¹H-n.m.r. studies of the isolated activation segment from pig procarboxypeptidase A

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The isolated activation segment (asA) from pig pancreatic procarboxypeptidase A was studied by ¹H-n.m.r. spectroscopy over a wide range of solution conditions. Isolated asA shows many characteristics of compactly folded globular proteins, such as the observation of perturbed positions for resonances from methyl groups, α -carbon atoms, histidine residues and the tyrosine residue. The single tyrosine residue (Tyr-70) exhibits a very high pK_a , and both histidine and tyrosine residues show slow chemical modification (deuteration and iodination). In contrast, asA shows rapid NH exchange. Analysis of the spectra by pH titration and nuclear Overhauser effects revealed several residue interactions. Quantitative analysis of deuterium and tritium exchange allowed the assignment of the histidine C-2-H resonances to their respective residues in the sequence. His-66, the closest to the sites of proteolytic attack in the proenzyme, is shown to be the most accessible to solvent in procarboxypeptidase A. It was also shown that asA is thermally very stable ['melting' temperature (T_m) 88 °C] and requires a high urea concentration for denaturation (6.25 M, at pH 7.5). Evidence is presented for some degree of conformational flexibility in the premelting range, a feature that could be ascribed to the preponderance of helical secondary structure and to the lack of disulphide bridges. The free solution structure of asA is probably unchanged when it binds to carboxypeptidase A.

INTRODUCTION

The monomeric form of pig pancreatic procarboxypeptidase A (PCPA), because of its simplicity, constitutes a good model for studying different properties of this class of proenzymes [1–3], particularly those related to the inhibition and activation processes. To gain insight into these processes, we have isolated the activation segment of PCPA (asA) [2] and determined its primary structure [4]. The asA is a folded 94-residue peptide [2,5], with a high content of α -helix [5,6], and with two potential α -helix-loop- α -helix structural motifs along the sequence [6,7]. This protein fragment also adopts a conformation that is highly resistant to denaturation [5,8,9].

The presence of the asA domain is probably the main reason for the low intrinsic activity displayed by the proenzyme and the slow proteolytic activation of it, since isolated asA shows strong inhibitory power towards carboxypeptidase A (CPA) (K_i approx. 2 nM [2]). However, it is uncertain whether the conformation of asA is the same in the isolated form and when integrated within PCPA. Thus, when the folding–unfolding processes of isolated asA and asA bound to the active enzyme were studied by electrophoresis in urea-gradient polyacrylamide gels [8] and scanning microcalorimetry [9] a different behaviour was observed for the two forms.

In order to define the limits within which the asA maintains its conformation and set the molecular basis for understanding the relationships between its conformation and inhibitory power, we have further studied the asA by n.m.r. spectroscopy. This study also aims to find the optimum conditions and to establish internal references for subsequently determining the detailed folding of asA in solution by two-dimensional n.m.r. The results show that isolated asA adopts a compact globular conformation

that is maintained over a wide range of solution conditions of pH, ionic strength, denaturant concentrations and chemical modifiers. However, evidence is also found showing that asA possesses some degree of conformational flexibility. Isotope-exchange studies followed by n.m.r. and h.p.l.c. have additionally allowed us to assign resonances from individual histidine residues within the sequence of asA and given information of significance for the activation process with regard to the accessibility to solvent of the regions of asA containing histidine residues, both in the isolated form and within the proenzyme.

