A FLUORESCENT 3'-ADP-ANALOG: INTERACTION WITH OXIDATIVE PHOSPHORYLATION AND THE ADENINE NUCLEOTIDE CARRIER

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1. Introduction

Previous studies [1–3] have shown that 3'-esters of ADP are effective inhibitors of oxidative phosphorylation and photophosphorylation, exhibiting K_i <1 μM. Besides the use of 3'-derivatives for photolabeling [2–6] with an azido-bearing substituent, the high affinity of these analogs to nucleotide binding sites encouraged us to synthesize fluorescent derivatives in order to study interactions with coupling factors F_1 or CF_1 for example. One of the most potent inhibitors of energy-linked phosphorylation was 3'-*O*-naphthoyl-1-ADP, which exhibits fluorescent properties at low wavelength. This derivative could be converted to a more suitable fluorescent analog by adding a dimethylamino-group to the naphthalene ring, yielding 3'-*O*-(5-dimethylaminonaphthoyl-1-)-ADP, in the following termed 'F-ADP'.

This report describes its inhibitory and fluorescent properties with submitochondrial particles from beefheart, demonstrating that this analog by its fluorescence response differentiates between the inner and outer side of the coupling membrane and that it also interacts with the adenine nucleotide carrier. The affinity to both systems, F_1 and carrier, as well as the fluorescence response is highly different.

2. Materials and methods

Submitochondrial particles (SMP) were prepared from aliquots of deep-frozen beefheart mitochondria, stored for several weeks as in [3]. Oxidative phosphorylation was measured by incorporation of ^{32}P into ATP using a hexokinase trap [3]. Fluorescence spectra were recorded using a Schoeffel ratio-spectrofluorimeter. Fluorescence titrations were carried out in the same instrument or in an Eppendorf-fluorimeter with appropriate filter selection.

In these experiments submitochondrial particles were suspended in 0.25 M sucrose, 20 mM Tris–HCl (pH 7.4) at 25°C. 5-Dimethylamino-1-naphthoic acid was prepared from naphthalene-1-carboxylic acid and esterified with adenine nucleotides as in [3], using carbonyldiimidazole for activation [7]. The resulting 3'-esters termed F-ADP and F-ATP (indicating their fluorescence capabilities) were purified chromatographically and used as the sodium salts, dissolved in water. Bonkrecic acid (BKA) was a generous gift from Professor M. Klingenberg (Munich). All other chemicals were obtained p.a. from commercial sources.

3. Results

Figure 1 shows absorption and fluorescence spectra of F-ADP in water and dioxane. The fluorescence spectrum is identical with that of 5-dimethylamino-1-naphthoicacidmethylester, which has been synthesized as a reference compound. The strong fluorescence in apolar media is extremely sensitive to solvent polarity and dipole interactions as seen from the dramatic effect of water added to the dioxane solution of the probe. The quantum yield in aqueous solution is 0.018 and increases in ethanol to 0.096, in dioxane to 0.280. When added to SMP, a large fluorescence enhancement of F-ADP is observed with an emission spectrum identical to that in dioxane. SMP-bound F-ADP shows a quantum yield of 0.45 suggesting that in addition to polarity conformational constraint of the bound molecule may be responsible for enhancing its fluorescence intensity.

