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Fluorescence studies of nucleotide interactions with bovine adrenal chromogranin A

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The binding of the fluorescent probe bis-ANS to chromogranin A, the major protein of adrenal chromaffin vesicles, caused a marked enhancement and blue shift in the fluorescence emission spectrum. The emission maximum shifted from 515 nm to 480 nm and the yield increased approx. 75-fold upon addition of 10 μM chromogranin A to 1 μM bis-ANS. Adenine nucleotides had clear effects on the bis-ANS fluorescence signal, while other nucleotides such as GTP, UTP and CTP had no discernible effect. Specifically, ATP caused a decrease in the fluorescence, whereas ADP and AMP caused a fluorescence increase. These results indicate adenine nucleotide binding to chromogranin A. Substitution of ATP with ε-ATP, an ATP derivative with a modification on the six-membered ring of the adenine base, failed to reduce the fluorescence intensity. Therefore, it was concluded that adenine bases play an important role in the chromogranin A-adenine nucleotide interaction.

Introduction

Adrenal secretory vesicles (chromaffin granules) contain 0.5–0.6 M catecholamines and high concentrations of ATP (130 mM), magnesium (10 mM) and calcium (10–40 mM) [1]. These ions are believed to interact within the granule, possibly forming complexes which result in lowered vesicular osmotic pressure. The association of ATP with catecholamines and divalent cations is well documented [2–4]. In addition, direct interactions between ATP, catecholamines and the major intravesicular protein chromogranin A, have been proposed [2] but the nature of these interactions remains unclear. Chromogranin A is a very acidic protein with a p*I* of 4.5 to 5.0, constituting 40% of intravesicular protein [1], and it undergoes Ca^{2+}-induced conformational changes and shows a strong affinity for calmodulin in the presence of calcium (unpublished data). We have recently developed a protocol yielding 5–10 mg of highly purified chromogranin A from 50 g of bovine adrenal medulla, which has greatly facilitated our studies on the interaction of chromogranin A with other vesicle constituents. The studies described in this report utilize fluorescence spectroscopy to monitor the interaction of chromogranin A with specific ligands.

Bis-ANS (4,4′-bis-1-phenylamino-8-naphthalene sulfonate) is a fluorescent probe first described by Rosen and Weber [5]. This probe exhibits a very low quantum yield in aqueous solvents but a marked enhancement in yield (and blue shift in its emission maximum) in apolar solvents and upon binding to a number of proteins. Bis-ANS is thought to bind to hydrophobic sites on proteins and also demonstrates a strong affinity for nucleotide binding sites. The latter phenomenon has been observed, for example, with dehydrogenases [6], protein kinase A [7], myosin-ATPase [8] and tubulin [9]. These observations prompted us to investigate whether bis-ANS would interact with chromogranin A and provide indirect evidence for hydrophobic sites and/or nucleotide binding sites on this protein.

Experimental procedures

Materials. 4,4′-Bis-1-phenylamino-8-naphthalene sulfonate) (bis-ANS) was a gift from Dr. G. Weber of the University of Illinois at Urbana-Champaign, and 1,N^6-

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ethanoadenosine 5′-triphosphate (ε-ATP) was purchased from Molecular Probes (Oregon). Other nucleotides were from Sigma.

Preparation of chromogranin A. Bovine chromaffin vesicles were isolated by a slight modification of the method of Smith and Winkler [10]. Fresh bovine adrenal glands were placed on ice and medullae were removed within 1 h of killing. Medullae were minced and homogenized with 2 × 15 s bursts in a Waring blendor with 5 vols. of buffer containing 0.3 M sucrose, 15 mM Tris-HCl (pH 7.5) and 0.1 M NaCl (buffer A). Homogenates were centrifuged at 800 × *g* for 10 min and the supernatant was recentrifuged at 16000 × *g* for 20 min. The 16000 × *g* pellet was resuspended in 5 vols. of ice-cold buffer A and homogenized with 3 strokes of a Teflon/glass Potter-Elvehjem homogenizer. Homogenates (10 ml) were layered on a 15 ml cushion of 1.8 M sucrose, 15 mM Tris-HCl (pH 7.5), 0.1 M NaCl and centrifuged for 1 h at 125000 × *g*. Highly purified vesicles were obtained in the pellet of this centrifugation. The vesicles were lysed by suspension of 40 vols. of 15 mM Tris (pH 7.5) followed by freezing and thawing. Soluble proteins were separated from the membranes by centrifugation at 40000 × *g* for 30 min. Using the soluble proteins, intact homogeneous chromogranin A was purified using a calmodulin affinity column as described elsewhere.

