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Interaction of DAPI with pepsin as a function of pH and ionic strength

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Abstract

The fluorescent probe 4′,6-diamidino-2-phenylindole (DAPI), extensively used with nucleic acids, has also recently been used with membranes and proteins (Favilla et al., Biophys. Chem., 46 (1993) 217–226 and Mazzini et al., Biophys. Chem. 52 (1994) 145–156, respectively).

The spectroscopic changes of DAPI observed, namely a considerable enhancement of fluorescence, induced circular dichroism (CD) and absorbance spectral shifts, have been exploited to study the binding of this dye to both native and alkali denatured pepsin. Fluorescence and CD titrations show that nearly two molecules of DAPI bind to either native or alkali denatured pepsin with pH and ionic strength dependent K_d values, whereas absorbance titrations evidentiate an interaction characterized by a lower affinity and a larger number of binding sites. Therefore two kinds of interaction are proposed: a specific one, involving both hydrophobic and electrostatic forces; and a non-specific one, involving surface protein negative charges only.

Finally, the behaviour of DAPI as a competitive inhibitor and the remarkable effect of pepstatin A, a specific inhibitor of pepsin, on both the CD and fluorescence spectra of DAPI in the presence of pepsin, show the involvement of the protein active site in the interaction. © 1997 Elsevier Science B.V.

Keywords: Pepsin; 4′,6-diamidino-2-phenylindole (DAPI); Binding; Fluorescent probe; Circular dichroism

1. Introduction

4′,6-diamidino-2-phenylindole (DAPI) is a synthetic drug belonging to the class of aromatic diamidino compounds, the structure of which is shown in Scheme 1.

The dye, initially studied for its trypanocidal activity [1], has become widely used mainly as a DNA probe, because of its large increase of fluorescence quantum yield upon binding to double helical DNA [2,3]. The occurrence of two modes of interaction between DAPI and DNA has been suggested: the first one as a very strong non-intercalative minor groove binding ligand, preferentially interacting with AT-rich regions [4–6]; the second one as an inter-

Abbreviations: DAPI: 4′,6-diamidino-2-phenylindole; CD: circular dichroism

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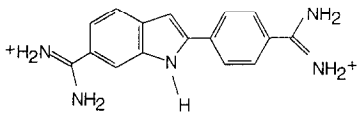

Scheme 1. Chemical structure of 4′,6-diamidino-2-phenylindole.

calative ligand with GC or mixed AT and GC DNA sequences [7]. In addition, DAPI has also been found to interact with AU-rich sequences in RNA [8].

DAPI has also been employed as a fluorescent probe with other systems: with tubulin, to monitor either microtubule assembly [9] or digestion of this protein by subtilisin [10], with sarcoplasmic reticulum vesicles to study the Ca^{2+} pump function [11], and more recently as a specific inhibitor of serine proteases [12].

During the past few years we have studied the binding of this dye to proteins, as well as to phospholipid bilayers, by exploiting its large enhancement of fluorescence in order to evaluate the binding parameters [13–15]. From these investigations it was clear that DAPI is able to interact only with acidic proteins or proteins above their isoelectric points. This can be ascribed to the fact that DAPI is positively charged at any pH below 12, due to the presence of its two diamidino groups, thus bringing to the fore the importance of electrostatic forces in the interaction with those proteins. However, as we have already seen from the study of the interaction of DAPI with phospholipid bilayers [14], it was clear that the hydrophobic moiety of the dye also plays an important role in the interaction. It therefore seemed interesting to us to see whether the hydrophobic moiety of the dye is also involved in the interaction with proteins.

Among a few proteins preliminarily considered, stomach porcine pepsin was found to interact with DAPI by both electrostatic and hydrophobic contributions, as previously described in a concise report [16]. In order to better assess the role played by the electrostatic terms, we have investigated this system in more detail by performing experiments at several different buffer concentrations. As a consequence, the possible residual interaction still present at high ionic strength should be attributed to the hydrophobic terms.

Moreover, since pepsin undergoes an important conformational transition from the native (at acidic pH) to the denatured state in a narrow pH range (between 6 and 7), this protein appears to be a suitable test system for checking the ability of DAPI to behave as a structural probe.

Finally, in order to achieve information about the binding site of DAPI, we have studied the possible effect of a specific inhibitor of pepsin (pepstatin A) on the binding of DAPI as well as the possible inhibitory effect of DAPI itself on the enzyme activity.

The kinetics of the irreversible transition to the so-called 'alkali denatured' state of pepsin, occurring at about pH 7, as well as its partial refolding upon re-acidification, both monitored by DAPI, is described in the following paper [17].

2. Materials and methods

Twice crystallized porcine stomach pepsin and pepstatin A were purchased from Sigma Chem. Co., whereas 4′,6-diamidino-2-phenylindole (DAPI.2-HCl) was obtained from Fluka and used without further purification. The enzyme activity assay was done using the synthetic peptide Phe-Gly-His-*p*-nitro-Phe-Phe-Ala-Phe methyl ester, from Bachem Feinchemikalien, as substrate.

