THE EFFECT OF LIGANDS ON THE INTERACTION OF 8-ANILINO-1-NAPHTHALENE SULFONATE WITH PYRUVATE KINASE OF *NEUROSPORA*

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1. Introduction

Fluorescent dyes such as 8-anilino-1-naphthalene sulfonate (ANS) and related compounds have frequently been employed as sensitive probes of the hydrophobic regions of a variety of proteins (for a review, see ref. [4]). A striking property of *N*-arylaminonaphthalene sulfonate derivatives is that whereas in aqueous solutions extremely weak fluorescence is exhibited, on interaction with nonpolar solvents, hydrophobic regions of a protein [12] and ionic interactions [1], a marked increase occurs in the fluorescence quantum yield, accompanied by a blue-shifted emission maximum. Extensive investigations of the interaction of ANS with BSA, initially conducted by Weber's group [16, 18] and Stryer [14], openened up a new era in protein chemistry and studies of structure/function relationships in macromolecules. In addition, ANS is increasingly being used in experiments on biological membranes and energy-induced transport. Energy-linked structural changes in mitochondrial membranes and the response of sonicated mitochondrial fragments toward substrates and energy yielding compounds has been analysed on the basis of fluorescence of ANS bound to the membrane [5]. And structural transitions in *E. coli* membrane vesicles associated with transport have been elucidated by characterization of ANS binding and properties of the ANS–membrane complexes [13].

The mode of interaction of individual proteins with ANS is usually quite distinct – binding may occur either at a few specific sites, or, in a completely non-specific manner. For instance, it has been demonstrated to bind specifically to the hemin site in apohemoglobin and apomyoglobin [14]; to a large number of sites in phosphofructokinase [2]; to substrate binding sites in transaldolase [3] and firefly luciferase [7]; and in the vicinity of the active site in cholinesterase [6]. The binding properties of ANS can often provide significant insights in mapping the ligand binding regions of a particular protein, provided the latter are interrelated with ANS binding sites.

In this communication evidence for interaction of ANS with *Neurospora* pyruvate kinase (PK) in relation to the sites binding substrates and the allosteric effector, is presented.

2. Materials and methods

PK was isolated from *N. crassa* (FGSC strain no. 533) and purified by a slight modification of the previously published procedure [9]. The enzyme preparation was homogeneous by the criteria of sedimentation velocity and electrophoresis on polyacrylamide gels. Enzyme assays were performed spectrophotometrically, as described earlier, by coupling with a lactate dehydrogenase system.

Protein determinations were carried out by the procedure of Lowry et al. [11] with BSA as a reference

Highly purified magnesium salt of ANS was purchased from Pierce Chemical Co. The remainder of the reagents were products of Sigma.

Fluorescence measurements were made with an Amicon–Bowman spectrophotofluorometer equipped with an X–Y recorder. The light source was a Xenon arc lamp. Measurements were made in 1 cm light path fused quartz cells of 4.8 ml capacity, in a final volume

of 1.0 ml. The cell holder was maintained at 20°C, or as specified, by circulating cold water through the system. The cuvette temperature was monitored by means of a thermocouple (Yellow Springs Instruments Co., Model 42 SC Telethermometer). Exciting light of 290 nm was used for monitoring the intrinsic protein fluorescence and 420 nm for ANS fluorescence. Emission spectra were obtained by recording fluorescence from 300 nm–650 nm for protein fluorescence and from 450 nm–580 nm for ANS–PK complexes. The fluorescence intensities determined from the emission maxima of individual spectra were expressed in arbitrary units. The instrument was standardized with a quinine sulfate solution. The emission spectra reported here are direct tracings from the originals and have not been corrected for instrument response.

3. Results and discussion

3.1. *Effect of ANS on the intrinsic protein fluorescence*

On excitation with light of 290 nm, PK exhibits fluorescence with an emission maximum of 353 nm (fig. 1). The emission spectrum is characteristic of tryptophanyl fluorescence and suggests that these residues are situated on the exterior of the protein, in a polar environment. As shown in fig. 1, addition of increasing amounts of ANS led to a quenching of this fluorescence with the concurrent appearance of a second emission peak with a maximum at 500 nm. On addition of still higher concentrations of ANS the tryptophan fluorescence can be quenched completely, but the intensity of fluorescence in the 500 nm region does not increase proportionately. It tends to reach a plateau around the level depicted in fig. 1. The cross-over point is located at approximately 445 nm. It has been observed previously that the intrinsic protein fluorescence of *Neurospora* PK is quenched to an extent of 50% by FDP [8]. Quenching by FDP to varying extents has also been reported for PK of yeast [10] and rabbit muscle [15]. It would be reasonable to conclude that ANS binding might be occurring at site/s close to, or closely related to, the FDP binding site.

