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ADP AND ATP TRANSPORT IN MITOCHONDRIA

EVIDENCE FOR A METAL-ION INVOLVED IN TRANSPORT CATALYSIS BY USE OF METALLOCHROMIC INDICATORS

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This study introduces a new class of active-site directed probes with respect to ADP and ATP transport catalysis in rat liver mitochondria. The anionic monoazo dyes, e.g., *p*-(2-hydroxy-1-naphthylazo)naphthol-sulfonic acid, are competitive inhibitors of carrier-mediated ADP uptake (K_i 20–30 μM). The azo dyes also can displace the same amount of carrier-specific bound ADP as does carboxyatractyloside. Two essential substructures could be derived from a structure-activity study. Firstly, a sulfonic acid group in the *para* position relative to the azo bridge which becomes neutralized upon binding by a specifically located positive charge of the carrier protein. This electrostatic binding component, which presumably is represented by a strategic arginyl residue, seems to be essential for substrate binding as well as inhibitor binding. The second structural requirement for effective inhibition was found to be the *o*-hydroxy or *o,o'*-dihydroxyazo system, which is known to form stable complexes with metal ions by chelation. Experiments on prevention and reversal of dye-mediated inhibition revealed that the metal-chelating properties are responsible for the effects observed. In addition, using bovine serum albumin or the synthetic polymer Kollidone, inhibition could be prevented as well as abolished. It is postulated that a metal ion, possibly Mg^{2+}, which is bound to the carrier protein plays an essential role for transport catalysis. The metal ion is assumed to form a functional ternary complex, i.e., a metal bridge complex between the carrier protein and its substrate.

Introduction

The carrier-mediated transport of ADP and ATP across the inner mitochondrial membrane represents a key process in the cellular energy supply of aerobic organisms (for review see Refs. 1 and 2).

Our studies with chemically modified adenine nucleotide analogues provided a detailed description of the basic steric, contact and structural elements which are prerequisites for carrier binding and additionally for subsequent transport catalysis. This approach substantiated the remarkable specificity of this transport system and led to a direct insight into the molecular mechanism of ADP and ATP translocation [3–5].

The findings with *syn*-structured 8-bromo-ADP [6], conformationally restricted ADP analogues [7] and substitution-inert Cr(III)- and Co(III)-ADP complexes (Boos and Schlimme, unpublished data) as well as with phosphate-modified analogues (for review see Ref. 3) did not exclude a metal-nucleotide interaction in the course of the translocation process.

From studies on the cation-dependency of the ADP, ATP exchange reaction, on the other hand,

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the concept arose that the free nucleotides rather than their Mg complexes are the substrates for the translocase [8–11].

Recently, our studies on the interaction of anthraquinone- and naphthylazo dyes with membrane bound nucleotide-dependent processes in mitochondria [12,13] led to the discovery of metal-chelating anionic monoazo dyes as active site directed probes with respect to ADP, ATP transport catalysis. Some results of this work have been presented in part as a poster contribution [14]. Besides their usefulness in this study, these dyes are commonly used as metallochromic indicators for complexometric determinations in analytical chemistry [15].

Materials and Methods

o-Hydroxybenzeneazo-β-naphthol (11) was prepared from diazotized *o*-aminophenol and β-naphthol according to [16]. 1-(3-Hydroxy-4-methyl-2-phenylazo)-2-naphthol-4-sulfonic acid (12) was prepared from diazotized 1-amino-2-naphthol-4-sulfonic acid and *o*-cresol according to Ref. 17. Compound 7 (Orange I) was a product of Fluka AG. All other dyes investigated were purchased from EGA Chemie. Carboxyatractyloside was obtained from Boehringer, [^{14}C]ADP from New England Nuclear and Kollidone (polyvinylpyrrolidone K 10) from Serva.

