the protein structure displays two continuous domains, referred as B and C, by analogy with α-chymotrypsin, stabilized by one and three disulfide bonds respectively (10,11). The active center is builded up from residues of both domains.

METHODS

Pig pancreatic elastase was prepared according to Shotton et al. (12). Enzymatic assays were performed with N-acetyl-L-trialanine methylester and binding experiments with elastin previously solubilized

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by treatment with sodium borohydride (14). Two different ways were used for the preparation of the two fragments. In method I, domain C was protected by binding of the enzyme to insoluble elastine and then a limited proteolysis by trypsin was allowed during 40 minutes at 4°C. In method II, fragments 16-125 and 126-245 were obtained by treatment of either N-acetyl-[^{14}C]-elastase or fluorescamine labeled elastase with trypsin immobilized on an insoluble matrix. Purification, in both methods was monitored by fractionation in a Sephadex G 100 column equilibrated in 6M guanidine-HCl, in 50 mM acetate buffer, pH 5.0. The isolated proteins or their fragments are further purified by high pressure liquid chromatography and characterized by their content in disulphide bonds, their N- and C-terminal groups and by finger printing (14).

Circular dichroïsm measurements were used as conformational probe of the native structure. The return of immunochemical properties of the C fragment was followed by competition between C fragment and the whole native protein for purified antielastase antibodies (13).

RESULTS

1- Spontaneous refolding of fragments corresponding to separate domains.

By using method I for the separation of the two domains, it was possible to obtain fragment 126-245 devoid of further proteolysis. We have not succeeded to obtain intact fragment 16-125 ; thus the unfolding-refolding process was investigated only with domain C. The corresponding fragment was denatured in 6M guanidine-HCl or 8M urea under reducing conditions (100 mM β-mercaptoethanol in 100 mM borate buffer, pH 8) to disrupt the disulfide bridges (6 free SH groups are thus titratable by 5,5' dithiobis-(2-nitrobenzoïc acid). By removal of denaturing and reducing agents (during a stepwise dialysis), this fragment refolds spontaneously. The dichroic spectrum indicates that this domain behaves as a refolded protein structure (14). Moreover, as illustrated in figure 1, the refolded fragment pushes out the whole elastase from the elastase antibody complex, with a replacement of 1 mole by 1 mole. The refolded domain recovers its ability to bind elastin. Its affinity for solubilized elastin was measured by equilibrium dialysis. The K_d value of 2.5 10^{-6}M thus determined is identical to that obtained for the refolded entire molecule for this solubilized form of the substrate (14).

These results, indicate that domain C is able to fold independently to reach a stable conformation very close to the conformation of this domain in the "native" entire molecule.

2 - In vitro complementation of fragments.

For this set of experiments method II was used. Figure 2 shows the separation and purification of the two fragments, 16-125 and 126-245; the analysis of disulphide content allowed us to identify them with domain

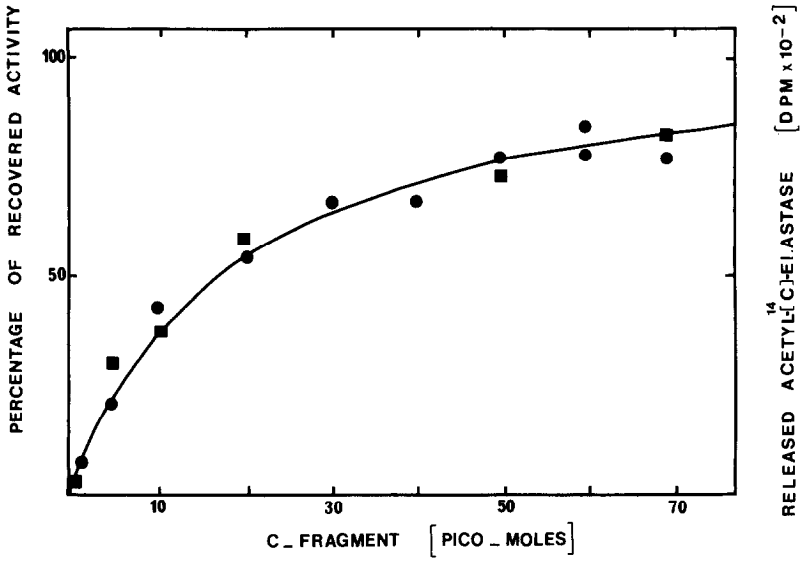

Figure 1 Competitive displacement of native N-acetyl-|^{14}C|- elastase from the specific enzyme-antielastase complex, by increasing amounts of restructured C-fragment.
(●) amount of N-acetyl -|^{14}C|-elastase displaced from the antigen-antibody complex.
(■) enzymatic activity of the released enzyme.