Mono- and bi-sodium phosphate, sodium chloride and sodium formate salts were from Merck or C. Erba. All solutions were prepared using deionized water, further purified with a Milli-Q Millipore purification system.

2.1. Purification of commercial pepsin

Although the commercial sample was a twice crystallized preparation, we found the presence of an impurity through a preliminary chromatographic check. We threrefore decided to purify it further by anion exchange chromatography (Fig. 1), according to the following procedure: 2 ml of 1 mM protein ($\sim$ 70 mg) were dissolved in 50 mM phosphate buffer at pH 5.6 and injected into a 6 ml anion exchange Resource Q column (Pharmacia) using a Waters 650 protein purification system. Fractions containing pepsin (peak 2) were pooled, concentrated and dialysed under negative pressure in a micro protein

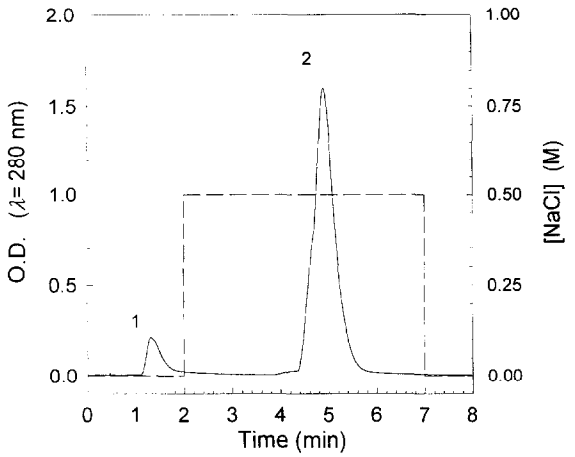

Fig. 1. Purification of commercial pepsin by anion exchange FPLC. 70 mg twice crystallized porcine stomach pepsin from Sigma was dissolved in 2 ml of 50 mM phosphate buffer at pH 5.6 (buffer A), injected into a Waters 650 protein purification system and purified through a 6 ml anion exchange Resource Q column (Pharmacia) equilibrated in buffer A, using a stepwise gradient of NaCl, as shown. Peak 1: impurity; peak 2: pepsin.

dialysis concentrator apparatus (Micro-ProDiCon Model 115, Spectrum) against a 5 mM phosphate buffer at pH 5.5 and 4°C and finally stored in the freezer. Spectroscopic analysis shows that peak 1 is to be attributed to an impurity present in the commercial preparation, possibly deriving from partial autolysis of pepsin, since its amount does not change with storage time when the protein is dissolved in the buffer under the conditions we used.

In order to reduce autolysis as much as possible, purified pepsin was alkali denatured under conditions of high ionic strength which favour a rapid and complete process [18], namely by incubating a concentrated protein stock solution (about 2 mM) for several minutes in 250 mM phosphate at pH 7.2.

2.2. Absorbance, fluorescence and CD measurements

Absorbance and fluorescence measurements were carried out with a Jasco 7850 spectrophotometer and a Perkin-Elmer LS-50 spectrofluorimeter, respectively, both themostatted at 20°C. In fluorescence, the optical density of the samples used for fluorescence was ≤ 0.1, at the excitation wavelength.

Because of the shift observed in the absorbance spectrum of DAPI in the presence of pepsin with respect to that of DAPI alone, as shown in Fig. 2, all fluorescence titration data referred to the absorbance of DAPI alone at the excitation wavelength. A similar shift of about 10 nm is typical of DAPI bound to proteins, as already observed [13,15].

Circular dichroism spectra were recorded on a thermostatted Jasco J500-A spectropolarimeter, using a cell pathlength of 5 cm and 1 cm for type I and type II titrations, respectively.

2.3. Titrations

As explained in detail in a previous paper [19], two types of titration (called type I and type II, respectively) were performed with each of the techniques used (fluorescence, CD and absorbance). With type I titrations, performed with constant DAPI and variable pepsin concentrations, both the fluorescence quantum yield and the dissociation constant of DAPI bound to the highest affinity site are derived. The Scatchard-like plots were built up using ΔF_{max} values, obtained by extrapolation at infinite pepsin concentration from double reciprocal plots ($1/\Delta F$ vs $1/[P]_{tot}$), to calculate the parameters $\alpha = [DAPI]_{bound}/[DAPI]_{total} = \Delta F/\Delta F_{max}$ and $[P]_{free} = [P]_{tot} - \alpha[L]_{tot}$ (where $[P]_{free}$ and $[P]_{tot}$ refer to free and total pepsin concentration, respectively). On the other hand, from type II titrations, performed at

