Reconstitution of Inhibitor Binding Properties of the Isolated Adenosine 5′-Diphosphate,Adenosine 5′-Triphosphate Carrier-Linked Binding Protein†

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ABSTRACT: We studied the binding of carboxyatractylate (CAT) and bongkrekate (BKA) to the solubilized ATP,ADP carrier-linked binding protein, which had been incorporated into liposomes. After solubilization with 3-lauramido-*N*,*N*-dimethylpropylamine oxide the binding protein had largely lost its affinity and binding capacity for both CAT and BKA. On incorporation into phospholipid vesicles, CAT binding was restored to a considerable extent (3.5 μmol of CAT/g of protein), reaching the original affinity as observed in mitochondria ($K_d = 10^{-8}$ M). With high amounts of CAT and under the influence of ADP the binding can be increased to 6.8 μmol of CAT/g of protein, indicating a movement of binding sites in the liposomal membrane. The binding of BKA was also reconstituted with high affinity ($K_d = 8 \times 10^{-8}$ M) and to the same extent (6.4 μmol of BKA/g of protein). As in the case of intact mitochondria, this reconstituted binding depends on the presence of ADP. This dependence on ADP has an apparent $K_m = 7$ μM, similar to the carrier affinity for ADP in intact mitochondria. The reorientation model of Klingenberg for the ADP,ATP carrier implicating an ADP-catalyzed transition between the CAT binding form (c state) and BKA binding form (m state) in the inner mitochondrial membrane has been confirmed in this reconstituted system.

The isolation of the ADP,ATP carrier-linked binding protein from beef heart mitochondria free of ligands (Krämer et al., 1977) permits a more direct investigation of the binding properties of this protein than is possible with the isolated ligand–carrier complexes (Riccio et al., 1975b). In the previous publication the incorporation of the binding protein into liposomes was reported. In the present study the binding of the specific inhibitors CAT[1] and BKA to the incorporated protein is investigated. Binding of these ligands can be considered to reflect highly specific properties of the protein, and therefore a reconstitution of these abilities would represent an important step toward the reconstitution of the original carrier properties.

Interaction of these ligands with the original mitochondrial membranes has been thoroughly studied (Klingenberg, 1974; Vignais, 1976; Klingenberg et al., 1973, 1975; Lauquin and Vignais, 1976). In particular the specific influence of BKA and ADP on the binding characteristics of the ADP,ATP carrier in the inner mitochondrial membrane has been intensively investigated (Erdelt et al., 1972; Klingenberg and Buchholz, 1973; Lauquin and Vignais, 1976). These results provide good criteria for the reconstitutional success in terms of functional and conformational properties of the isolated ADP,ATP carrier-linked binding protein. Preliminary results have already been published (Klingenberg et al., 1977). During the progress of this work reconstitution of adenine nucleotide transport has been published by Shertzer and Racker (1976). These authors used a nonpurified preparation as shown in the work of Serrano et al. (1976) (Figure 1B) and thus are unable to provide data based on molecular parameters such as carrier sites, ligand binding, etc.

Materials and Methods

All materials used in this work were the same as those in the preceding paper (Krämer et al., 1977).

Protein determination, assay for LAPAO, preparation of liposomes, isolation of the ADP,ATP carrier-linked binding protein, and incorporation of this protein into liposomes were also carried out as described in the preceding paper (Krämer et al., 1977). In order to verify the relative homogeneity of the liposomes used in this work, the vesicles were separated on Sepharose 4B. The elution diagram (Figure 1) shows a distribution of liposomal populations similar to those described in publications on liposome preparations (Huang and Thompson, 1974; Hauser and Irons, 1972). In some experiments liposomes preloaded with CAT, with ADP, or with both were used. After sonication of the phospholipids in the presence of these substances the external ADP and CAT were removed by dialysis against the sonication buffer or by passage through a Dowex-formate column.

Analysis of the binding properties of the isolated binding protein was performed with three different methods. *Equilibrium dialysis* was carried out with a "Dianorm" apparatus (Dr. Weder, ETH Zürich). The buffers and ligands used are indicated in the corresponding experiments. This apparatus could only be used in long-lasting experiments, as for example in the ligand displacement studies, because the time required for equilibration is longer than 6 h at room temperature due to the presence of the detergent LAPAO.

Membrane filtration was performed by sucking the sample through two membrane filters (Sartorius-Membranfilter GmbH, Göttingen), one with a 0.2-μm pore size on top and the other with 0.01 μm below. With this combination both liposomes and solubilized binding protein can be retained on the filters, the protein probably mainly due to adsorption to the filter material. Some difficulties arise because of the unspecific adsorption of BKA to the filter material. For BKA-binding

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[1] Abbreviations used are: CAT, carboxyatractylate; BKA, bongkrekate; LAPAO, 3-lauramido-*N*,*N*-dimethylpropylamine oxide.

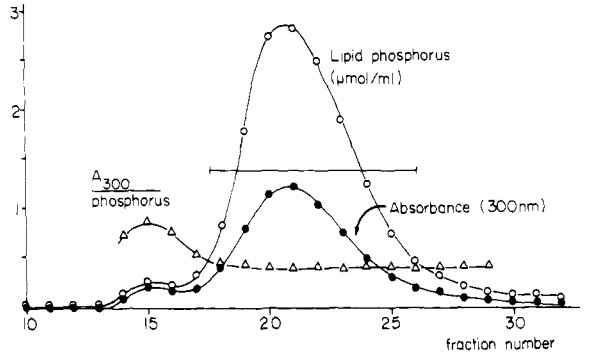

FIGURE 1: Elution diagram of egg-yolk phospholipid vesicles on Sepharose 4B. Liposomes were prepared as described under Materials and Methods. Absorbance at 280 nm and lipid phosphorus were measured in the eluate. The region with a constant A_{280}/phosphate ratio (⊢⊣) represents a relatively homogeneous liposome population, which accounts for about 85% of total liposomes.