C and B respectively. Peak I has, after reduction, six-SH groups titratable by 5,5'dithiobis-(2-nitrobenzoic acid), peak II has two SH groups.

Reassociation was performed without reduction of disulphide bridges by incubation of the two isolated and purified fragments in 6 M guanidine HCl; then denaturing agent was eliminated and purification of the resulting mixture was monitored on a Sephadex G 100 column equilibrated at pH 8.0 (figure 2c). A peak eluted as the native elastase was obtained with a yield of 30% in protein. The complemented protein had the same dichroic spectrum as the native one (14). The enzymatic activity although weak is however significant (k_{cat} = 1.4 sec^{-1}) and corresponds to about 2% of the activity of the native enzyme on acetyl-L-trialanine methylester, at 25°C, 100 mM KCl and pH 8.5. It was checked that the cleaved protein before separation of the domains displays an activity of about 60%. Therefore the weak activity of the complemented enzyme has to be essentially attributed to an incomplete conformational readjustment of the domains upon complementation.

DISCUSSION

The present data clearly show that one, at least, of the two domains of elastase, can refold independently to reach a stable conformation.

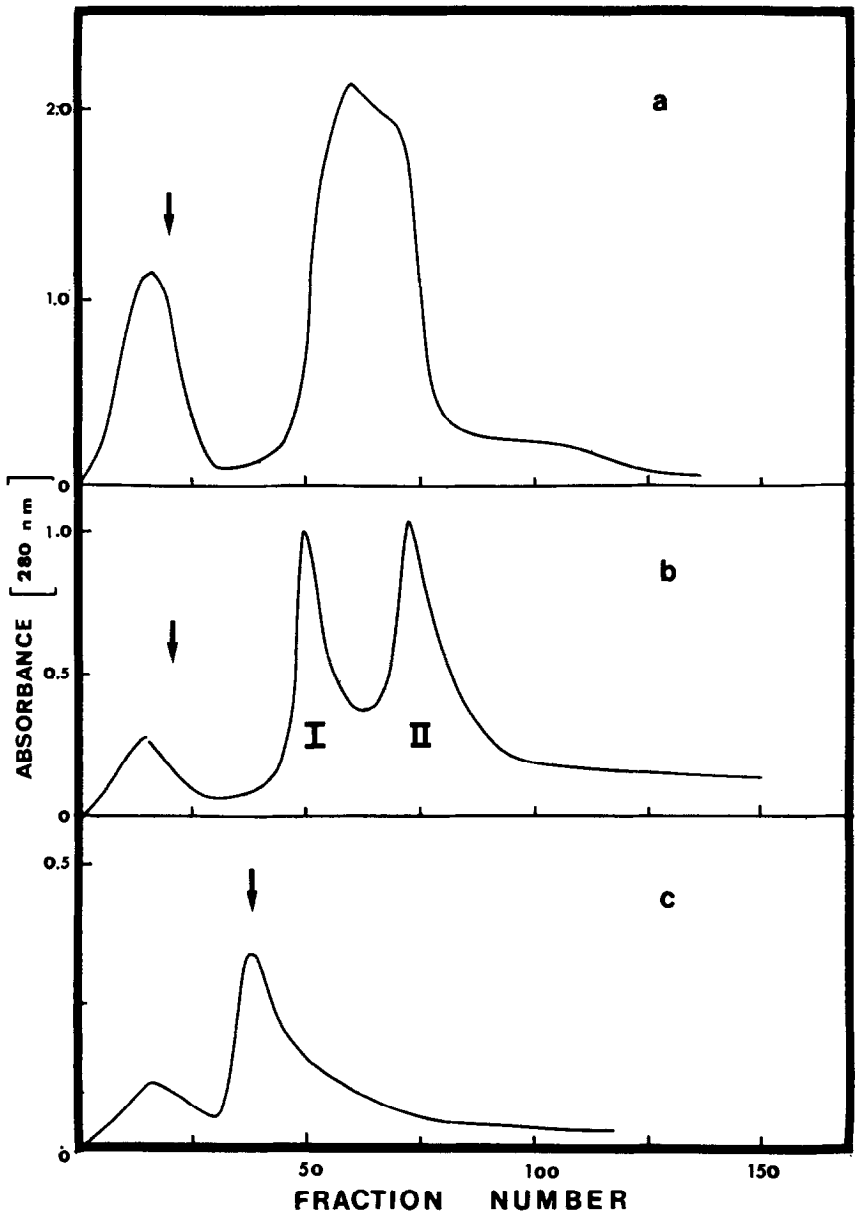

Figure 2 Elution profile of proteins from a Sephadex G 100 column.
a. N-acetyl-$|^{14}C|$- elastase after limited proteolysis in a column equilibrated in 6M guanidine-HCl (100 mM acetate buffer, pH 5.0).
b. Purification of protein fragments (obtained as illustrated in a) by repeated chromatographic runs in the same conditions.
c. Reassociation of fragments obtained in b. After incubation at pH 8, the proteins were fractionated in the column without guanidine-HCl, at pH 8.0 (borate buffer, 50 mM). The arrows indicate the position corresponding to the elution of the intact protein.

This conformation is very close to the conformation of the domain in the "native" entire molecule, since this fragment

1) exhibits the dichroic properties of a structured protein,
2) displaces native elastase from the antigen-antibody complex,
3) binds soluble elastin with the same affinity as the native elastase.

