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The Interaction of 4′, 6-Diamidino-2-phenylindole (DAPI) with Pepsin

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We recently found that the fluorescent dye DAPI, well-known for its use with nucleic acids, is also able to interact with proteins as well as ordered phospholipids assemblies. The interaction of DAPI with pepsin under different conditions of pH and ionic strength was studied with fluorescence and circular dichroism techniques. From a comparison of the results obtained, the interaction appears to be rather tight and specific, dependent on both electrostatic and hydrophobic forces, and able to probe the tridimensional conformation of the protein.

KEY WORDS: 4′, 6-Diamidino-2-phenylindole (DAPI); pepsin, fluorescence, circular dichroism, binding.

INTRODUCTION

DAPI (4′,6-diamidino-2-phenylindole) has gained wide popularity as a fluorescent probe for double-stranded nucleic acids and chromosome staining in cytology. More recently a large amount of structural information has been obtained by both X-ray diffraction [1] and NMR [2] studies on DAPI–DNA complexes. In the past 2 years, our group has accumulated evidence that the dye is also able to interact with proteins [3] as well as ordered phospholipid structures [4].

Upon binding to these macromolecular assemblies, DAPI exhibits a large increase in its fluorescence quantum yield, as it also does with DNA. The binding can be thought to arise mainly from electrostatic effects between the cationic dye and negative residues on proteins or phospholipid bilayers. However, as happens with DNA, important contributions to the binding apparently derive also from hydrophobic interactions, particularly in the case of phospholipid systems, where an almost-complete absence of ionic strength effects has been found [4].

From a number of preliminary investigations on various proteins (e.g., albumins, dehydrogenases, and hemocyanins), the binding of DAPI to them appears to be considerably dependent on the ionic strength. Actually, the binding of DAPI appears to be dependent largely on the net charge on the protein, being appreciable only with proteins above their isoelectric point. In this report we describe the interaction of DAPI with purified porcine stomach pepsin, as deduced from fluorescence and circular dichroism investigations. It turns out that the binding is driven not only by electrostatic but also by hydrophobic forces and that DAPI is a sensitive structural probe for pepsin.

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EXPERIMENTAL

Protein Purification

Twice-crystallysed pepsin (EC 3.4.23.1) from Sigma Chemical Co. was purified with a Waters Advanced Pro-

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tein Purification System using a Protein Pak Glass DEAE 5PW column (Fig. 1).

Only fractions relative to peak II, containing active pepsin, were used after dialysis against 5 m*M* phosphate, pH 5.5, at 4°C. All measurements at pH 7.0 were performed on completely inactivated pepsin, as measured according to the classical assay based on hemoglobin digestion [5].

Fluorescence Measurements and Titrations

Spectra were collected at two pH's (5.5 and 7.0) and ionic strengths (20 and 100 m*M* sodium phosphate) using a computer-driven Perkin-Elmer LS-50 spectrofluorimeter.

Time-resolved fluorescence measurements were carried out using a single-photon counting apparatus described previously [4], equipped with a nitrogen flash lamp pulsed at 20 kHz (199F Edimburgh Instruments), a stop photomultiplier (Philips XP2020 Q), and fast electronics (NIM). Data deconvolution was performed according to a nonlinear least-squares fitting analysis [6].

Titrations at constant DAPI and variable pepsin concentrations were performed according to the progressive dilution method [7]. Data elaboration was performed as described elsewhere [8].

Circular Dichroism

CD spectra were collected with a computer-driven Jasco J500 spectropolarimeter, using a 0.5-cm-path-length cell thermostated at 20°C.

RESULTS AND DISCUSSION

Representative fluorescence spectra of DAPI, with and without pepsin, at two pH's and ionic strengths, are shown in Figs. 2 and 3. Whereas the absorbance spectra of DAPI bound to pepsin are only slightly red-shifted and hypochromic (data not shown), the corresponding fluorescence spectra are largely blue-shifted and of a much higher intensity, compared to those of DAPI alone under identical conditions.