Binding and translocation measurements. Mitochondria were prepared from rat liver (male Wistar rats Bor: WISW, SPF TNO; 150–200 g) according to Ref. 18. Protein was determined by the biuret method. Mitochondria (2.5 mg protein) were incubated at 5°C in a medium of 70 mM sucrose, 210 mM mannitol, 1 mM triethanolamine, 40 μM Ap_5A (P^1,P^5-di(adenosine-5-)pentaphosphate) and oligomycin (4 μg/mg protein), pH 7.2, in a total volume of 290 μl. Separation of mitochondria from the incubation mixture (50 μl aliquots) was done by centrifugation through a silicone oil layer (AR-200, Wacker Chemie) in microvials using a Beckman Microfuge. The radioactivity in the denatured (15% $HClO_4$) sediment was measured with a liquid scintillation counter using Instagel II (Packard). Differentiation between carrier-linked, i.e., inhibitor-sensitive (specific), and noncarrier-linked, i.e., inhibitor-insensitive (unspecific), binding as well as exchange (uptake) with the endogenous adenine nucleotide pool was performed in analogy to Ref. 19.

Results

Effect of azo dyes (1–22) on mitochondrial carrier-mediated [^{14}C]ADP transport

From the data in Table I it is evident that addition of certain azo dyes, i.e., compounds 1, 2, 9, 13 and 19–22, prevent [^{14}C]ADP uptake as well as carrier-specific binding of [^{14}C]ADP (Expt. 2). The remaining amount of bound [^{14}C]ADP corresponds to that obtained by preincubation of mitochondria with the well known specific inhibitor carboxyatractyloside (Expt. 3). Furthermore, these azo dyes also can displace the same amount of carrier-specific bound [^{14}C]ADP as does carboxyatractyloside (Expts. 4, 5). All other dyes investigated revealed neither significant uptake inhibition nor displacement of [^{14}C]ADP.

Kinetic analysis of [^{14}C]ADP transport inhibition

Inhibition studies carried out with two representative azo dyes revealed a competitive type of inhibition of mitochondrial [^{14}C]ADP uptake (cf. Table II). The potency of the inhibitory action is

TABLE I

EFFECT OF AZO DYES ON MITOCHONDRIAL [^{14}C]ADP TRANSPORT

Incubation of mitochondria was done as described in Methods and Materials and terminated (c) by centrifugation through a silicone oil layer. 60 μM [^{14}C]ADP; carboxyatractyloside (CAT) and dyes (1, 2, 9, 13, 19–22) at 500 μM. The differences of Expts. 1 and 5, 1 and 4, respectively yield inhibitor-sensitive (carrier-specific) binding of [^{14}C]ADP, that of Expts. 5 and 3, 4 and 2, respectively carrier-mediated uptake. Inhibitor insensitive (carrier-unspecific) binding is given by Expts. 2 and 3. Data represent mean ± S.E. for four separate determinations; range of confidence is 95%.

Expt.	Sequence of additions	[^{14}C]ADP (nmol/mg protein)
1	ADP–30 s–c	3.52 ± 0.13
2	Dye–30 s–ADP–30 s–c	0.25 ± 0.02
3	CAT–30 s–ADP–30 s–c	0.23 ± 0.02
4	ADP–30 s–Dye –30 s–c	1.66 ± 0.07
5	ADP–30 s–CAT–30 s–c	1.60 ± 0.08

TABLE II

KINETIC ANALYSIS OF [^{14}C]ADP TRANSPORT INHIBITION

Sampling for rate measurements in the presence of dyes was performed within the linear phase of [^{14}C]ADP uptake as in Ref. 4. Values given were derived from a Lineweaver-Burk plot; 13, 40 μM; 19, 40 μM; K_m (ADP) 11 μM, V_{max} 7.35 nmol/min per mg.

Dye	K_i (μM)	$K_{m'}$ (μM)	V_{max} (nmol/min per mg)	Type
13	23	29	7.69	competitive
19	18	34	8.20	competitive

obvious from the K_i values, which have the same order of magnitude as the K_m of ADP. Compounds 1, 2, 9, 20, 21 and 22 competitively inhibit [^{14}C]ADP uptake to the same extent (data not shown).

Structure-activity relationships of the azo dyes investigated

The azo dyes investigated can be subdivided in four classes according to their chemical constitution and are listed in Tables III–VI, respectively. Those azo dyes, which act as inhibitors of mitochondrial [^{14}C]ADP uptake are framed by dotted lines.

Table III. Compounds 1 and 2 are potent inhibitors. Both dyes are hydroxylated in the *ortho* position as well as sulfonated in the *para* position with respect to the azo bridge. Desulfonation of the phenyl moiety (5) or sulfonation of the naphthol component (3, 5) inactivate the dyes. A change of the hydroxyl group from *ortho* to *para* (7) also leads to a loss of the inhibitory property. The food dye (8) is inactive due to a phenol instead of a naphthol substituent.

