L. (1974) *Nature (London) 249*, 224-227.
Shearer, R. W. (1977) *Prev. Detect. Cancer*, [*Proc. Int. Symp.*], *3rd., 1976, 1 (Part 1)*, 53-68.
Siegal, G. P., Quinlan, T. J., Moses, H. L., & Getz, M. J. (1979) *J. Cell. Physiol. 98*, 283-298.
Sippel, A. E., Hynes, N., Groner, B., & Schütz, G. (1977) *Eur. J. Biochem. 77*, 141-151.
Supowit, S. C., & Rosen, J. M. (1980) *Biochemistry 19*, 3452-3460.
Tedeschi, M. V., Colbert, D. A., & Fausto, N. (1978) *Biochim. Biophys. Acta 521*, 641-649.
Thompson, E. B., Tomkins, G. M., & Curran, J. E. (1966) *Proc. Natl. Acad. Sci. U.S.A. 56*, 296-303.
Tomozawa, Y., & Sueoka, N. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 6305-6309.
Van Ness, J., Maxwell, I. H., & Hahn, W. E. (1979) *Cell (Cambridge, Mass.) 18*, 1341-1349.
Wilkes, P. R., Birnie, G. D., & Paul, J. (1979) *Nucleic Acids Res. 6*, 2193-2208.
Winick, M., & Noble, A. (1965) *Dev. Biol. 12*, 451-466.
Young, B. D., Birnie, G. D., & Paul, J. (1976) *Biochemistry 15*, 2823-2829.

Chemical Modifications of Atractyloside and Bongkrekic Acid Binding Sites of the Mitochondrial Adenine Nucleotide Carrier. Are There Distinct Binding Sites?†

Marc R. Block, Guy J. M. Lauquin, and Pierre V. Vignais*

ABSTRACT: The nature of the binding sites for two specific inhibitory ligands of the ADP/ATP carrier in beef heart mitochondria has been investigated by means of chemical modifications with 2-hydroxy-5-nitrobenzyl bromide (HNB), a rather selective reagent for tryptophanyl residues, and 2,3-butanedione and phenylglyoxal, two reagents which modify arginyl residues. Atractyloside binding, but not bongkrekic acid binding, was rapidly inactivated by HNB. Atractyloside binding was also rapidly inactivated by butanedione and phenylglyoxal whereas bongkrekic acid binding was only slowly inactivated by these reagents. In all cases, inactivation decreased the number of high affinity binding sites without significant modification of the K_d value of the remaining sites; furthermore, specific protection of atractyloside or bongkrekic acid binding was afforded by preincubation with the homologous ligand. Inhibition of atractyloside binding by HNB was accompanied by the binding of HNB to the ADP/ATP carrier protein, as shown by examination of the spectral properties of the purified protein; protection against HNB inhibition by preincubation of mitochondria with atractyloside was correlated with a decrease in the amount of bound HNB. The sulfonium salt of HNB did not interfere with atractyloside binding. As HNB is a penetrable reagent in contrast to its sulfonium salt, the chemical reactivities of the two compounds being similar, it is inferred that the amino acid residues modified by HNB are located in the hydrophobic region of the ADP/ATP carrier molecule. Both atractyloside and bongkrekic acid bindings were inhibited by phenylglyoxal and butanedione, but the binding of atractyloside was inactivated at least 3 times faster than that of bongkrekic acid by these reagents. The reaction order with respect to phenylglyoxal concentration was 1 for inactivation of atractyloside binding and 2 for inactivation of the bongkrekic acid binding. Inactivation of atractyloside and bongkrekic acid binding by phenylglyoxal was studied as a function of the specific binding of [^{14}C]phenylglyoxal, sensitive to atractyloside and bongkrekic acid, respectively. Complete inactivation of atractyloside binding required the incorporation of 1 mol of [^{14}C]phenylglyoxal per mol of carrier dimer (M_r 60000), indicating a mechanism of half-site reactivity for the atractyloside site of the ADP/ATP carrier. Full inactivation of bongkrekic acid binding required at least twice as much phenylglyoxal. The data are compatible with the hypothesis that the ADP/ATP carrier protein is an asymmetric protein spanning the inner mitochondrial membrane, its asymmetry being reflected by distinct preexisting binding sites for atractyloside and bongkrekic acid.