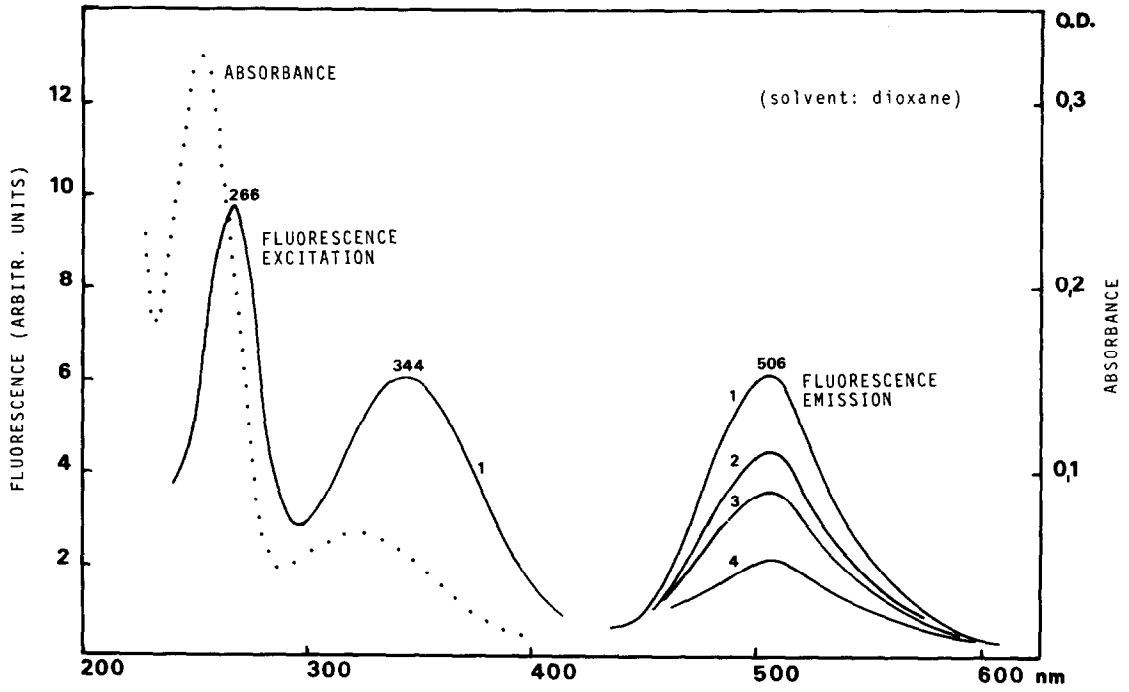

Fig.1. Absorbance and fluorescence spectra of F-ADP. (. . .) Absorbance spectrum of a solution of 2.15×10^{-5} M F-ADP in 0.1 M K-phosphate buffer (pH 7.2). (–) Fluorescence of 2.15×10^{-5} M F-ADP in dioxane; left, excitation spectrum; right, emission spectrum; (1) absolute dioxane; (2) 2.2 vol. % H_2O, (3) 3.6 vol. % H_2O; (4) 5.4 vol. % H_2O in dioxane.

From fluorescence titrations it emerged that no binding (accompanied by fluorescence) occurs to synthetic phospholipid vesicles, to intact mitochondria, mitoplasts or to bovine serum albumin, whereas high affinity binding with large fluorescence increase was observed only with submitochondrial particles (K_d 4–9 μM). As an important conclusion it follows that only at the inner surface of the mitochondrial membrane a binding site is available which generates high fluorescence of the analog. A K_d 42 μM was found for the F-ATP analog. Surprisingly, also the respective F-AMP shows binding with concomitant fluorescence increase and almost the same affinity as F-ADP. In contrast, free 5-dimethylamino-1-naphthoicacid or F-adenosine did not show any binding. Thus, the presence of the phosphate moiety is essential for specific binding, guiding the fluorophore to its proper position in the membrane.

Figure 2 shows the inhibitory properties of F-ADP and the corresponding AMP- and ATP-analogs on oxidative phosphorylation, closely resembling those found for 3′-*O*-naphthoyl-1-adenine nucleotides [3], with a half-maximal inhibition at about 0.5 μM