This behavior appears to be quite general for DAPI with all macromolecular systems investigated thus far (proteins, phospholipids, and nucleic acids), possibly suggesting similar binding modes of DAPI or similar chemical environments about it.

The binding of DAPI to pepsin is affected by both pH and ionic strength. At a constant ionic strength, the fluorescence intensity of DAPI bound to pepsin is lower at pH 7.0 than at 5.5, under otherwise identical conditions. This result, due to a looser binding at pH 7.0 than pH 5.5 (see below), may appear rather surprising if one

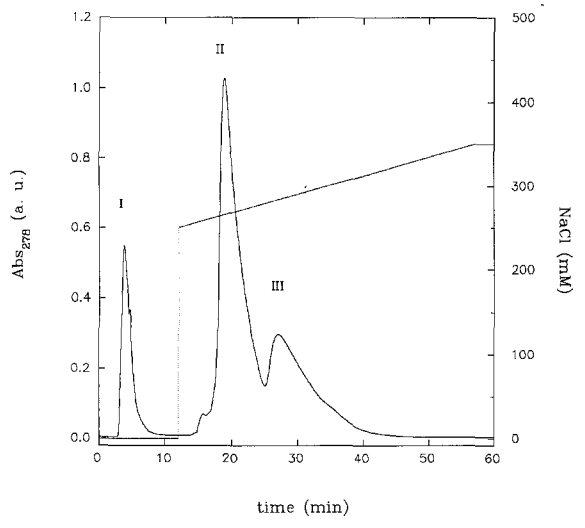

Fig. 1. FPLC of commercial crystalline porcine stomach pepsin. Ten milligrams of protein dissolved in 1 ml of 0.1 *M* phosphate buffer at pH 5.5 was chromatographed using the system described under Experimental, with the same buffer as eluant A and 0.1 *M* phosphate plus 1 *M* NaCl pH 5.5 as eluant B.

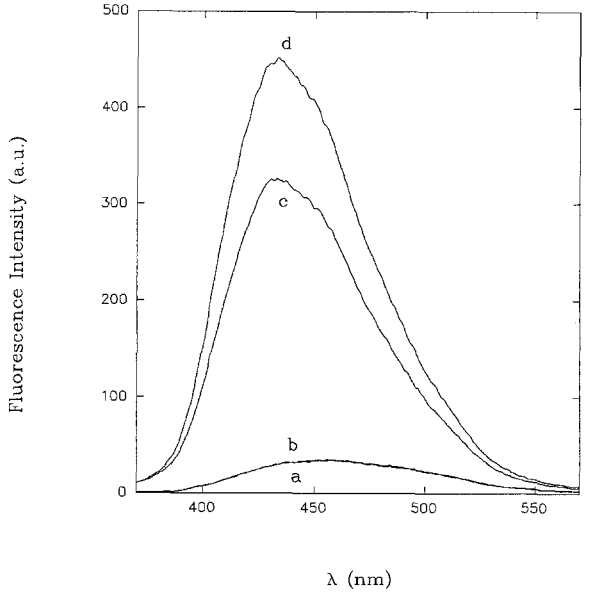

Fig. 2. Fluorescence spectra of 5 μ*M* DAPI (excited at 350 nm) at pH 5.5. (a, b) DAPI alone; (c, d) in the presence of 75 μ*M* pepsin. Phosphate buffer is 20 m*M* in b and d and 100 m*M* in a and c.

