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Mitochondrial Adenine Nucleotide Carrier. Investigation of Principal Structural, Steric, and Contact Requirements for Substrate Binding and Transport by Means of Ribose-Modified Substrate Analogues†

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ABSTRACT: A selected series of fourteen ribose-modified adenine nucleotide analogues was prepared and characterized as their α-^{32}P- or U-^{14}C-labeled compounds. The capacity of rat liver mitochondria for adenine nucleotide carrier-linked (specific) binding and carrier-mediated transfer across the inner mitochondrial membrane as well as the amount of noncarrier-linked (unspecific) binding of these analogues was determined at 5 °C by means of an inhibitor (atractyloside) stop method and compared with the natural substrates ADP and ATP. Kinetic data of carrier-specific bound analogues were evaluated from Dixon plots and indicate these analogues as competitive inhibitors for mitochondrial [U-^{14}C]AD(T)P uptake. The findings confirm the distinct substrate specificity of the carrier system. By means of the analogues, an experimental proof of the two-step nature of mitochondrial adenine nucleotide translocation, i.e., carrier-specific binding (recognition) and transport, was obtained. Furthermore, the findings provide a detailed description of the basic steric, contact, and structural elements which are prerequisite for carrier-specific binding (A) and additionally for subsequent transport (B): (A) (1) an anti- or syn-positioned β-*N*-glycosyl-linked purine base; (2) a S- or N-type sugar pucker; (3) a cis disposition of the C(4′)–C(5′) bond with respect to the heterocycle; (B) (1) a nonfixed anti-positioned purine base with a *N*-glycosyl torsion angle of approximately −20°; (2) a S-type sugar pucker; (3) a gauche–gauche orientation of the exocyclic C(5′)–O(5′) group; (4) a trans-positioned, i.e., C(2′) ribo hydroxyl, group, which presumably "triggers" the induction of carrier-mediated transport.

The transfer of ADP and ATP across the inner mitochondrial membrane represents a key process of energy supply in aerobic eucaryotic cells. This transfer is catalyzed by a membrane integral lipoprotein, i.e., the ANP[1] carrier. This protein recently has been isolated from mammalian (Brandolin et al., 1974; Riccio et al., 1975; Shertzer & Racker, 1976; Bojanovski et al., 1976) and yeast mitochondria (Boulay et al., 1979) as well as partially characterized and reconstituted.

However, an understanding in which way substrate and transporter protein interact at the molecular level is still pending. One approach is the use of substrate analogues, which proved to be of exceptional utility in the investigation of ligand–receptor interactions, especially in the field of enzyme-catalyzed reactions (Yount, 1975).

Translocation experiments carried out with a selected series of substrate analogues should provide a detailed description of chemical and structural features, which are of potential importance to mitochondrial transport. Moreover, it should be possible to prove experimentally the two-step nature of the process by means of analogues, which are bound to the carrier but inappropriate for transportation.

We decided to investigate ribose-modified adenine nucleotide analogues, as slight modifications at the ribose moiety, which represents the central molecule part, affect the overall nucleotide structure in a decisive manner.

Recently, on the basis of our findings (Boos et al., 1976a,b) on the binding characteristics, photoaffinity labeling of the carrier–substrate binding region with substrate analogues was achieved (Schäfer et al., 1977).

Experimental Procedures

Materials. [U-^{14}C]ATP (196 mCi/mmol), [U-^{14}C]2′dATP (**2d**) (450 mCi/mmol), and [^{32}P]phosphorus oxychloride (1–50 mCi/mmol) were purchased from Amersham, England. Yeast hexokinase (EC 2.7.1.1), orthophosphoric monoester phosphohydrolase (EC 3.1.3.1), and atractyloside were from Boehringer, Germany; 5′-ribonucleotide phosphohydrolase (EC 3.1.3.5) and 3′dAdo (**3a**) were from Sigma Chemicals, Ger-