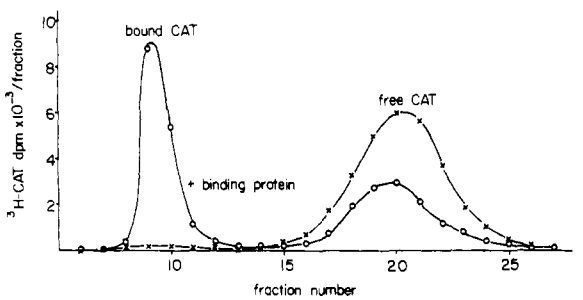

FIGURE 2: CAT binding to liposome incorporated protein. Elution diagram of [^{3}H]CAT added to pure liposomes and to liposomes with incorporated binding protein. Separation in bound and free CAT on Sephadex G75 (7 × 300 mm).

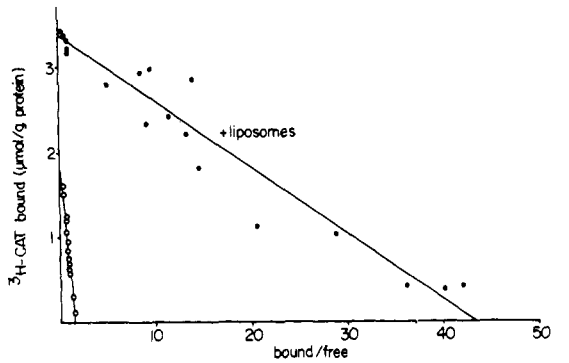

FIGURE 3: [^{3}H]CAT binding to solubilized and incorporated binding protein. Mass action plot. [^{3}H]CAT was added to solubilized and to liposome incorporated ADP,ATP carrier-linked binding protein; for experimental data see Table I.

TABLE I: CAT Binding to the ADP,ATP Carrier Protein in Mitochondria, in the Solubilized State and after Incorporation into Liposomes.[a]

CAT binding to[b]	In buffer[b]	C_0[c] (μmol/g)	K_d (nM)
Beef heart mitochondria	1	14–18	12
	2	9.5	15
Solubilized binding protein			
− liposomes	2	1.6	310
+ liposomes	2	3.5[d]	10

[a] Binding analysis by Sephadex gel filtration or by membrane filtration as described under Materials and Methods. [b] Buffer 1: 0.25 M sucrose, 0.5 mM EDTA, 2.5 mM $MgCl_2$, and 20 mM morpholinopropanesulfonic acid (pH 6.8). Buffer 2: 0.1 M Na_2SO_4, 1 mM EDTA, 10 mM morpholinopropanesulfonic acid (pH 7.5). [c] Maximum binding corrected for pure binding protein (details in the text). [d] Can be increased to 6.8 μmol/g of binding protein (cf. Figure 4).

experiments, therefore, we prefer the following gel filtration method.

For the very tightly binding inhibitors CAT and BKA a *gel filtration technique* with Sephadex G75 can be used. Figure 2 shows a separation of CAT bound to liposome-incorporated protein from free CAT. Liposomes alone with CAT are applied to another G75 column as a control. A clear separation of bound and free CAT is achieved and free CAT is not bound at all to the liposomes. A similar elution pattern is obtained with BKA, but only when working at a pH above 7.3, because then BKA is completely ionized and becomes less lipophilic. Otherwise BKA appears in the void volume with the LAPAO micelles. Binding experiments are usually performed with two of the methods described above.

Results

I. Reconstitution of CAT Binding to the Isolated ATP,ADP Carrier-Linked Binding Protein

The first attempts to demonstrate the reconstitutional activity of the isolated ADP,ATP carrier-linked binding protein are carried out with the highly specific and tightly binding inhibitor CAT. ^{3}H-Labeled CAT was added either to the solubilized binding protein or to the binding protein incorporated in phospholipid vesicles (Krämer et al., 1977). The binding characteristics (Figure 3) are shown in a mass action plot (Scatchard, 1949). These data are compared with the binding of CAT to beef heart mitochondria (Table I).

The values for binding of CAT to intact mitochondria are taken from Klingenberg et al. (1975) and the maximum binding is based on the content of pure binding protein. The calculation of binding to mitochondria takes into account that the CAT binding protein accounts for about 9–10% of the total mitochondrial protein (Riccio et al., 1975a,b), which is identical with a mol wt 30 000 polypeptide described with the same abundancy by Capaldi et al. (1973), Boxer (1975), and Boxer et al. (1977). The data for the binding protein preparation, isolated by the batch procedure (Krämer et al., 1977), are related to 70% content of CAT binding protein. On this basis the values of the CAT binding to mitochondria and isolated protein can be compared. The CAT binding to mitochondria is also tested with the same high ionic strength buffer used for the binding to the solubilized protein.