MATERIALS AND METHODS

Preparation of the activation segment

Monomeric PCPA was isolated from the acetone-dried powder of pig pancreas as in ref. [10]. The isolated asA was obtained by limited proteolysis of the zymogen by trypsin followed by chromatography on DEAE-Sepharose in the presence of 7 M-urea, as described in ref. [2]. After removal of urea by dialysis, asA was concentrated by chromatography on small DEAE-Sepharose columns, freeze-dried and stored at –20 °C. Some preparations of asA contained several sharp resonances from organic contaminants in the ¹H-n.m.r. upfield spectrum, and these were removed by isoelectric precipitation of the protein at pH 4.5 followed by redissolution in the buffer of analysis. This method was also used to eliminate water and replace it by ²H₂O in some samples. Alternatively, asA was obtained by means of reverse-phase h.p.l.c. Samples (300 μ l) of a PCPA solution at 2 mg/ml in 40 mM-Tris/HCl buffer, pH 7.5, were treated with trypsin at a PCPA/trypsin ratio of 40:1 (w/w) at 25 °C for 5 min. After addition of 0.25 % (v/v) trifluoroacetic acid, samples

Abbreviations used: asA, activation segment of procarboxypeptidase A; PCPA, procarboxypeptidase A; CPA, carboxypeptidase A; RCS resonance, ring-current-shifted resonance.

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were loaded on to an Ultrapore C-3 reverse-phase column (Beckman) that had been equilibrated with 0.05 % trifluoroacetic acid. Elution of isolated asA used a gradient from the initial conditions up to 40 % (v/v) acetonitrile in 0.045 % trifluoroacetic acid over 35 min, with the use of curve no. 4 of an M680 (Waters) gradient controller at a flow rate of 1 ml/min. Acetonitrile was evaporated under vacuum and samples were freeze-dried.

N.m.r. spectroscopy

A Bruker 270 MHz spectrometer equipped with quadrature detection and a variable-temperature accessory, operating with double-precision accumulation in the Fourier-transform mode, was used. Normally, 2000 transients were averaged by using a 90° pulse (12 μ s), preceded by a gated irradiation of 0.3 s to diminish the solvent (water) peak. A delay time of 200 μ s and a sampling time of approx. 0.6 s was used. Resolution enhancement was performed by convolution difference [11]. Difference Overhauser spectra were obtained as previously described [12]. Generally 100 000–400 000 scans were required to observe a clear Overhauser effect. In most experiments chemical shifts were referenced to the ^1H signal from 2,2-dimethyl-2-silapentane-1-sulphonic acid run in parallel spectra. All samples were dissolved in 99.8 % (v/v), $^2\text{H}_2\text{O}$ in the appropriate buffer, and all the spectra were obtained at 25 °C unless otherwise stated. For certain n.m.r. spectra the isolated asA was dissolved in $^2\text{H}_2\text{O}$ at 3–5 mg/ml and after the pH had been adjusted to 8.5, incubated in these conditions for 2 h at room temperature. Under these conditions most of the slowly exchanging low-field proton resonances (NH protons) were removed. Subsequently, the protein sample was equilibrated at the desired pH either with NaO^2H or ^2HCl or by addition of deuterated buffer.

pH titration of different residues of asA was made by addition of small portions of NaO^2H solution to the sample and the pH readings were taken in the n.m.r. tube with a 3 mm-diameter combination microelectrode. All pH values refer to the reading uncorrected for the presence of $^2\text{H}_2\text{O}$. Subsequent n.m.r. spectra were obtained at 25 °C, in the presence of an external standard of 1 mM-tetramethylammonium chloride. The shifts were made relative to 2,2-dimethyl-2-silapentane-1-sulphonic acid by adding 3.17 p.p.m. The titration curves were derived by fitting the experimental data to theoretical sigmoidal-type curves by using a non-linear-regression analysis programme.

The analysis of the kinetics of isotopic exchange of amide NH protons was carried out by dissolving the sample in 5 mM-potassium phosphate buffer, pH 6.7, in $^2\text{H}_2\text{O}$ with or without salt added and recording a series of spectra (15 min each) at fixed periods of time at 25 °C.

Histidine ^2H and ^3H exchange

Histidine deuteration was carried out at 25 °C, 40 °C and 50 °C in 0.1 M-NaCl/5 mM-potassium phosphate buffer, pH 7.5, in the same n.m.r. tube. Measurement of the degree of deuteration of the C-2 protons of histidine residues was carried out after adjusting the sample temperature to 25 °C (1 h each run).

