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Morphology of the procarboxypeptidase A-S6 complex

A solution X-ray scattering study

B. Kerfelec [1], C. Chapus [1*], and P. Vachette [2]

[1] Centre de Biochimie et de Biologie Moléculaire du CNRS, BP 71, F-13402 Marseille Cedex 9, France
[2] Laboratoire d'Utilisation du Rayonnement Electromagnétique, Université Paris Sud, F-91405 Orsay, France

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Abstract. Bovine pancreatic procarboxypeptidase A is secreted as a non-covalent association of three different proteins (pro CPA-S6). The free native subunits can be obtained by dissociation of the complex by dimethylmaleylation. Moreover, two specific binary complexes resulting from the high affinity of procarboxypeptidase A (subunit I) for its other two partners (subunits II and III) can also be obtained.

In order to better understand the function of the association, an investigation of the morphology of the ternary complex by solution X-ray scattering has been carried out. The radii of gyration of all the molecular species have been obtained and the experimental results have been interpreted in terms of compact objects of simple shape. The various components correspond to globular particles as shown by the value of the ratio $Rg/M^{1/3}$. This is confirmed by the moderate anisotropy of the simple geometric shapes determined using an assumed value of 0.3 g H_2O/g protein for the hydration. The distances between the centres of gravity of pairs of species strongly suggest that the components are in the closest distance configuration or close to it. However, the binary complex I-III appears to be more open than the complex I-II. Finally, a model of the interaction between carboxpeptidase A and its activation peptide has been constructed by comparing the hypothetical geometric model of subunit I to the crystallographically determined structure of carboxypeptidase A.

Key words: Procarboxypeptidase A, protein complex, X-ray solution scattering, activation

Introduction

In contrast to most secreted proteins which are monomeric, bovine pancreatic procarboxypeptidase A is secreted as a non-covalent ternary complex with a sedimentation coefficient of 6S (proCPA-S6) (Keller et al. 1956). The procarboxypeptidase A (subunit I), central element of the complex, is specifically bound at two distinct sites, to a chymotrypsinogen of the C-type (subunit II) (Peanasky et al. 1969) and to an inactive protein (subunit III) (Puigserver and Desnuelle 1975; Kerfelec et al. 1986). No interaction between subunits II and III has been observed.

The same ternary complex has only been found in two other ruminant species, sheep and goat (Kerfelec et al. 1985), suggesting that the presence of such a complex might be related to the particular features of the digestion of the ruminants. In most species studied so far, pancreatic procarboxypeptidase A is either monomeric (Marchis-Mouren et al. 1961; Reek et al. 1970; Lacko and Neurath 1970; Kobayashi et al. 1981; Kerfelec et al. 1985) or associated with a functionally different protein described as zymogen E in the pig (Kobayashi et al. 1981) or as a probable chymotrypsinogen of the C-type in the sei whale (Yoneda 1980).

The native free subunits can be obtained from the ternary complex by a reversible two-step dissociation process, using a mild chemical treatment which induces an electrostatic repulsion between the subunits (Kerfelec et al. 1984). Subunit III is first released during this treatment showing that it is less tightly bound to subunit I than subunit II. The remaining binary complex (I-II), which has also been described in vivo (Brown et al. 1963), is further dissociated by increasing the reagent concentration. On the other hand, an artificial binary complex I-III can be formed by mixing equimolar amounts of subunits I and III (Kerfelec et al. 1985).

The specific function of the three subunits in the complex and the physiological significance of the association are not fully understood. Subunits I and II are zymogens which, by trypsin activation, are converted into active enzymes playing a complementary function

Abbreviations: pro CPA, procarboxypeptidase A; pro CPA-S6 (or T.C.), ternary complex with a sedimentation coefficient of 6S; CPA, carboxypeptidase A

in the degradation of proteins and peptides. By contrast, although structurally related to the pancreatic serine protease family, subunit III is apparently devoid of any specific enzymatic activity (Puigserver and Desnuelle 1975; Wicker and Puigserver, 1981; Kerfelec et al. 1986). The inactivity of the protein might result mainly from the lack of the two N-terminal hydrophobic residues characteristic of the active form of the serine endopeptidases. Subunit III shows a striking sequence homology with human protease E (Shen et al. 1987) suggesting that subunit III could be a truncated protease E. It should be emphasized that the two proteins may belong to a new pancreatic serine endopeptidase sub-family.