Table I shows the drastic effects of incorporation into liposomes on the binding of CAT to the isolated protein. After solubilization with the detergent the binding affinity of the isolated protein decreases about 20- to 30-fold and the maximum binding becomes relatively low. After insertion into phospholipid membranes, the maximum binding is increased twofold, reaching one-third of the binding of CAT to mitochondria in the same buffer. More pronounced is the restoration of binding affinity to the original value measured in mitochondria.

In these binding experiments, carried out with moderate CAT concentrations (up to 5 μM) and relatively short incubation times (10 min for CAT binding), added ADP has no effect on the observed maximum binding and K_d of the CAT binding. When using high concentrations of CAT and longer times, the CAT binding could be increased further (Figure 4). The ADP effect succeeds within several minutes only if the

TABLE II: BKA Binding to the ADP,ATP Carrier Protein in Mitochondria, in the Solubilized State, and after Incorporation into Liposomes.[a]

BKA binding to	ADP[b]	Binding affinity[c] High C_0 (μmol/g)	High K_d (μM)	Low C_0 (μmol/g)	Low K_d (μM)
Beef heart mitochondria	+	10–18[d]	0.01[e] −0.04		
Solubilized binding protein					
− liposomes	−	0		0.2	2
+ liposomes		0.2	0.1	0.2	1.5
− liposomes	+	0.7	0.08	3.75	1.5
+ liposomes		6.4	0.08	0	

[a] As described under Materials and Methods. Only Sephadex gel filtration could be used for binding analysis in the case of BKA. [b] ADP concentration in incubation buffer, 50 μM. [c] Maximum binding corrected for pure binding protein (cf. Table I). [d] According to Weidemann et al. (1970). [e] According to Lauquin and Vignais (1976).

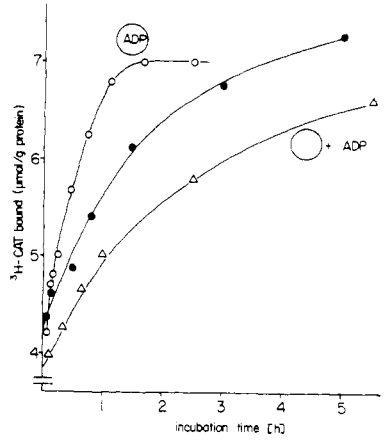

FIGURE 4: Influence of ADP on CAT binding capacity. In each experiment externally 50 μM CAT was added. Additional 80 μM ADP was present in the inner (ADP) or outer (○ + ADP) space where indicated. By semilogarithmic plots (not shown) the half-times of binding increase were determined, $t_{1/2}$ = 85 min (without ADP), 65 min (external ADP), and 18 min (internal ADP).

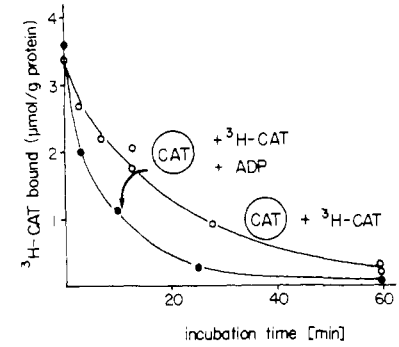

FIGURE 5: Interaction of the incorporated binding protein with CAT in the external and internal space of liposomes. Liposomes with internal CAT (70 μM) and protein were mixed at time 0. After the indicated time intervals [^{3}H]CAT (1 μM) is added and 3 min later analyzed by membrane filtration. The half-times as determined in semilogarithmic plots (not shown) were $t_{1/2}$ = 16 min (without ADP) and 5 min (with ADP).

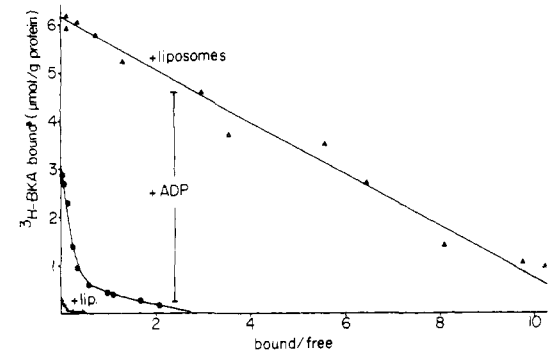

FIGURE 6: [^{3}H]BKA binding to solubilized and incorporated binding protein. Mass action plot. [^{3}H]BKA was added to solubilized and to liposome incorporated binding protein (ADP, when added, 50 μM).

CAT concentration is increased considerably. Without the addition of ADP this increase in the CAT binding has a long half-time of about 90 min. It is only slightly but significantly accelerated by external ADP. Internal ADP, incorporated into the liposomes during sonication, shortens the process of additional binding to a half-time of about 20 min. In all these experiments the basic binding without ADP, of about 3.5 μmol of CAT/mg of binding protein, remained unaffected. This binding type is too fast to be resolved kinetically with the methods used here.

For further elaboration of this effect the CAT distribution between the two spaces was reversed by preloading the liposomes with high CAT concentrations. Trace amounts of labeled CAT were added externally so as to monitor the available binding sites at the outside of the vesicular membrane. Figure 5 shows that after mixing the binding protein with the liposomes the initial reconstituted binding capacity for external CAT of about 3.5 μmol/g of protein decreases during incubation time down to less than one-tenth. The presence of ADP (now in the external space) causes considerable acceleration of the observed binding decrease.

