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KINETIC STUDIES ON INTERACTION OF (Na,K)-ATPase WITH CIBACRON BLUE F3GA AS PROBE OF THE NUCLEOTIDE FOLD

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Cibacron Blue F3GA (Cb) effectively and reversibly inhibits the activity of (Na,K)-ATPase. Its inhibitory effect does not occur through occupation of the ouabain binding site, but presumably results from Cb-occupation of one catalytic site not competitively attracting ATP. Cb also inhibits ouabain binding to (Na,K)-ATPase. Its inhibitory effect is competitively antagonized by ATP proving accomodation of Cb in the ATP binding site. - If one admits Cb as a suitable analytical tool for the detection of a supersecondary structure folding pattern, the findings suggest that the ATP binding site is lined by ß-pleated sheets flanked by α-helices thus providing an environment that funnels ATP to the catalytic site.

Although (Na,K)-ATPases has attracted wide interest as a model energy-converting enzyme (1,2), the relation between its structure and function are but partially explored (3-7 and references cited therein). The two catalytic peptides of the tetrameric enzyme contain two ATP-binding sites and two ouabain-binding sites that show under appropriate conditions anticooperative communication resulting in induced geometric asymmetry of the sites (8-11). The analysis of the secondary structure of the protein components by infrared spectroscopy in the amide band region revealed that 16 - 22 % of peptide groups form highly ordered α-helical regions and 18 - 34 % form ß-pleated sheets with an antiparallel packing of 3 or 4 chains (12). This information prompted the idea that the ß-pleated sheets and the α-helices could be packed in domains containing central ß-pleated sheets flanked by α-helices. Such supersecondary structure folding pattern is known for many enzymes to be involved in forming catalytic sites that accomodate adenine nucleotides and Cibacron Blue (Cb). This sulfonated polyaromatic blue dye is known to be potentially

Abbreviations: (Na,K)-ATPase, sodium- and potassium-activated adenosine triphosphatase (EC 3.6.1.3); Cb, Cibacron Blue F3GA.

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useful for probing nucleotide binding sites in numerous proteins [for references cf. (13,14)]. The question of whether the dye interacts with (Na,K)-ATPase and is a suitable tool for the elucidation of the folding pattern of the peptide chains involved in forming the ATP binding site will be tackled in the present paper for the first time.

MATERIALS AND METHODS

ATP from Boehringer (Mannheim) was used as imidazole salt. [^{3}H]ouabain (2.28 Ci/mmole) from Isocommerz (Berlin) was labeled on the butenolide ring by hydrogen-tritium exchange (15). Cibacron Blue F3GA from Ciba-Geigy was a mixture of the meta isomer with the probably active para isomer (16), more than 90 % pure and used without further purification. All other chemicals were of analytical reagent grade. The (Na,K)-ATPase preparations were obtained by established procedures as described by Walter (17) for guinea-pig kidney, by Matsui and Schwartz (18) for guinea-pig heart muscle (omitting here the deoxycholate-treatment) and human heart muscle, and by Samaha (19) for human brain cortex omitting the LiCl-treatment. Protein was determined by the procedure of Bensadoun and Weinstein (20). The assay medium for estimating (Na,K)-ATPase activity contained 2 mM ATP, 4 mM Mg, 80 mM Na, 5 mM K, 80 mM imidazole-HCl (pH 7.4), 0.1 mM EDTA, 0.3 mM phosphoenolpyruvate, 0.2 mM NADH, 9 IU pyruvate kinase and 9 IU lactate dehydrogenase, all in a volume of 2 ml. After addition of aliquots of the enzyme preparations, the decrease of extinction at 334 nm (21) was recorded at 37 °C. Any inhibitory effect of Cb on the auxiliary enzymes did not affect the sufficiency of the optical test. The ATPase activity suppressed by 0.1 mM ouabain was taken as (Na,K)-ATPase activity amounting to 46 (25 °C), 6, 8, and 114 µmole/mg•hr, respectively, in the membranous enzyme preparations from the four sources given in the above-chosen order. The binding of [^{3}H]ouabain to (Na,K)-ATPase from human brain cortex and the dissociation of the complex formed were followed at 37 °C in 40 mM imidazole-HCl buffer solution (pH 7.4) under various conditions specified in the legends to tables and figures. The incubation was terminated by removal of the membrane fragments from the incubation medium by vacuum filtration on glas fibre filters (Whatman GF/C). The filters were washed twice with ice-cold buffer solution, sucked dry and measured for radioactivity in a scintillation spectrometer. The proportion of nonspecific [^{3}H]ouabain binding assessed by omitting ATP, Mg, Na or Mg, P_i from the medium amounted to less than 6 % of saturation binding, and was corrected for so that all data given in the tables were specific bindings. The calculation of the kinetic parameters was carried out with a parameter optimizing program. All experiments were repeated at least twice showing similar results as those presented here.