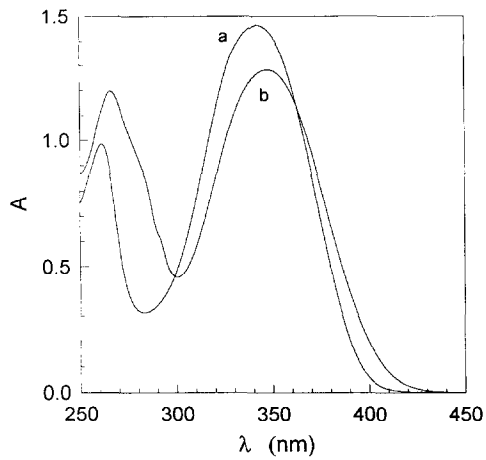

Fig. 2. Absorbance spectra of DAPI. Spectra of 65 μM DAPI: (a) alone and (b) with 15.4 μM pepsin, at 20°C and in 5 mM phosphate buffer pH 5.0.

constant pepsin and variable DAPI concentrations, the total number of classes and binding sites can be obtained.

2.4. Inhibition and biological activity assays

The displacement of DAPI from pepsin by the highly specific inhibitor pepstatin A was studied by monitoring both fluorescence and CD spectral changes of the dye, using twice an excess of the inhibitor over pepsin in a 50 mM phosphate buffer at both pH 5.5 and pH 7.2.

The biological activity of pepsin was evaluated by measuring the initial cleavage rate of the substrate Phe-Gly-His-*p*-nitro-Phe-Phe-Ala-Phe methyl ester at 37°C in 50 mM sodium formate buffer at pH 4, according to the spectrophotometric method of Medzihradszky et al. [20]. In order to evaluate the kind of inhibition, as well as the inhibition constant, of DAPI, the activity assays were performed at dye concentrations which varied from 200 to 10 μM.

3. Results

3.1. Spectroscopic titrations

The binding of DAPI to both native (pH 5.7) and alkaline denatured pepsin (pH 7.2) was investigated by fluorescence, circular dichroism and absorbance by performing several titrations to evaluate the effect of protein structural conformation on the binding parameters. Furthermore, titrations at each pH were performed at several phosphate concentrations in order to assess the electrostatic contribution to the interaction observed.

3.1.1. Fluorescence

Double reciprocal plots and Scatchard-like plots, as derived from type I titrations at pH 5.7 and pH 7.2, are shown in Figs. 3 and 4 respectively. At each pH, five different phosphate buffer concentrations (from 200 to 12.5 mM at pH 5.7 and from 100 to 5 mM at pH 7.2) were used. The values of all the dissociation constants, deduced from this set of titrations as well as from that of type II (not shown), are summarized in Table 1. Though the affinity of DAPI to pepsin is strongly dependent on both pH and phosphate buffer

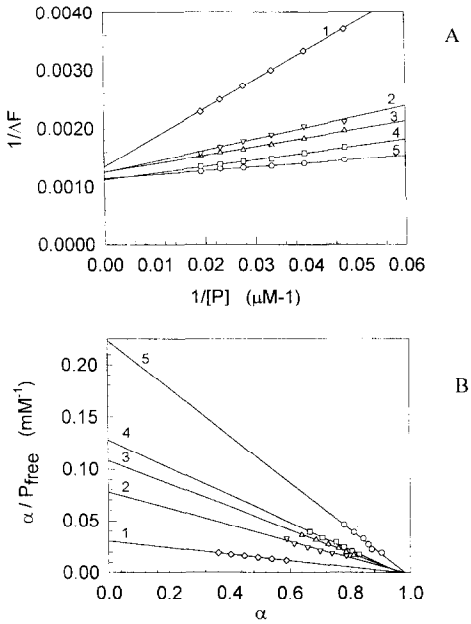

Fig. 3. Fluorescence titrations at pH 5.7 as a function of phosphate concentration. Conditions used: 5 μM DAPI mixed with pepsin, varied from 52 to 21 μM, in (1) 200, (2) 100, (3) 50, (4) 25 and (5) 12.5 mM phosphate buffer (λ_{ex} 360 nm; λ_{em} 433 nm; 20°C). (A) double reciprocal plots; (B) Scatchard-like plots. For the meaning of α and P_{free} see under Materials and methods and ref. [19].

concentration, the fluorescence quantum yield enhancement factor Q, defined as

$$Q = \frac{\text{quantum yield of DAPI}_{\text{bound}}}{\text{quantum yield of DAPI}_{\text{free}}}$$

and n, the number of binding sites, are almost invariant ($Q = 34 \pm 2$ and 34 ± 1; $n = 2.4 \pm 0.3$ and 2.2 ± 0.3 at pH 5.7 and 7.2, respectively). On the other hand, the strong dependence of the binding constants on the phosphate buffer concentration, observed with both native and alkaline denatured protein, can be well explained by electrostatic effects involved in the interaction between DAPI and pepsin.