Histidine tritiation was carried out at 37 °C in 20 mM-Tris/HCl buffer, pH 8.0. In the case of PCPA, 2 mg of protein was incubated at 2.5 mg/ml in the incubation solution containing tritiated water at a concentration of 0.45 Ci/ml. At 24, 48 and 72 h, 250 μ l portions (625 μ g of protein) were withdrawn and treated with trypsin at a PCPA/trypsin ratio of 40:1 (w/w) and incubated for 5 min at 25 °C. The activation segment was isolated from this digest as described above. In the case of isolated asA, incubation was performed at a 0.65 mg/ml in the same buffer containing the same tritium concentration as with the proenzyme. At 24, 48 and 72 h, 250 μ l portions (162 μ g of protein) were

withdrawn and immediately subjected to h.p.l.c., as described above, to eliminate non-incorporated radioactivity.

The samples of tritiated asA isolated from both PCPA and asA were digested with bovine trypsin [L-tosylphenylalanyl-chloromethane ('TPCK')-treated] at 37 °C. Trypsin was added as two portions at the beginning and at the middle of a 2 h period to reach a final asA/trypsin ratio of 25:1 (w/w). The resulting peptide mixture was loaded on to a μ Bondapak C_{18} reversed-phase column equilibrated with 0.1 % trifluoroacetic acid.

Elution of the individual peptides was achieved with a gradient from the initial conditions to 80 % (v/v) acetonitrile in 0.09 % trifluoroacetic acid over 120 min at a flow rate of 1 ml/min. Isolated peptides were dried *in vacuo*, redissolved in scintillation solution free of tritium and counted for radioactivity, with corrections for counting efficiency.

Chemical modifications

Samples of asA were iodinated by addition of portions of 0.05 M- I_2 /0.25 M-KI in $^2\text{H}_2\text{O}$ to the protein solutions in 10 mM-sodium tetraborate buffer, pH 9.0, at 25 °C. After 1 h standing at 25 °C, n.m.r. spectra (30 min running time) were recorded. A similar timing and procedure was followed when additional portions of iodinating reagent were added. Finally, the pH was adjusted to 6.4 and the n.m.r. spectrum was recorded again. Portions of the samples at an I_2 /asA molar ratio of 8:1 were analysed by electrophoresis in urea-gradient polyacrylamide gels.

Denaturation studies

Urea denaturation studies were performed by stepwise addition of solid deuterated urea (Sigma Chemical Co.) to the solution of isolated asA at 3–5 mg/ml in 20 mM-potassium phosphate buffer, pH 6.5 or 7.5, in 99.8 % $^2\text{H}_2\text{O}$, followed by n.m.r. analysis at 25 °C after each addition (approx. 70 min interval between additions). The pH of the sample was checked before each spectrum. Thermal denaturation studies were carried out on isolated asA at 5 mg/ml in 0.1 M-NaCl/99.8 % $^2\text{H}_2\text{O}$.

Electrophoresis on urea gradients

Polyacrylamide gels with transverse urea gradients (0–9 M) were prepared according to Creighton's procedure [13,14] in 45 mM-Tris/25 mM-boric acid/0.78 M-EDTA, pH 8.6. Electrophoresis was carried out at 300 V for 5 h at 22 °C. Gels were stained by diffusion with 0.1 % Coomassie Blue.

RESULTS AND DISCUSSION

Effect of pH and salt concentrations

Several features that indicate a globular folded conformation for the isolated activation segment (asA) of PCPA are apparent in its ^1H -n.m.r. spectrum at intermediate pH (i.e. pH 6.5), as shown in Fig. 1. Thus at low field the C-2-H resonances of the three histidine residues of the protein show a particular and different chemical shift; the C-3-H + C-5-H and C-2-H + C-6-H proton resonances from the single tyrosine residue of the molecule are slightly shifted upfield with respect to the random-coil position, and several putative C_{α} -H proton resonances are spread from 5.0 to 6.55 p.p.m., i.e. far from their usual unperturbed position at about 4.5 p.p.m. At high field other important conformationally dependent features are the series of ring-current-shifted (RCS) resonances, probably from methyl protons, located between 0 and 1.0 p.p.m.