The organization of the subunits in the complex has no influence on the rate of activation of the two zymogens (Chapus et al. 1987). It must be pointed out that, although also resulting from a tryptic attack, the mechanism of activation of subunit I is more complicated than that of subunit II. The very large activation peptide generated during subunit I activation is not released and exerts a very strong inhibitory effect on the newly formed CPA (Chapus et al. 1987) as already observed during the activation of porcine pro CPA (San Segundo et al. 1982). Therefore, in vitro, very long incubation times are required to reveal the carboxypeptidasic activity. The in vivo mechanism leading to the suppression of the inhibitory effect of the activation peptide has not been elucidated.

The association of the subunits allows a full expression of the activity of the resulting enzymes. Subunits II and III have been shown to stabilize to a certain extent the newly formed CPA. Similarly, subunit II exhibits a higher catalytic efficiency when it is associated. Moreover, the presence of subunits II and III probably leads to the protection of subunit I against denaturation in the acidic environment of the duodenum of the ruminants (Kerfelec et al. 1986).

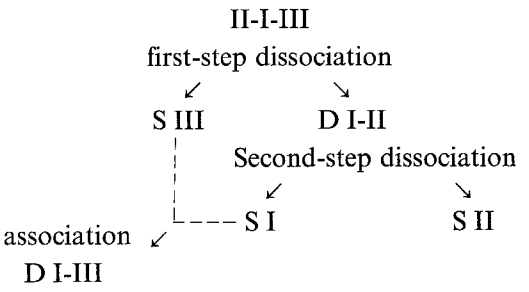
The organization of the subunits in the bovine ternary complex is reminiscent of that found in the mouse 7S nerve growth factor complex occurring in the adult male rat submandibular gland (Isackson et al. 1984). This complex, which is a hexamer, contains three types of subunits, two active subunits (β and γ) and an inactive serine protease-like subunit (α) the function of which remains obscure. As in the bovine ternary complex, one of the subunits, the β subunit, responsible for the nerve growth activity, is the central element of the complex and is specifically bound, at two distinct sites, to the other two subunits.

In order to get more information on the quaternary shape of the bovine pro CPA S6 complex, a small angle X-ray scattering investigation of all the molecular forms, free or associated subunits, has been carried out. Moreover, a simple geometric model describing the interaction of CPA with its activation peptide has been deduced from the comparison of the hypothetical structure of subunit I with the crystallographically determined structure of CPA.

Materials and methods

Preparation of the various molecular forms

The bovine ternary complex (pro CPA S6) was purified from an acetone-dried powder as previously reported (Puigserver et al. 1972; Kerfelec et al. 1984). The various molecular species were obtained from the complex as summarized in the following scheme:



The ternary complex was dissociated according to a two-step procedure using 2,3-dimethylmaleic anhydride as previously described (Kerfelec et al. 1984). After removal of the dimethylmaleyl groups by dialysis, all the molecular species (free subunits and binary complex I-II) were further purified by chromatography on DE-Trisacryl as previously reported (Kerfelec et al. 1985). The artificial binary complex I-III was obtained by filtration, on Ultrogel AcA 54 of a mixture containing equimolar amounts of subunits I and III (Kerfelec et al. 1985). All the purified molecular species were dialyzed against 50 m*M* *Tris*/HCl, pH 7.5, containing 0.1 *M* NaCl, then concentrated in an Amicon cell equipped with a PM 10 membrane and finally filtered through a Millipore HA filter (0.22 μm).

The concentration of the protein solutions was estimated from their absorbance at 280 nm using an absorption coefficient $A^{1\%}$ of 18 for the ternary complex, 20 for subunit II and the binary complex I-III, 16.2 for the binary I-II complex, 14 for subunit I and 18.8 for subunit III.

X-ray scattering experiments

X-ray scattering curves were recorded on the small angle scattering instrument D24 in the synchrotron radiation laboratory L.U.R.E. at Orsay (Depautex et al. 1987). The cross-section was of 0.5 mm × 2.0 mm at the sample level and 0.5 mm × 1.0 mm at the detector level. All solutions were studied in the same flattened quartz capillary 0.9 mm thick. The capillary was extensively flushed with buffer to rinse it between two successive samples.