The mitochondrial ADP/ATP carrier is specifically recognized and inhibited by two natural inhibitors, atractyloside and bongkrekic acid. These two inhibitors are characterized by a number of puzzling binding properties. They both bind specifically to the ADP/ATP carrier protein. They compete with each other for binding; yet, atractyloside is a nonpenetrant inhibitor which inhibits ADP/ATP transport when externally added to mitochondria whereas bongkrekic acid inhibits ADP/ATP transport only under conditions which favor its penetration into the matrix space of mitochondria (for review, cf. Vignais, 1976). In inside-out submitochondrial particles, the reverse situation holds (Lauquin et al., 1977), indicating that the binding asymmetry of the ADP/ATP carrier with respect to atractyloside and bongkrekic acid is not really due to the different permeability of the mitochondrial membrane to the two inhibitors but is inherent to the topography or the functioning of the carrier. Two hypotheses have been formulated to take into account the binding properties of atractyloside and bongkrekic acid. In the first case (Block et al., 1979), it is assumed that the carrier protein spans the membrane and possesses a binding site for atractyloside on the outer (cytosolic) face and another one for bongkrekic acid on the inner (matrix) face; the interaction between the two sites would then be indirect. In the second alternative (Klingenberg, 1976), it is supposed that atractyloside and

† From the Laboratoire de Biochimie (CNRS/ERA No. 903 et INSERM U.191), Département de Recherche Fondamentale, Centre d'Etudes Nucléaires de Grenoble 85X, 38041 Grenoble cedex, and Faculté de Médecine de Grenoble, France. *Received April 3, 1980.*

0006-2960/81/0420-2692$01.25/0

bongkrekic acid bind to the same site as ADP or ATP on the carrier and that this site is alternatively oriented to the outside and the inside. One way to discriminate between the two hypotheses is to test the effect of specific chemical modifications on the binding efficiency of the carrier with respect to atractyloside and bongkrekic acid. The protein modifying reagents 2-hydroxy-5-nitrobenzyl bromide (HNB),[1] a rather selective reagent for tryptophan (Koshland et al., 1964), and 2,3-butanedione and phenylglyoxal, two selective modifiers of arginine residues (Riordan, 1973; Takahashi, 1968), were selected on the basis that they are true inactivating reagents, inhibiting binding of atractyloside or/and bongkrekic acid in an all-or-none process without altering the affinity for these ligands under conditions of partial inhibition. Although it cannot be excluded that the chemical modifiers used reacted at a distance from the atractyloside and bongkrekic acid sites, the all-or-none quality of their inhibitory effect supports the idea that they react at the atractyloside or bongkrekic binding acid sites. The results presented in this paper suggest that atractyloside and bongkrekic acid binding sites differ by at least a sequence of amino acids.

Experimental Procedures

Materials. [7-^{14}C]Phenylglyoxal was obtained from the Commissariat à l'Energie Atomique, Saclay, France. HNB was purchased from Pierce, phenylglyoxal from Fluka, and butanedione from Aldrich.

Beef heart mitochondria were prepared according to Smith (1967). Inside-out submitochondrial particles were routinely obtained by sonication of frozen beef heart mitochondria as described by Beyer (1967).

Partial purification of chemically modified ADP/ATP carrier protein from beef heart mitochondria was performed by hydroxylapatite chromatography of a Triton extract of mitochondrial particles (Krämer & Klingenberg, 1979). The yield of purification was similar for the unliganded control and the modified ADP/ATP carrier protein, indicating similar chromatographic behavior with the batch of hydroxylapatite used in the present experiments (Bio-Gel HTP, Bio-Rad, batch 18384). Protein concentration was determined by the Lowry method.

Protein Modifications. Routinely the mitochondrial suspension was incubated at 20 or 25 °C for an appropriate period of time with the reagent. The mitochondria were separated from the reagent by centrifugation. The resuspended mitochondria were used for binding assays with [^{3}H]atractyloside and [^{3}H]bongkrekic acid.