The role of substrates and ligands in modification of the ANS-induced quenching was explored by including ADP, PEP, and FDP in the system and adding increments of ANS, as before. Quenching by ANS was

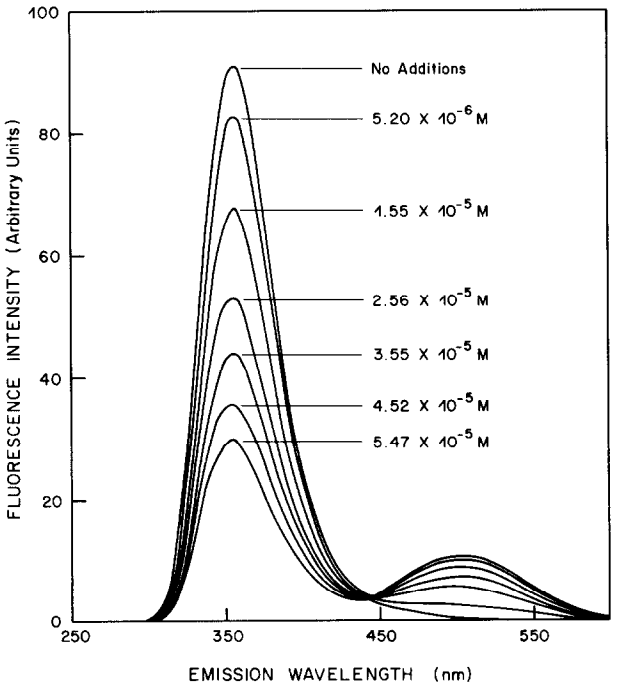

Fig. 1. Quenching of intrinsic protein fluorescence by ANS binding to pyruvate kinase. Basic system: 0.1 M phosphate buffer, pH 7.5 and 81 μg PK in a final volume of 0.995 ml. Excitation λ was 290 nm. Emission spectra recorded at 20°C from 300–550 nm. Spectrum 1, buffer and PK. ANS was added in 0.005 ml increments successively; the final concentrations of ANS are given beside each spectrum.

evaluated in terms of the quantity $\Delta I/I$, where ΔI is the change in fluorescence on addition of ANS, and I, the relative fluorescence intensity of the enzyme in the buffer. Plots of $\Delta I/I$ versus ANS concentration were not significantly different whether or not 5×10^{-4} M PEP, ADP, ATP or FDP was present in the system. Double reciprocal plots of ΔI vs. ANS concentration yielded an approximate dissociation constant of 0.11 mM for ANS. Quenching by ANS appeared to be independent of pH in the range 6.5–9.0 using 0.1 M phosphate buffer.

3.2. *Interaction of ANS with nonpolar regions of PK*

Interaction of ANS with hydrophobic regions of PK was evaluated by fluorescence of ANS on excitation at 420 nm. Free ANS, in 0.1 M phosphate buffer, pH 7.5, showed an emission maximum at 515–517 nm. Included in fig. 2 are spectra showing interaction of

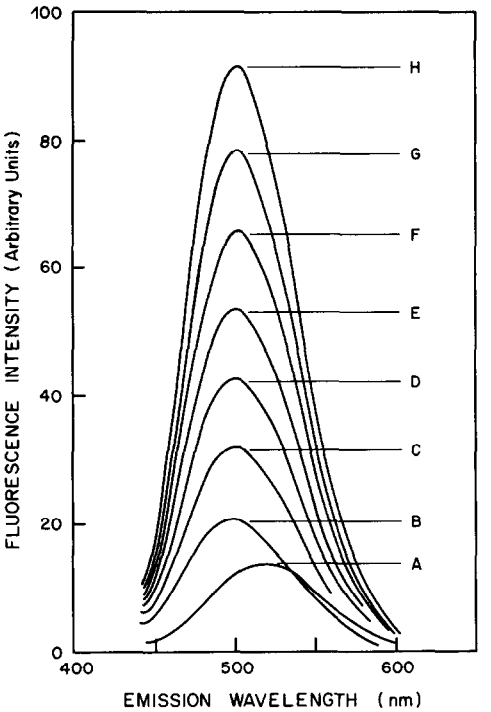

Fig. 2. Fluorescence of ANS on binding to PK as a function of ANS concentration: Excitation λ, 420 nm. emission recorded from 440 nm–600 nm; A) 10^{-3} M ANS in 0.1 M phosphate buffer, pH 7.5, no PK; spectra B to H were derived from systems containing 121.5 μg of PK and ANS concentrations as follows: B) 1.96×10^{-4} M; C) 3.85×10^{-4} M; D) 5.66×10^{-4} M; E) 7.4×10^{-4} M; F) 10^{-3} M; G) 1.18×10^{-3} M; and H) 1.43×10^{-3} M.