A linear position sensitive detector with delay-line readout was used with an entrance window 3 mm wide. The data-acquisition system, developed at the E.M.B.L. (Heidelberg and Hamburg) has already been described (Bordas et al. 1980). The sample to detector distance was 800 mm with a corresponding channel width of $ds = 1.65 \times 10^{-4}$ Å^{-1}/channel. The scattering parameter is $s = 2 \sin \theta/\lambda$, where 2θ is the angle through which the X-rays are scattered, and the wavelength $\lambda = 1.608$ Å (K-edge of Co). The counting time was 1,600 s.

Background scattering from slits, residual air path, capillary tube and buffer was measured, normalized and subtracted from the scattering of the protein solution. The radius of gyration and the intensity at the origin were obtained from a linear regression on a Guinier plot ($\ln[i(s)]$ vs s^2) of the background corrected intensities (Guinier and Fournet 1955; Luzzati 1960).

The normalized intensity extrapolated to zero-angle is related to the Mr of the scattering species (Luzzati 1960). A solution of 4.7 mg/ml aspartate transcarbamylase (ATCase) from *E. coli* was used as a reference to derive a value for the relative molecular weight of the various species. ATCase is a very stable protein which presents no detectable interaction in solutions of low to moderate concentration (<15 mg/ml) (Moody et al. 1979; Hoover et al. 1983; Weber 1968). It has a molecular weight of 306 kDa and a partial specific volume of 0.739 cm^3 g^{-1}.

Partial specific volumes were derived from the amino-acid sequences and from the values for individual amino-acids previously published (Cohn and Edall 1941).

We tried to record data at large angles which could have given an estimate of the hydrated volume of each species and allowed us to calculate the distance distribution function. However, the scattered intensity was very weak at large angles and high concentrations were required which could not be reached. A hydration value was thus assumed so as to derive an estimate of the volume.

Determination of simple geometric shapes

If we know the radius of gyration Rg and the volume V of a particle, we can consider equivalent geometrical shapes depending on two parameters, such as ellipsoids of revolution (semi-axes a and b) or circular cylinders (height H and diameter D). The values of the parameters are then derived from the expressions of Rg and V:

ellipsoid: $R^2g = (2a^2 + b^2)/5$ $\quad V = 4\pi a^2 b/3$

cylinder: $R^2g = D^2/8 + H^2/12$ $\quad V = \pi D^2 H/4$

In each case, two solutions are obtained corresponding to prolate (elongated) and oblate (flattened) shapes.

The parallel axes theorem

Let us consider a complex Dij of two particles Si and Sj. Let Rgi and Rgj be the radii of gyration of the two isolated components, $Rgij$ the radius of gyration of the complex and dij the distance between the centres of gravity of Si and Sj. The parallel axes theorem gives the following relationship between the three radii of gyration and the distance d:

$$R^2gij = \alpha i R^2gi + \alpha j R^2gj + \alpha i \alpha j d^2 ij,$$

where $\alpha i = Mi/(Mi + Mj)$ with Mi the molecular weight of Si.

In the case of equivalent ellipsoids or cylinders, the shortest distance between the centres of gravity, without interpenetration, corresponds to a contact along the small axes for ellipsoids, along a generating line for prolate cylinders and across the section for oblate cylinders with the line joining the centres of gravity perpendicular to the large axes. Among the various combinations of those simple geometrical shapes in this closest distance configuration, dij is largest for two elongated ellipsoids and smallest for two flattened cylinders (Fig. 1). It should be stressed that a rotation of one of the particles around the line joining the centres of gravity would not modify the radius of gyration of the complex since it leaves d^2ij unchanged.

Results and discussion

Morphology of the free or associated components

Scattering spectra of all three monomers, of the two binary complexes I-II and I-III and of the ternary complex were recorded at several concentrations ranging from 2 to 17 mg/ml. Guinier plots are shown in Fig. 2 while the values of the radii of gyration are plotted in Fig. 3 as a function of concentration. The values extrapolated to infinite dilution are given in Table 1. Using ATCase as a standard, the values of the molecular weight of the various species derived from the intensity at the origin agreed within 20% with the corresponding value derived from the amino-acid sequence. The values of the ratio $Rg:M^{1/3}$ are given in Table 1. They all lie in the range 0.6 to 0.75 observed for globular proteins (lysozyme, bovine serum albumin).