We had not still succeeded to demonstrate directly the spontaneous refolding of domain B which may be only postulated. If this process is experimentally evidenced the formation of nucleation centers followed by a rapid folding of the polypeptide chain around these nucleus to generate domains becomes a plausible model of the folding of elastase. In a last step during the folding process,two "building blocks" have to correctly interact to allow the expression of the full activity of the enzyme. The"right"interdomain interaction seems only realized when the polypeptide chain of elastase is not disrupted. The complementation of the domains obtained upon cleavage of bond 125-126 leads to a protein in which the "right coupling" between domains necessary for the optimization of the catalytic efficiency of the enzyme seems prevented.

The properties of the protein obtained by complementation of the two fragments underline the importance of the interactions between domains to refine and modulate the functional conformation of an enzyme. These probably very small but decisive rearrangements are hindered when the polypeptide chain is disrupted in the complemented enzyme.

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REFERENCES

(1) Edelman, G.M., (1970) Biochemistry 9, 3197-3205.
(2) Wetlaufer D.B. (1973) Proc. Natl. Acad. Sc. USA 70, 697-701.
(3) Kuntz I.D. (1975) J. Am. Chem. Soc. 97, 4362-4366.
(4) Rao S.T.& Rossmann M.G. (1973) J. Mol. Biol. 76, 241-256.
(5) Schulz G.E. (1977) Angew. Chem. Int. Ed. Engl. 16, 23-32.
(6) Liljas A. & Rossmann M.G. (1974) Ann. Rev. Biochem. 43, 475-507.
(7) Slaby I. & Holmgren A. (1975) J.B.C. 250, 1340-1347.
(8) Teale J.M. & Benjamin D.C. (1976) J. Biol. Chem. 251, 4603-4608. and J. Biol. Chem. 251, 4609-4615.
(9) Högberg-Raibaud A. and Goldberg M.E. (1977) Proc. Natl. Acad. Sc. USA 74, 442-446.
(10) Shotton D.M. & Hartley B.S. (1970) Nature 225, 802-806.

(11) Shotton D.M. & Watson H.C. (1970) Nature 225, 811-816.
(12) Shotton D.M. in Methods in Enzymology , vol. XIX, S.P. Colowick & N.O. Kaplan, A.P. NY (1970) 113-140.
(13) Ghélis C., Schechter A.N., Tempete M., Hatzfeld A. & Yon J.M. to be published.
(14) Ghélis C. , unpublished results.