From the above indicators, the folded globular conformation of asA is maintained from pH 13.0 to pH 5.7 (see Fig. 2). Analysis of the conformation from pH 5.7 to pH 3.0 is hindered by macroscopic aggregation of the peptide, probably as a result of its isoelectric neutralization. Below pH 3.5 asA has an inter-

Fig. 1. 270 MHz ^1H -n.m.r. spectrum of the isolated asA of pig pancreatic PCPA in $^2\text{H}_2\text{O}$ at 5 mg/ml at pH 6.5 and 25 $^\circ\text{C}$

RCS methyl peaks and several resolved resonances are labelled. The insets show expanded regions of the spectrum. The change in the downfield spectrum due to hydrogen/deuterium exchange, and at two different times after dissolution, is also shown.

Fig. 2. pH titration of several resonances of isolated asA in $^2\text{H}_2\text{O}$ at 5 mg/ml at 25 $^\circ\text{C}$

Titration was performed by addition of small portions of NaOH solution, and correction of the measured pH for the presence of salts was made in the upper alkaline range. All pH values refer to uncorrected readings for the presence of $^2\text{H}_2\text{O}$.

mediate solubility, but under these conditions shows a significant loss of folded structure, as seen by the coalescence of the above-mentioned perturbed resonances. This situation is completely reversible after a change to neutral or alkaline pH.

As judged by the various perturbed resonances, an increase in salt concentration of the medium from 20 mM- to 1 M-NaCl had little effect on the peptide fold either at low or at high pH (results not shown). Moreover, the increase in salt concentration promoted aggregation of the asA near its isoelectric point,

widening the range of pH over which it aggregates. This can be explained by the very high content of apolar residues in asA (48%) [2,4] and to the possible existence on its surface of apolar regions designed to bind to CPA.

Titration of specific residues

Fig. 2 shows the titration of the C-2-H resonances from the three histidine residues. A non-linear least-squares analysis leads to pK_a values of 6.49, 6.43 and 6.40 for the resonances labelled

His-I, His-II and His-III respectively. Application of the same approach to C-4-H singlets at approx. 7.2 p.p.m. yields a pK_a of 6.50. The closeness between all pK_a values hinders the use of this parameter for the pairing of the resolved histidine C-4-H resonance with the other C-2-H resonances. All these values are similar to those shown by histidine residues in random-coil proteins [15]. This fact suggests that the histidine residues of asA are in similar microenvironments and initially points to the possibility of their being surface residues. However, their different chemical shifts and their low rate of isotope exchange and chemical modification (see below) do not accord with a location on the surface.

The lack of pH-dependence for several resonances located between 5.0 and 6.55 p.p.m. is also of interest. This spectral pattern frequently arises from hydrogen-bonded C_{α} -H protons included in β -sheet structures [16], such as those from bovine pancreatic trypsin inhibitor [16,17] or from the interconnected loops of the EF-hand Ca^{2+} -binding proteins [18,19]. In this respect it is worth mentioning that asA contains about 11% of β -structure [5], and that it shows folding propensities similar to the members of the latter family of proteins [6]. On the basis of these observations, we provisionally assign the perturbed resonances to C_{α} -H protons located in the inner part of β -sheets. The only exception to those assignments would be the resonance that shifts from 6.7 to 6.1 p.p.m. on going from pH 5.7 to 8.0, which could belong to a highly perturbed C-4-H proton from a histidine residue.