Assuming a hydration value of 0.3 g H_2O/g protein, a typical value for soluble proteins, one can derive an estimate of the hydrated volume of the particle:

$$V = M[\bar{v} + \alpha \bar{v}_{H_2O}]/N_A,$$

where $\bar{v}$ is the partial specific volume of the protein, α the hydratation of the particle and N_A the Avogadro number. Simple geometrical shapes depending on two parameters, such as cylinders (height H and diameter D) or ellipsoids of revolution (semi-axes a and b) can

Table 1. Radii of gyration of the various species

Species	S I	S II	S III	D I-II	D I-III	T. C.
Molecular weight	45.127	25.838	25.824	70.965	70.951	96.789
R_g (Å)	24.1 ± 0.7	21.5 ± 0.7	21.0 ±0.3	27.4 ± 0.3	29.4 ± 0.3	32.1 ± 0.3
$R_g/MW^{1/3}$	0.68	0.73	0.71	0.66	0.71	0.70

S I, subunit I; S II, subunit II; S III, subunit III; D I-II and D I-III, binary complexes between subunits I and II and subunits I and III, respectively; T. C., ternary complex.
The molecular weights have been derived from the amino-acid sequences

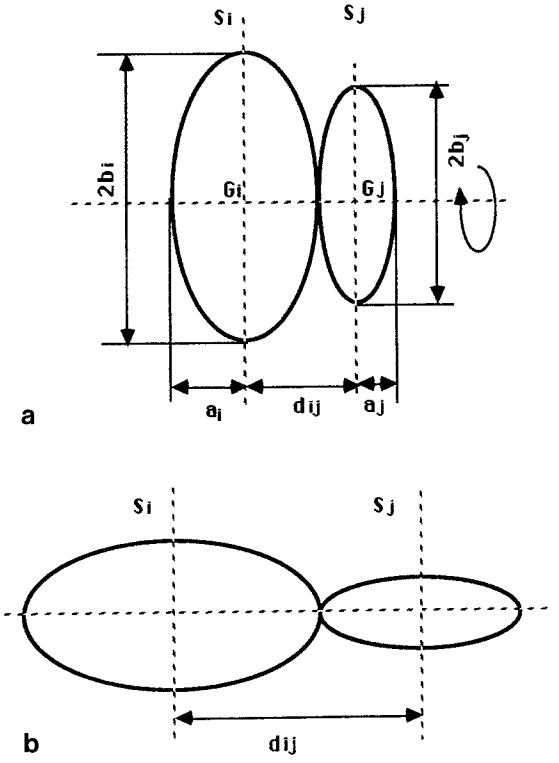


Fig. 1. Some geometrical representations. **a** Two elongated ellipsoids Si and Sj in the closest distance configuration. ai, bi and aj, bj are the semi-axes of the particles Si and Sj; dij is the distance between the centres of gravity of the two particles. The curved arrow indicates that one particle is free to rotate with respect to the other one around the line joining the two centres of gravity Gi and Gj without modifying the radius of gyration of the complex. **b** The same ellipsoids in the most extended conformation. Both diagrams **a** and **b** could be drawn for any combination of cylinders or ellipsoids

