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STUDIES OF THE ADP/ATP CARRIER OF MITOCHONDRIA WITH FLUORESCENT ADP ANALOGUE FORMYCIN DIPHOSPHATE

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Summary

The ADP/ATP carrier was studied by a fluorescent substrate, formycin diphosphate which is the only fluorescent ADP analogue to bind. Its low quantum yield, short decay time and spectral overlap with tryptophan has as yet prevented its wider use.

By incorporating fluorescent acceptors of formycin diphosphate fluorescence, anthracene-maleimide and vinylanthracene, into the membrane, these difficulties were circumvented. Only bound formycin diphosphate transfers energy to the probes so that the secondary emission of these probes is a measure for membrane-bound formycin diphosphate.

The fluorescent transfer is inhibited by ADP, bongkrekate and carboxyatractylate whether added before or after incubation of formycin diphosphate showing that only binding to the adenine nucleotide carrier is measured. It also shows directly that the earlier demonstrated ADP fixation by bongkrekate is indeed a displacement into the matrix.

The fluorescence decay time of the bound formycin diphosphate is measured as 1.95 ns compared to 0.95 ns of the free formycin diphosphate, indicating that formycin diphosphate is bound at the carrier in a non-polar environment.

The depolarization decay time was found to be larger than 15 ns, indicating that carrier-bound formycin diphosphate is immobile within this time period.

Introduction

Fluorescence can be considered as a powerful tool for studying functional membrane proteins such as carriers, and thus for gaining insight into the

Abbreviations: FoDP, formycin diphosphate.

molecular mechanism of translocation. The understanding of such a mechanism is particularly advanced in the ADP/ATP carrier of mitochondrial membranes (see ref. 1), and it seemed desirable to use fluorescence for further study of the ADP/ATP carrier. A fluorescent label was required which specifically probes the ADP/ATP carrier and does not bind to the many other proteins of the mitochondrial membrane.

For this purpose we looked for a fluorescent ADP analogue which still can bind and translocate on the ADP/ATP carrier. In view of the specificity of the carrier, highly fluorescent analogues such as the 2-aminopurine isomer or etheno-ADP [2] cannot be used since they are not bound and translocated even to a marginal extent (unpublished results). A genuine fluorescent ADP analogue with nearly unaltered structure is formycin diphosphate (FoDP). The fluorescent FoDP differs from ADP only in an exchange of C against N between positions 7 and 8 of the purine moiety. It is still actively translocated at about one-third of the rate of ADP [1].

However, we encountered a number of drawbacks of the formycin nucleotide: low quantum yield [3], short decay time, and interference with tryptophan fluorescence. It appears that these problems have prevented wider use in studies with ADP- and ATP-utilizing enzymes since its initial introduction [3,4].

In the present studies on the ADP/ATP carrier in mitochondria, these difficulties, resulting in deterioration of fluorescence polarization and fluorescence decay time measurements, were partially overcome. The strong interference of tryptophan could be circumvented by introducing secondary fluorescent probes which accept energy by fluorescence transfer from FoDP.

Material and Methods

Preparation of FoDP

First FoMP was prepared from formycin according to the procedure of Tsuchiya [5] as modified by Ward et al. [4] using $POCl_3$. Separation and purification of the FoMP was carried out using ion exchange on Dowex. The main fraction containing FoMP was purified on DEAE-32 cellulose with triethylamine bicarbonate. FoMP was identified by electrophoresis or thin-layer chromatography and determined enzymatically with adenylate kinase. The yield was up to 60% as referred to formycin.

Preparation of FoDP from FoMP followed essentially the method of Michelson [6,7]. For this purpose FoMP was transferred into the trioctylammonium salt. This salt was dissolved in dimethylformamide and dioxane and reacted with diphenylchlorophosphate under further addition of tributylamine. The activated FoMP was concentrated and purified by extraction with ether.