RESULTS

Inhibitory Effect of Cb on (Na,K)-ATPase Activity.

The dye showed a rather strong inhibitory effect on enzyme activity (table 1). The inhibition was complete within less than one minute. In contradistinction to the inhibitory effect of Cb on myosin subfragment 1 (22) and on catalytic subunit of protein kinase (23), the interaction of Cb with (Na,K)-ATPase did not result in a slowly reversible inhibition. The activity of the enzyme,

Table 1 - Comparison of the concentrations of ouabain and Cb required for half-maximum inhibition of (Na,K)-ATPase from various sources

Source of (Na,K)-ATPase	$K_{0.5}$ (μM)	
	Ouabain	Cb
Guinea-pig kidney	-	11 (25 °C)
Guinea-pig heart muscle	0.52	15
Human heart muscle	0.019	30
Human brain cortex	0.027	12

found after pre-incubation with 100 μM Cb in the presence of 4 mM Mg or 4 mM Mg and 2 mM ADP, as well as in the absence of effectors for 30 min at 37 °C and then 100-fold dilution of the pre-incubation mixture, corresponded to the activity allowed by the residual Cb concentration (not demonstrated). Unlike ouabain, Cb did not show species differentiae of inhibitory power (table 1). In addition, the degree of Cb-inhibition was not reduced by enhancement of the K concentration (table 2) as it is known for enzyme inhibition by ouabain (24). The Hill coefficient was definitely greater than unity indicating kinetic co-operativity of Cb interaction with the enzyme.

The limiting rate of ATP hydrolysis was progressively reduced with increasing Cb concentrations. However, Cb at any concentration studied did not change beyond experimental scattering the K_m(ATP) value characterizing the low-affinity site of the enzyme through which it works as Na/K antiporter. Moreover, the variation of the ATP concentration over a range covering the

Table 2 - Parameters characterizing the Cb-effected inhibition of (Na,K)-ATPase from human brain cortex

Enzyme effectors (mM)	Cb (μM)	K_m(ATP)[a] (mM)	V (μmole/mg min)	Inhibition[d] (%)	h
0.001 - 1 ATP, 2 Mg, 95 Na, 5 K	0	0.11[b]	1.37[b]	-	1.5
	10	0.07	0.66	41	
	20	0.10	0.40	70	
	30	0.14	0.35	77	
0.001 - 0.3 ATP, 2 Mg, 50 Na, 50 K	0	0.11[c]	1.01[c]	-	1.3
	10	0.10	0.54	42	
	30	0.12	0.26	75	

[a]Calculated from Lineweaver-Burk plot by linear regression for the low-affinity site of the enzyme; the parameter for the high-affinity site was not accessible through the method applied. [b]For 0.03 - 1 mM ATP. [c]For 0.01-0.3 mM ATP. [d]For whole concentration range of ATP.

high and low affinity sites of the enzyme (9) did not change the inhibitory power of Cb (table 2). Taken together, the findings suggested that Cb inhibited Na,K-ATPase in an uncompetitive manner towards ATP. As (Na,K)-ATPase contains two interacting catalytic sites (8-11) Cb might have developed its inhibitory effect through binding to one catalytic site, the geometry and phosphorylated state of which was not complementary to competitively attract ATP.

Inhibitory Effect of Cb on ouabain binding to (Na,K)-ATPase.

Unlike Mg and P_i, or Mg, ATP and Na, Cb did not promote, but it inhibited effectively ouabain binding to the enzyme. This effect was exploited to disclose the binding site of Cb on (Na,K)-ATPase.