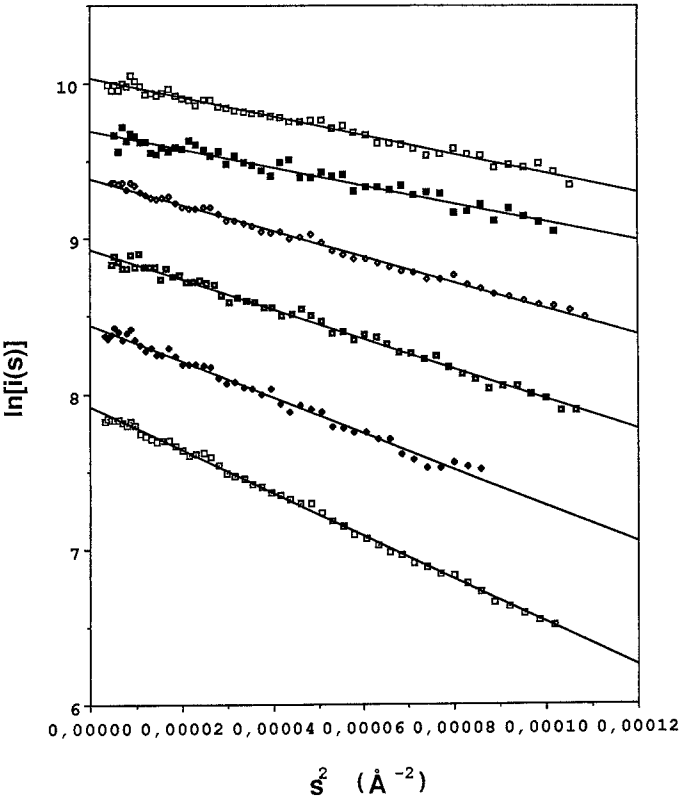


Fig. 2. Guinier plots of all the species studied. The curves have been arbitrarily displaced along the vertical axis for the sake of clarity. From top to bottom: subunit III, subunit II, subunit I, binary complex I-II, binary complex I-III and ternary complex

then be considered (see Material and Methods); the values of those parameters are shown in Table 2. All particles appear to be moderately anisometric, with values of the order of 2 to 3 (or their reciprocal values) for the anisometry parameter p defined in Table 2.

The distance $d12$ between the centres of gravity of subunits I (S I) and II (S II) in the binary complex I-II (D I-II) has a value of 30 Å as given by the parallel axes theorem (see Material and Methods). This lies in the range 24 Å–35 Å obtained with the simple geometrical shapes of Table 2 in the closest distance configuration (Fig. 1 a, Materials and Methods). By comparison, contacts along the large axes of the particles lead to values between 60 Å and 90 Å. Such an extended structure of D I-II can thus be ruled out (Fig. 1 b). In the case of D I-III a value of 38 Å is obtained, to be compared to the same 24–35 Å range, since S II and S III have a very similar size. Here too, an extended structure can be ruled out. This higher value of $d13$ suggests that S I and S III are not in the closest distance configuration, forming a somewhat more open binary complex than D I-II. It is worth noting that this is in qualitative agreement with the relative strength of association of the two binary complexes, D I-II being more stable than D I-III.

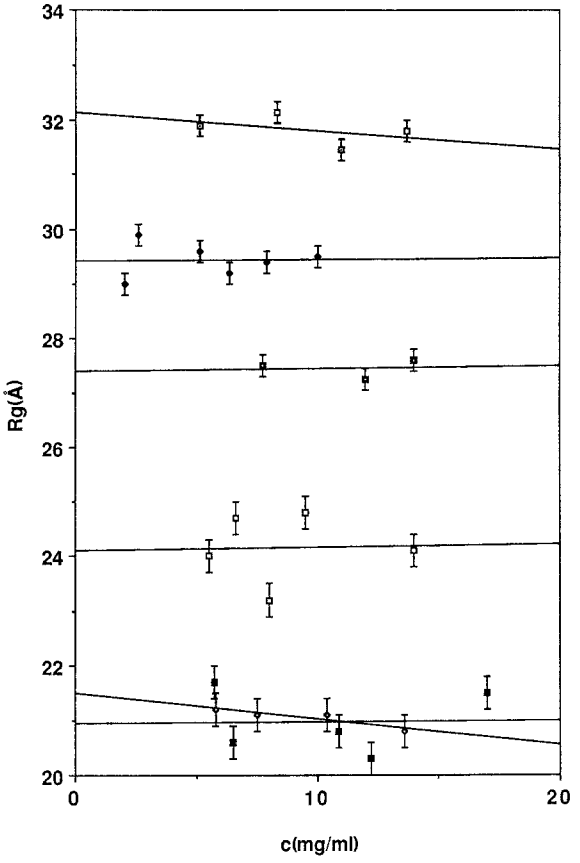


Fig. 3. Radii of gyration of each species as a function of concentration. The extrapolations to infinite dilutions are given in Table 1

The ternary complex can in turn be considered as the association of D I-II and S III or of D I-III and S II. Values of 43 Å and 37 Å are respectively obtained for the distances between centres of gravity. Again, this supports the view of a complex in the closest distance configuration or close to it. Indeed, if one considers, for instance, D I-III and S II as elongated ellipsoids in this closest distance configuration, the distance between the centres of gravity is 38.5 Å, close to the experimental determination, which leads to a radius of gyration of the ternary complex of 32.3 Å, within the error bars of the experimental value. The other combination (D I-II and S III) gives a value of 30.6 Å for the radius of gyration of the complex, slightly smaller than the experimental one.

These results show that the ternary complex can be built from the two binary complexes without modification of their proposed shapes. This is in agreement with previous biochemical data showing that, in the ternary complex, subunit III is also less tightly bound to subunit I than subunit II.