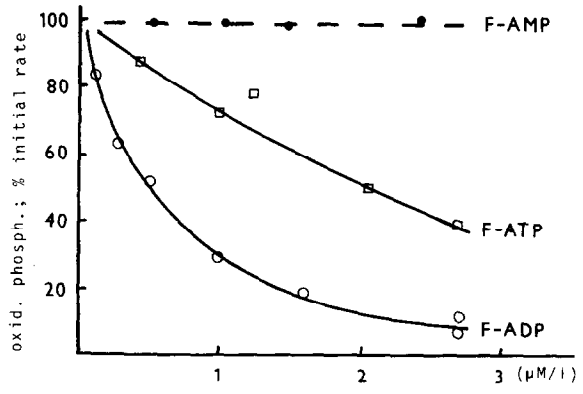

Fig.2. Effects of F-AMP, F-ADP, F-ATP on oxidative phosphorylation in SMP. Each sample contained 220 mM sucrose, 10 mM Tris (pH 7.2), 2.5 mM $MgCl_2$, 2.5 mM [^{32}P]potassium phosphate, 10 mM succinate, 10 mM glucose, 0.1 mM ADP; 70 units hexokinase, and the amount of inhibitor as indicated; the reaction was started by adding 0.16 mg/ml submitochondrial particles and terminated after 3 min at 30°C by $HClO_4$; ^{32}P-incorporation into ATP was analyzed as in [3].

F-ADP. The AMP-analog was totally inactive. F-ADP is not phosphorylated itself. The calculated K_i for F-ADP, however, is >1 order of magnitude lower than the K_d found from fluorescence titrations.

This discrepancy was resolved when it was detected that the fluorescence of SMP-bound F-ADP is specificially sensitive to bonkrecic acid (BKA), an inhibitory ligand of the adenine nucleotide carrier, which is also assumed to bind to a locus on the carrier system only exposed to the inner side of the mitochondrial membrane (reviewed [8,9]).

In fig.3 several subsequent additions of substrates and inhibitors demonstrate the general fluorescence response of F-ADP, bound to beefheart submitochondrial particles. Two important phenomena can be distinguished:

(i) The bound analog responds to energization and de-energization by a small (12–14% of total) reversible change of fluorescence.

(ii) The fluorescence is rapidly quenched by addition of BKA, and a slow and much less pronounced fluorescence decrease follows addition of atractylate.

If atractylate was present during preparation of the submitochondrial particles, this latter effect was absent. BKA lowers the fluorescence to ~20% of the initial value.

Figure 4 shows a normalized diagram of fluores-

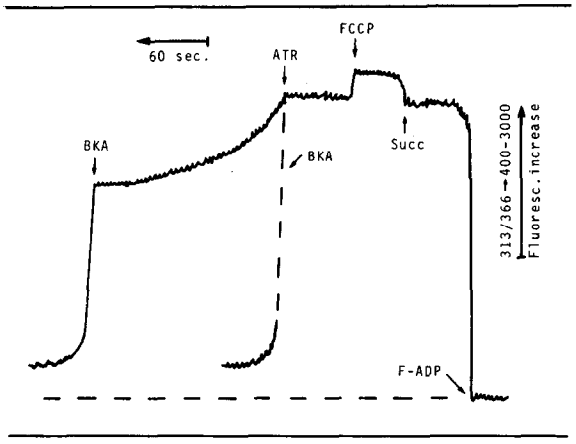

Fig.3. Fluorescence response of F-ADP bound to submitochondrial particles. SMP (2.3 mg/ml) were suspended in 0.25 M sucrose, 20 mM Tris (pH 7.2); where indicated 2.69×10^{-6} M F-ADP, 5×10^{-3} M succinate, 1.2×10^{-6} M FCCP, 7.4×10^{-6} M atractylate, or 3.2 nmol boncrecate were added. Fluorescence was recorded in an Eppendorf filter-fluorimeter.