Titration of the resonances from the single tyrosine residue of asA is also observed at high pH, as shown in Fig. 2. The pK_a value for the ionization of the tyrosine residue derived from these experiments is very high, over 11.8, and is in accordance with values obtained by u.v. absorbance and fluorescence spectrophotometric titration (M. Vilanova, unpublished work). This suggests that the tyrosine residue is located in a buried environment or is hydrogen-bonded to another residue. The upfield shift suffered by the tyrosine resonance after titration also allows one to observe the existence of unknown broad resonances (perhaps from phenylalanine residues or the tryptophan residue) hidden underneath the tyrosine C-3-H + C-5-H and C-2-H + C-6-H resonances at intermediate pH (see Fig. 5). It is notable that the changes observed in the n.m.r. spectrum of asA when titrated to pH 13.0 are fully reversed when the peptide is adjusted back to intermediate pH.

Changes in the upfield spectrum are also observed in the whole titration range. Most of the RCS methyl resonances between 0 and 0.6 p.p.m. display pH-dependent chemical shifts, as shown in Fig. 2. Some of these RCS resonances show a double-sigmoidal titration, each component related to the titration of the histidine residues and the tyrosine residue, a fact that suggests a complicated net of spatial relationships.

Exchange of NH protons

Analysis of the low-field spectra of asA at short intervals after dissolution in 2H_2O , at pH 6.5 and 25 °C, shows the rapid disappearance of several resonances, probably assignable to NH protons (amide backbone, histidine and tryptophan NH protons; see Fig. 1 inset). A rough estimate based on area measurements indicates the existence of ten slowly exchangeable NH protons, with an average half-life somewhat less than 4 h. Additionally, another five NH protons are observed to be very resistant to exchange by deuterium, with half-lives greater than several days. At 40 °C only the same highly resistant protons are visible after 1 h dissolution in 2H_2O . A similar behaviour is observed at pH 7.5, at both temperatures. The number of slowly exchangeable NH protons is surprisingly small, taking into account that folded asA contains about 50 NH backbone protons involved in α -

helices [5], a conformational feature that should decrease the exchange of these protons [17]. This suggests that isolated asA has a folded structure with a high degree of conformational flexibility that facilitates the hydrogen/deuterium exchange. Conformational flexibility has also been proposed for the isolated globular domains of other proteins rich in α -helix and that lack disulphide bridges, such as the very-lysine-rich H1/H5 histones [20–22] and the *lac* repressor head-piece [23].

Histidine isotope-exchange kinetics and sequence assignments

In Fig. 3(a) a tryptic peptide map of asA obtained by reverse-phase h.p.l.c. is shown, indicating the histidine-containing peptides. From the measurement of tritium incorporated into these peptides after incubation of isolated asA in tritiated water (at pH 8.0), the different exchange rates of the three histidine residues of asA were deduced (see Fig. 3b). The same experiments were also performed for asA when included within PCPA. The results, expressed as the fraction unexchanged versus time, show that the relative exchange rates are His-31 > His-66 > His-7 in isolated asA and His-66 > His-31 > His-7 in PCPA. Whereas His-66 presents the same exchange rate in both cases, both His-31 and His-7 incorporate much less tritium when asA is forming part of the whole proenzyme. It is clear that the latter two histidine residues are much less accessible to the solvent when they are included in the proenzyme. In contrast, His-66 probably does not suffer any environmental change when asA is severed from PCPA. This is in agreement with the fact that His-66 is the histidine residue closest to the region subjected to tryptic cleavage during activation [24], whereas the other two histidine residues (31 and 7) probably belong to a region interacting with CPA, thus making their accessibility to the solvent more difficult.

To assign histidine resonances in the 1H -n.m.r. spectrum to sequence positions, the above tritium exchange rates can be correlated with n.m.r.-measured deuterium exchange rates of isolated asA dissolved in deuterated water. The exchange rate of deuterium was found to be very dependent on temperature. Thus a very important increase in exchange rates was observed on going from 25 °C to 40 °C and to 50 °C (results not shown). This strong dependence of deuteration on temperature could be ascribed to the flexible character of this protein, favoured by its lack of disulphide bridges. In Fig. 3(c) we show a plot of the area of the histidine C-2-H peaks in the n.m.r. spectrum as a function of time at 40 °C, using the area of non-exchangeable aromatic peaks as a reference. The half-lives for the three histidine residues in the spectrum are 78, 35 and 57 h for His-I, His-II and His-III respectively. Comparing the relative rates of deuterium exchange with the results of tritium incorporation, the following peak assignment can be deduced: His-I is His-7, His-II is His-31 and His-III is His-66.