For phosphorylation the activated FoMP was dissolved in a mixture of tributylamine, pyridine, dimethylformamide and anhydrous H_3PO_4 at a ratio of FoMP : H_3PO_4 = 1 : 2. After 20 h at room temperature the reaction mixture was diluted with water and extracted with ether. The aqueous phases were pooled and concentrated. FoDP was purified on DEAE-cellulose 32 with a triethylamine bicarbonate gradient. The triethylammonium salt was converted

into sodium salt by addition of NaI in methanol. The yield of Na_2FoDP was about 25% as referred to FoMP. FoTP, which was also formed as a byproduct in this reaction, was also collected in other fractions of the DEAE-cellulose chromatography at a yield of about 5%.

9-Vinylanthracene was purchased from E. Merck and Co. and used without further purification. 9-Anthracene maleimide was synthesized by a method similar to that of Weltmann et al. [8]. First, 9-aminoanthracene was synthesized from anthracene according to Dimroth [9] and Meisenheimer [10], then 3 g of aminoanthracene were dissolved in 30 ml of tetrahydrofuran, and a solution of 1.6 g maleic acid anhydride in 15 ml tetrahydrofuran was added. After 24 h stirring at 0°C the yellow precipitate (9-anthracene-maleimide) was filtrated and washed with tetrahydrofuran.

1 g of 9-anthracene-maleimide was then added to a mixture of 20 g acetic acid anhydride and 60 mg sodium acetate. The suspension was stirred for 2 h at 75°C. The now clear and slightly yellow solution was cooled down and poured into 50 ml of concentrated $NaHCO_3$ solution. The precipitate was then dissolved in chloroform/dioxan/acetic acid (65 : 25 : 4) and purified by thin-layer chromatography. Out of the three fractions (5, 75, 20%) the second one inhibited the swelling of rat liver mitochondria in NH_4/P_i solution as effectively as NEM.

Fluorescence measurements

All fluorescence spectra were measured with a Hitachi spectrofluorometer MPF 2A.

Fluorescence titrations and measurements of fluorescence polarization were carried out with an apparatus consisting of a 450 W Xenon high-pressure lamp (Osram type XBO 450 W), an excitation monochromator and an emission monochromator (Bausch and Lomb, types 33-86-75 and 33-86-45). Emission was collected at 90° to the plane of excitation. A special quartz cuvette of 2 X 3 mm with 150 μl volume was used in a thermostated holder. Stray light was minimized by low pass ultraviolet-transmitting filters in the excitation light path (Schott UG 5) and high-pass filters (Schott KV) in the emission light path. The excitation light could be polarized by a Glass-Thompson calcite prisma (Halle, Berlin), the emission light by film polarizers (Käsemann, Oberaudorf, GFR).

For measurements of decay times the same optical set-up was used. The flashlamp was constructed according to Maletz [11]. The spark gap was enclosed in a brass chamber with 10-mm thick quartz windows under pressure of 30-bar purified N_2 with an addition of H_2 and He. Decay times were measured after a single photon counting principle [12,13]. The electronic circuitry for these measurements was purchased from Ortec according to the application note AN-35. A small part of the flashlight falls directly onto a photomultiplier (RCA 8575) which generates the start signal. Fluorescence light is monitored by a second photomultiplier (RCA 8850) which generates the stop signal. Start and stop signals are fed into a Time-to-Pulse Height Converter. The output signals are then analyzed by a Pulse Height Analyzer.

Evaluation

The evaluation of short decay times poses severe mathematical problems

because of the distortion of the decay curves by the finite duration of the flash. The difficulty of this problem has often been stated. Furthermore, some systematical errors may occur in fluorescence decay time measurements [14—17]. Isenberg and coworkers [18,19] claim that it is possible to deconvolute several decay times out of measured data, while for Knight and Selinger [20] deconvolution is a non-method for real data; they propose convolution and fitting. Ware et al. [21] use six and more decay times for fitting, but whether these decay times have any physical meaning remains open to discussion.

In most experiments there is some a priori knowledge as to how many and which decay times are to be expected. In many cases this knowledge is based on a physical model of the experiment which should be used to check all computing results. In our experiments a convolution procedure was applied first to test if fitting with these decay times, according to the physical model, is possible. This was accomplished by solving the so-called normal equations to find out the appropriate weights for a set of given decay times. If a fit was possible, this was optimized by slightly varying the decay times and — independently — by applying the method of Isenberg and Dyson [18]. The latter method was also used to find out if a fit is possible by a completely different set or more decay times.