Table IV. In this series of *o,o'*-dihydroxy azo dyes, only compound 9 is active. Again, a *para*-positioned sulfonic acid group as well a *ortho'*-positioned hydroxyl group are essential for an inhibitory action.

Table V. This class of naphthol-azo-naphthyl compounds conclusively shows that, although the hydroxyl group as well as the sulfonic acid group are in the right position, any additional sulfonation of the naphthol moiety leads to inactivation (food dyes 14, 15, 18).

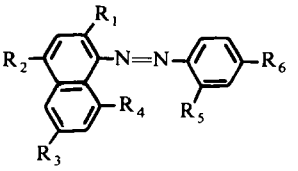
Table VI. Each of these azo dyes possesses a vicinal arrangement of hydroxyl groups as well as a relative to the azo bridge *para*-positioned sulfonic acid group. All of them are potent inhibitors.

Fig. 1 summarizes the inhibitor structures delineated from the structure-activity study. There are two major structural requirements for effective inhibition, namely: a *para*-positioned sulfonate

TABLE III

NAPHTHOLAZOPHENYL DYES

Compound



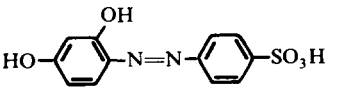
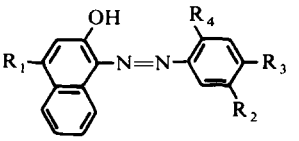
	R_1	R_2	R_3	R_4	R_5	R_6
1	OH	H	H	H	H	SO_3H
2	OH	H	H	H	CH_3	SO_3H
3	OH	H	SO_3H	H	H	H
4	OH	H	SO_3H	SO_3H	H	H
5	OH	H	H	H	H	H
6	OH	H	H	H	H	NO_2
7	H	OH	H	H	H	SO_3H
8						

TABLE IV

NAPHTHOLAZOPHENOL DYES

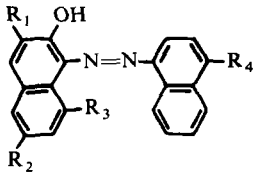
Compound

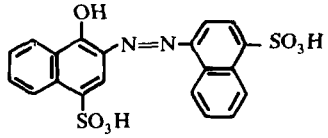


	R_1	R_2	R_3	R_4
9	SO_3H	CH_3	H	OH
10	H	SO_3H	H	OH
11	H	H	H	OH
12	SO_3H	CH_3	OH	H

TABLE V

NAPHTHOLAZONAPHTHYL DYES



	R_1	R_2	R_3	R_4
13	H	H	H	SO_3H
14	H	SO_3H	SO_3H	SO_3H
15	SO_3H	SO_3H	H	SO_3H
16	SO_3H	SO_3H	H	H
17	H	SO_3H	SO_3H	H
18				

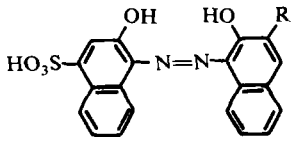
group and at least one *ortho*-positioned hydroxyl group relative to the azo bridge.

Evaluation of the mode of inhibitory action

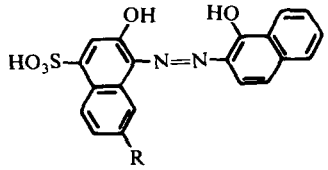
One of the essential substructures derived from the structure-activity study, i.e., the *o*-hydroxy or

TABLE VI

NAPHTHOLAZONAPHTHOL DYES



19	R = H
20	R = COOH



21	R = H
22	$R = NO_2$

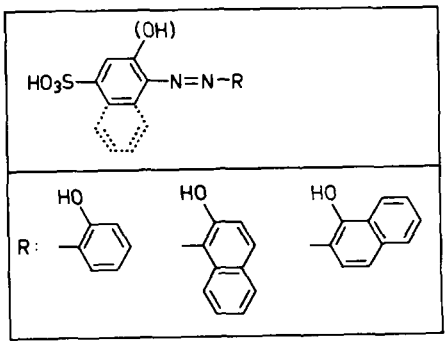


Fig. 1.

o,o'-dihydroxyazo system (cf. Fig. 1), is known to form stable complexes with metal-ions by chelation [20]. In order to find out whether the inhibitory effect of the azo dyes is due to their metal-complexing activity or not, a series of experiments was carried out (Table VII).