The possible objection that these results reflect simply an isotope dilution due to leakage of internal cold CAT is refuted by two arguments. First, even if all internal CAT is mixed with the externally added labeled CAT, the specific activity would be only decreased to about 50% of the original value due to the very small internal volume of sonicated liposomes. Secondly, control experiments with labeled internal CAT at the same concentration show that during the incubation time used in this experiment less than 1–2% of the internally trapped inhibitor leaks out after removal of the external CAT by passage through a Dowex-formate column, thereby generating a gradient of CAT concentration to the outside.

II. Reconstitution of BKA Binding to the Isolated ADP,ATP Carrier-Linked Binding Protein

With the system used for restoration of CAT binding to the isolated binding protein practically no interaction of the inhibitor ligand BKA with the protein is seen either in the solubilized or in the incorporated state (Figure 6 and Table II, lines

TABLE III: Comparison of Nucleotide Interaction with Intact BHM and with the Liposome Incorporated Binding Protein.

Nucleotide	Enhancement of BKA binding, apparent K_m (μM)[a]	Binding to BHM,[b] K_d (μM)
ADP	2	7
ATP	3	12
ITP	20	
AMP	800	

[a] The apparent K_m values are calculated as described in the text. [b] The values of nucleotide binding to mitochondria are calculated as described in the text.

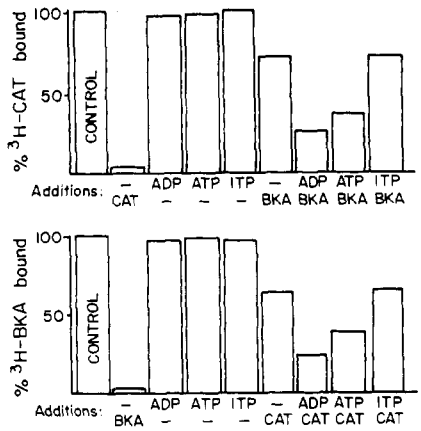

FIGURE 7: Interaction of CAT and BKA binding with other ligands. Displacement of bound [^{3}H]CAT respectively bound [^{3}H]BKA by the indicated ligands. [^{3}H]CAT and [^{3}H]BKA were bound to liposome incorporated binding protein. CAT and BKA as displacing ligands were added in about 100-fold excess (20–40 μM); nucleotides were added at 250 μM. Equilibrium dialysis was carried out in 0.5 M NaCl, 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 7.5) for 5 h at room temperature; both dialysis chambers contain identical liposome concentrations (because of unspecific adsorption of BKA), the only difference being the presence of binding protein.

2 and 3). Binding to the solubilized protein is virtually nil (in Figure 6 represented by the origin), and even after incorporation into liposomes only negligible amounts of this inhibitor ligand can be bound to the protein. From experiments with intact mitochondria (Klingenberg and Buchholz, 1973), however, it is known that ADP enhances the binding of BKA to the carrier. This effect is also seen with the isolated binding protein.

When adding ADP to the isolated carrier protein the binding of BKA is increased, but this effect is mainly due to an unspecific type of binding with a high K_d. An enhancement of high affinity binding after addition of ADP is observed only with the incorporated system. The degree of restoration of the binding affinity is difficult to assess by comparison with mitochondria (Table II). Only uncertain data about the K_d of BKA binding in mitochondria are available because of the high proportion of unspecific BKA binding in the lipid phase of the membrane (Klingenberg, 1976a). The maximum binding reaches about 40 to 50% of the average value found in the isolated BKA–protein complex, where the inhibitor is added before the solubilization of the mitochondria. Therefore, not only the binding of BKA is reconstituted, both with high affinity and to a substantial extent, but also, remarkably, the dependence on ADP as found in intact mitochondria. The binding of BKA to the incorporated protein shows no second phase of slow binding increase, in contrast to the situation with CAT binding as described above.

The stimulation of BKA binding by ADP has been studied with regard to nucleotide concentration. The obtained hyperbolic relationship between the binding enhancement and the ADP concentration and linear reciprocal plots (not shown) indicates a well-defined apparent K_m of ADP for the formation of the BKA–protein complex. The specificity of this enhancement effect was tested with certain other nucleotides (Table III), among which particularly ATP affects the BKA binding, whereas ITP has only a weak effect and AMP nearly no activity. The apparent K_m values for ADP and ATP are similar to the dissociation constant K_d of ADP and ATP to the carrier in beef heart mitochondria (Weidemann et al., 1970). Also, the specificity for the other nucleotides shows a similar pattern in intact mitochondria (see Table I of Pfaff and Klingenberg, 1968) and in the reconstituted system.

It is important to mention that in these experiments no binding of ADP whatever can be seen whether ADP is added either before, simultaneously, or after BKA.

In an additional experiment it was confirmed that the internal CAT (cf. Figure 5) can decrease BKA binding in a similar fashion as was shown above for the binding of external [^{3}H]CAT. Externally bound BKA in fact dissociates from the CAT-loaded liposomes in a manner analogous to the removal of [^{3}H]CAT (Figure 5). The estimated half-life is also very similar to that found for the dissociation of the [^{3}H]CAT–protein complex.