3.1.2. Circular dichroism

Type I and type II titrations were also performed using this technique, that is by exploiting the induced ellipticity of DAPI bound to pepsin. As an example, CD spectra of DAPI in the presence of variable pepsin (type I titrations) at pH 5.7 with three differ-

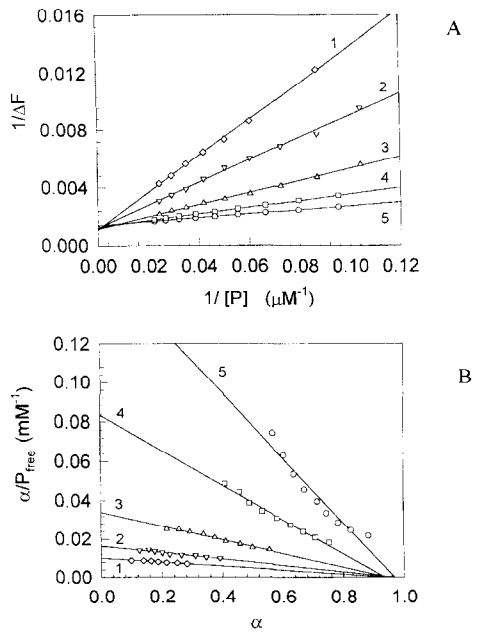

Fig. 4. Fluorescence titrations at pH 7.2 as a function of phosphate concentration. Conditions used: 5 μM DAPI mixed with pepsin, varied from 41 to 9 μM, in (1) 100, (2) 50, (3) 25, (4) 12.5 and (5) 5 mM phosphate buffer (λ_{ex} 360 nm, λ_{em} 433 nm, 20°C). (A) double reciprocal plots; (B) Scatchard-like plots. For the meaning of α and P_{free} see under Materials and methods and ref. [19].

ent phosphate buffer concentrations are shown in Fig. 5. The corresponding dissociation constants, as derived from double reciprocal plots and Scatchard plots (not shown), are in accordance with the previous ones obtained from fluorescence at the same pH (Table 1 and Fig. 6). The CD intensity below 310 nm, largely due to pepsin itself, does not affect the CD intensity of DAPI at longer wavelengths, where the binding parameters were estimated. As with fluorescence, nearly two molecules of DAPI are found to bind to pepsin at pH 5.7, whereas no data at pH 7.2 could be obtained since DAPI bound to alkali denatured protein is not dichroic.

Table 1
Dissociation constants of DAPI bound to pepsin, as obtained from CD and fluorescence titrations

Phosphate (mM)	K_d (μM)		
	Fluorescence		Circular dichroism
	pH 5.7	pH 7.2	pH 5.7
5	—	6.0	—
12.5	4.5	12.5	2.5
25	7.5	28.0	6.0
50	9.0	53.0	8.0
100	12.5	94.5	14.5
200	32.0	—	27.5

Experimental conditions are those described in the captions to Fig. 3Fig. 4Fig. 5. The mean values of n (number of binding sites), extrapolated from Scatchard plots, are 2.4 ± 0.3 and 2.2 ± 0.3 from fluorescence at pH 5.7 and 7.2, respectively, and 2.5 ± 0.1 from CD at pH 5.7.

3.1.3. Absorbance

A series of difference spectra, as obtained by subtracting the spectra of DAPI with pepsin from those of DAPI alone, and the relative absorbance

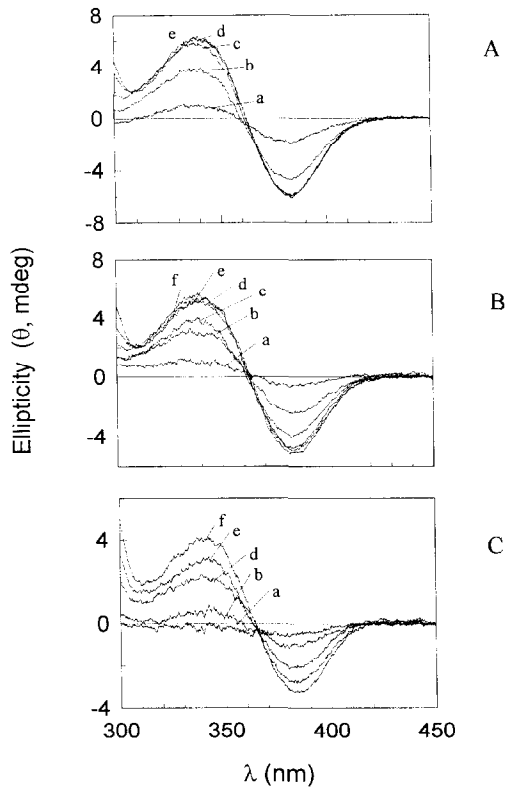

Fig. 5. Circular dichroism spectra of DAPI bound to pepsin: titrations at pH 5.7 as a function of phosphate concentration. Conditions used: (A) 12.5 mM, (B) 25 mM and (C) 100 mM phosphate buffer. 15 μM DAPI mixed with variable pepsin, as follows: (a) 1.7 μM, (b) 5.5 μM, (c) 9.6 μM, (d) 19.0 μM, (e) 30.8 μM and (f) 50.0 μM. Each spectrum was recorded at 20°C using a 5 cm pathlength cell, 4 s time constant and 20 nm min^{-1} scanning speed.