A stock solution of 0.1 M HNB was freshly prepared in anhydrous dimethylformamide. Aliquots of the HNB solution were added under vigorous stirring to the mitochondrial suspension at a concentration of 0.2 mg of protein/mL in 120 mM KCl, 20 mM Mes, pH 6.5, and 2 mM β-mercaptoethanol. Rapid mixing was essential because HNB is quickly hydrolyzed in water (the half-time of hydolysis is shorter than 1 min). After 30 min of incubation with the reagent, the particles were recovered by centrifugation and resuspended in the same saline buffer as above for binding assays with [^{3}H]-atractyloside and [^{3}H]bongkrekic acid. The incubation was terminated by centrifugation. The pellet of sedimented mitochondria was solubilized by 1 mL of 5% Triton X-100 and 0.5 M NaCl at 20 °C, and its radioactivity was counted by scintillation. The mixture was acidified with HCl to avoid quenching due to the strong yellow color of the nitrophenol moiety of HNB bound to mitochondria.

The HNB-labeled ADP/ATP carrier protein was extracted from mitochondria by Triton X-100 and purified by hydroxylapatite chromatography, as described for the unliganded carrier (Krämer & Klingenberg, 1979), and its absorption spectrum was recorded with a Cary Model 219 spectrophotometer after exhaustive dialysis.

Treatment of mitochondria with butanedione was carried out as described by Marcus et al. (1976) for modification of mitochondrial ATPase. A 0.1 M solution of butanedione was freshly prepared in 0.1 M borate buffer, pH 7.8. The pH was readjusted to 7.8 with 1 N NaOH. After a 5-min preincubation period of the mitochondrial particles (1 mg/mL) in 70 mM borate buffer, pH 7.8, and 50 mM KCl at 25 °C, a sample of the butanedione–borate solution was added. After appropriate incubation periods, 1-mL fractions were withdrawn and added to 4 mL of chilled 50 mM KCl and 70 mM Mes buffer (pH 6.5). The acidic pH and the low temperature were sufficient to stop the reaction (Riordan, 1973). Chemical modification by phenylglyoxal was conducted in the same manner as for butanedione, with mitochondria preincubated with borate buffer, except that phenylglyoxal was dissolved in methanol or dimethylformamide, and the borate buffer was made of 50 mM borate, pH 7.8, and 0.2 M sucrose.

[^{14}C]Phenylglyoxal Binding Assay. Particles were used at a final concentration of 1 mg of protein/mL in 50 mM borate buffer, pH 7.8, and 0.2 M sucrose and preincubated for 5 min at 25 °C in this buffer. At time zero, [^{14}C]phenylglyoxal was added at concentrations varying between 3 and 10 mM and incubated with the particles for periods of time ranging between 30 and 55 min at 25 °C. In the blanks run under similar conditions, 10 μM atractyloside or 10 μM bongkrekic acid was added prior to [^{14}C]phenylglyoxal. The reaction was stopped both by cooling to 0 °C and by lowering the pH to 6.6 by a 5-fold dilution in 50 mM KCl and 70 mM Mes, pH 6.5. After centrifugation, the sedimented particles were solubilized by Triton X-100, and the ADP/ATP carrier protein was purified on hydroxylapatite column as described. Triton X-100 was removed from the protein solution by precipitation of the protein in acetone at −20 °C overnight. Protein was solubilized with 10% $NaDodSO_4$ and 8 M urea and then diluted with water to 4% $NaDodSO_4$. The protein solution was then exhaustively dialyzed against 4 L of 3 M urea for 20 h at 28 °C at pH 6 to remove noncovalently bound [^{14}C]phenylglyoxal. By subtraction of the blanks, the [^{14}C]phenylglyoxal binding sensitive to atractyloside or bongkrekic acid could be determined.

[^{3}H]Atractyloside and [^{3}H]Bongkrekic Acid Binding Assays. [^{3}H]Atractyloside and [^{3}H]bongkrekic acid were prepared as previously described (Brandolin et al., 1974; Lauquin & Vignais, 1976). They were added at the appropriate concentrations to chemically modified mitochondria (1 mg of protein) in a standard saline buffer made of 120 mM KCl and 10 mM Mes, pH 6.5, except in the case of mitochondria chemically modified by butanedione or phenylglyoxal where the buffer was 50 mM KCl and 70 mM Mes, pH 6.5. The final volume was 5 mL. When [^{3}H]bongkrekic acid was used, the mitochondria were preincubated for 15 min at 25 °C in an hypotonic medium made of 0.05 M sucrose, 0.05 M phosphate buffer, pH 7.4, and 5 mM $MgCl_2$. This pretreatment substantially decreased low affinity binding without changing the specific high affinity binding (Lauquin & Vignais, 1976). Following chemical modification, the mito-

[1] Abbreviations used: HNB, 2-hydroxy-5-nitrobenzyl bromide; Mes, 2-(*N*-morpholino)ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate.