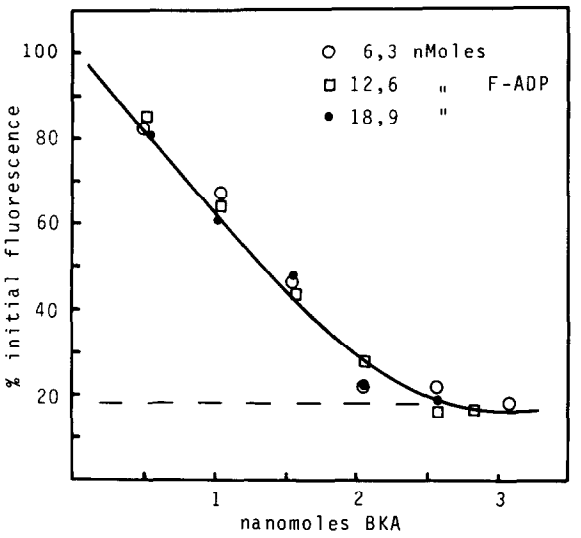

Fig.4. Quenching of F-ADP fluorescence by boncrecate. In individual experiments the indicated amounts of F-ADP were added to 2 ml SMP suspension to yield different initial fluorescence intensities, regarded as 100%. Then the fluorescence was titrated by 1 µl additions of a boncrecate solution until no further quench was observed. Protein was 0.9 mg/ml.

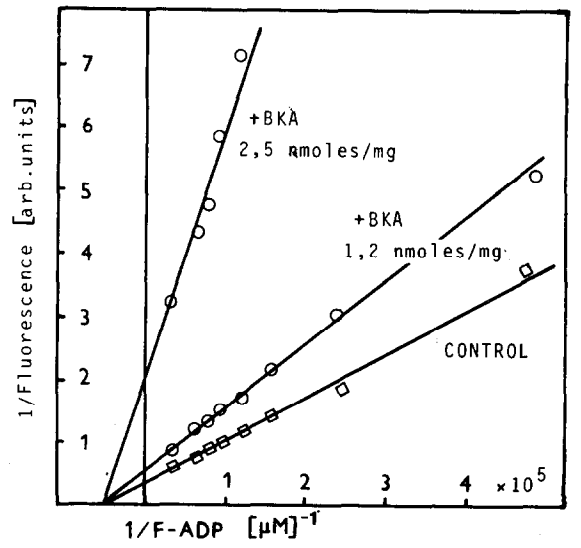

Fig.5. Reciprocal plot of fluorescence titration of SMP particles by F-ADP; effect of BKA. SMP 0.75 mg/ml were suspended in 0.25 mM sucrose, 20 mM Tris–HCl (pH 7.4). Fluorescence increase was titrated with F-ADP in absence or in presence of the indicated amounts of boncrecate. The height of the fluorescence signal (arbitrary units) versus concentration of F-ADP was evaluated.

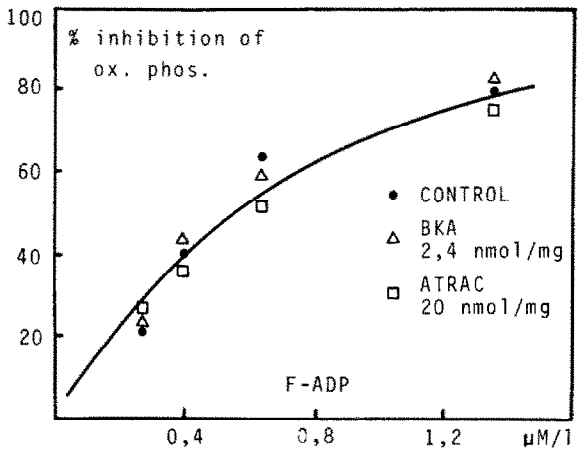

Fig.6. Effect of F-ADP on oxidative phosphorylation in SMP. Relative inhibition in absence or in presence of the indicated amounts of boncrecate or atractylate. Experimental conditions as given in fig.2.

cence-quench titrations by BKA. Independent of the initial fluorescence, i.e., of the amount of F-ADP initially bound, always the same amount of BKA/mg protein is required to induce maximum quench. This suggests a non-competitive phenomenon which is in agreement with the results of fig.5, giving fluorescence titrations of beefheart SMPs in presence of different amounts of BKA.