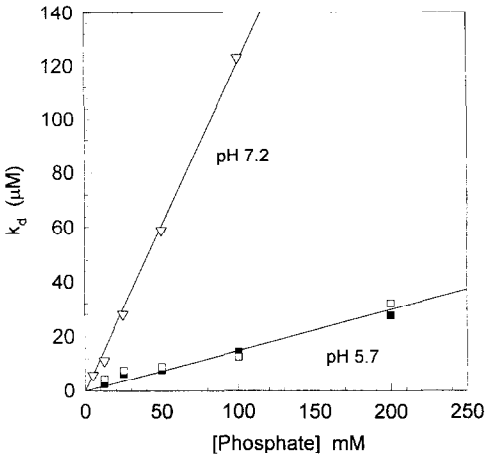

Fig. 6. Binding of DAPI to pepsin: dissociation constants as a function of phosphate concentration: (□—□) pH5.7 and (▽—▽) pH7.2, from fluorescence titrations; (■—■) pH5.7, from CD titrations. Experimental conditions as described in the captions to Figs. 3, 4 and 5, respectively.

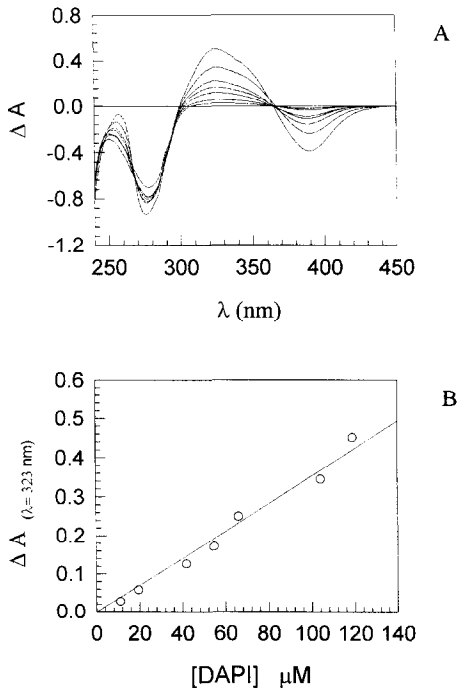

Fig. 7. Absorbance titrations. Absorbance difference spectra, obtained by subtracting the spectra of DAPI with pepsin from those of DAPI alone, at several DAPI concentrations, are shown in (A). The ΔA values read at 323 nm were used to plot the titration curve shown in (B). DAPI was varied from 10 to 120 μM, and pepsin was held constant at 15 μM, in 20 mM phosphate at pH 5.0 and 20°C.

values at 323 nm for a series of DAPI concentrations are shown in Fig. 7A and 7B, respectively. Since the data reported in Fig. 7B do not show any tendency to saturate, even at the highest concentration of DAPI used (120 μM, above which solutions developed turbidity), this kind of titration monitors an interaction quite different from those detected by fluorescence and CD.

3.2. Salt effect

In order to investigate thoroughly the effect of ionic strength on the binding of DAPI to pepsin, a series of measurements as a function of NaCl concentration, from 0.1 to 2 M, have been performed.

The intensity of the fluorescence spectra of DAPI, with pepsin at pH 5.5, decreases as NaCl concentration increases (Fig. 8A), reaching a plateau at 1 M NaCl, which is about three times higher than that of

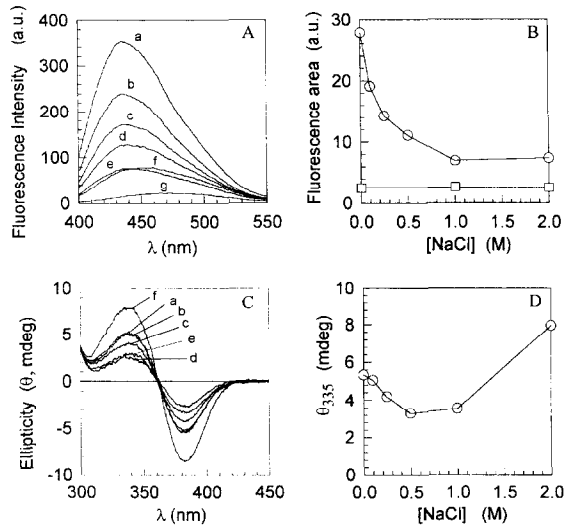

Fig. 8. Fluorescence and CD of DAPI in the presence of pepsin as a function of NaCl concentration. (A) Fluorescence spectra of 15 μM DAPI with 28 μM pepsin in 25 mM phosphate buffer at pH 5.7 (λ_{ex} 380 nm) at several salt concentrations: (a) 0, (b) 0.1, (c) 0.25, (d) 0.5, (e) 1.0, (f) 2.0 M NaCl. The spectrum of DAPI alone (g) is independent of salt concentration. (B) Fluorescence areas versus salt concentration, as derived from previous plot (A): (○—○) DAPI with pepsin; (□—□) DAPI alone. (C) Circular dichroism spectra: conditions used as in (A). (D) Ellipticity at 345 nm versus salt concentration, as derived from previous plot (C).