III. Specific Interactions of Different Ligands with the Isolated ADP,ATP Carrier-Linked Binding Protein

The simultaneous interaction of both inhibitors CAT and BKA with the protein should give additional information concerning relative affinities and other factors which determine the binding specificity. This approach was first used in experiments where one bound labeled ligand was displaced by the other unlabeled ligand (Riccio et al., 1975b). For this purpose, either [^{3}H]CAT or [^{3}H]BKA was bound to the isolated and liposome incorporated binding protein and the sensitivity of this binding to various additions of CAT, BKA, ADP, ATP, and ITP in excess was tested (Figure 7). Both CAT and BKA are displaced readily by the unlabeled homologous ligand. The nucleotides ADP,ATP and ITP alone have no substantial effect. Under the influence of an excess of added BKA, the amount of bound CAT decreases about 30%, but with the simultaneous addition of BKA and ADP, CAT is displaced to a much larger extent. ATP in combination with BKA has a somewhat smaller effect and ITP does not stimulate this process at all compared with the addition of BKA alone.

A similar pattern is seen with bound BKA. This time ADP or ATP supports the displacement by CAT of the bound BKA. The relatively large effect of CAT in removing BKA is due to the small amount of ADP already present in the incubation medium that had been added previously in order to promote the binding of BKA to the incorporated protein.

It is possible to resolve this displacement process kinetically by means of membrane filtration and gel chromatography. The kinetics of the displacement of bound BKA by added CAT in the presence of ADP (cf. Figure 7) can be expected to be strongly dependent on liposome incorporation. The displacing agent CAT is added either to the BKA–protein complex, to the liposome-incorporated BKA–protein complex, or to the liposome-incorporated binding protein to which BKA was bound after insertion into the phospholipid membrane (Table IV). Figure 8 represents situation c of Table IV. The kinetics of BKA removal (line $C_1 + C_2 + C_3$ in Figure 8) can be resolved

TABLE IV: Kinetic Solution of Displacement of Bound [^{3}H]BKA by an Excess of Added CAT.[a]

Binding proteins	Kinetic portion	[^{3}H]BKA displaced		Half-time (min)
		μmol/g	% of total	
(a) BKA-protein complex	1	10.4	100	90
(b) BKA-protein complex + liposomes	1	2.4	22	∞
	2	3.5	32	16
	3	5.0	46	6
(c) Binding protein + liposomes	1	1.4	20	∞
+ BKA	2	2.0	30	18
	3	3.4	50	5
(d) Binding protein + liposomes	1	3.0	50	∞
(with internal ADP)	2			
+ BKA	3	3.1	50	10

[a] [^{3}H]BKA is bound to the binding protein in the indicated state. An excess of CAT (50 μM) is added together with ADP (50 μM). In experiment d additional ADP in the internal space of the liposomes was present.

TABLE V: Binding of [^{3}H]CAT after Displacement of Bound BKA from the Incorporated Binding Protein.[a]

Binding protein	External ADP (μM)	Rebound CAT (μmol/g)	Half-time
BKA-protein complex + liposomes	0	5.0	>5 h
	100	5.5	18 min
Binding protein + liposomes	1-2	3.2	>5 h
(with internal	9	2.9	2.5 h
ADP)	25	3.3	40 min
	90	2.9	22 min
+ BKA	100	3.3	16 min

[a] [^{3}H]CAT (20 μM) was added to liposomes with incorporated BKA loaded binding protein.

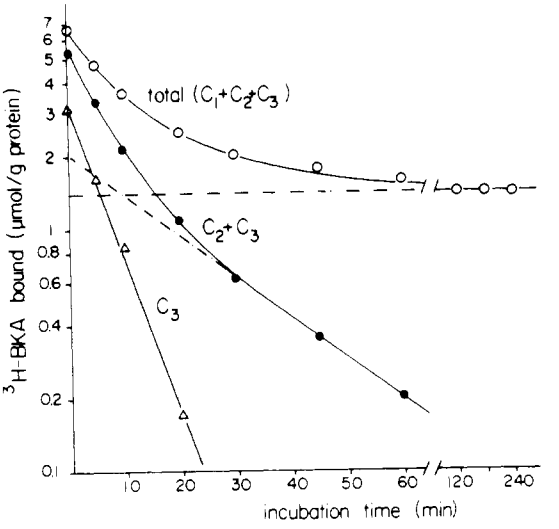

FIGURE 8: Displacement of [^{3}H]BKA bound to liposome incorporated binding protein by added CAT. An excess of CAT (50 μM) is added to liposome incorporated binding protein, which was first saturated with [^{3}H]BKA. Residual bound BKA was determined after the indicated incubation time with CAT. In curve C_2 + C_3 the residual nondisplaceable part of the total bound [^{3}H]BKA (portion C_1) is subtracted. Further subtraction of portion C_2 (linear part of curve C_2 + C_3) leads to curve C_3.

in three different components on the basis of first-order kinetics: C_1, a stable component that cannot be detached even by very long incubation times (20% of total binding), and two readily displaceable portions of BKA binding with short half-times (C_2 (30%) with a half-time of 18 min and C_3 (50%) with a half-time of 5 min). The other experiments in Table IV are carried out and analyzed in the same way as shown in Figure 8.

As seen in Table IV most of the BKA bound to liposome-incorporated binding protein (components 2 and 3) is displaced with identical half-times whether the ligand BKA is bound to the protein before solubilization of the mitochondria or after incorporation of the binding protein. In the free BKA-protein complex the half-time is longer, probably due to hindered accessibility of the CAT and BKA binding sites in the detergent-protein micelle. In this case CAT is able to detach all the bound BKA. After incorporation into the phospholipid membrane, the BKA binding is kinetically divided into three different portions (50, 30, and 20%) with different half-times (5 min, 18 min, ∞). The additional presence of ADP also in the internal volume enhances the nondisplaceable portion of BKA up to 50%. Now the removable part follows simple first-order kinetics containing only C_3.