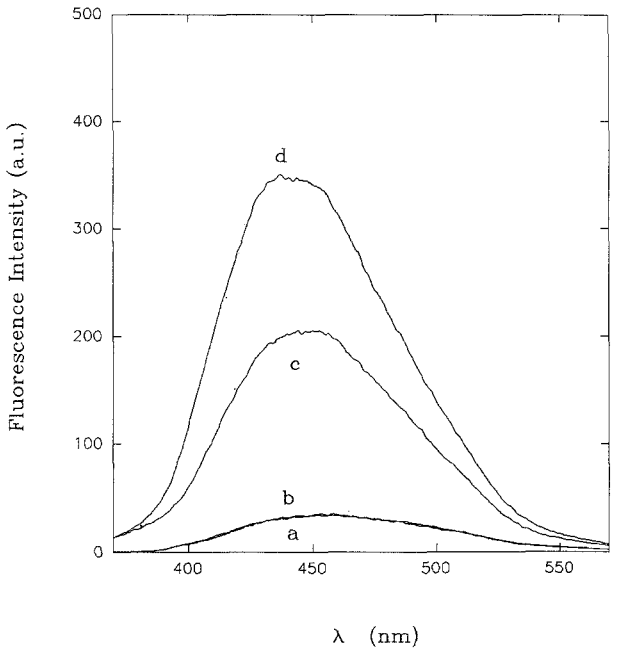

Fig. 3. Fluorescence spectra of 5 μ*M* DAPI (excited at 350 nm) at pH 7.0. Other conditions as stated in the legend to Fig. 2.

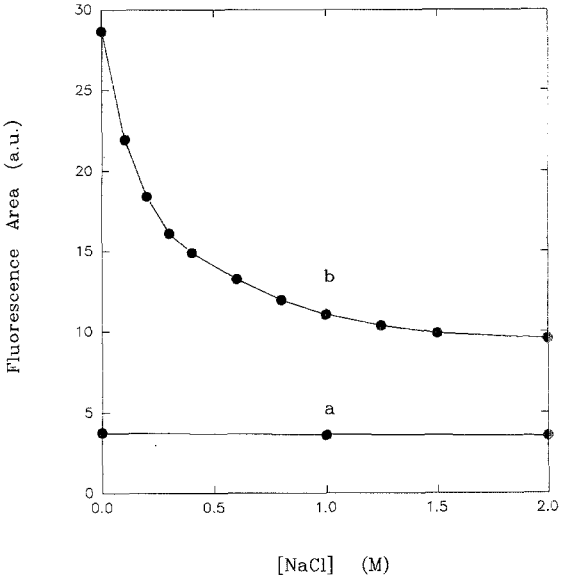

Fig. 4. Effect of NaCl concentration on the fluorescence of DAPI: (a) 5 μ*M* DAPI and (b) 5 μ*M* DAPI plus 28 μ*M* pepsin, both in 100 m*M* phosphate at pH 5.5.

considers only electrostatic effects involved in the interaction. In fact, the protein is more electronegatively charged at pH 7, whereas DAPI has two positive net charges deriving from its two amidino groups at both pH's. This effect therefore implies the involvement of other forces, hence a structural specificity between DAPI and pepsin, which apparently depends upon the different structure of pepsin at the two pH's.

At a constant pH, the effect of an increasing ionic strength is a decrease in fluorescence intensity of DAPI bound to pepsin, more remarkable at pH 7.0 than 5.5.

This effect can be seen as due to an increase in the apparent dissociation constant by the electrostatic screening of salt counterions on both DAPI and pepsin. On the other hand, even at a very high ionic strength, the fluorescence enhancement of DAPI is still remarkable, thus suggesting that forces, other than electrostatic ones, play a role in the interaction (Fig. 4).

From fluorescence decay measurements few changes in either preexponential terms or lifetimes of DAPI bound to pepsin were found at different pH's and ionic strengths (Table I). On the other hand, from titrations at the two pH's a decrease in the quantum yield of DAPI bound to pepsin (from 0.5 at pH 5.5 to 0.3 at pH 7.0, taking a quantum yield of 0.03 for DAPI alone [9]) as well as an increase in the dissociation constants (from about 10 μ*M* at pH 5.5 to about 25 μ*M* at pH 7.0) was obtained.