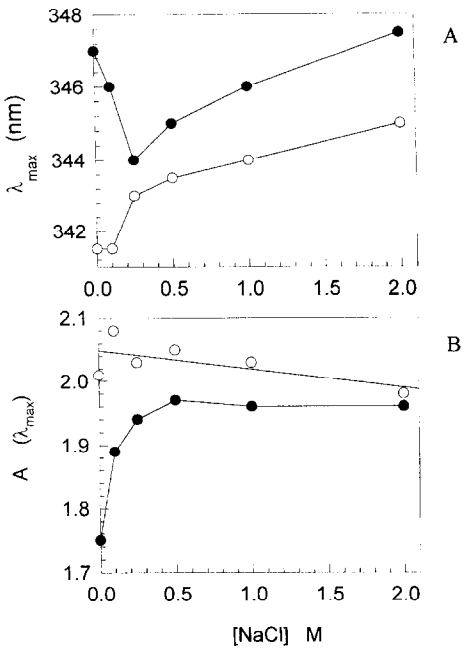

Fig. 9. Effect of NaCl on the absorbance spectrum of DAPI: (A) λ_{max} absorbance value and (B) optical density for 72 μM DAPI alone (○—○) and with 10 μM pepsin (●—●) in 25 mM phosphate buffer at pH 5.5; 1 cm pathlength cell, 20°C.

DAPI alone (Fig. 8B). The spectral shape is unchanged until very high concentrations (2 M NaCl) are reached, where the maximum, nearly 20 nm red-shifted (spectrum f), is more similar to that of DAPI alone (spectrum g).

The intensity of the CD spectra of DAPI with pepsin at the same pH decreases with NaCl concentration, as with fluorescence, but it increases again above 0.5 M with no change of spectral shape (Fig. 8C and 8D).

At pH 7.2, the fluorescence intensity of DAPI with denatured pepsin is about one-half of that at pH 5.7 in the absence of NaCl and decreases with salt concentration to a final value similar to that at pH 5.5 (not shown).

Finally, the absorbance spectra of DAPI in the presence of pepsin at pH 5.5 as a function of NaCl concentration show a small maximum wavelength shift of about 3 nm (Fig. 9A) and a little change of intensity of about 10% (Fig. 9B). The maximum wavelength shift relative to DAPI with pepsin reaches a minimum value at about 0.25 M NaCl, beyond which it increases again. In contrast, the shift observed with DAPI alone shows no minimum but increases with salt, showing a trend similar to that observed with pepsin. On the other hand, the intensity of DAPI with pepsin reaches a saturation value at about 0.25 M NaCl, whereas that of DAPI alone is almost unaffected by salt.

3.3. Inhibition assays

In order to investigate whether pepstatin A, a well known inhibitor of pepsin which binds very tightly into the active site, could affect the binding of DAPI, spectral measurements have been performed with and without the inhibitor. Furthermore, assays of the

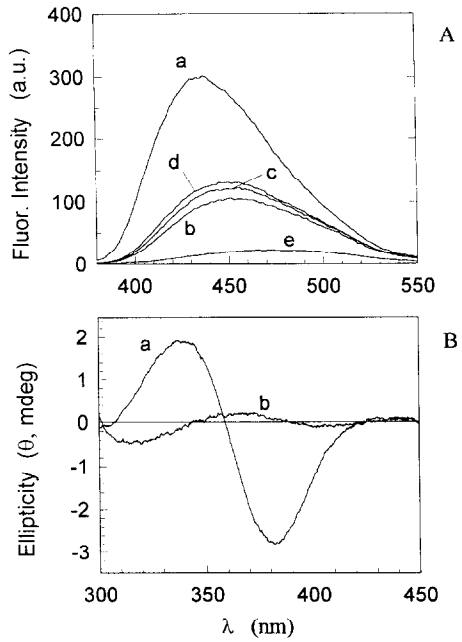

Fig. 10. Effect of pepstatin A on fluorescence and CD of DAPI in the presence of pepsin. (A) Conditions used for fluorescence spectra were 50 mM phosphate buffer at either pH 5.5 or pH 7.2, 20°C, 4.8 μM DAPI, 25.4 μM pepsin, 50 μM pepstatin A. The spectra of DAPI were recorded as follows: (a) with pepsin, pH 5.5; (b) with pepsin and pepstatin A, pH 5.5; (c) with pepsin, pH 7.2; (d) with pepsin and pepstatin A, pH 7.2. As a control, the spectrum of DAPI alone is shown in (e) (no change was observed with either pepstatin A or pH). (B) CD spectra of 77 μM DAPI: (a) with 33 μM pepsin; (b) with 33 μM pepsin and 62.5 μM pepstatin A, in 50 mM phosphate buffer at pH 5.5, 20°C, 0.4 cm pathlength cell.

enzymatic activity of pepsin, using a synthetic substrate, have been made with and without DAPI, with the aim of finding out whether DAPI could behave as an inhibitor itself.