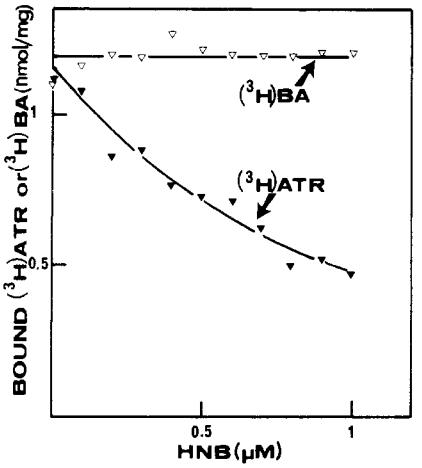

FIGURE 1: Effect of increasing concentration of HNB on [^{3}H]-atractyloside and [^{3}H]bongkrekic acid binding to beef heart mitochondria. The preincubation with different concentrations of HNB for 30 min at 20 °C was performed as described under Experimental Procedures. After centrifugation, the mitochondrial pellets were resuspended in saline medium for binding assays with [^{3}H]bongkrekic acid ([^{3}H]BA) and [^{3}H]atractyloside ([^{3}H]ATR) (cf. Experimental Procedures). The concentrations of [^{3}H]BA and [^{3}H]ATR used were 0.4 and 0.6 μM, respectively. At these concentrations, the high affinity binding sites for BA and ATR are saturated.

chondria were incubated with either [^{3}H]atractyloside or [^{3}H]bongkrekic acid. After a 40-min incubation at 0 °C, the mitochondria were collected by centrifugation and the pellets treated with 1 mL of 5% Triton X-100 and 0.5 M NaCl at 20 °C. Radioactivity was estimated by liquid scintillation.

Results

Effect of HNB on Atractyloside and Bongkrekic Acid Binding. Under the binding conditions described (cf. Experimental Procedure), the atractyloside and bongkrekic acid high affinity binding sites of beef heart mitochondria were saturable; the saturation plateau of the binding curves corresponded to 1.0–1.2 mol of high affinity sites per mg of protein, and the K_d values were between 20 and 30 nM [see Block et al. (1979)]. As shown in Figure 1, HNB (up to 1 mM) inhibited the binding of atractyloside without altering the binding of bongkrekic acid. Above 1 mM HNB, some inhibition of bongkrekic acid binding was observed. However, this might be due to a nonspecific detergent effect observed with high concentrations of HNB. As HNB is a penetrable reagent, it is expected to reach the bongkrekic acid sites located on the inner face of the mitochondrial membrane. However, to demonstrate that the lack of inhibition of bongkrekic acid binding by HNB was due to a lack of amino acid residue(s) reactive to HNB, and not to inaccessibility, the effect of HNB on the binding of bongkrekic acid to inside-out submitochondrial particles was tested; no inhibition was observed for concentrations of HNB lower than 1 mM, which corroborates that HNB does not react with the bongkrekic acid site.

Interestingly, HNB decreased the total number of atractyloside sites without altering the K_d value for the remaining sites (Figure 2). This points to a true inactivation effect of HNB which inhibits irreversibly the atractyloside site by chemical modification of amino acid(s) located at this site.

Dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium, a water-soluble derivative of HNB of similar specificity (Horton & Tucker, 1970), was tested in the same range of concentrations as HNB but did not exhibit any significant effect on atractyloside binding. As HNB has the ability to penetrate membranes whereas the sulfonium salt of HNB does not, it may be inferred that the amino acid residue(s) modified by HNB

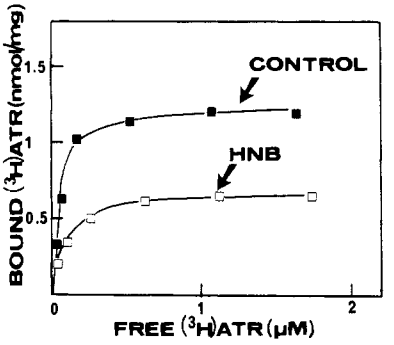

FIGURE 2: Atractyloside binding to control and HNB-pretreated mitochondria. The conditions were as described under Experimental Procedures. The final concentration of HNB was 0.8 mM.