Table I. Values of Lifetime (ns) and Normalized Preexponential Factors for DAPI Alone and in the Presence of Pepsin[a]

	pH 5.5				pH 7.0			
	τ_1	α_1	τ_2	α_2	τ_1	α_1	τ_2	α_2
DAPI + pepsin	1.23	0.43	2.57	0.57	1.02	0.55	2.92	0.45
DAPI	0.26	0.92	2.72	0.08	0.28	0.92	2.56	0.08

[a]DAPI, 5 μ*M*; pepsin, 32 μ*M*. All measurements were performed at 20°C in 100 m*M* phosphate buffer. Similar values were obtained in 20 m*M* phosphate.

These results imply a pH-dependent binding mechanism, since the quantum yield and dissociation constant values are different at the two pH's. At the same time, however, the constancy of dynamic fluorescence parameters suggests the presence of a static quenching, more efficient at pH 7.0 than at pH 5.5. In comparison, no change in fluorescence intensity, preexponentials, or lifetimes of free DAPI with pH or ionic strength was found.

As mentioned above, the observed increase in the dissociation constant with pH has to be related to a major degree of specificity of DAPI for the native structure of pepsin. Since hydrophobic cations have recently been described as inhibitors of pepsin [10], this may the case also for DAPI, given its affinity for native pepsin.

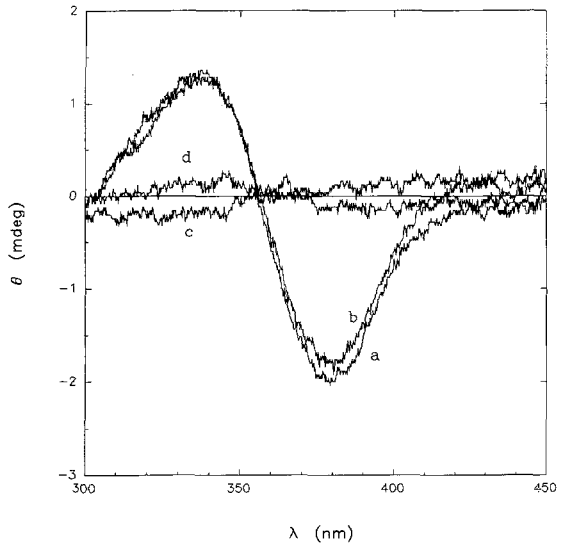

Fig. 5. CD spectra of 32 μ*M* DAPI in the presence of 32 μ*M* pepsin, in 20 m*M* and 100 m*M* phosphate buffer. (a, b) At pH 5.5; (c, d) at pH 7.0.

The results described thus far, obtained by fluorescence, can be compared with those obtained by CD. In Fig. 5 the CD spectra of DAPI bound to pepsin are shown at different pH's and ionic strengths. It is evident that DAPI becomes dichroic only at pH 5.5, i.e., with native pepsin, thus giving further evidence of specific binding.

Since the CD spectrum is split into two bands of opposite sign, typical of exciton effects, one possible explanation is that two or more DAPI molecules bind near each other on the protein surface with appropriate geometry, under the conditions used for CD measurements (1:1 dye protein ratio). Alternatively, the two bands could arise from a single geometry, with two optically active transitions of similar energy.

Furthermore, the fact that the intensity of the CD spectrum at pH 5.5 is unaffected by ionic strength suggests that the hydrophobic part of the interaction is probably responsible for the observed optically active transitions. Therefore, while fluorescence can monitor the binding of DAPI to either native or denatured pepsin, even if with a different sensitivity, CD is more selective since only the binding of DAPI to native pepsin can be monitored.

In conclusion, although we are investigating this interaction between DAPI and pepsin in more depth, it would be interesting to get more detailed structural information with other techniques of intrinsically higher resolution, such as NMR and X-ray diffraction.

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