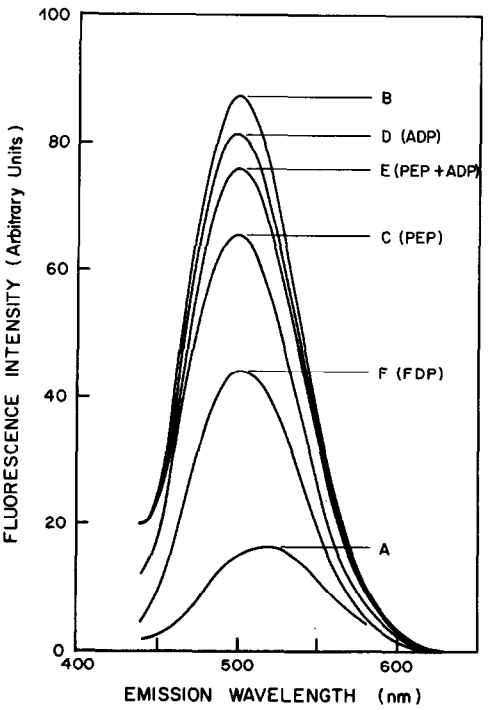

Fig. 3. Effect of substrates and allosteric ligands on fluorescence of ANS–PK complex. Excitation λ, 420 nm. All spectra were derived from systems containing 0.1 M phosphate buffer, pH 7.5 and 10^{-3} M ANS with ligands added as indicated. 121.5 μg of PK was present where indicated: A) 10^{-3} M ANS, no PK; B) ANS + PK; C) ANS + PK with 10^{-3} M PEP; D) ANS + PK with 10^{-3} M ADP; E) ANS + PK with 10^{-3} M PEP and ADP; F) ANS + PK with 10^{-3} M FDP.

ANS with PK – spectra B–H show a gradual increase in fluorescence as a function of ANS concentration at a constant level of PK. The fluorescence intensity attained a saturation value at about 1.5×10^{-3} M ANS. Plots of reciprocal fluorescence versus reciprocal ANS concentration gave a value for K_{diss} of about 3×10^{-3} M. Relative to the emission of free ANS, a blue shift of the emission maximum by 15–17 nm was observed on binding of ANS to PK. This is a direct reflection of the presence of ANS molecules in a nonpolar environment. The increase in relative quantum yield of ANS on binding to PK is significant but not as high as that reported for some other proteins, i.e., BSA [17].

In fig. 3 spectra are presented illustrating the effect of ADP, PEP, and FDP on the fluorescence of ANS–PK complex. Spectra A and B represent the fluorescence of free ANS and ANS–PK complex, respectively. On addition of 10^{-3} M ADP there was a small decrease of ANS–PK fluorescence. PEP alone produced a far greater effect and and intermediate value was obtained when ADP and PEP were present simultaneously. The maximum amount of quenching was brought about by the presence of FDP; furthermore, ADP and PEP were not able to displace FDP. Quenching of ANS fluorescence by ligands can also be expressed as the ratio ΔI/I, where ΔI is the change in fluorescence of ANS–PK complex on addition of one of the ligands and I, the fluorescence intensity of ANS–PK. ΔI/I values for fluorescence of ANS–PK in the presence of ADP, PEP, ADP + PEP and FDP were 0.07, 0.25, 0.15 and 0.5, respectively.

The relative quantum yield of ANS–PK complex

and the degree of quenching by FDP were observed to be temperature and pH dependent. For instance, at 15°C the relative quantum yield of ANS–PK complex was found to be about 3 times the value at 22°C, while at 38°C it was significantly lower. Quenching by FDP showed temperature and pH optima around 22°C and pH 8.0. At temperatures below 17°C, FDP afforded extremely low levels of quenching, ANS binding, on the other hand, was observed to be relatively high. The extent of quenching of the ANS–PK fluorescence, under different conditions, can be utilized as a direct measure of FDP binding to PK.

From the foregoing experiments it is apparent that at low concentrations ANS binds to PK resulting in a complete quenching of the tryptophanyl fluorescence, showing energy transfer from aromatic residues to the bound dye. A similar interaction has also been documented for BSA and ANS [14]. At higher concentrations of ANS, binding can be detected to nonpolar regions of the protein which appear to be connected, predominantly, with the FDP binding sites, but also to a moderate extent, with the substrate sites and, finally, to certain areas of the protein which are apparently unrelated to any of the ligand binding regions.

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