Morphology of the complex CPA-activation peptide

Subunit I is the precursor of carboxypeptidase A. A proteolytic cleavage yields the active enzyme with a molecular weight of 34.368 Da and the so-called activation peptide with the remaining 10.760 Da. Carboxypeptidase A has been studied by crystallographic techniques. In an early paper reporting the low resolution (5 Å) electron density map (Lipscomb et al. 1968), the dimensions of the equivalent ellipsoid are given: 52 Å × 44 Å × 40 Å. Adding 1.5 Å to the three semi-axes to account for hydration water leads to a value of 0.29 g H_2O/g protein, very close to the value of 0.3 g H_2O/g protein used previously.

If we approximate the activation peptide by a sphere, its radius is calculated to be 16.5 Å with a corresponding radius of gyration of 12.7 Å. Let us position this sphere on a tangent to the ellipsoid of carboxypeptidase A along one of the three axes. The radius of gyration of the complex is easily determined using the parallel axes theorem. Values between 23.8 Å and 25.0 Å are obtained, depending on the point of contact. Those values are very close to the experimental determination of 24.1 Å. Finally, if we append the activation peptide on a tangent along the longest axis of the carboxypeptidase, the overall dimensions of the assembly are 88 Å × 47 Å × 43 Å, reasonably close to the dimension of the elongated ellipsoid proposed for subunit I in Table 2 (Fig. 4).

All this suggests that the activation peptide forms a well defined domain clearly distinct from the active part of the molecule and also supports an elongated model for subunit I. The hypothesis of a folded structural domain for the activation peptide is strongly supported by biochemical studies. It must be emphasized that the bovine and porcine activation peptide, which are highly homologous, are very resistant to proteolysis. In this respect, the porcine peptide was found to possess a high content of secondary structure (Avilès

Table 2. Dimensions of simple geometrical shapes

Particle	Elongated models		Flat models	
	Ellipsoid 2a × 2a × 2b (Å) p = b/a	Cylinder H × D (Å) p = H/D	Ellipsoid 2a × 2a × 2b (Å) p = b/a	Cylinder H × D (Å) p = H/D
S I	40 × 40 × 92 2.3	70 × 38 1.8	74 × 74 × 27 0.36	23 × 66 0.35
S II	31 × 31 × 85 2.7	65 × 29 2.2	66 × 66 × 19 0.29	16 × 60 0.27
S III	32 × 32 × 82 2.6	63 × 30 2.1	66 × 66 × 20 0.30	17 × 58 0.29
D I-II	48 × 48 × 102 2.1	78 × 45 1.7	84 × 84 × 33 0.39	28 × 74 0.38
D I-III	45 × 45 × 115 2.6	88 × 42 2.1	91 × 91 × 28 0.31	24 × 81 0.30
T. C.	50 × 50 × 125 2.5	95 × 47 2.0	99 × 99 × 32 0.32	27 × 88 0.31

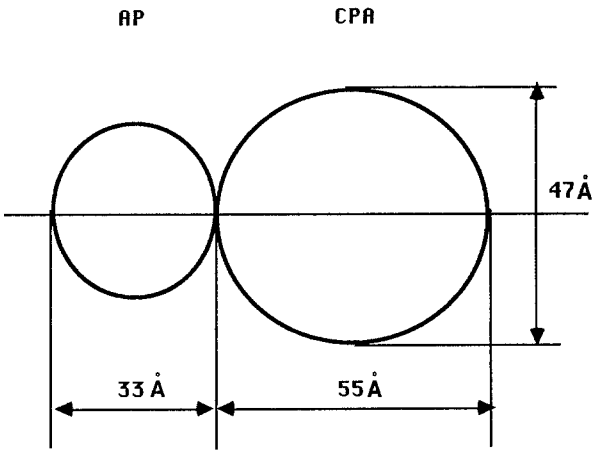


Fig. 4. Geometrical model of the complex CPA-activation peptide. Schematic representation of the complex of the carboxypeptidase A (*CPA*) and its activation peptide (*AP*) forming the subunit I

et al. 1982, 1985), suggesting that it is rather compact. Moreover, both peptides can reversibly inhibit the carboxypeptidasic activity, thus implying that they are not submitted to an extensive irreversible unfolding when they are removed from their enzyme part.

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