Iodination of histidine residues and the tyrosine residue

Particular changes in the n.m.r. spectrum of isolated asA were observed after addition of I_2/KI at pH 9.0, as shown in Fig. 4. No spectral changes were observed at low concentrations of iodination reagent, e.g. 2 equivalents of I_2 per mol of protein. However, when the concentration of reagent was doubled, the disappearance of the resonance at 7.4 p.p.m. is the most apparent differential feature. This resonance is preliminarily assigned to a C-2-H proton of a tryptophan residue, in view of its spectral location, its being capable of substitution by iodine and its constant chemical shift with pH. At 8 mol of I_2 added per mol protein, partial modification can be seen of the histidine C-2-H (resonances I, II and III), of the C-4-H resonance at about 6.9 p.p.m. (which lies on top of the tyrosine C-2-H + C-6-H resonances at pH 9.0) and the low-field tyrosine resonance. Under these conditions a strong decrease in the intensity of the

Fig. 3. Histidine isotope-exchange kinetics

In (a) is shown a tryptic peptide map of asA obtained by h.p.l.c. on a reverse-phase column as described in the Materials and methods section. Histidine-containing peptides are labelled with their position in the sequence. (b) Tritium exchange in histidine residues of asA as measured by h.p.l.c.: continuous lines and Δ , $\square$ and $\circ$ symbols refer to the quantification made on isolated asA, and broken lines and $\blacktriangle$, $\blacksquare$ and $\bullet$ symbols to asA within PCPA; Δ and $\blacktriangle$, His-7; $\square$ and $\blacksquare$, His-31; $\circ$ and $\bullet$ His-66. (c) Deuterium exchange of histidine residues of asA as measured by n.m.r.: the same symbols as in (b) are used, and correspond only to experiments made on isolated asA. In both cases, the fraction unexchanged is plotted on a logarithmic scale versus time. See the Materials and methods section for incubation conditions.

Fig. 4. Effect of iodination on the conformation of isolated asA

(a) A portion of the downfield spectrum, showing the changes in the histidine and tyrosine side-chain resonances at different iodine/asA ratios. (b) Electrophoresis on transverse gradients of urea (0-9 M) of isolated asA before (i) and after (ii) treatment with iodine at 8 mol of I_2 /mol of asA. The protein at 5 mg/ml in 10 mM-sodium tetraborate, pH 9.0, was treated with different relative quantities of a freshly prepared I_2 /KI mixture in 2H_2O . The spectra or the electrophoretic analyses were taken after 1 h incubation at room temperature.

RCS resonances 2 to 4 (between 0.3 and 0.6 p.p.m.) was also observed, a possible indication of unfolding of the peptide as a result of the chemical modification.

Unexpectedly, analysis at intermediate and high pH (pH 6.5 and 9.0) of the heavily iodinated sample indicated that RCS resonance 1 maintained its intensity and spectral position. To check the overall folding of both samples, they were analysed by electrophoresis in transverse gradients of urea (0–9 M), as shown in Fig. 4(b). This experiment demonstrated that the iodinated and the non-iodinated asA are both folded and exhibit a similar unfolding with urea concentration. This behaviour suggests that on iodination asA suffers only local changes in tertiary structure.

The above experiments suggest that the histidine residues and single tyrosine residue of isolated asA are neither fully exposed surface residues nor fully buried, since their chemical modification is accomplished at intermediate relative concentrations of iodination reagent as compared with other proteins having similar conformational features [25,26]. Furthermore, these modifications do not heavily distort the overall folding of the molecule.