Table I: Effect of Atractyloside and Nucleotides on Inhibition of Atractyloside Binding by HNB[a]

additions	[^{3}H]ATR binding (nmol mg^{-1})	additions	[^{3}H]ATR binding (nmol mg^{-1})
none	1.04	ADP + HNB-Br	0.62
HNB-Br	0.55	AMP + HNB-Br	0.50
ATR + HNB-Br	0.95	AMPNP + HNB-Br	0.70
ATP + HNB-Br	0.65	GDP + HNB-Br	0.54

[a] Beef heart mitochondria (1 mg of protein in 5 mL of 120 mM KCl and 10 mM Mes, pH 6.5, and 2 mM β-mercaptoethanol) were preincubated at 20 °C for 10 min with atractyloside (1 μM) or nucleotides (50 μM). Then HNB in dimethylformamide was added under vigorous stirring to a final concentration of 0.8 mM. After 30 min of incubation at 20 °C, the suspension was centrifuged at 20000*g* for 5 min. The mitochondria pellet was resuspended in 5 mL of 120 mM KCl and 10 mM Mes, pH 6.5, with 1 μM [^{3}H]atractyloside and then incubated for 40 min at 0 °C. After centrifugation, the pellet was solubilized by Triton X-100 and assayed for radioactivity as described under Experimental Procedure.

are located in the hydrophobic region of the ADP/ATP carrier molecule.

Although HNB is rather selective for tryptophan, it may also react with cysteinyl residues and tyrosyl residues (Koshland et al., 1964). Since the mitochondria were supplemented with β-mercaptoethanol, the readily accessible cysteinyl residues located in the outer region of the carrier, in the mitochondrial membrane, can be excluded as possible candidates for HNB modification. Therefore, tryptophanyl or tyrosyl residues in an hydrophobic environment and cysteinyl residues not readily accessible to β-mercaptoethanol are potential candidates for HNB modification leading to a loss of atractyloside binding capacity.

Preincubation of mitochondria with atractyloside prior to HNB addition resulted in full protection of atractyloside binding sites (Table I). ATP, ADP, and AMPNP showed a limited, but significant, protective effect; in contrast, AMP and GDP were totally ineffective. These latter data are typical of the well-known specificity of ADP/ATP transport for ADP, ATP, and closely related analogues (Vignais, 1976).

To ascertain that inactivation of atractyloside binding is due to the direct binding of HNB to the ADP/ATP carrier protein in mitochondria, the carrier protein was purified from HNB-treated mitochondria, exhaustively dialyzed, and its spectral properties were examined after alkalinization of the medium. The spectrum showed a peak at 410 nm that is typical of the covalently bound phenolic chromophore (Naik & Horton, 1973). When mitochondria were preincubated with an excess of atractyloside prior to treatment with HNB, the purified