The results in Table VII show that: (a) Mg^{2+} protects against dye-mediated inhibition (Expts. 1, 2, 3); (b) EDTA abolishes Mg^{2+}-protection (Expt. 4); (c) preincubation with EDTA has no effect on dye mediated inhibition (Expt. 5, basic experiment C); (d) dye-Mg complexes are inactive (Expt. 6, basic experiment B); (e) EDTA regains inhibition by destroying the dye-Mg complex (Expt. 7); (f) dye-Mg complexes do not displace carrier-bound [^{14}C]ADP (Expts. 8, 9, basic experiment C); (g) preincubation with albumin or Kollidone prevents the inhibition exerted by the dye (Expt. 10, basic experiment B); (h) albumin and Kollidone abolish dye-mediated inhibition (Expt. 11).

Discussion

The data presented conclusively show that some of the investigated monoazo dyes are active-site directed probes, i.e., they interact specifically with the mitochondrial adenine nucleotide carrier protein. This is obvious from their competitive nature of inhibition with respect to carrier-mediated [^{14}C]ADP uptake as well as from their ability to displace carboxyatractyloside-sensitive, i.e., carrier-specific bound, [^{14}C]ADP.

In addition, these dyes have besides their colour a unique experimental advantage when compared to the known inhibitors atractyloside and

TABLE VII

PREVENTION AND ABOLITION OF INHIBITION

Incubation of rat liver mitochondria was done as described in Methods and Materials and terminated (c) at the indicated time by centrifugation through a silicone oil layer. 60 μM [^{14}C]ADP; carboxyatractyloside (CAT), dyes, $MgCl_2$, EDTA, each 500 μM. 1 : 1 dye-Mg; bovine serum albumin, Kollidone, each 1 mg. Data represent mean ± S.E. for four separate determinations; range of confidence is 95%. n.d., not determined.

Expt.	Sequence of additions	[^{14}C]ADP (nmol/mg protein)		[^{14}C]ADP (nmol/mg of protein)
A	ADP——30 s–c	3.52 ± 0.13	A–B = Inhibitor sensitive (carrier-specific) binding	1.92
B	ADP–30 s–CAT–30 s–c	1.60 ± 0.08	B–C = Carrier-mediated uptake	1.37
C	CAT–30 s–ADP–30 s–c	0.23 ± 0.02	C = Inhibitor insensitive (carrier-unspecific) binding	0.23

					Compound: 9	13	19
1	Dye ——	1 min——	ADP——	30 s——c	0.26 ± 0.02	0.22 ± 0.02	0.28 ± 0.02
2	Dye ——1 min–Mg^{2+}——	1 min——	ADP–30 s–	CAT—30 s–c	1.38 ± 0.07	1.20 ± 0.07	1.24 ± 0.06
3	Mg^{2+} ——1 min–dye——	1 min——	ADP–30 s–	CAT—30 s–c	1.34 ± 0.07	1.12 ± 0.06	1.30 ± 0.06
4	Mg^{2+} ——1 min–dye–1 min–	EDTA–1 min–	ADP–30 s–	CAT—30 s–c	0.81 ± 0.06	0.70 ± 0.04	0.95 ± 0.05
5	EDTA——1 min–dye——	1 min——	ADP–30 s–	CAT—30 s–c	0.26 ± 0.02	0.23 ± 0.02	0.37 ± 0.02
6	Dye-Mg——	1 min——	ADP–30 s–	CAT—30 s–c	1.41 ± 0.08	1.32 ± 0.07	1.45 ± 0.08
7	Dye-Mg–1 min–EDTA——	1 min——	ADP–30 s–	CAT—30 s–c	0.55 ± 0.05	0.52 ± 0.05	0.52 ± 0.06
8			ADP–30 s–	dye—30 s–c	1.67 ± 0.08	1.68 ± 0.08	1.71–0.07
9			ADP–30 s–	dye-Mg–30 s–c	3.74 ± 0.10	3.80 ± 0.12	3.78 ± 0.11
10	Albumin;–1 min–dye—— Kollidone	1 min——	ADP–30 s–	CAT—30 s–c	n.d.	n.d.	1.58 ± 0.07
11	Dye——1 min–albumin;– Kollidone	1 min——	ADP–30 s–	CAT—30 s–c	n.d.	n.d.	1.56–0.06

carboxyatractyloside. That is, their inhibitory action can be prevented as well as abolished by albumin or the synthetic polymer Kollidone, whereby the latter commonly is used as a blood plasma substitute.