The possibility of whether the loss of BKA on addition of CAT is accompanied by a rebinding of CAT was also investigated. For this purpose in displacement experiments analogous to that described in Figure 7 and Table IV, the ^{3}H labeling was reversed, i.e. [^{3}H]CAT was added to unlabeled BKA bound to the protein. The data show (Table V) that CAT binds with nearly the same half-time (at 100 mM ADP, 16 min) as BKA is displaced (case d in Table IV, 10 min). This confirms the equivalence of these two processes. The relatively low extent of rebinding compared with the originally bound BKA is probably due to the known instability of the protein not liganded with CAT or BKA (Krämer et al., 1977). The protein appears to be particularly labile in the presence of ADP (Klingenberg et al., 1975a), a situation which may interfere in the ADP-dependent displacement process of BKA by CAT. Nevertheless, the recovery of about 50% binding demonstrates that in fact a real displacement has occurred. It is also shown in Table V that the rate of the displacement is strongly dependent on the concentration of ADP, in agreement with Figure 7. Therefore, in all displacement experiments 50 μM ADP was added.

Further information about the relative binding characteristics of CAT and BKA to the incorporated protein can be obtained with simultaneous addition of CAT and BKA. As shown with moderate concentrations of CAT, BKA binds twice as much as CAT to the incorporated binding protein (columns a and b in Figure 9). When adding CAT and BKA in various concentrations only part of the total available binding sites can be occupied (columns c-f). Again in columns c and e, where

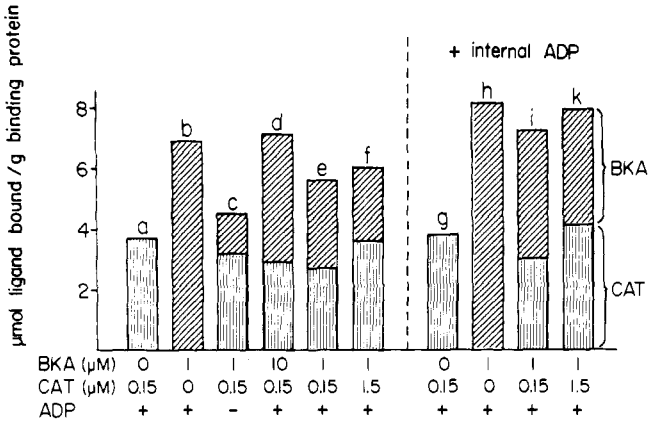

FIGURE 9: Competitive binding of simultaneously added CAT and BKA. Binding was analyzed by equilibrium dialysis (conditions as in Figure 7). External ADP (100 μM) was present where indicated; additional ADP (80 μM) in the internal volume was present in the last four experiments.

CAT and BKA are added in the concentration ratio of their dissociation constants of 1:7 (cf. Tables I and II), the effect of added ADP on BKA binding is seen, although in these lengthy equilibrium dialysis experiments it is not as high as in the gel filtration binding assays (Figure 5). Increasing the concentration of CAT, about 50% of the total binding sites are saturated by this ligand, whereas BKA fills up the remaining sites only when increasing the concentration still tenfold. However, unspecific binding with low affinity may interfere in this case.

These results are further elaborated by using liposomes preloaded with internal ADP. If now CAT and BKA are added in sufficiently high concentrations, all accessible binding sites are saturated with a ratio of 1:1 for CAT:BKA.

Discussion

The putative adenine nucleotide carrier protein of beef heart mitochondria was first isolated as a CAT-protein complex by Riccio et al. (1975a,b). The tightly bound ligand CAT stabilized the protein during solubilization by detergents. After removal of CAT the solubilized binding protein becomes irreversibly denatured. The question arises as to whether the substitution of detergent surrounding the protein by a phospholipid membrane again stabilizes the protein against denaturation after removal of CAT. This approach, however, is only one aspect of determining the influence of phospholipid membranes on the ADP,ATP carrier-linked binding protein. More interesting is to start from the other side, i.e. with the ligand-free protein, and to study the reconstitution of the ligand binding.

The preceding paper (Krämer et al., 1977) describes the isolation of the ligand-free form of the ADP,ATP carrier protein, which turns out to be relatively labile. Nevertheless, by a short-cut batch procedure for isolation, it can be obtained largely undenatured. The influence of phospholipid membranes on the properties of this protein is tested in the present paper.

Incorporation of the protein into liposomes protects the binding protein partially against denaturation. This is demonstrated by the fact that after 2 h of storage the binding capacity of the solubilized protein decreases by about 50%, whereas after the same storage time the incorporated protein exhibits only slightly diminished binding (experiments not shown).

The protein solubilized with LAPAO has a weak binding capacity for CAT and BKA. After incorporation into phospholipid vesicles, striking restoration of the binding capacity is achieved accompanied by a reconstitution of high affinity binding. The dissociation constants of CAT and BKA even reach values identical with those in the original mitochondria. It is of particular importance that starting with the free binding protein, transition between the two states of the carrier protein can be achieved. These two conformational states have been postulated from studies of the action of BKA and ADP on the ADP,ATP carrier in intact mitochondria (Scherer and Klingenberg, 1974; Klingenberg, 1974), in which the binding sites are directed either to the outside (c state) or to the inside (m state) of the inner mitochondrial membrane. They differ in their relative affinities for the two ligands CAT (binds preferably to the carrier in the c state) and BKA (prefers the m state). The transition between the two states is possible only in the presence of ADP.