In all cases the computing results were checked as to whether, firstly, the fit was satisfactory, and secondly, the result conformed to the physical model of the experiment. The convolution methods rule out a physical model, if no fit can be accomplished. On the other hand, a good fitting result does not guarantee that the found decay times are really present in the probes.

Results

The excitation and emission spectra of FoDP are compared to those of beef heart mitochondria in Fig. 1. There is a strong overlap of both the excitation and emission spectra of FoDP with those of mitochondria. The fluorescence of mitochondria is due mostly to tryptophan which partically coincides with that of FoDP in the excitation. The excitation wavelength of 310 nm had to be chosen since it gives a compromise between a sufficient absorbance of formycin and discrimination against tryptophan. This is all the more important as in most studies the FoDP concentration is considerably lower than in Fig. 1. With the chosen concentrations of mitochondria and FoDP and at the maximum excitation wavelength of FoDP (295 nm) the emission intensities of mitochondria and FoDP are equal (at 340 nm). By exciting at 310 nm the ratio of the emission intensities of FoDP and mitochondria changes to 5.

To get acquainted first with the technical problems, the interaction of FoDP with pure enzymes was studied. In the literature no corresponding studies are reported, probably because of the strong overlap of the fluorescence spectra of formycin and tryptophan. We studied the binding of FoDP to various kinases using fluorescence [22]. However, conclusive results were obtained only with myokinase because this protein contains no tryptophan. The fluorescence intensity of FoDP is not changed on binding to myokinase. Therefore, fluorescence polarization was applied to determine the immobilization of FoDP by

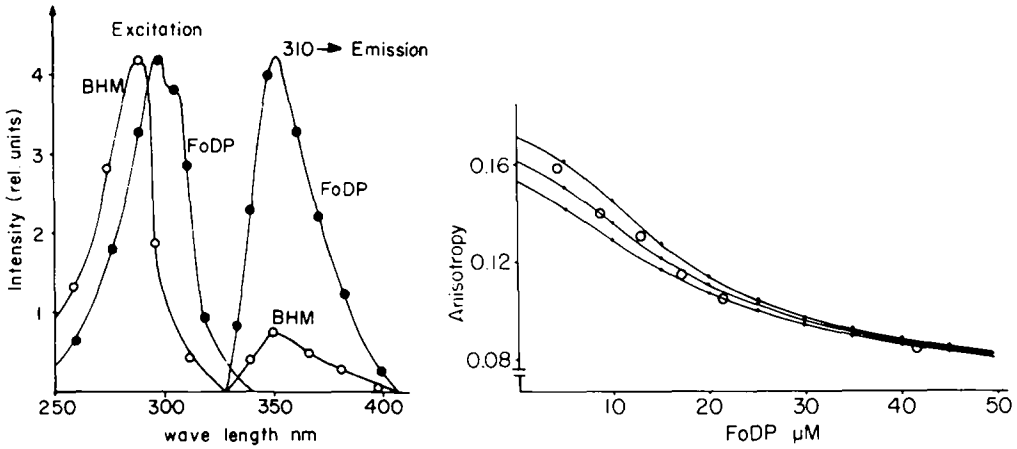

Fig. 1. Excitation spectra (at emission 350 nm) and emission spectra (at excitation 295 nm) of beef heart mitochondria and FoDP. Concentrations of mitochondria and FoDP were so chosen that both excitation spectra have the same maximal height. Excitation at 295 nm gives a nearly complete overlap of both emission spectra. By exciting at 310 nm the emission intensity of FoDP and mitochondria has a ratio of 5. The medium contained 20 mM Tris-HCl, pH 7.2, and 0.25 M saccharose.

Fig. 2. Adenylate kinase is titrated with FoDP and the resulting fluorescence anisotropy (310 → 350 nm) is measured. Conditions: 50 mM K^+-HEPES, pH 8.0/0.2 M tetramethylammoniumchloride/1 mM dithioerythrol. The measured curve is compared with calculated FoDP anisotropy-concentration functions using the indicated dissociation constants for FoDP.

the binding. The measured fluorescence anisotropy is plotted in Fig. 2 as a function of FoDP concentration. The measured data are compared to calculated anisotropy-concentration functions, using various dissociation constants for FoDP. The best fit is obtained with a $K_d = 2\ \mu M$. This value agrees with that determined for ADP binding to myokinase.