As seen from fig.6, neither BKA nor atractylate affect the inhibition of oxidative phosphorylation by increasing concentrations of F-ADP, yielding in all cases half maximum inhibition at 0.5–0.6 μM as previously determined (100 μM ADP present).

Thus it is obvious, that the large fluorescence signal of F-ADP which titrates with K_d 4–9 μM (depending on the SMP preparation) indicates an interaction of the analog with the carrier protein. The analog binds to F_1 in the membrane with much higher affinity ($K_1 \ll 1$ μM), and emites only little fluorescence. This has been confirmed by preliminary studies with isolated beefheart F_1. After depletion of endogenous nucleotides, a high affinity site for F-ADP with $K_m \sim 10^{-8}$ M was detected (G.S. et al. unpublished) may be assigned to one of the 'tight' binding sites.

4. Discussion

It has been concluded from results with 3′-esters of ADP [2,3] that their binding site on F_1 should contain a rather hydrophobic cavity accepting the 3′-substituent. Thus, the question arises, why the high-affinity inhibitor F-ADP only exhibits very weak fluorescence when bound to the coupling factor. Two reasons may be considered:

(i) The site is much less hydrophobic than previously assumed.

(ii) The fluorescence of the probe is quenched, e.g., by π–π interactions of the aromatic system.

Indeed, especially those analogs show very high affinities to the coupling ATPase which are bearing an aromatic 3′-substituent capable of such interactions. The aromatic amino acid tyrosine, which is present in the catalytic center [10], may allow such interactions with the analog. In fact, in weakly solvating aprotonic solvents tyrosine was revealed to be an excellent quencher of F-ADP fluorescence. It may be presumed, therefore, that in the catalytic site of F_1, F-ADP sensitizes this tyrosyl residue.

It is of particular interest, that most of the fluorescence signal results from binding of F-ADP to the adenine nucleotide carrier. The presence of the dimethylamino-group in the fluorophore is essential for this interaction, because a corresponding fluorescence enhancement is totally absent with 3′-*O*-naphthoyl-1-ADP, which emits at shorter wavelengths. Most likely the specific environment of this group influences its interaction with the aromatic π-system, being responsible for the high fluorescence when bound to the carrier. The dramatic quench of fluorescence by BKA is suggested to indicate a conformational transition of the carrier, which may be resolved kinetically in the future. Loss of fluorescence yield may result from a sudden exposure of the bound probe to the aqueous space or from conformational constraint on the fluorophore. From an analogous experiment with a spinlabeled analog (K. Suhl, G.S., unpublished, [11])) it seems unlikely, that bound F-ADP is released from the carrier by the action of BKA.

Energization of submitochondrial particles had a small but rapid effect on fluorescence of F-ADP. It is suggested that this effect also results from carrier-bound F-ADP, indicating an energy controlled transition of the carrier. This conclusion is based on the finding that in the presence of BKA the energy-linked fluorescence transition was absent. It supports the conclusions in [8,9] that the function of the carrier is modulated by the energy-state of the mitochondria.

As to the slow quenching of F-ADP fluorescence by atractylate it should be emphasized that this effect was small and in many cases almost absent. It is regarded as a slow diffusion of atractylate to its specific binding site, presumably in leaky sonic particles. Therefore, by the differential effects of atractylate and bonkrecate on F-ADP-fluorescence, the quality or 'inside/out scrambling' of submitochondrial particles, or the 'sideness' of reconstituted systems containing the carrier protein may be conveniently monitored.

In addition to formycin-diphosphate, recently introduced as a carrier-binding fluorescent analog [12], F-ADP is a promising new tool for the investigation of nucleotide interactions on the adenine nucleotide transport system.

Acknowledgements

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