Fluorescence measurements. Steady-state fluorescence measurements were carried out using either an SLM 8000C (SLM-AMINCO, Urbana, Illinois) spectrofluorometer or a Hitachi-Perkin-Elmer MPF 44-A spectrofluorometer. Excitation and emission bandpasses were set at 4 nm. All fluorescence measurements were made at 22°C using a dual pathlength 'T-cuvet' with the short pathlength (2 mm) oriented towards the excitation side to minimize inner filter effects. The buffer was 15 mM sodium acetate (pH 5.5) and 1 mM $MgCl_2$ unless noted otherwise.

Time-resolved measurements. Lifetime measurements were carried out using a home-built multifrequency and phase modulation fluorometer based on the Gratton design [11]. Excitation of bis-ANS was accomplished by using the 364 nm line of an argon ion laser (Spectra Physics Model 2035-3.55), and emissions at wavelength greater than 400 nm were observed through Schott KV 399 cut-on filters.

Other methods. Concentration of bis-ANS in ethanol was determined spectrophotometrically at 385 nm using 16670 $M^{-1} \cdot cm^{-1}$ as the extinction coefficient. Protein determination was according to Bradford [12].

Results

Fig. 1 shows the marked enhancement and blue-shift in the emission of bis-ANS upon binding to chromogranin A. The emission due to 1 μM bis-ANS, in buffer, shown in curve a, exhibits a maximum near 520 nm. Upon introduction of 10 μM chromogranin A the emission maximum shifts to 480 nm (curve b) and the yield (calculated from the areas under the curves) increases approx. 75-fold (the ordinates for curves a and b are accordingly different). The binding of bis-ANS to chromogranin A was then studied by titration experiments. Fig. 2 shows the titration of 10 μM chromogranin A with bis-ANS, the curve demonstrates a marked enhancement of fluorescence intensity and a saturation effect.

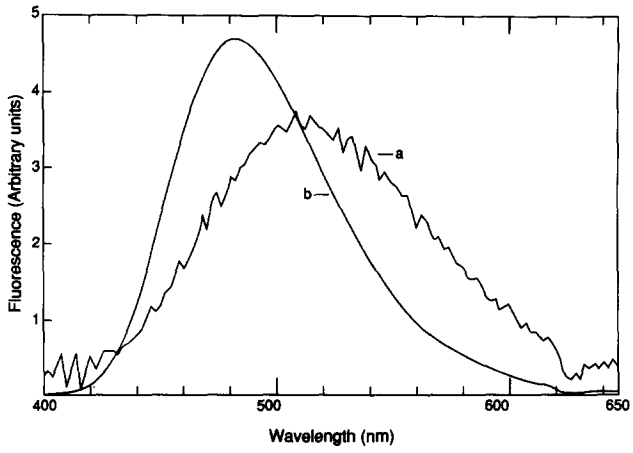

Fig. 1. Fluorescence emission spectra showing the increase in quantum yield upon binding of bis-ANS to chromogranin A. Fluorescence emission spectra of either 1 μM bis-ANS only (a) or 1 μM bis-ANS plus 10 μM chromogranin A (b). Spectrum (a) is drawn on an expanded scale. Excitation was at 365 nm.

Experiments designed to ascertain the effects of nucleotides on bis-ANS binding were then carried out. Fig. 3 shows the effect of various nucleotides, specifically, ATP, ADP, AMP, GTP, UTP and CTP upon the fluorescence of a solution of 45 μM bis-ANS and 3 μM chromogranin A. All adenine nucleotides had clear effects on the bis-ANS fluorescence signal; specifically ATP led to a decrease in this fluorescence (this decrease leveled off at about 6 mM ATP) whereas ADP and AMP led to an increased fluorescence. The other

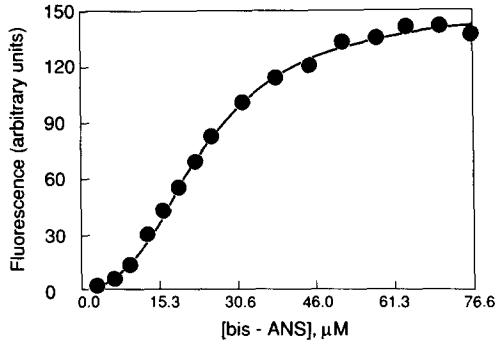

Fig. 2. Titration of 10 μM chromogranin A with bis-ANS; the bis-ANS concentrations indicated represent total fluorophore concentrations.