Similar measurements with creatine kinase, hexokinase and pyruvate kinase also show fluorescence anisotropy — however, of such a low degree that only rough estimates on the dissociation constants are possible, which range at about 50 μM.

In similar titrations of beef heart mitochondria with FoDP, no change of fluorescence anisotropy was observed even at low concentrations of FoDP. The reason for this failure is the strong interference of tryptophan fluorescence which is considerably larger at the wavelength chosen than the contributions from the free and bound FoDP. Only the bound FoDP would increase the fluorescence anisotropy. More concentrated suspensions of mitochondria which are used to increase the ratio of bound to free FoDP gave further depolarization due to scattering artifacts. Therefore, we searched for a method where the signal is dependent only on the amount of bound FoDP and where the complications of polarization anisotropy measurements due to the strong tryptophan interference are avoided.

This became possible by using the energy transfer resulting from bound FoDP to fluorescent probes bound in the membrane. Secondary emission of these probes was used as a measure for the bound FoDP. The probe had to fulfill the following requirements: The absorption spectrum should overlap

strongly with the emission spectrum of FoDP. However, the absorption must be low at the excitation wavelength to be used for FoDP, 310 nm, in order to avoid primary excitation of the probe. Excitation and quantum yields of the probe should be large. The probe should be non-polar in order to dissolve in the lipid membrane. It should be excited only by carrier-bound FoDP and not be free FoDP. A non-polar membrane-attached probe fulfills these conditions, whereas a water-dissolved probe could take up energy from the free FoDP.

Two derivatives of anthracene, 9-vinylanthracene and 9-anthracene-maleimide concur largely with these conditions. Both are strongly non-polar and 9-anthracene-maleimide binds also covalently to SH groups which are present in the membrane and in the ADP/ATP carrier as well. The overlap of the absorption and emission spectra of 9-anthracene-maleimide and FoDP are shown in Fig. 3. At the excitation wave number of 32 250 cm^{-1} (corrresponding to 310 nm), the absorbance of 9-anthracene-maleimide is only low. The absorption spectra of 9-vinylanthracene is very similar.

In order to avoid organic solvents, the probes were applied to the mitochondria according to the method of Shinitzky [23] by absorbing the probes

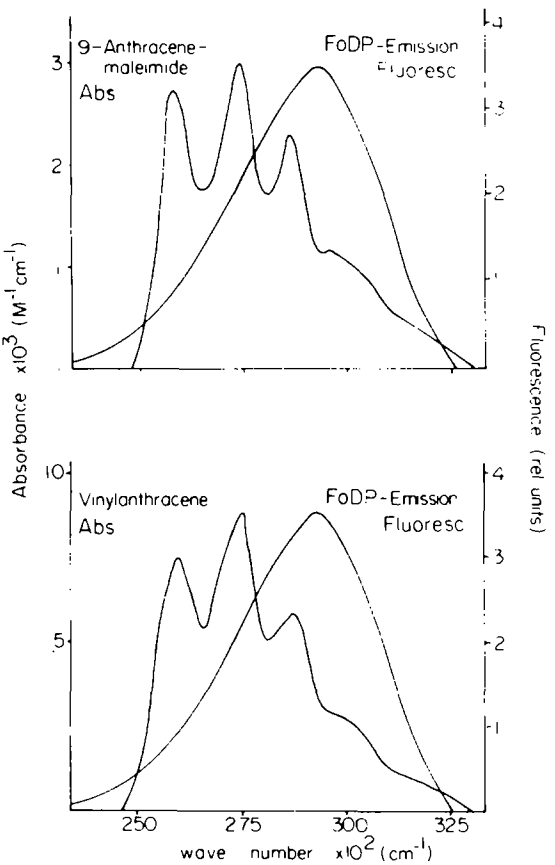

Fig. 3. Spectral overlap of the absorption spectra of 9-vinylanthracene and 9-anthracene-maleimide in ethanol with the emission spectra of FoDP in 20 mM Tris-HCl, pH 7.2.