The course of dissociation of [^{3}H]ouabain from the complexes with the enzyme from two sources, revealed by addition of excess unlabeled ouabain or of Cb in concentrations sufficient for complete inhibition of (Na,K)-ATPase activity, did not differ as to rate and completeness (fig. 1). The $K_{0.5}$(Cb) value determined at ouabain concentrations near to or tenfold higher than the $K_{0.5}$

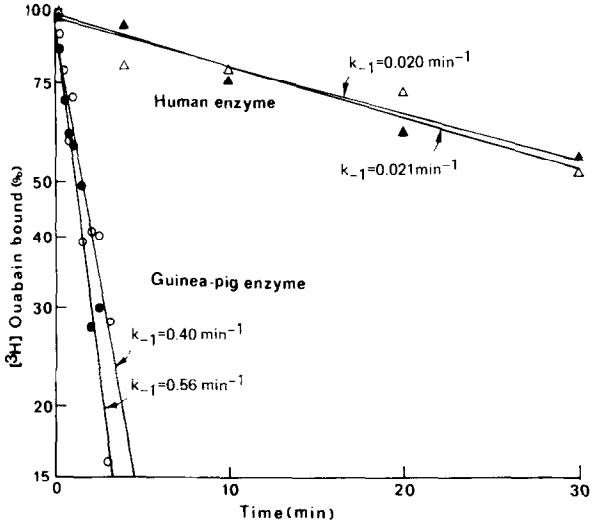

Figure 1 - Dissociation of [^{3}H]ouabain-complexes of (Na,K)-ATPase from human brain cortex or from guinea-pig heart muscle revealed by addition of high concentrations of unlabeled ouabain (open signs) or of Cb (filled signs). The complexes were formed by pre-incubating human enzyme for 30 min and guinea-pig enzyme for 10 min at 37 °C with 0.1 µM [^{3}H]ouabain in the presence of 4 mM Mg and 2 mM P_i. After adding 0.1 mM ouabain or 0.2 mM Cb at zero time, 1.5 ml aliquots of incubation mixture containing 149 µg protein (human enzyme) or 235 µg protein (guinea-pig enzyme) were analyzed for remaining enzyme-bound [^{3}H]ouabain at the time indicated.

Table 3 - Cb influence on initial rate (v_o) and equilibrium value (EO_∞) of Mg-plus P_i-supported [^{3}H]ouabain binding to (Na,K)-ATPase from human brain cortex.

Cb (μM)	0.03 μM Ouabain		0.3 μM Ouabain	
	v_o (pmole/mg•min)	EO_∞ (pmole/mg)	v_o (pmole/mg•min)	EO_∞ (pmole/mg)
0	24 ± 1.2	178 ± 3	262 ± 22	364 ± 6
0.5	21 ± 1.6	171 ± 4	220 ± 11	352 ± 4
1.0	17 ± 1.0	157 ± 3	186 ± 11	342 ± 5
2.0	13 ± 0.6	133 ± 2	114 ± 14	306 ± 12
5.0	5 ± 0.4	77 ± 2	46 ± 7	161 ± 10
10.0	2 ± 0.1	35 ± 1	20 ± 4	53 ± 4

The enzyme preparation (57 μg protein) was incubated for 0 - 60 min or 0 - 8 min at the lower or the higher ouabain concentration, respectively, in 1 ml of a medium containing 4 mM Mg and 2 mM P_i. The course of ouabain binding to the enzyme fitted well with an interaction scheme in which the association is a bimolecular process, whereas the dissociation is a monomolecular one, and all enzyme molecules able to bind the glycoside have the same affinity for ouabain. The values of v_o and EO_∞ were thus calculated by using the expressions described earlier to be valid for the characterized simple binding model (25). The errors given represent the deviations of the indicated calculated parameters from a fitted curve comprising 8 experimental data. Nonlinear regression according to the expression

$$\text{Cb-effected inhibition of } v_o \text{ and } EO_\infty = \frac{[Cb]^h}{K_{0.5}^h + [Cb]^h}$$

yielded the following parameters: from the Cb influence on the initial rates of ouabain binding $K_{0.5}(Cb) = 2.0 \pm 0.1$ μM and $h = 1.4 \pm 0.05$ at 0.03 μM ouabain, and $K_{0.5}(Cb) = 1.8 \pm 0.1$ μM and $h = 1.4 \pm 0.1$ at 0.3 μM ouabain; from the Cb influence on the equilibrium values of ouabain binding $K_{0.5}(Cb) = 4.1 \pm 0.2$ μM and $h = 1.5 \pm 0.03$ at 0.03 μM ouabain, and $K_{0.5}(Cb) = 4.4 \pm 0.8$ μM and $h = 1.8 \pm 0.15$ at 0.3 μM ouabain. Determined in a separate experiment, but with the same enzyme preparation [(Na,K)-ATPase activity = 112 μmole/mg•hr], the $K_{0.5}$(ouabain) value amounted to 0.037 μM and the limiting value of ouabain binding was 453 pmole/mg.