Inter-proton Overhauser effects

Overhauser effects on the aromatic proton resonances are observed when the upfield RCS methyl resonances are specifically irradiated, as shown in Fig. 5. Mixed Overhauser effects are observed in some of these experiments, probably because of the difficulty of irradiating a single resonance without affecting the adjacent ones. This is particularly severe for RCS resonances 3 to 5, whose effects are not discussed here. However, comparison of differential Overhauser spectra at intermediate and high pH (7.3 and 11.8 respectively) help in the assignment of particular contributions for the other RCS resonances. Thus, on irradiation of RCS resonances 1 and 1', very clear Overhauser signals emerge at 7.2–7.4 p.p.m. in the region of non-pH-titrating aromatic resonances, still unassigned but probably ascribable to the single tryptophan residue or to phenylalanine residues. On irradiation

Fig. 5. Inter-proton Overhauser effects after selective irradiation of RCS resonances

Nuclear Overhauser difference spectra of isolated asA were obtained at two different pH values, 7.3 and 11.8, at 25 °C. After irradiation at RCS 1/1' or 2/2' (broken arrows), Overhauser effects were observed for the indicated resonances from aromatic residues (continuous arrows) in the downfield region of the spectrum.

of RCS resonances 2 and 2', clear Overhauser signals appear at the spectral positions of Tyr-70 C-3-H + C-5-H and C-2-H + C-6-H resonances in addition to another broad signal in the region of non-titrating aromatic resonances, probably again from phenylalanine residues or the tryptophan residue. The Overhauser signal in the position of tyrosine resonances observed on irradiation of RCS resonance 2 cannot arise from residual irradiation on adjacent RCS resonance peaks since this signal is not obtained when direct irradiation is performed on RCS resonance 3 or 4 or 1, as already stated. It can thus be concluded that the single tyrosine residue of the protein is in close proximity to the methyl group of the unknown apolar residue that generates RCS resonance 2, and is probably that responsible for its upfield shift.

Urea and thermal denaturation

At pH 6.5, addition of urea up to 2 M induces some significant changes in the spectrum: RCS resonance 1 shows a continuous displacement to lower field (Fig. 6b), whereas the remaining RCS resonance peaks remain largely unchanged in both position and area. In addition, one of the histidine C-2-H peaks (His-II, corresponding to residue 31) moves upfield in this interval of urea, whereas, for example, His-III and the tyrosine peak at 6.8 p.p.m. remain unchanged (Fig. 6a). Beyond 2 M-urea (4–8 M) the major unfolding transition occurs, and this can be monitored by comparing total area of the RCS resonance peaks with that of the total methyl peak area (Fig. 6c). At pH 7.5, higher concentrations of urea are required to induce similar spectral changes in asA. From the analysis of the denaturation curves it was found that the mid-point was 5.5 M-urea at pH 6.5 and 6.25 M-urea at pH 7.5.

The above values are in accord with those deduced from electrophoresis of asA in transverse urea gradients (0–9 M), at pH 8.6, from which a denaturation mid-point of 6.2 M can be calculated (see Fig. 4b). A careful analysis of the electrophoretic profile reveals a small and continuous decrease in mobility of asA in the 0–4.5 M-urea range, whereas an abrupt and sigmoidal decrease in mobility, with a single inflexion point, is observed in the 4.5–9 M-urea range. The former effect can be attributed to a small increase in the hydrodynamic volume of asA at the lower urea concentration range. The major electrophoretic change is indicative of a rapidly equilibrating two-state unfolding [14]. This interpretation of a rapid equilibrium between the folded and denatured conformations of asA is in full agreement with that derived from n.m.r. experiments, in which a broadening of histidine C-2-H resonances is observed between 4 M- and 6 M-urea, that is at the middle of the denaturation transition (results not shown).

Another denaturant of asA, increase in temperature, promotes a downfield shift for RCS resonance 1 similar to that observed for urea denaturation. As previously described [5], a downfield displacement of RCS resonance 1 is observed from 25 to 70 °C, a pre-melting range as judged by comparison of the denaturation of asA seen by n.m.r. and by differential scanning calorimetry [9].