A systematic study with a variety of monoazo dyes led to a very defined structure-inhibitor activity relationship which can be used to specify the molecular nature of interaction. Two functional determinants could be characterized. Firstly, a sulfonic acid group in the *para* position relative to the azo bridge (cf. Fig. 1). Without an anionic charge in this very position, neither carrier-specific binding nor inhibition takes place. As the relative position of the sulfonate group is important in determining the potency of inhibition, one can assume that the interaction between the anionic dye and the transport system involves a structured electrostatic binding component. In this context, it is interesting to note that the most inhibitory active part of the atractylates is represented by the atractyligenin moiety, which bears one negative charge in the form of a carboxyl group [21,22]. From the anthraquinonoid inhibitors it is known that their minimum structure for effective inhibition also bears a sulfonic acid group [12]. Furthermore, the studies with substrate analogues revealed that one negative charge at the C-5′ position of adenosine is sufficient for cytosolic carrier-specific binding [23]. This is in line with recent findings of Block et al. [24], who detected a strategic arginine residue at the cytosolic substrate binding site.

The second structural requirement for effective inhibition was found to be the *o*-hydroxy- or *o,o′*-dihydroxyazo system (cf. Fig. 1). From the data on prevention and reversal of inhibition (cf. Table VII) as well as from the structure-activity study (see Results) it is evident that the chelating properties, i.e., the metallizable substituents of these azo dyes, are responsible for the observed effects. The fact that EDTA does not act as an inhibitor of ADP uptake primarily should be due to steric hindrance of its hexadentate complex formation. However, bidentate aminopolycarboxylic acids, e.g., iminodiacetic acid, revealed no inhibition, either (data not shown). Thus, these findings as well as the presented data strongly suggest that dye-mediated inhibition is based on a concerted event involving both the neutralization of a specifically located positive charge and the formation of a bi- or tridentate dye-metal complex (cf. Fig. 2).

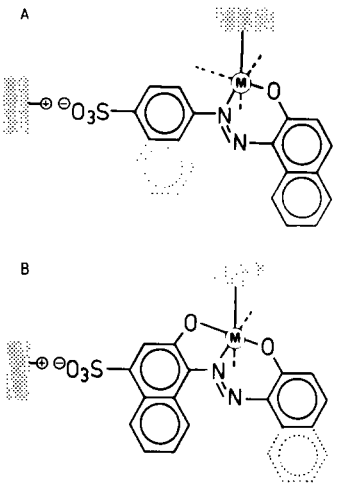


Fig. 2. Schematic representation of the dye-metal-carrier protein interactions. (A) Bidentate complex with inhibitor structures of *o*-hydroxyazo dyes; (B) tridentate complex with inhibitor structures of *o,o′*-dihydroxyazo dyes. The metal ion is shown in an octahedral configuration. Dotted areas represent carrier protein regions.

Furthermore, it is reasonable to assume that these specific inhibitor-protein interactions reflect those concerning the substrate, as it is known that ADP forms α,β bidentate metal complexes through a six-membered chelate ring whereby one negative charge remains at the terminal phosphate. It is therefore postulated that a metal ion, possibly Mg^{2+}, which is covalently bound at the carrier protein, plays an essential role in transport catalysis. This metal ion is assumed to form a functional ternary complex, i.e., a metal bridge complex between the carrier protein and its substrate. In this context, it should be mentioned that for substrate binding the free forms of ADP and ATP have to be present [25]. Further support for a functional ternary complex arises from the findings (Boos and Schlimme, unpublished data): (a) stable Cr(III)- and Co(III)-[^{14}C]ADP complexes, which can not exchange ligands, and therefore cannot form a metal bridge complex, are not transported, and (b) coupling of these azo dyes to an adenosine moiety via the C-5′ position yields potent bifunctional inhibitors. Based primarily on these as well

as on the findings with substrate analogs we proposed a new model for mitochondrial adenine nucleotide translocation [26].

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