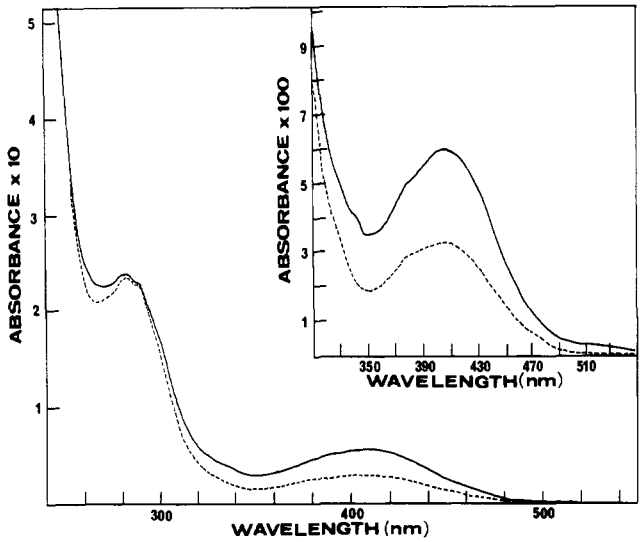

FIGURE 3: Absorption spectrum of ADP/ATP carrier purified from HNB-treated mitochondria. Effect of pretreatment with atractyloside. Two parallel assays were carried out. In the first one, beef heart mitochondria (20 mg of protein in 100 mL of 120 mM KCl, 20 mM Mes, pH 6.5, and 2 mM β-mercaptoethanol) were incubated for 30 min at 0 °C before addition of HNB (one mM final concentration). The second assay was similar except that the medium was supplemented with atractyloside (6 nmol/mg of protein). The two suspensions of mitochondria were incubated with HNB at 20 °C under vigorous stirring for 30 min. On small aliquots, it was determined that atractyloside binding in the control was inactivated by HNB to an extent of 26% and in atractyloside-pretreated mitochondria to an extent of 5%. The difference, 21%, therefore referred to specific inactivation of the atractyloside site. The remaining suspensions were centrifuged. The mitochondria recovered by centrifugation were washed twice and solubilized by a mixture of 4% Triton X-100 and 0.5 M NaCl. The ADP/ATP carrier protein was purified by hydroxylapatite chromatography as described under Experimental Procedures. Triton was removed by acetone precipitation of the protein overnight at −20 °C. Protein was solubilized in 10% $NaDodSO_4$ and 8 M urea and diluted with water to 4% $NaDodSO_4$. Free HNB was removed by dialysis under stirring against 4 L of 3 M urea for 20 h at 28 °C. The two protein solutions were adjusted to 0.1 mg of protein/mL in a medium made of 3% $NaDodSO_4$, 3 M urea, and 0.1 N NaOH. Differential spectra were recorded against a solution of 3% $NaDodSO_4$, 3 M urea, and 0.1 N NaOH.

ADP/ATP carrier exhibited a spectrum in which the 410-nm peak was markedly depressed, in agreement with the expected protective effect of atractyloside on the binding of HNB to the atractyloside site of the carrier (Figure 3). However, the 410-nm peak was not completely abolished, indicating that, in addition to HNB-sensitive amino acids which play a critical role in atractyloside binding, there are HNB-reactive amino acids located at a distance from the atractyloside site. In the experiment of Figure 3, HNB inactivated the control mitochondria by 26%; the atractyloside-preincubated mitochondria were inactivated by only 5%. On the basis of an ϵ_{410nm} of 18900 M^{-1} cm^{-1} for HNB (Barman & Koshland, 1967), the difference in absorption of the HNB-modified carrier proteins, nonprotected and protected by atractyloside, was used to estimate the number of amino acid residues modified when the atractyloside site is fully inactivated. The calculation yielded a value of about 2 mol of amino acid residue per mol of ADP/ATP carrier of M_r 30000, after tentative extrapolation to 100%.

Effects of Butanedione and Phenylglyoxal on Atractyloside and Bongkrekic Acid Binding. Incubation of beef heart mitochondria with 2,3-butanedione in borate buffer resulted in inhibition of both atractyloside binding and bongkrekic acid binding. In the case of partial inhibition, the total number of atractyloside and bongkrekic acid sites was decreased