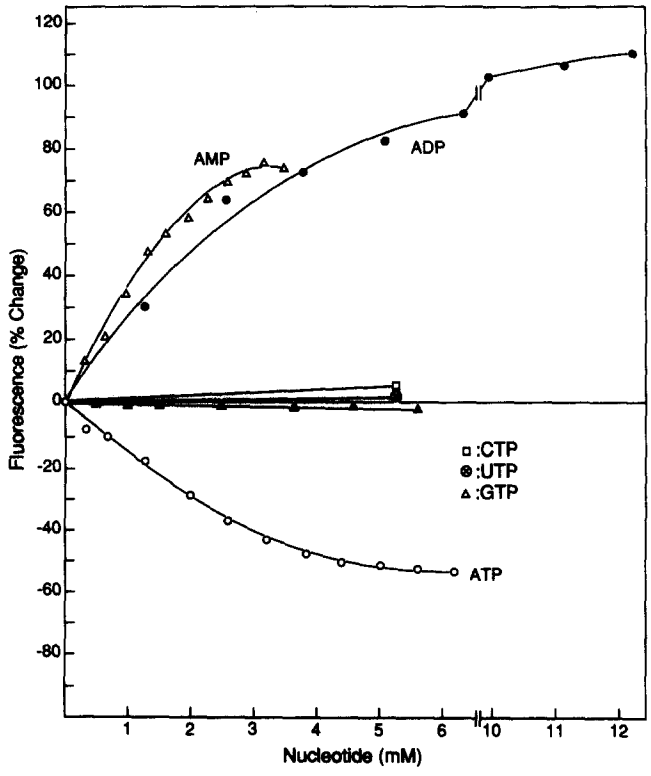

Fig. 3. Fluorescence titration of chromogranin A with various nucleotides. Titration of chromogranin A (3 μM) in the presence of saturating level of bis-ANS (45 μM) with nucleotides was carried out by adding 1–2 μl increments of 200 mM nucleotide (ATP, ADP, AMP, CTP, UTP and GTP). Excitation, 420 nm; emission, 480 nm. Buffer, 15 mM sodium acetate (pH 5.5).

nucleotides had no discernible effect on the bis-ANS fluorescence.

To investigate the ATP effect more fully, the spectra shown in Fig. 4 were obtained. Bis-ANS (50 μM) and chromogranin A (10 μM) gave the fluorescence spectrum shown in curve a of Fig. 4A. Upon addition of 5 mM ATP, the fluorescence intensity decreased resulting in the spectrum shown in curve b, Fig. 4A. Normalization of those spectra, as shown in Fig. 4B, however, indicates that the emission maxima and bandwidths are identical. Lifetime data were also obtained on these two solutions (Fig. 5). The lifetime data on the aforementioned bis-ANS/chromogranin A solution indicated a heterogeneous emitting population which was analyzed according to a distributional model [13,14]. The resulting Lorentzian distribution curves for the bis-ANS/chromogranin A solutions with and without ATP are shown in Fig. 5. No significant difference was observed; in both cases, a distribution centered around 7.5 ns with a width of about 3.8 ns was obtained. The chi-square values for both analyses were less than 2.

Discussion

The fluorescent probe bis-ANS is known to fluoresce intensely in apolar environments yet only weakly in water [5]. The large fluorescence increase seen upon introduction of chromogranin A to a solution of bis-ANS (Fig. 1) suggests the presence of apolar sites on chromogranin A. The binding curve shown in Fig. 2 demonstrates saturability, suggesting a limited number of spectroscopically detectable bis-ANS binding sites on chromogranin A. Precise calculation of the number of binding sites and their respective dissociation constants is, however, complicated by the fact that the quantum yield of each bis-ANS band is not necessarily identical. The sigmoidal binding curve resulting from the titration of 10 μM CGA with bis-ANS, apparent in Fig. 2, could be the result of either (1) cooperative binding of bis-ANS molecules which have similar quantum yields when bound or (2) simple binding of multiple bis-ANS molecules which have lower quantum yields for the stronger binding sites compared to the weaker binding sites. Much more detailed binding studies are required to sort out these potential complexities. Such analyses are, however, beyond the scope of this report, which is intended mainly to demonstrate that bis-ANS binds to CGA and can be displaced specifically by ATP. We may note that, in the absence of such detailed information, we have chosen to plot intensity vs. total, not free, bis-ANS concentration (Fig. 2); hence, we have not attempted to assign a simple dissociation constant to these binding data.