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[1] Abbreviations used: ANP, adenine nucleotide (ADP, ATP); 2′dAdo, 9-(β-D-2′-deoxyribofuranosyl)adenine (**2a**); 3′dAdo, 9-(β-D-3′-deoxyribofuranosyl)adenine (**3a**); ara-Ado, 9-(β-D-arabinofuranosyl)adenine (**4a**); xylo-Ado, 9-(β-D-xylofuranosyl)adenine (**5a**); rox-Ado, ribose-oxidized adenosine, 2,2′-[1′-(9-adenyl)-1′-(hydroxymethyl)]dioxodiethyl ether (**6a**); rro-Ado, ribose-ring-opened adenosine, 2,2′-[1′-(9-adenyl)-1′-(hydroxymethyl)]dihydroxydiethyl ether (**7a**); 2′,3′-methoxy-Ado, 2′,3′-*O*-methoxymethylidene-9-(β-D-ribofuranosyl)adenine (**8a**); 2′,3′-isoprop-Ado, 2′,3′-isopropylidene-9-(β-D-ribofuranosyl)adenine (**9a**); 2′,3′-ddAdo, 9-(β-D-2′,3′-dideoxyribofuranosyl)adenine (**10a**); 3′-*O*-methyl-Ado, 3′-*O*-methyl-9-(β-D-ribofuranosyl)adenine (**11a**); lyxo-Ado, 9-(α-L-lyxofuranosyl)adenine (**12a**); αAdo, 9-(α-D-ribofuranosyl)adenine (**13a**); 8,2′-*O*-cyclo-Ado, 8,2′-anhydro-8-oxy-9-(β-D-arabinofuranosyl)adenine (**14a**); 8,3′-*O*-cyclo-Ado, 8,3′-anhydro-8-oxy-9-(β-D-xylofuranosyl)-adenine (**15a**); $(Et)_3NH^+HCO_3^-$, triethylammonium bicarbonate.

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many; αAdo (**13a**) was from Calbiochem, Germany; 2′,3′ddATP (**10d**) was from P-L Biochemicals, Inc., Milwaukee, WI. Generous gifts were as follows: xylo-Ado (**5a**) from Dr. R. K. Robins, ICN, Irvine, CA; 3′-*O*-methyl-Ado (**11a**) from Dr. J. W. Daly, NIH, Bethesda, MD; lyxo-Ado (**12a**) from Dr. R. Sanchez, Calbiochem, San Diego, CA; 8,2′-*O*-cyclo-Ado (**14a**) and 8,3′-*O*-cyclo-Ado (**15a**) from Dr. M. Ikehara, University of Osaka, Japan.

Chemical Syntheses. The nucleoside analogues (**3a**, **4a**, **5a**, **10a**, **11a**, **12a**, **13a**, **14a**, and **15a**) were chemically phosphorylated with [^{32}P]phosphorus oxychloride according to Sowa & Ouchi (1975). Separation of the reaction mixture was achieved on a DEAE-52-cellulose column (3 × 80 cm) using a linear gradient of 0–0.3 M $(Et)_3NH^+HCO_3^-$, pH 7.5, 4 °C, and 2.4-L total volume. The α-^{32}P-labeled nucleoside triphosphates were prepared starting with 20 μmol of the corresponding [^{32}P]-5′-monophosphate according to Hoard & Ott (1965) with the following modifications (Boos et al., 1978a): the tri-*n*-octylammonium salts of nucleoside 5′-monophosphate as well as pyrophosphate were used instead of the less soluble pyridinium salts. During activation with 1,1′-carbonyldiimidazole, the reaction mixture was stored under vacuum (over P_2O_5/NaOH), thus leading to higher yields. Chromatographic separation of the products was achieved on a DEAE-52-cellulose column (3 × 80 cm) using a linear gradient of 0.2–0.35 M $(Et)_3NH^+HCO_3^-$, pH 7.5, 4 °C, and 2.0-L total volume.

[U-^{14}C]rox-ATP (**6d**) and [U-^{14}C]rro-ATP (**7d**) were synthesized as published (Boos et al., 1975). [U-^{14}C]-2′,3′-Methoxy-ATP (**8d**) and 2′,3′-isoprop-ATP (**9d**) were prepared by a modified procedure according to Chládek (1968). ATP (**1d**; 67 μmol) was dissolved in trimethyl orthoformate (2 mL), dimethyl sulfoxide (4 mL), and dioxane (0.4 mL). Concentrated H_2SO_4 (20 μL) was added and the reaction mixture was stirred for 48 h at 24 °C. **9d** was synthesized as outlined for **8d**; however, only 0.4 mL of trimethyl orthoformate and 2.55 mL of acetone were used additionally. Chromatographic separation on DEAE-52-cellulose was achieved as described above after neutralization with pyridrine.

The sodium salts of the nucleotide analogues were obtained by passing through a cation-exchange column (Dowex 50 WX4, Na^+ form). Specific activities centered around 0.5 μCi/μmol of nucleotide. Purity of the investigated nucleotide analogues was controlled by TLC (thin-layer chromatography) and LC (high-pressure liquid chromatography) according to Stahl et al. (1973). Total phosphate analysis was done according to Zilversmit & Davies (1950). Radioactivity was measured with a liquid scintillation counter (Packard Tricarb 544) using Aquasol (New England Nuclear). Absorbance scans were recorded on a Shimadzu UV-200 spectrophotometer.