3.3.1. Effect of pepstatin A

The presence of pepstatin A markedly reduces the fluorescence intensity of DAPI bound to pepsin at pH 5.5, with a red shift of about 20 nm (Fig. 10A; compare spectra a and b), whereas at pH 7 the inhibitor has no effect on fluorescence (compare spectra c and d), as expected, since it does not bind to the denatured protein. The CD spectrum of DAPI, at pH 5.5, is almost undetectable in the presence of the inhibitor (compare spectra a and b in Fig. 10B). The CD effect of pepstatin A was tested only at pH 5.5 because no ellipticity of DAPI was observed near neutrality, as already mentioned above.

3.3.2. Biological activity assays

The Dixon plot of $1/v_0$ (reciprocal of initial velocity) versus substrate concentration provides a way of identifying the type of inhibition and of determining the value of the inhibition constant. We have deduced competitive inhibition by DAPI, since the straight lines a and b, relative to two different concentrations of DAPI, intercept at an ordinate value of $1/v_{max}$ (Fig. 11). The extrapolated high value of inhibition constant (about 270 μM) was expected, considering the reduced affinity of the dye at the relatively low pH (~ 4.0) used for the activity assays.

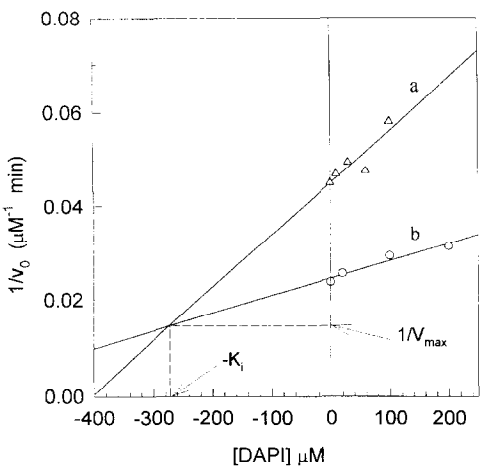

Fig. 11. Dixon plot relative to inhibition of pepsin by DAPI. The initial rate was measured at two different substrate concentrations: (△—△) 50 μM and (○—○) 150 μM, in the presence of variable concentrations of DAPI (from 10 to 200 μM) and 23 nM pepsin. The coordinates of the intersection point between the straight lines a and b give the values of the inhibition constant K_i and the reciprocal of the maximum rate v_{max}.

4. Discussion

The study of the interaction of DAPI with pepsin has given evidence for appreciable spectroscopic changes of DAPI, which have been exploited not only to measure the binding parameters but also to monitor the conformational state of the protein, thus revealing a possible use of this dye as a structural probe for pepsin.

A series of titrations has been performed on the basis of the observed spectroscopic changes, namely a large fluorescence intensity enhancement, an induced CD spectrum (only below pH 6) and an absorbance shift.

From fluorescence titrations (some of which are shown in Figs. 3 and 4) the main results obtained are the following:

(a) the dissociation constants are strongly dependent on phosphate concentration (Table 1);

(b) the quantum yield enhancement of DAPI bound ($Q \approx 30$) and the number of binding sites (~ 2) are approximately the same for either native (pH < 6.0) or alkaline denatured pepsin (pH > 7.0).

In this interaction a central role is certainly played by the electrostatic forces, considering the strongly acidic character of pepsin (pI < 1 [21]), which has a very large number of negatively charged residues (30 aspartic and 13 glutamic acid side chains), and the positive charges of the two amidino groups of the dye (Scheme 1). On the other hand, although the net negative charge on the protein is higher at pH 7.2 than 5.5, the binding constants measured at pH 5.5 are always larger than those measured at pH 7.2, at the same ionic strength, suggesting the involvement of other specific, i.e. not only electrostatic, interactions. This conclusion is well supported by the fact that the so-called alkaline denaturation occurs concomitantly with the ionization of some buried carboxyl groups of aspartic or glutamic acid residues exhibiting anomalously high pK values [22].

The weaker binding of DAPI to denatured pepsin could be explained by considering that the alkaline denaturation of the pepsin, by releasing the electrostatic strain on the protein surface, brings about an increment of the surface area as well as of negative charges, with a probable net decrease of the surface charge density. The fact that the same binding stoichiometry is observed at either pH and is limited to only two molecules of DAPI per protein molecule suggests that the specificity of the binding sites of DAPI to the protein is retained, even when the protein is partially denatured. Considering that the alkaline denaturation has been suggested to involve unfolding of the N-terminal lobe (residues 1–172) [22], one could infer that the binding sites are probably located on the C-terminal lobe.