Another property of bis-ANS is its affinity for nucleotide-binding sites of numerous proteins [6–9]. Decrease of fluorescence upon addition of ATP (Fig. 3)

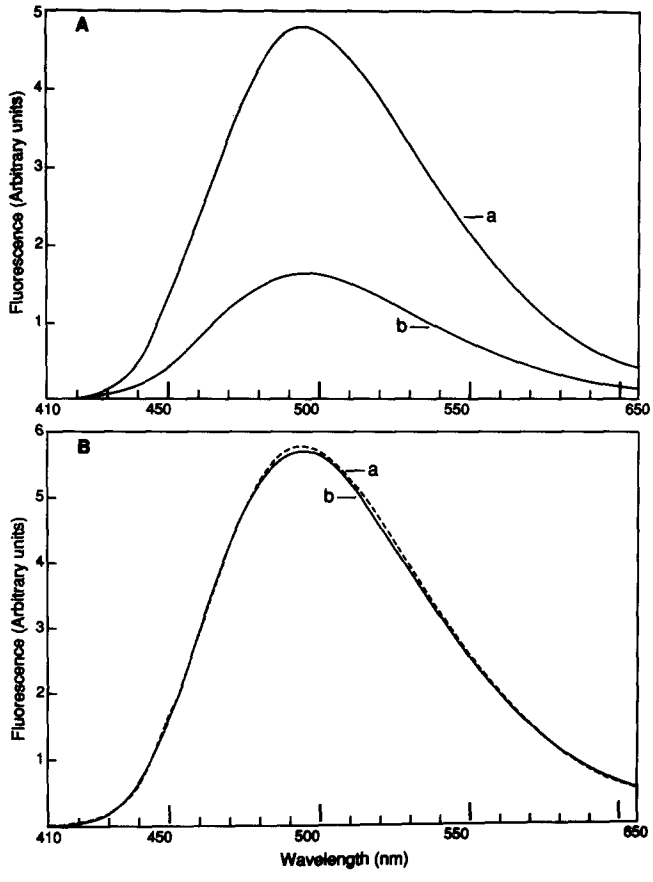

Fig. 4. Reduction of fluorescence intensity by ATP. (A) Spectra (a) was obtained with 10 μM chromogranin A, 45 μM bis-ANS, and spectra (b) was obtained 20 min after addition of 6 mM ATP to 10 μM chromogranin A, 45 μM bis-ANS. Fluorescence intensity decreased immediately after addition of ATP, but the rate of decrease became slower as time went. Excitation and emission were 400 nm and 480 nm, respectively. (B) Overlay of spectra (b) drawn on an expanded scale on spectra (a).

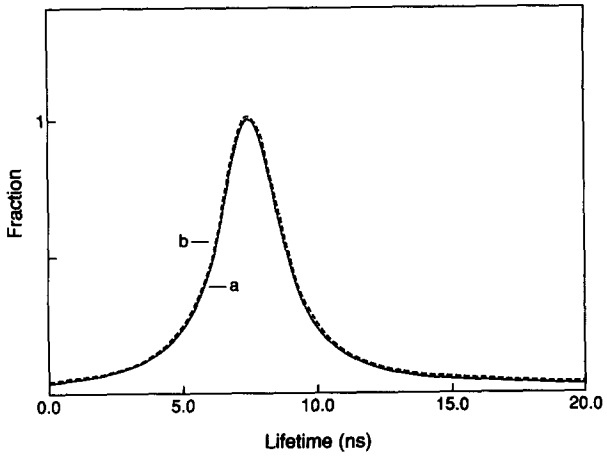

Fig. 5. Distribution analysis for bis-ANS bound to chromogranin A. Distribution analysis for 80 μM bis-ANS bound to 10 μM chromogranin A in the absence (solid line) and presence (dashed line) of 5 mM ATP. Data were analyzed using a Lorentzian distribution model. In both cases the center lifetime is 7.5 ns and the widths are near 3.8 ns. The reduced chi-square value for this fit was near 1.5.