Enzyme Assays. [α-^{32}P]- or [U-^{14}C]monophosphates were digested with 5′-ribonucleotide phosphohydrolase (EC 3.1.3.5). Incubation assay was as follows: 50 μL of diethylbarbituric acid (40 mM), pH 8.0, 0.1 unit of 5′-ribonucleotide phosphohydrolase, 12 h at 37 °C, and 50 nmol of nucleotide. Enzymatic degradation of nucleoside 5′-triphosphates with yeast hexokinase (EC 2.7.1.1) (Boos et al., 1976c) yielded the [α-^{32}P]- or [U-^{14}C]nucleoside 5′-diphosphates. Treatment with alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) yielded the nucleosides. Incubation assay: 0.1 unit of alkaline phosphatase, 50 μL of 0.1 M Tris buffer, pH 8.0, 20 μL of 0.05 M $MgCl_2$, and 50 nmol of nucleotide, 4 h at 37 °C.

Translocation Measurements (Inhibitor Stop Method). Mitochondria were prepared from rat liver (male Wistar rats; 150–200 g weight) following published procedures (Hagihara, 1960). Protein was determined by the biuret method. Mitochondrial adenine nucleotide translocation studies were carried out at 5 °C according to Pfaff & Klingenberg (1968). Differentiation between carrier-linked, i.e., atractyloside-sensitive (specific), and noncarrier-linked, i.e., atractyloside-insensitive (unspecific), binding as well as between binding and exchange with the endogenous adenine nucleotide pool was performed as described (Boos et al., 1975) by using 40 μg of atractyloside per mg of mitochondrial protein. Mitochondria (2.5 mg of protein) were incubated in a medium containing 70 mM sucrose, 210 mM mannitol, and 1 mM triethanolamine, pH 7.2, in a total volume of 250 μL at 5 °C; for further details in assaying see Boos et al. (1975). Separation of mitochondria from the incubation mixture was done by centrifugation through a silicone oil layer (AR-100; Wacker Chemie, München, Germany) in microvials using a Beckman Microfuge. In inhibition experiments, sampling for rate measurements was performed within the linear phase (4–15 s) of [^{14}C]adenine nucleotide uptake according to Boos et al. (1975) at 5 °C.

Results

Syntheses. The described synthetic procedure of the chemical phosphorylation of a variety of nucleoside analogues leads to satisfactory yields as well as specific activities. Futhermore, utilizing a modified procedure, cyclization of the cis-positioned C(2′)- and C(3′)-hydroxyl groups of the ribose moiety yielded in a one-step reaction two valuable analogues (**8d** and **9d**). The homogeneity of the products was substantiated by thin-layer and high-pressure liquid chromatography (Table I).

Mitochondrial Binding and Exchange of Ribose-Modified Adenine Nucleotides. The capacity of rat liver mitochondria for carrier-linked (specific) binding and carrier-mediated exchange across the inner mitochondrial membrane as well as the amount of noncarrier-linked (unspecific) binding of adenine nucleotide analogues was determined by means of the atractyloside-differentiation technique.

Figure 1 shows the concentration dependency of binding properties of the natural substrates under equilibrium conditions at 5 °C. Saturation of the carrier-specific binding sites is achieved at an external [U-^{14}C]ANP concentration of 250 μM with 1.6 ± 0.2 nmol/mg of mitochondrial protein.

The experimental evidence (see Table II) indicates that isomerization of the cis diol groups at the C(2′) and (C3′) position of the natural substrate into the corresponding trans compounds (ara-ANP, **4c** and **4d**; xylo-ANP, **5c** and **5d**) results in drastically decreased specific binding and total loss of carrier-mediated transmembrane exchange. Oxidation and subsequent reduction of the cis diol system (rox-ANP, **6c** and **6d**; rro-ANP, **7c** and **7d**) gives rise to analogues which show only weak specific binding (**7c** and **7d**) and no transport activity. Loss of both vicinal hydroxyl groups as in 2′,3′ddANP (**10c** and **10d**) leads to increased unspecific binding and no transport. Substitution of the C(2′)- or C(3′)-hydroxyl group by a hydrogen atom (2′dANP, **2c** and **2d**; 3′dANP, **3c** and **3d**) does not affect carrier-specific binding very strongly.

The 3′-deoxy derivative is so far the only ribose-modified analogue able to substitute for ANP as a substrate in transmembrane exchange. This is further substantiated by the fact that 3′dADP (**3c**) was shown to act as a substrate in oxidative phosphorylation of rat liver mitochondria (Boos et al., 1976a,b).