on glass wool and then mixing the mitochondria with the coated glass wool. For the best effects the mitochondria were loaded with 9-vinylanthracene to the extent of 20 μmol/g protein. 9-Anthracene-maleimide was absorbed at considerably lower concentration (2 μmol/g protein) since higher amounts damage the membrane. A marked incorporation of 9-anthracene-maleimide requires addition of substrates and valinomycin whereas uptake of 9-vinylanthracene is independent of these additions. The effect of valinomycin is reminiscent of the incorporation of another maleimide derivative, *N*-(*N*-acetyl-4-sulfomoylphenyl)maleimide, into SH groups of mitochondria [24]. Apparently the high pH and alkalination of the matrix space in the presence of valinomycin and active respiration are responsible for the increased incorporation of the maleimide derivatives. In addition, ADP is required for the activation of the SH groups of the ADP, ATP carrier [25].

The fluorescence of 9-anthracene maleimide incorporated into beef heart mitochondria can be used to measure the binding of FoDP by radiation less energy transfer. This method is based on the fact that only membrane-bound and not free FoDP can be expected to interact with the membrane-imbedded anthracene labels. The secondary emission of the anthracene probes should be proportional to the amount of FoDP bound. The basic fluorescence of the anthracene in the absence of FoDP results from the energy transfer of the tryptophan.

In Fig. 4 the fluorescence increment is measured as dependence on the FoDP concentration. At about 20 μM FoDP the fluorescence reaches a maximum. No fluorescence increase is found when bongkrekate or carboxyatractylate are added before FoDP. Carboxyatractylate and bongkrekate as inhibitors of the adenine nucleotide transport should block the binding site for FoDP since they have been shown to remove ADP from the carrier with high binding affinity [26–29].

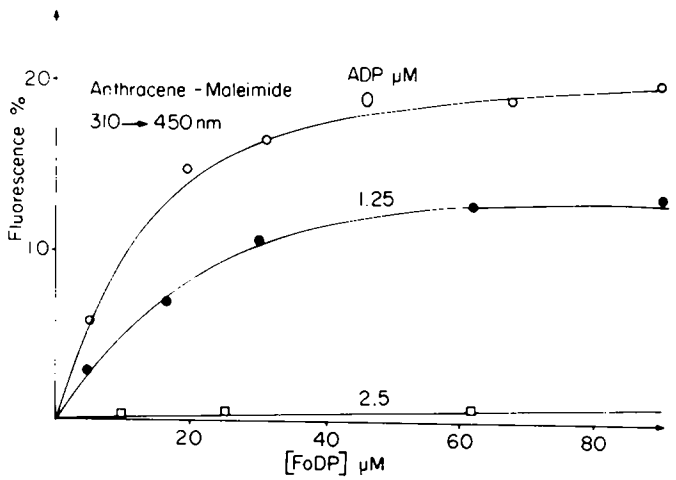

Fig. 4. Fluorescence transfer of bound FoDP to the anthracene labels. Beef heart mitochondria (2 mg/ml) are labelled with 9-anthracene-maleimide. Fluorescence is excited at 310 nm and measured at 450 nm. On addition of FoDP, bound FoDP molecules transfer their fluorescence to the labels from which the secondary emission is measured. Fluorescence transfer can be initiated by adding ADP, bongkrekate or carboxyatractylate. Conditions: 20 mM Tris-HCl, pH 7.2/0.25 M saccharose.

Also ADP suppresses fluorescence already at low concentrations; for example, at 2.5 μM ADP, fluorescence is suppressed by more than 90%. ADP competes effectively with FoDP due to its better interaction with the carrier [1,2]. Further evidence for the specificity of binding comes from the fact that no fluorescence increase is measured with FoMP, in accordance with the inability of AMP to bind to the carrier [26]. From the concentration dependence, the dissociation constants can be calculated for FoDP, K_d = 5 μM, and from the competition with ADP, for ADP, K_d = 0.2 μM. These calculations are based on competitive binding equilibration of ADP and FoDP with the carrier and are resolved by computer fitting of the concentration function by varying the K_d for ADP and FoDP.