implied displacement of bis-ANS by ATP. The fact that ATP cannot displace the probe completely suggests that not all bis-ANS sites are ATP binding sites, or that one or more of the bis-ANS binding sites has much higher affinity than does ATP. The increase in fluorescence upon addition of ADP or AMP (Fig. 3) could be due to a net increase of bis-ANS binding to chromogranin A, a combined effect of bis-ANS displaced by adenine nucleotides which is more than compensated by additional bis-ANS bound to chromogranin A as a result of a conformational change induced by adenine nucleotide binding. Changes in fluorescence intensity could be induced only by adenine nucleotides, while other nucleotides, specifically, GTP, UTP and CTP, had no apparent effect. The only structural difference between ATP and GTP is the six-membered ring of the purine base, suggesting that the purine ring is important to the binding. Fluorescence emission before and after addition of ATP were identical (Fig. 4). These results clearly demonstrated that ATP interacts with chromogranin A but the observed decrease in bis-ANS fluorescence could be due to either displacement of bis-ANS from the protein or a decrease in the quantum yield of the bound bis-ANS. The superposition of the normalized spectra before and after addition of ATP and the invariance of the lifetime data (Fig. 5) suggest that the decrease in the bis-ANS signal in the presence of ATP is not due to simple quenching or subtle alterations in the bis-ANS environment which affect the quantum yield. This conclusion seems reasonable since alterations in the quantum yield should affect the lifetime and emission maximum. The simplest explanation is that ATP displaces some fraction of the bound bis-ANS. On the other hand, one cannot at this time rule out the possibility that ATP binds at a completely separate site and that the release of bis-ANS is due to a free energy coupling mechanism as opposed to direct competition. The importance of the normal purine ring to the binding process was also tested by employing ε-ATP, a fluorescent ATP derivative with a modification on the six-membered ring of adenine base, in lieu of ATP [15]. Substitution of ATP with ε-ATP in the identical experiment described in Fig. 4 failed to reduce the fluorescence intensity (not shown). Also, the polarization of the ε-ATP fluorescence did not increase upon addition of the protein, suggesting the lack of significant binding. Failure of ε-ATP to replace ATP supports the idea that the six-membered ring of adenine is directly involved in the interaction with chromogranin A.

Interaction of ATP with catecholamines had been studied earlier using nuclear magnetic resonance (NMR) spectroscopy [2–4]. Upon dilution of an aqueous solution containing 0.5 M norepinephrine and 0.125 M ATP with water, Maynert et al. [16] observed precipitation of norepinephrine-ATP complex at a ratio of 4:1 at pH 7.0. They also noticed that the presence of Mg^{2+} or

Ca^{2+} increased the solubility of the complex. While studying the interactions among ATP, Mg^{2+} and epinephrine at acidic pH values, Tuck and Dallas [3] found that Mg^{2+} was tightly bound by the β- and γ-phosphate groups of ATP. Moreover, in the ternary complex the aromatic ring of epinephrine overlapped the purine ring of ATP and there appeared to be an ionic interaction between the protonated amino group and α-phosphate of ATP. The presence of ATP-catecholamine complex in granules [4] and in intact adrenal medulla [17] has also been reported. However, this conclusion was received with reservation. Based on carbon-13 intensities of ATP and epinephrine of intact chromaffin vesicles, Sharp and Richard [18] concluded that epinephrine and ATP are not directly complexed in intact chromaffin vesicles. On the basis of NMR relaxation data which had been used to characterize molecular motion and intermolecular complexes in the aqueous environments of chromaffin vesicles [19], they suggested electrostatic interactions of highly charged chromogranin A with ATP and catecholamine. They noticed that epinephrine and ATP have significantly different rotational correlation times in the granules, which was understood to mean that these two are not in a simple binary complex state. Daniels et al. [2] reported a ternary complex formation among epinephrine, ATP and chromogranin A based on NMR relaxation time measurements. In their previous studies, they noted that epinephrines exchange rapidly enough for them all to experience the same chemical environment both in the vesicles [20] and in intact adrenal medulla [17].

In the catecholamine-ATP complex, the aromatic ring of catecholamine overlaps the purine ring of ATP [2,3] while the protonated amino group of catecholamine interacts electrostatically with α-phosphate of ATP [4]. Although the identities of the amino acids of chromogranin A which form bonds with ATP are not yet known, the interaction described in this report could allow the formation of a ternary complex between chromogranin A, ATP and catecholamine, all of which are abundant in the chromaffin vesicles.

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