In the present work CAT binds readily to the isolated and incorporated ADP,ATP carrier-linked binding protein (Tables I and II), whereas BKA needs the simultaneous presence of ADP for binding, as expected from the situation in intact mitochondria. Furthermore, ADP is also necessary for the displacement of bound CAT by BKA and BKA by CAT (Figure 7). Both the stimulation of BKA binding and the displacement of bound ligands have similar nucleotide specificities as the adenine nucleotide carrier in the inner mitochondrial membrane for binding and transport. Finally, the apparent K_m of ADP and ATP for the formation of the BKA-protein complex is closely similar to the K_d of this nucleotide for binding to the adenine nucleotide carrier (Table III). These experimental data led to the following conclusions. (1) The binding protein, when isolated with LAPAO, is mainly in the c state, which agrees with its antigenic properties, as shown in the preceding paper (Krämer et al., 1977). (2) The interconversion of the two states by ADP action can be described in the following sequence:

$$\begin{array}{ccc} \text{CAT–protein (c state)} & \rightleftharpoons & \text{CAT + protein (c state)} \\ & & \text{ADP} \uparrow\downarrow \text{ADP} \\ \text{BKA–protein (m state)} & \rightleftharpoons & \text{BKA + protein (m state)} \end{array}$$

(3) In all the experiments with the liposome-incorporated system the binding of CAT and BKA is mutually exclusive, similar to the situation in mitochondria.

It is notable that also in the reincorporated state no binding of ADP to the isolated protein can be detected. Possibly the binding of ADP is so weak ($K_d > 10^{-4}$) that it cannot be detected with the present methods. In any case it is demonstrated that at least in this reconstituted system a stable ternary complex between BKA, ADP, and carrier protein is not seen.

In this context the question arises whether the incorporated protein is inserted unidirectionally into the membrane. It is known in several cases that liposome-incorporated proteins are oriented in the membrane (Hilden et al., 1974; Knowles and Racker, 1975; Eytan et al., 1976). The present experiments, as discussed below, permit identification of binding sites on definite sides of the vesicular membrane.

The binding data (Tables I and II, Figure 8) show that CAT can saturate only half of the binding sites available for BKA (Figure 9), i.e. BKA reaches also the portion not accessible for CAT. Maximum filling of the binding sites with BKA requires ADP in the internal volume of the liposomes. These findings can be explained by assuming that about half of the binding sites are situated on the outside and half on the inside of the vesicles. The outside binding sites are accessible to the im-

permeant ligand CAT and also for BKA, whereas only the membrane-permeable ligand BKA can bind to the inner binding sites.

CAT binding to the incorporated protein, however, is not restricted to one-half of the sites of BKA binding if high concentrations of CAT are used, as shown in Figure 4. In contrast with the initial fast binding of CAT (3.5 μmol/g of protein), the appearance of this second type of binding is extremely slow and can be accelerated by ADP. The assumption that this increase of CAT binding sites reflects the turning of binding sites from inside to outside is supported by the finding that ADP can accelerate this process substantially only when present in the internal volume of the liposomes. Therefore, ADP has two clearly separate effects on the binding protein in the reconstituted system. First, it catalyzes the interconversion between c state and m state and, secondly, it enables a movement of CAT or BKA binding sites across the phospholipid membrane.

One possible objection to this interpretation, namely that high concentrations of CAT simply increase the binding capacity of the incorporated protein, is refuted by the results shown in Figure 5, where in liposomes with high internal concentration of CAT, the amounts of externally accessible binding sites for [^{3}H]CAT are decreased. This effect, though proceeding in the opposite direction, also depends on ADP and has similar half-times as the "forward" direction. In addition, the binding for external [^{3}H]BKA is diminished. Therefore, internal CAT is able to move the binding sites to the inside of liposomes, a process analogous to that observed with externally added CAT (Figure 6). This provides further evidence for an ADP-catalyzed reorientation of the binding site between the two faces of the membrane.

Further experimental support is given by the kinetic resolution of the displacement process (Table IV and Figure 8). Whereas CAT is able to detach all the bound BKA from detergent–protein micelles of solubilized BKA–protein complex, kinetically distinguishable types of binding can be detected in liposomes. When an excess of CAT is added to the liposome-incorporated protein with bound BKA, 50% of the bound ligand is displaced relatively quickly, presumably representing the fraction of bound BKA accessible to CAT from the outside of the liposomes. A further 30% of the BKA is detached with a slower half-time, probably accounting for those binding sites turned to the outside of the membrane by the high excess of CAT. The remaining 20% of the binding sites are not affected by the displacing ligand. This last portion is considerably increased to 50% by using liposomes with internal ADP, accompanied by the disappearance of component 2. This is in agreement with the competitive binding experiments (Figure 9) when CAT and BKA are added simultaneously. Internal, but not external, ADP tightens the binding of BKA to the carrier protein to such an extent that CAT is unable to pull the binding site to the outside.

It is not yet clear whether this shift of binding sites corresponds to a real movement of the protein or to a change in binding affinities on both sites produced by an interconversion of protein conformations.