The fluorescence enhancement by FoDP can also be partially or fully reversed by the subsequent addition of bongkrekate, carboxyatractylate and ADP, as shown in Fig. 5, which presents the decrease of fluorescence as a function of the inhibitor concentration. The amounts required for the complete reversal are considerably higher than those added prior to FoDP. The fluorescence increment of 9-vinylanthracene cannot be fully repressed by the inhibitors, in contrast to that of 9-anthracene-maleimide.

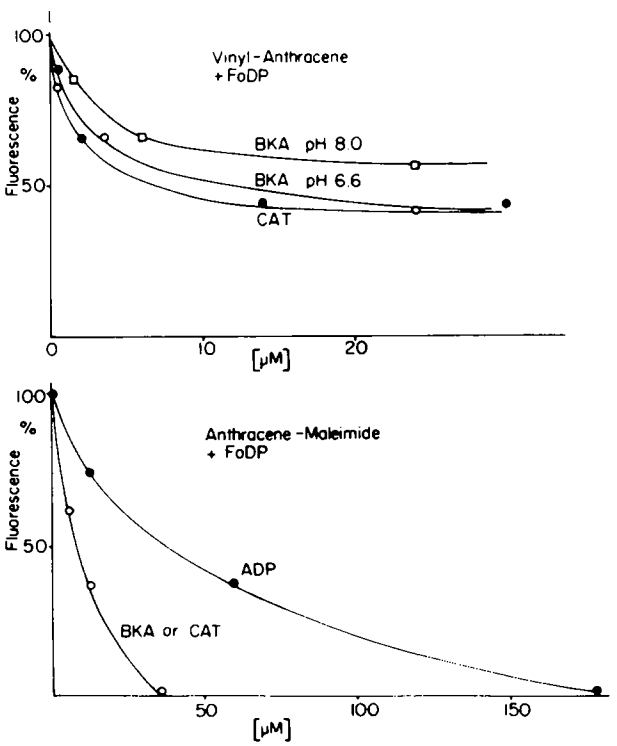

Fig. 5. Reversal of fluorescence transfer by inhibitors. Mitochondria are labelled with 9-anthracene-maleimide or 9-vinylanthracene and a nearly saturating concentration of FoDP is added. The fluorescence transfer between bound FoDP and the labels is measured (excitation 310 nm, emission 450 nm). By the addition of bongkrekate, carboxyatractylate or ADP the bound FoDP is removed from the binding site and the fluorescence transfer is inhibited.

This interesting difference may be explained if one assumes that 9-anthracene-maleimide is localized at the surface of the membrane attached to the SH groups while 9-vinylanthracene is a deeply imbedded probe. Therefore, 9-vinylanthracene should sense the more deeply in the membrane located FoDP, in contrast to 9-anthracene-maleimide. The residual fluorescence could then be attributed to FoDP located on the inner face of the inner membrane, which is, however, not bound to the carrier but possibly to the binding sites of the ATP synthase complex.

All these findings are not affected by the fluorescence transfer from tryptophan to the probes. This transfer represents a great part of the absolute signal, but it is not changed by adding FoMP, ADP, bongkrekate and carboxyatractylate. If ADP does not change this transfer, the same should apply for FoDP. It means furthermore that (a) the relative distribution of tryptophan and the probes remains constant in orientation and distance, and (b) is not changed by a redistribution of aromatic groups or paramagnetic ions.

Fluorescence decay time measurement

Fluorescence decay times give information on the chemical environment and the mobility of the fluorophore. Because of interference by tryptophan, decay time measurements are superior to direct quantum yield measurements, since they are able to discriminate between the contribution of different fluorophores. Bound FoDP is immobilized and in a different environment which may result in a change of the fluorescence decay time. Therefore, it should be possible to detect directly the fluorescence of bound FoDP without the mediation of 9-anthracene maleimide.