Methylation of the C(3′)-hydroxyl group (**11c** and **11d**) results in weak carrier-specific binding and complete inactivity in transmembrane exchange. Chemical blocking of both vi-

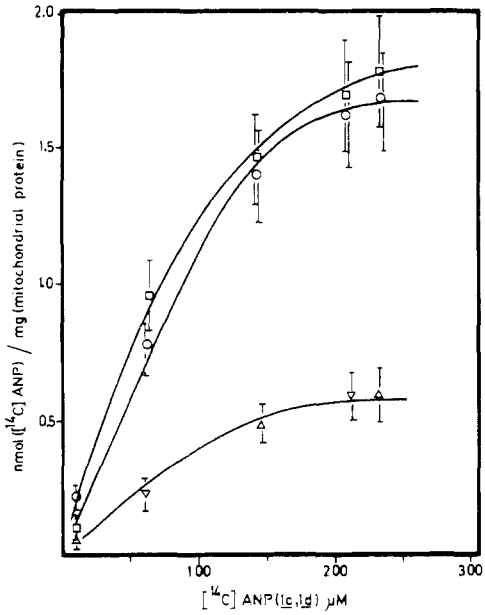


FIGURE 1: Binding properties of [U-^{14}C]ANP (**1c** and **1d**) (initial addition of the triphosphate; see also Table II, footnote *a*) to rat liver mitochondria. (○) Carrier-specific binding; (□) carrier-mediated transmembrane exchange; (▽) carrier-unspecific binding. Equilibrium conditions at 5 °C. Vertical bars indicate the deviation from the average with a range of confidence of 95%.

cinal ribose hydroxyl groups as in 2′,3′-methoxy-ANP (**8c** and **8d**) and 2′,3′-isoprop-ANP (**9c** and **9d**) results in a total loss of carrier-specific binding. Unspecific binding is increased due to the introduction of apolar methyl groups. Linkage of the cis-positioned hydroxyl groups with the C(8) position of the purine moiety via an oxygen bridge as in 8,2′-*O*-cyclo-ANP (**14c** and **14d**) and 8,3′-*O*-cyclo-ANP (**15c** and **15d**) brings about the loss of exchangeability. Inversion of the ribose configuration at the C(4′) or C(1′) position (**12c** and **12d**; **13c** and **13d**) gives rise to substrate analogues which show only weak specific binding (**12c** and **12d**) and no transport activity.

Effect of Substrate Analogues on the Uptake of [U-^{14}C]-ANP by Rat Liver Mitochondria. By use of the outlined inhibitor stop method, the mitochondrial [U-^{14}C]ANP uptake was measured by manual handling in forward exchange experiments at 5 °C and found to be linear within the first 4–15 s (data not shown). Recently published data (Nohl & Klingenberg, 1978) support this kinetic pattern. During the initial phase of the translocation process, unspecific binding sites are saturated proportional to the external [U-^{14}C]ANP concentration. Sampling for rate measurements in the presence of substrate analogues was performed within the first 4–15 s of [U-^{14}C]ANP uptake. These studies were carried out with substrate analogues which were found to bind specifically to the adenine nucleotide carrier system, i.e., **2c** and **2d**; **4c** and **4d**; **7c** and **7d**; **14c** and **14d**, or those showing an unusual high unspecific binding, i.e., **8c** and **8d**; **9c** and **9d**. K_I and V_{max} (Table III) were derived from Dixon plots (Figure 2) and controlled arithmetically by using the Lineweaver–Burk linearization with a range of confidence of 95% in the regression analysis according to Stahl et al. (1974).

All the analogues studied in inhibition experiments turn out to inhibit the [U-^{14}C]ANP uptake competitively. The K_I values have about the same order of magnitude as K_m of ANP = 41 (29–83) μM (Boos et al., 1975). The competitive inhibitory nature is furthermore substantiated by the fact that V_{max} of [U-^{14}C]ANP uptake with 7.0 (5.7–9.1) nmol min^{-1} (mg of protein)$^{-1}$ (Boos et al., 1975) is not affected significantly under the presence of substrate analogues.