Recently, reconstitution of ADP,ATP transport in artificial membranes was published by Shertzer and Racker (1976). Repetition of their work in our laboratory and also their own evidence showed that during this preparation the ADP,ATP carrier protein has not been purified in a well-defined manner. Through demonstrating reconstitution of nucleotide exchange it shows only small rates and highly variable reproducibility. In view of the undefined protein preparation it is not possible to derive from these reconstitution studies any molecular parameters which are necessary to elucidate the properties of the reconstituted carrier protein.

One problem in identifying the observed artificial movement processes with the original translocation process is the large difference of the transfer times. The highest transfer rate of binding sites in the liposomal system, the ADP-catalyzed increase of the CAT binding, has a half-time of 5 min, whereas in mitochondria this time should be <0.05 s, as estimated from the turnover number (Klingenberg, 1976a). We believe that this difference can be explained more on a quantitative basis rather than on fundamental differences. Although still present, the interaction of ADP with the isolated protein may be severely impaired. This would agree with the inability to determine ADP binding even to the incorporated protein.

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Oxidative Titrations of Reduced Cytochrome aa_3: Correlation of Midpoint Potentials and Extinction Coefficients Observed at Three Major Absorption Bands†

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ABSTRACT: Anaerobic oxidative titrations of purified cytochrome aa_3 were monitored at three wavelengths (444, 604, and 820 nm), in both the absence and the presence of carbon monoxide. Computer simulation of each titration curve was utilized to ascertain the midpoint potentials of the four oxidation-reduction centers of the enzyme. For experiments performed under nitrogen, two components were found to titrate with low potential (heme a_L = 220 mV, Cu_L = 240 mV) and two with high potential (heme a_H, Cu_H = 340 mV), consistent with results obtained previously in reductive titrations. Unequal heme extinction coefficients were observed at 444 nm. Oxidation by either potassium ferricyanide or 1,1′-bis(hydroxymethyl)ferricinium ion showed that the low potential heme component contributed 75% of the absorbance change at 444 nm. At 820 nm, the entire absorbance change could be attributed to a single, low potential copper component. Midpoint potentials calculated for the carbon monoxide complexed enzyme agreed with previously reported values. The copper components retained the values observed under nitrogen, while the titratable heme group gave an apparent midpoint potential of 260 mV. These results enable us to assign absorbance changes at various wavelengths to specific redox components of cytochrome aa_3.

In a previous paper we presented midpoint potentials and extinction coefficients for the metal ion centers of cytochrome aa_3 observed at 604 nm (Schroedl and Hartzell, 1977). This absorbance band has been extensively studied and the absorbance changes accompanying oxidation and reduction have been correlated with those measured at other wavelenths and with changes in the electron paramagnetic resonance spectrum of the protein (Hartzell and Beinert, 1976). We report here an examination of direct chemical, oxidative titrations of reduced cytochrome aa_3, as monitored at the 444, 604, and 820 nm absorption bands, performed in the presence and absence of carbon monoxide atmosphere.

Experimental Procedure

Continuous anaerobic oxidative titrations were performed at ambient temperature on a purified, phospholipid-depleted preparation of cytochrome aa_3 (Hartzell and Beinert, 1974). A specially designed apparatus patterned after that of Foust et al. (1969) was used. Solutions contained 4-30 μM cytochrome aa_3 ($\Delta\epsilon_{604}^{R-O}$, 24 mM^{-1} cm^{-1}; $\Delta\epsilon_{444}^{R-O}$, 168 mM^{-1} cm^{-1} (Van Gelder, 1966); and $\Delta\epsilon_{820}^{R-O}$, 2.8 mM^{-1} cm^{-1} (Griffiths and Wharton, 1961) (all $\Delta\epsilon$ values are expressed as two heme *a* per cytochrome aa_3)), 0.1 M potassium phosphate buffer, pH 7.5, and either 0.2% (w/v) sodium cholate or 0.2% (w/v) Triton QS-30 as a solubilizing agent. The enzyme was reduced with a 5-15% equivalent excess of NADH (P-L Biochemicals) with 5 μL of 0.01% (w/v) phenazine methosulfate as mediator catalyst. Potassium ferricyanide and HMF (Strem Chemicals, Inc.) were standardized using ϵ_{420} = 1.02 mM^{-1} cm^{-1} (Ibers and Davidson, 1951) and titration of cytochrome *c* ($\Delta\epsilon_{550}^{R-O}$ = 21.1 mM^{-1} cm^{-1} (Van Gelder and Slater, 1962)) solutions, respectively. A more thorough description of the experimental procedure can be found elsewhere (Schroedl and Hartzell, 1977). Carbon monoxide (Matheson Gas Products) was freed of oxygen by passage over a bed of Ridox (Fisher Scientific Co.).

Spectra were recorded on a Cary 17 ratio recording spectrophotometer. Change in absorbance was plotted against oxidizing equivalents per cytochrome aa_3 (ΔA vs. equiv). All

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[1] Abbreviations used: PMS, phenazine methosulfate; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride; HMF, 1,1′-bis(hydroxymethyl)ferricinium cation; $E^{o\prime}$, midpoint potential value; $\Delta\epsilon_\lambda^{R-O}$, reduced minus oxidized extinction coefficient at designated wavelength; NHE, normal hydrogen electrode; a_h, high potential heme *a*; Cu_H, high potential copper; a_L, low potential heme *a*; Cu_L, low potential copper; IR, infrared.