Measurements of the fluorescence decay times were only possible after eliminating the major fluorescence contribution of tryptophan by measuring the differences between two decay curves with and without FoDP. These difference measurements pose considerable technical difficulties and rely in particular on the stability of the sample and the apparatus, over the 1—2 h accumulation times. The drift of the apparatus can be checked by a stable sample such as 9-vinylanthracene in ethanol. Both decay curves agree within the statistical error. In order to ensure stability of the mitochondrial solution, these measurements were only performed at low temperature between 0 and 5°C. Occasionally interfering fluorescence drift by NADH was avoided by uncoupling, which causes oxidation of NADH.

In Fig. 6 a typical experiment of fluorescence decay curves with FoDP is shown. In the first part, the decay curves of the total fluorescence and the difference decay curves, calculated from FoDP plus mitochondria minus mitochondria alone, are shown. In the second part, these difference decay curves are shown for FoDP in the presence and absence of carboxyatractylate. The fluorescence decay of free FoDP only is measured in the presence of carboxyatractylate and computed as $\tau = 0.95$ ns. In the absence of carboxyatractylate, $\tau = 1.95$ ns is computed. It can be concluded that the decay time of FoDP when bound to the ADP/ATP carrier is markedly increased. This indicates that the FoDP bound to the ADP/ATP carrier is in a different chemical environment than free FoDP.

The differentiating procedure of measuring the decay time of FoDP was also

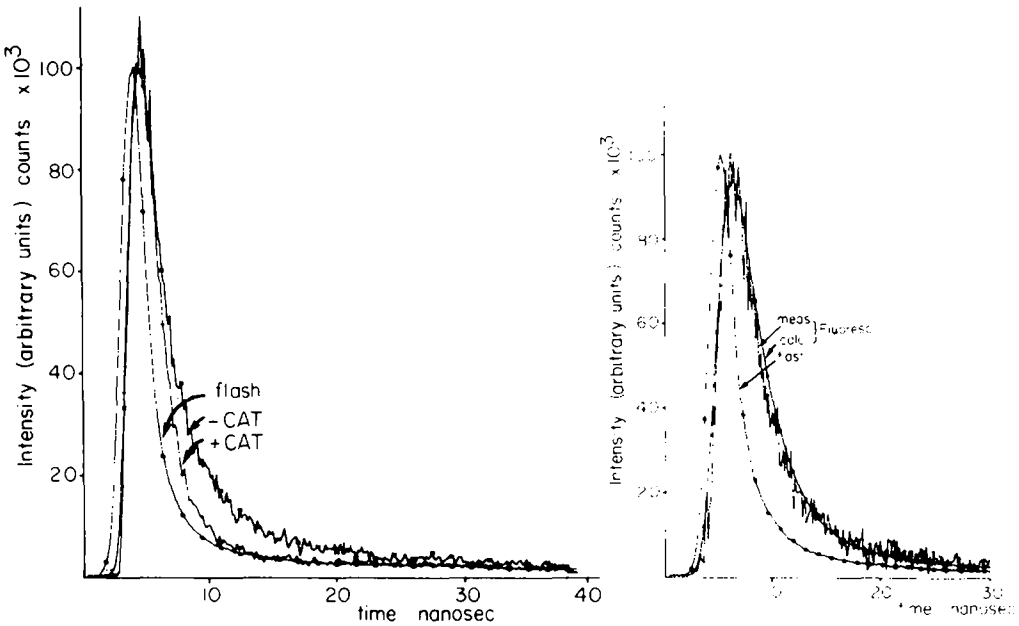

Fig. 6. Fluorescence decay of bound FoDP. The fluorescence decay curve of mitochondria (2 mg/ml) in Tris-HCl, pH 7.2/ 0.25 M saccharose, with and without FoDP (2 μM), is alternately measured and the two curves are collected and finally subtracted. In A, these difference curves are compared in the presence and absence of carboxyatractylate. The decay curve in the presence of carboxyatractylate can be fitted assuming a decay time of 0.95 ns as free FoDP. In the absence of carboxyatractylate the decay time is increased to 1.95 ns, as can be seen in B.

applied to measure depolarization times. In this case the stability problem is more aggravated since measurements have lasted considerably longer, due to a 90% attenuation of the light intensity by the two polarizators. Therefore, the limit ofaccuracy was nearly reached. Within the attainable accuracy, the fluorescence curves of emission, polarized vertical and parallel to the exciting light, were equal. Depolarization time is therefore either considerably smaller or larger than 2 ns. Only the latter case is possible for a molecule the size of FoDP when it is immobilized by binding.