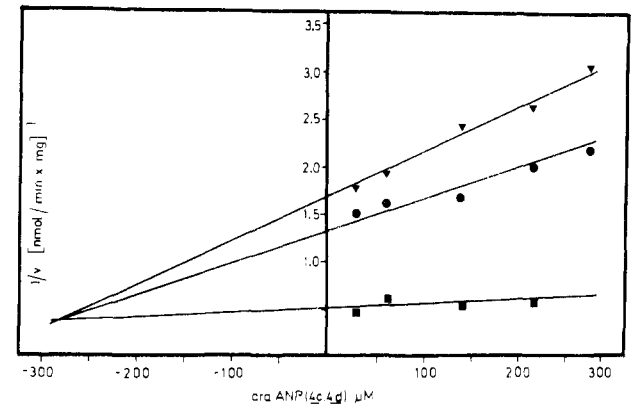


FIGURE 2: Effect of ara-ANP (**4c** and **4d**) on the [U-^{14}C]ANP (initial addition of the corresponding triphosphates; see also Table II, footnote *a*) uptake by rat liver mitochondria; Dixon plot. (▼) [U-^{14}C]ANP, 6.04 μM, r = 0.998; (●) [U-^{14}C]ANP, 23.07 μM, r = 0.972; (■) [U-^{14}C]ANP, 48.15 μM, r = 0.969. K_I = 282 (232–350) μM; V_{max} = 2.63 (1.16–3.40) nmol min^{-1} (mg of protein)$^{-1}$ at 5 °C. r = correlation coefficient. Numbers in parentheses are the range of confidence (95%) for graphic determination (Stahl et al., 1974).

It is thus evident that the analogues investigated compete for the same carrier-specific binding site as the natural substrates during the initial phase of mitochondrial [U-^{14}C]ANP uptake. Though the analogues **8c** and **8d** exhibit no carrier-specific binding in translocation experiments (cf. Table II), they act in an apparent "competitive" manner with respect to [U-^{14}C]ANP uptake. This may be due to the highly lipophilic character of these analogues leading to incorporation into the outer surface of the inner mitochondrial membrane, thus affecting the [U-^{14}C]ANP uptake. An analogous effect was shown for the anesthetic butacaine (Fayle et al., 1975).

Discussion

The investigated mitochondrial carrier system is highly specific for ATP and ADP. None of the naturally occurring ribo- or deoxyribonucleoside 5′-*O*-tri(di)phosphates of guanine, hypoxanthine, uracil, thymine, and cytosine are substrates for mitochondrial nucleotide transport (Schlimme et al., 1977).

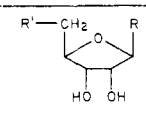
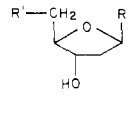
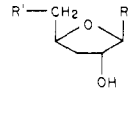
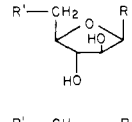
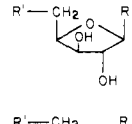
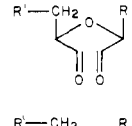
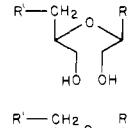
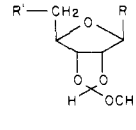
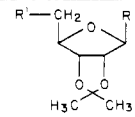
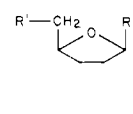
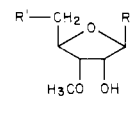
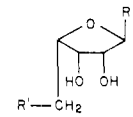
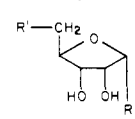
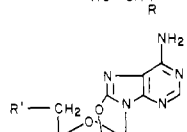
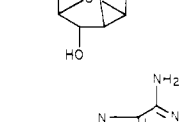
This study with a series of ribose-modified analogues confirms the substrate specificity of the mitochondrial adenine nucleotide carrier system and provides a detailed description of the chemical and structural properties which are essential for carrier-specific binding and transmembrane exchange.

Prerequisite for carrier-specific binding and carrier-mediated transport of adenine nucleotides is a definitive substituent configuration at the ribofuranosyl ring system. Isomerization, cyclization, or substitution by hydrogen of the C(2′) ribo hydroxyl group still allows specific binding but excludes transfer. Moreover, data presented indicate a decisive functional importance of the C(2′) ribo hydroxyl group with respect to transmembrane exchange. Further prerequisites for transport catalysis are, besides an intact furanosyl ring system, a β-*N*-glycosyl-linked purine base as well as a cis-positioned C(4′)–C(5′) group. The C(3′)-hydroxyl group has to be trans positioned and can be replaced by hydrogen (cf. Figure 3).

Besides these findings of the influence and importance of the substituent configuration at the ribose moiety on the interaction with the transport system, the data presented allow a detailed description of the basic steric factors involved in substrate binding and transport.