(ouabain) value did not differ (table 3); this finding excluded competition of Cb for the ouabain binding site.

The inhibitory effect of Cb on ouabain binding to (Na,K)-ATPase was much reduced when ATP or ADP was included in the binding-supporting medium containing Mg and P_i, and when Mg, ATP and Na was used instead of Mg and P_i to promote ouabain binding (table 4). Moreover, the Cb-effected inhibition of both initial rate and equilibrium value of ouabain binding to the enzyme became progressively decreased by an enhancement of the ATP concentration (table 5). The plots $1/v_o$ or $1/[EO]_\infty$ against 1/[ATP] showed no linearity (not demonstrated). Thus, the [ATP]-dependence of the inhibitory effect of Cb on ouabain binding could not be classified as a simple competition between Cb and ATP for one catalytic site.

Table 4 - Inhibitory effect of Cb on [^{3}H]ouabain binding to Na,K-ATPase from human brain cortex when nature and combination of enzyme effectors were varied

Effectors (mM)	Bound [^{3}H]ouabain (pmole/mg protein)		Cb-inhibition of binding (%)
	No Cb	2 μM Cb	
4 Mg, 2 P_i	284	103	64
4 Mg, 2 ATP	135	117	13
4 Mg, 2 ATP, 10 Na	277	266	4
4 Mg, 2 ATP, 10 K	96	93	4
4 Mg, 2 P_i	240	88	63
4 Mg, 2 P_i, 10 Na	179	55	69
4 Mg, 2 P_i, 2 ADP	42	35	17
4 Mg, 2 P_i, 2 ATP	144	129	10

The enzyme preparation (51 μg protein) was incubated with 0.03 μM [^{3}H]ouabain in the presence of the indicated effectors in a total volume of 4.5 ml for 30 min.

DISCUSSION

Cibacron Blue was already reported to inhibit the proton-translocating ATPase of mitochondria from yeast (26) and the energy transfer in the chloroplast system (27). The Cb-effected inhibition of proton-translocating ATPase isolated from chloroplasts is clearly competitive to ATP on the active site (27). Cb also strongly interacts with the ATP binding site of (Na,K)-ATPase as shown in the present paper. As discussed earlier with respect of Cb interaction with nucleotide coenzyme binding sites of various enzymes (13,28), Cb can assume a conformation resembling that of bound nucleotides, in which the anthraquinone

Table 5 - Influence of the ATP concentration on the Cb-effected inhibition of initial velocity (v_o) and equilibrium value (EO_∞) of [^{3}H]ouabain binding to (Na,K)-ATPase from human brain cortex

ATP (μM)	Per cent inhibition of parameters	
	v_o	EO_∞
2.8	75	48
28.1	57	27
281	46	19

The enzyme preparation (57 μg protein) was incubated in 3 ml of a medium containing 0.1 μM [^{3}H]ouabain, 56 μM Cb, 0.25 mM Mg, 150 mM Na and the ATP concentrations indicated. In the absence of Cb, v_o amounted to 141, 161, and 169 pmole/mg·min and EO_∞ was 272, 322, and 311 pmole/mg at 2.8, 28.1, and 281 μM ATP, respectively.

ring and the sulfonate groups in rings D and C of Cb mimick the adenine and phosphate moiety of the nucleotides, respectively. In (Na,K)-ATPase, the anthraquinone moiety and the sulfonate moieties of Cb could thus be accomodated by the adenine and phosphate subsites in the ATP binding site.

If one admits Cb as a suitable analytical tool for the detection of a supersecondary structure folding pattern (cf. Introduction), our findings suggest that the ATP binding site of (Na,K)-ATPase is lined by ß-pleated sheets flanked by α-helices thus providing an environment that funnels both ATP and Cb to the catalytic site [for detailed discussion cf. (13)]. With regard to the function of (Na,K)-ATPase, such supersecondary structure folding pattern could easily accomodate the Gibbs energy changes involved in energy transfer through the enzyme protein and thus play a central role in energy conversion as hypothesized by Baltscheffsky for the mechanism of coupling ATPases many years ago (29).

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