Discussion

Among the fluorescent analogues of adenine, formycin comes much closer to the structure of adenosine than, for example, the ethano derivatives. Not only the highly specific ADP/ATP carrier of the mitochondria, but also numerous other adenine nucleotide-utilizing enzymes reject the etheno derivatives but accept formycin nucleotides. Early hopes in the literature [3,4] of utilizing the fluorescent properties of formycin in studying the interaction of adenine nucleotides with protein have not been fulfilled, since its fluorescence has several disadvantages, making an evaluation difficult. The absorption overlaps partially and the emission completely with tryptophan. Its fluorescence yield is relatively low and the decay time therefore short (less than 1 ns). The spectral

overlap, in particular, prevents a quantitative evaluation of binding of formycin nucleotides.

These difficulties have in part been overcome by introducing fluorescent probes which accept fluorescence only from bound FoDP and not from the excess of free FoDP. It is not necessary to know the exact localization of the fluorescent probe relative to the bound FoDP for studying the concentration dependence of FoDP binding. Of the two anthracene derivatives, the maleimide form (9-anthracene-maleimide), being an SH reagent, binds more specifically to proteins than the non-covalently absorbed vinylanthracene, which is absorbed mostly in the lipid phase. Therefore, the maleimide derivative has a strong response to FoDP and is more sensitive to removal of FoDP by inhibitors. The dissociation constant measured by this method for FoDP is about one order of magnitude larger than that for ADP, in accordance with observations that the translocation rate with FoDP is about 5 times slower than that with ADP. In contrast, the binding to adenylate kinase is comparably unspecific; FoDP is accepted with the same affinity as ADP. This is in accordance with the reported unspecificity of ADP binding in the absence of Mg^{2+} to this enzyme.

The equivalence of bongkrekate and carboxyatractylate in suppressing the fluorescence of formycin diphosphate is of particular interest if one recalls that bongkrekate apparently increases the binding of ADP to mitochondria opposite to the action of carboxyatractylate [30,31]. Therefore, it was first concluded that under the influence of bongkrekate, ADP becomes tightly fixed to the carrier in a ternary bongkrekate-carrier-ATP complex, resulting in an inhibition of dissociation and of transport. It was then realized in very detailed studies that first this ADP migrates on the carrier to the inner side of the membrane, where it is displaced by bongkrekate from the carrier [31—33]. ADP is then trapped in the matrix because the carrier is blocked by bongkrekate, so that it appears as tightly fixed. The fluorescence results confirm directly that the formycin diphosphate (instead of ADP) is displaced by bongkrekate and, although being trapped, becomes free, similar as with carboxyatractylate. The non-bound nature of the trapped ADP has been previously demonstrated in a different manner by showing that this ADP is freely available to intramitochondrial P_i-transfer reactions [29].

Although the measurement of FoDP binding may be exploited by studying the interaction of the inhibitors bongkrekate and carboxyatractylate with the adenine nucleotide carrier, these studies give no direct insight into the nature of the binding site. This is only possible by those properties of fluorescence that are changed by the chemical and physical surroundings of the binding site, such as decay time or rotational diffusion.

The measurements indicate that the decay time of FoDP is more than doubled by binding. Because of the limited solubility of FoDP in nonpolar solutions, it is impossible to study the relation between the environmental polarity and decay time of FoDP. FoDP is only slightly soluble in ethanol, where we find a decay time of about 2.8 ns. The increased decay time by binding may thus be interpreted as a binding in a nonpolar environment, where FoDP is relatively immobile. This is in accordance with the measurements of the fluorescence depolarization.

Both the immobility and the hydrophobic environment can be reconciled

with a tight binding of the substrate FoDP to a macromolecular carrier, in accordance with the reorientation and gated-pore mechanism derived for the ADP/ATP carrier [34]. It excludes a mere diffusion of FoDP in a water-filled pore. Since the movements of the FoDP-protein complex are in the millisecond time range, the translocation turnover cannot be measured by fluorescence.

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