On the basis of NMR, ORD, and CD studies (Sundaralingam, 1969, 1975; Ts'o, 1974; Davies, 1978), adenine nucleotides can be considered in terms of three structural features: (a) pseudorotation (puckering) of the ribose with an equilibrium between N-type [C(3′)endo, C(2′)exo] and S-type [C-

Table 1: Chromatographic Properties of Investigated Analogues **1-15** (a, R' = OH; b, R' = HPO_4^-; c, R' = $HP_2O_7^{2-}$; d, R' = $HP_3O_{10}^{3-}$; R = Adenine)

compd	no.	R_f values[a] A	B	C	R_t values (min)[b] D	E	F
	**1a**	0.18	0.49		22.0		
	1b			0.52		2.6	13.2
	1c			0.35		5.7	28.0
	1d			0.16		34.0	96.4
	**2a**	0.30					
	2b			0.48			
	2c			0.31		*c*	
	2d			0.17			
	**3a**	0.31					
	3b			0.50			
	3c			0.33		*c*	
	3d			0.17			
	**4a**	0.15					
	4b			0.47			12.4
	4c			0.33			29.2
	4d			0.13			52.4
	**5a**	0.28					
	5b			0.46			18.8
	5c			0.30			47.6
	5d			0.15			70.8
	**6a**		0.64				
	6b					2.6	
	6c			*d*		5.2	
	6d					29.2	
	**7a**		0.57				
	7b						
	7c			*d*			
	7d						
	**8a**	0.36			117.0		
	8b						
	8c			0.48			
	8d			0.39			
	**9a**	0.36			42.0		
	9b						
	9c			0.48			
	9d			0.39			
	**10a**	0.28					
	10b			0.50			
	10c			0.38			
	10d			0.18			
	**11a**	0.42					
	11b			0.56			
	11c			0.44			
	11d			0.22			
	**12a**	0.16					17.6
	12b			0.46			46.4
	12c			0.31			89.6
	12d			0.13			
	**13a**		0.54				
	13b			0.58			
	13c			0.42			
	13d			0.19			
	**14a**	0.22					
	14b			0.47		7.4	
	14c			0.29		15.4	
	14d			0.11		39.2	
	**15a**	0.18					
	15b			0.33			15.2
	15c			0.16			31.6
	15d			0.04			79.6

[a] TLC systems: (A) 1 M ammonium acetate–ethanol (1:8 v/v)-cellulose plates (G1440/LS 254; Schleicher & Schüll, Dassel, Germany); (B) as above except cellulose plates from Merck, Darmstadt, Germany; (C) 0.75 M KH_2PO_4, pH 4.1, and PEI-cellulose F 1440 (Schleicher & Schüll, Dassel, Germany). [b] LC systems: (D) column, 300 × 5 mm; flux rate, 8 mL/h; 25 bar; 24 °C; stationary phase, Nucleosil 5 SA (Macherey & Nagel, Düren, Germany); mobile phase, 0.4 M ammonium formate, pH 5.0; detection system, Zeiss PMQ IV, 258 nm; (E) column, 300 × 5 mm; flux rate, 21 mL/h; 67 bar; 24 °C; stationary phase, Jonex SB (Macherey & Nagel, Düren, Germany); mobile phase, 0.1 M $LiNO_3$ and 0.02 M KH_2PO_4, pH 2.6; (F) column, 300 × 5 mm; flux rate, 11 mL/h; 85 bar; 24 °C; stationary phase, Nucleosil 5 SB (Macherey & Nagel, Düren, Germany); mobile phase, 0.3 M $LiNO_3$ and 0.02 M KH_2PO_4, pH 2.7. [c] Boos et al. (1976c). [d] Boos et al. (1975).

(2′)endo, C(3′)exo] conformers; (b) exocyclic group C(5′)–O-(5′) orientation with three possible rotamers (gauche–gauche; gauche–trans; trans–gauche); (c) syn–anti position of the planar purine base to the quasi-planar ribose moiety described by the torsion angle ϕ_{CN} [projections O(1′)–(C1′) and N-(9)–C(8)] which is defined to −30 ± 45° for an anti conformation and to 150 ± 45° for a syn conformation. These three major structural parameters describing the overall nucleotide conformation were shown to be in solution for ANP predominantly anti (ϕ_{CN} = −20°), C(2′)endo–C(3′)exo, and gauche–gauche (Haschemeyer & Rich, 1967; Davies & Danyluk, 1974; Lee et al., 1975).