From CD titrations, the obtained dissociation constants and number of binding sites at pH 5.5 are very similar to those from fluorescence (Fig. 5 and Table 1). The CD spectrum of bound DAPI is composed of two nearby bands of opposite sign, typical of two identical chromophores assembled in a stacking dimer, giving rise to an exciton splitting [23]. Therefore, whereas at native pH both CD and fluorescence reveal the same interaction between DAPI and protein, at neutral pH, with denatured protein, CD of DAPI is almost completely abolished. This loss of CD signal can be related to a total relaxation of the structural chiral constraints exerted on DAPI by the native protein, without, however, preventing the binding of the dye.

These spectral changes of DAPI have also been exploited to study the kinetics of alkaline denaturation, as well as the partial refolding occurring upon reacidification of pepsin, as described in the following paper [17].

In order to acquire further information, we have performed absorbance titrations: curves of ΔA versus DAPI concentration show no tendency towards saturation, in the range investigated, as shown in Fig. 7 (above this range no measurements are possible since precipitation occurs). This result can be rationalized by assuming that the absorbance technique is sensitive only to purely electrostatic interactions, established between the amidine positively charged moieties of DAPI and the negative groups on the pepsin surface, probably involving a much larger number of DAPI molecules. Precipitation of the protein, and the consequent absence of saturation, is probably due to a progressive reduction of the net charge of the protein–DAPI complex. At the same time, the presence of an isosbestic point at 366 nm does suggest that only two species are responsible for the observed absorbance behaviour, namely free and bound DAPI.

The existence of two different binding modes of DAPI has also been proposed by several authors to explain the interaction of this dye with DNA [24,25]. In this case too the spectroscopic data have been interpreted in terms of a first highly energetic binding mode, characterized by high specificity for AT, AU and IC clusters, and of a second binding mode with lower affinity, attributed to the electrostatic attraction between the dye's positive charges and the negative phosphate groups of the polynucleotide backbone.

The involvement of specific interactions between DAPI and pepsin is well supported by the fluorescence and CD of the dye, as a function of salt concentration. In fact, whereas the decrease of fluorescence intensity, observed at acidic pH between 0 and 2 M NaCl (Fig. 8A and 8B), can be well accounted for by ionic strength effects, the relatively high residual value, at 2.0 M salt, suggests that hydrophobic interactions are still present, since under these conditions all electrostatic interactions should be negligible. A similar pattern was also observed with CD (Fig. 8C and 8D), the intensity of which decreased progressively with salt concentration, reaching a minimum between 0.5 and 1 M, then rising again. This increase can be rationalized by assuming that the presence of a high salt concentration forces the protein into a more compact state, thus inducing DAPI to bind with an even more distorted, i.e. dichroic, conformation. This conformational change of DAPI, at 2 M salt concentration, can be related to the remarkable redshift (about 20 nm) observed in the corresponding emission spectrum of DAPI (compare spectra e and f in Fig. 8A).

The behaviour of the absorbance spectra of DAPI in the presence of pepsin, as a function of NaCl concentration, shown in Fig. 9, is different from those observed in either fluorescence or CD and is well explained by ionic strength effects. In fact, the optical density of DAPI at the maximum wavelength reaches a plateau value, similar to that of DAPI

alone, at relatively low ionic strength (0.25 M NaCl) (Fig. 9B), thus confirming the essentially electrostatic character of the interaction monitored by this technique. At the same time, small changes of λ_{max} of both bound and free DAPI have been observed in the whole range of salt concentration investigated. Changes observed below 0.25 M NaCl can be related to the displacement of DAPI by salt, as described above, whereas those occurring above this salt concentration can be related to small changes of the ground energy levels of DAPI, induced by increasing salt concentrations.

In the presence of pepstatin A and pepsin at pH 5.5, a considerable decrease in fluorescence intensity and the disappearance of the CD of DAPI are observed (Fig. 10). These effects can be interpreted as being due to a displacement of DAPI from its specific binding site, which could therefore be located near the active site. The relatively high value of the residual fluorescence of DAPI can be tentatively explained by assuming that the dye is only partially displaced from its site by the inhibitor. The new binding mode is probably characterized by a microenvironment of higher polarity (redshift similar to that found at pH 7.2), in which the dye is apparently bound in a more flexible way (CD is almost completely lost).

The involvement of the enzyme active site is also suggested by the results obtained from the activity assays (Fig. 11), since DAPI appears to behave as a competitive inhibitor, with a dissociation constant of 270 μM, with respect to the substrate used.

To summarize, our spectroscopic studies have shown the existence of different modes of interaction of DAPI with native pepsin: a large number of dye molecules appear to be involved in non-specific electrostatic interactions on the protein surface, as monitored by absorbance; whereas only two DAPI molecules bind in a specific way, probably near the active site, as deduced by fluorescence and CD. In addition, a comparison with the different results obtained at pH 7, where the protein is partially denatured, suggest that this dye can be used as a structural probe to monitor conformational changes in pepsin, such as those induced by pH and ionic strength. This suggestion has been largely exploited in the following paper [17].

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