The observation of a peak of continuously changing chemical shift is similar to the n.m.r.-spectral effects reported for the thermal denaturation of histones H1 and H5 [20,21], of calbindin [19], of calmodulin [27] and of the *lac* repressor head-piece [23]. It is noteworthy that all these proteins show important similarities to asA: a single globular domain of small size, a high content of α -helix and a lack of disulphide bridges. Crane-Robinson & Privalov [22] suggested that the continuous spectral displacement observed in the thermal denaturation of histones H1 and H5 could be related to considerable side-chain motions in the protein and to the increase of these motions with unfolding. In the case of calbindin and calmodulin, this displacement has been

Fig. 6. Addition of urea to isolated asA

(a) Low-field spectrum showing upfield displacement of the HisII resonance between 0 M- and 2 M-urea and the complete unfolding at higher urea concentrations. (b) Observed changes in spectral position and area of upfield RCS resonances with change in urea concentration. (c) Quantification of area decrease underneath the upfield RCS methyl resonances at increasing concentrations of denaturant at two different pH values, 6.5 (○) and 7.5 (●). The percentage of RCS area with respect to the total apolar resonances area is plotted versus urea concentration.

attributed to the quite mobile internal structure of the protein and to the expansion of the tertiary fold with temperature [19,27]. In accordance with this, isolated asA shows significant temperature-induced side-chain mobility and conformational expansion in the pre-melting range.

The above conformational effects at low and intermediate concentrations of denaturants are probably restricted to certain regions of asA, since the spectral displacements were observed for only a limited number of resonances. In contrast, a high temperature is required for the disruption of the overall folding of asA, a fact that recalls the behaviour of calmodulin [18,27] and calbindin [19], proteins with folding propensities similar to asA [6]. The very high content of apolar residues in all these proteins may be responsible for their high thermostability [28], and their folding in packed α -helices should facilitate their flexibility and pre-melting conformational transitions [29]. In this respect, it is also worth mentioning that these proteins do have a small percentage of β -structure, which could have an important contribution to the stability by acting as rigid parts within the conformation [30]. In the particular case of asA, this hypothesis is substantiated by the constancy of chemical shifts in the urea and thermal pre-melting ranges for those resonances located between 5.0 and 6.55 p.p.m. that have been assigned to $C_{(\alpha)}$ -H protons involved in β -structures.

CONCLUSIONS

The isolated activation segment of PCPA (asA) in solution presents many characteristics of a stable globular fold over a range of pH from 5.7 to 13 and from 10 mM to 1 M salt concentration. The isolated asA also shows a high conform-

ational stability against temperature ['melting' temperature (T_m) 88 °C] and urea denaturation (midpoint from 5.5 M- to 6.25 M-urea, according to pH). In the absence of disulphide bridges, this high stability can be largely ascribed to its high content of apolar residues.

The thermostability of asA is not the consequence of a high overall rigidity, since it shows a rapid hydrogen/deuterium exchange in most of its NH groups at room temperature. Of particular interest are the small conformational transitions that have been detected under pre-melting conditions. This behaviour is probably due to the folding of the protein largely in α -helices.

It seems likely that the intermediate flexibility of asA, reflected in fairly rapid NH exchange rates and in the pre-melting conformational transitions, may play an important role in its binding to CPA and in the inhibition of its activity. However, because of its high stability, it is unlikely that the overall conformation observed for asA in free solution suffers drastic changes after this binding. The previous evidence showing a different denaturation of asA in the isolated form and within the proenzyme [8,9] must be related more to the co-operative action of CPA than to a different conformation of the segment in the two forms.

Isotope-exchange measurements show that the exposure to solvent of His-66 is little affected by the detachment of asA from CPA. In contrast, His-7 and His-31 are significantly altered, suggesting that they lie close to the interface between the two components of the proenzyme, CPA and asA.

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