The results show that restriction of the pseudorotation by cyclization of the vicinal hydroxyl groups as in 2′,3′-methoxy-ANP (**8c** and **8d**), 2′,3′-isoprop-ANP (**9c** and **9d**), 8,2′-*O*-cyclo-ANP (**14c** and **14d**), and 8,3′-cyclo-ANP (**15c** and **15d**) as well as abolition of the ribofuranose pucker (rox-ANP, **6c** and **6d**; rro-ANP, **7c** and **7d**) prevents the transmembrane exchange. The findings with ara-ANP (**4c** and **4d**), which exhibits an anti, C(2′)exo–C(3′)endo, gauche–gauche conformation (Remin et al., 1976), and xylo-ANP (**5c** and **5d**), which prefers an anti, C(2′)exo–C(3′)endo, gauche–trans conformation (Remin et al., 1976), as well as 3′dANP (**3c** and **3d**), which exists predominantly in an anti, C(2′)endo–C(3′)exo conformation with equally populated rotamers (Davies & Danyluk, 1974), point out the importance of the exocyclic group orientation and the pseudorotation for carrier-mediated transport. The data suggest that a high population of a S-type ribose conformation and gauche–gauche rotamers are necessary for transmembrane exchange.

Introduction of a covalent linkage via a bridging oxygen atom between C(2′) of the sugar moiety and C(8) of the purine ring fixes the adenine base in 8,2′-*O*-cyclo-ANP (**14c** and **14d**) in the syn–anti boundary region or "high anti" conformation with ϕ_{CN} = −122° (Ikehara et al., 1974; Boos et al., 1978b). In 8,3′-*O*-cyclo-ANP (**15c** and **15d**) the heterocycle is centered by the anhydro linkage in an anti position with ϕ_{CN} = −72° (Ikehara et al., 1972; Boos et al., 1978b). From the data presented, it is evident that a covalent fixation of the nucleobase in an anti position impedes substrate binding, whereas exclusion of a covalently fixed anti positioning of the adenine moiety still allows carrier-specific binding. Transmembrane

Table II: Binding Properties of Ribose-Modified U-^{14}C- or α-^{32}P-Labeled Nucleotide Analogues to the Mitochondrial Adenine Nucleotide Carrier System

	properties [nmol/(mg of mitochondrial protein)][a]		
compd	spec binding	exchange	unspecific binding
2c, 2d	0.52 ± 0.06[b]	-[c]	0.71 ± 0.09
3c, 3d	1.01 ± 0.03	1.01 ± 0.13	1.90 ± 0.23
4c, 4d	0.20 ± 0.02	-	1.26 ± 0.15
5c, 5d	0.15 ± 0.02	-	0.81 ± 0.10
6c, 6d	-	-	1.42 ± 0.16
7c, 7d	0.21 ± 0.03	-	1.58 ± 0.19
8c, 8d	-	-	6.50 ± 0.82
9c, 9d	-	-	6.50 ± 0.82
10c, 10d	0.62 ± 0.06	-	5.17 ± 0.65
11c, 11d	0.11 ± 0.01	-	2.81 ± 0.35
12c, 12d	-	-	1.80 ± 0.22
13c, 13d	-	-	1.42 ± 0.21
14c, 14d	0.36 ± 0.04	-	3.42 ± 0.42
15c, 15d	-	-	1.78 ± 0.21

[a] The extramitochondrial nucleotide analogue concentration was varied in a range up to 250 μM the same as for the natural substrates **1c** and **1d** in Figure 1. The values reported were taken at 250 μM external nucleotide analogue concentration, i.e., at saturation conditions for **1c** and **1d** at 5 °C. Due to the fact that during the incubation phosphoryl transfer reactions take place, even in the presence of oligomycin, all experimental findings are expressed as ANP instead of the appropriate nucleoside triphosphates added at the beginning of the translocation experiments. [b] Values reported are taken from four independent experiments; range of confidence is 95%. [c] Dashes indicate no significant binding or exchange within the range of confidence of 95%.

Table III: Inhibition Constants of Substrate Analogues and Maximum Velocities for Mitochondrial [U-^{14}C]ANP Uptake in the Presence of Substrate Analogues

compd	K_I[a] (μM)	V_{max}[a] [nmol min^{-1} (mg of protein)$^{-1}$]
2c, 2d	134 (73-171)[b]	5.00 (3.29-7.58)[b]
7c, 7d	90 (45-128)	7.10 (4.40-14.20)
8c, 8d	43 (11-86)	4.17 (2.38-9.09)
14c, 14d	227 (185-291)	5.00 (3.15-7.52)

[a] Inhibition constants (K_I) and maximum velocities (V_{max}) were determined as shown in Figure 2 for ara-ANP (**4c** and **4d**) at 5 °C. [b] Range of confidence (95%) for graphic determination (Stahl et al., 1974).

exchange, however, primarily depends on a nonfixed nucleobase within the narrow confines of the anti region with ϕ_{CN} approximately -20°. This is furthermore substantiated by the fact that the syn-structured ANP analogues 8-bromo-ANP (Schlimme & Stahl, 1974) with ϕ_{CN} = +120° (Rao & Sundaralingam, 1970) and 8-azido-ANP (Schäfer et al., 1976) are specifically bound but not transported. The inhibition constant of 8-bromo-ANP, K_I = 44 (28-89) μM (Schlimme & Stahl, 1974), and the presented data show that conformational changes about the *N*-glycosyl bond are not accompanied by a significant change in K_I. Anti- or syn-structured analogues thus have no detectable effect on the competition of carrier-specific binding.

From CPK-model studies it follows that the N(1)–C(6) ($-NH_2$) amidine system of anti-structured nucleotides overlaps with the N(7)–C(5)–C(6) ($-NH_2$) system of syn-type analogues. N(7) thus might substitute for N(1) in carrier-specific binding (Schlimme et al., 1979). As described above, for ANP transport catalysis the purine base has to approximate to the degree of anti conformation possessed by the natural substrate. The energy needed for such a syn–anti interconversion is in the range of 26 kJ/mol at room temperature (Rhodes & Schimmel, 1971). Due to the small energy barrier, it is not excluded that the transport protein can select and bind specifically a minor constituent of the conformers in solution, e.g., anti of preferentially syn-structured 8-BrANP. However, binding of 8-BrANP in an anti conformation excludes due to electrostatic repulsion a gauche–gauche orientation of the exocyclic group, which was shown to be a further prerequisite for transport catalysis.

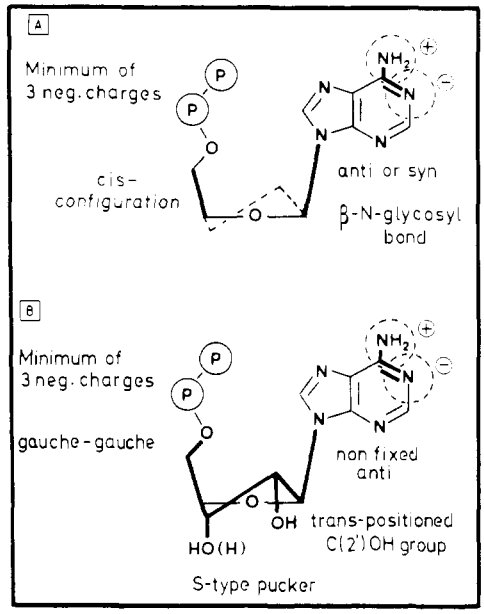


FIGURE 3: Schematic representation of basic steric, contact, and structural elements of adenine nucleotides prerequisite for carrier-specific binding (A) and transport (B). Deficiency (+) and excess (−) of π-electron charge distribution.

On the basis of the available information on the conformation of ribose-modified adenine nucleotides and their behavior in mitochondrial carrier-mediated transport, an attempt is made to indicate schematically the basic steric, contact, and structural elements prerequisite for carrier-specific binding (Figure 3A) and for subsequent transport (Figure 3B).

All three major structural parameters, i.e., exocyclic group orientation, ribose pucker, and syn–anti conformation, make preponderant contributions to carrier-specific binding and transmembrane exchange, whereby the latter one is "triggered" by a trans-positioned C(2′)-hydroxyl group, presumably via hydrogen-bonding interactions.

Modifications of the phosphate chain have only moderate influence on carrier-specific binding and exchange with adenine nucleotides of the endogenous pool, provided that the number of negative charges along the phosphate moiety is unchanged (Schlimme et al., 1973, 1977). Structural modifications of the adenine base are tolerated by the carrier with respect to binding and exchange as long as an "adenine-like" π-electron charge distribution of the N(1)–C(6) ($-NH_2$) amidine system (Figure 3) is retained (Schlimme et al., 1977, 1979).

The discussed "trigger" function of the hydrogen-bonding interaction fits into the concept of a "gated pore" mechanism as the mode of mitochondrial ANP transport. The entering substrate probably "triggers" a conformational change of the carrier protein from a strained to a relaxed state. The energetization of the latter one could be achieved by the desolvation energy liberated during the binding process of the substrate and additionally by the energy (approximately 8 kJ/mol) made available by hydrogen bridge formation. These considerations are based on the fact that substrate analogues still bearing the functional C(2′) ribo hydroxyl group, e.g., rro-ANP (**7c** and **7d**), are not transported, i.e., fail to restrain the conformation of the protein due to an approximately 10

kJ/mol lower binding energy compared to the natural substrate as calculated from K_I values and K_D for ADP.

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