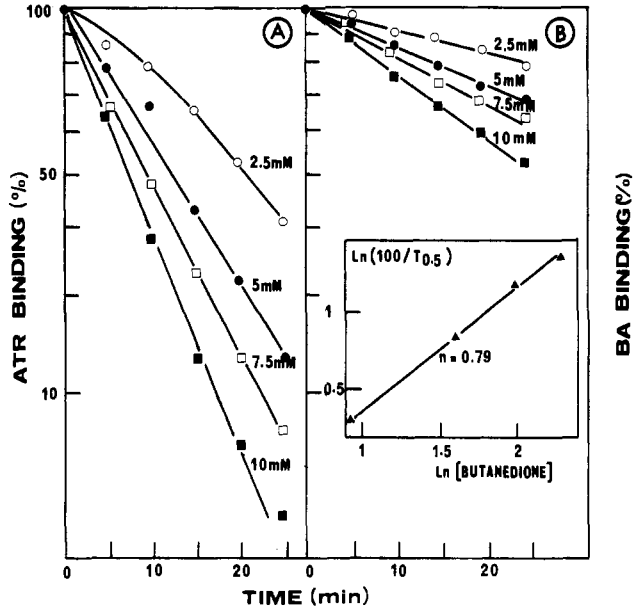

FIGURE 4: Semilogarithmic plot of the inhibition time course of atractyloside and bongkrekic acid binding at different butanedione concentrations. The incubation medium contained 70 mM borate buffer (pH 7.8), 50 mM KCl, 1 mg/mL mitochondrial protein, and various concentrations of butanedione as indicated in the figure. After incubation at 25 °C for the times indicated, 1-mL aliquots were withdrawn and diluted with 4 mL of cold 50 mM KCl and 70 mM Mes, pH 6.5, supplemented with 0.6 μM [^{3}H]atractyloside (A) or 0.4 μM [^{3}H]bongkrekic acid (B), concentrations corresponding to the high affinity saturation plateau. At time zero, the amounts of bound atractyloside and bongkrekic acid were 1.2 and 1.1 nmol/mg of protein, respectively. Bongkrekic acid binding was stable through the incubation period whereas atractyloside binding slowly decreased to 1.0 nmol/mg of protein. Appropriate corrections were made for this decrease. The percentage of binding was related to a control experiment without butanedione. The inset shows the determination of the order of the reaction of inhibition of bongkrekic acid binding with respect to butanedione; the slope of the plot gives an n value of 0.79.

without significant alteration of the K_d value. The K_d values for atractyloside and bongkrekic acid binding were between 20 and 30 nM for control mitochondria, mitochondria modified by butanedione up to 80% inactivation, and mitochondria modified by phenylglyoxal up to 60% inactivation. As shown in Figure 4, atractyloside binding was inhibited 3–4 times faster than bongkrekic acid binding. Since bongkrekic acid binds to the inner face of the mitochondrial membrane, it could be argued that the lower inhibitory efficiency on bongkrekic acid binding was caused by a restricted accessibility of butanedione to bongkrekic acid sites. Inside-out submitochondrial particles were therefore used since the bongkrekic acid sites in these particles are exposed to the outside. However, the inhibitory efficiency of butanedione on bongkrekic acid binding was as low with inside-out submitochondrial particles as with intact mitochondria. Examination of data in Figure 4 revealed a lag in the inactivating effect of low concentrations of butanedione on atractyloside binding. Because of this anomaly, further treatment of the kinetics of inactivation to investigate, for example, the reaction order with respect to butanedione was not possible. On the contrary, inactivation of bongkrekic acid binding followed first-order kinetics. The plot of the log of the reciprocal of the half-time of inactivation against the log of butanedione concentration (Levy et al., 1963) yielded a straight line with a slope of 0.79 (inset, Figure 4). Two other experiments gave slope values of 0.81 and 0.89. These data suggest that 1 mol of butanedione inactivates 1 mol of bongkrekic acid binding site.

Table II: Effect of Preincubation with Ligands on the Inactivating Effect of Butanedione on Atractyloside and Bongkrekic Acid Binding Sites[a]

ligand added prior to butanedione treatment	half-time of inactivation (min)	
	ATR binding	BA binding
none	15	30
ATR	>300	21
BA	nd	>120
ADP	18	57

[a] Beef heart mitochondria (30 mg/mL) in 120 mM KCl and 10 mM Mes, pH 6.5, were preincubated with atractyloside (ATR) (3 nmol/mg of protein), bongkrekic acid (BA) (3 nmol/mg of protein), and ADP (100 nmol/mg of protein) at pH 6.5 for 5 min at 25 °C. The suspension was then diluted in 70 mM borate buffer, pH 7.8, and 50 mM KCl to a final concentration of 1 mg/mL and maintained at 25 °C. At time zero, butanedione was added to a final concentration of 5 mM for inactivation assays of atractyloside binding and to a final concentration of 10 mM for inactivation assays of bongkrekic acid binding. The reaction was terminated by both cooling to 0 °C and lowering the pH to 6.6 by a 5-fold dilution of suspension in 50 mM KCl and 70 mM Mes, pH 6.5. [^{3}H]Atractyloside or [^{3}H]bongkrekic acid was then added at a final concentration of 1 μM and left for 40 min at 0 °C. After centrifugation, the pellet was treated with Triton X-100 for determination of radioactivity. It is noteworthy that conditions of preincubation do not affect the further binding assays. (nd, not determined.)

As shown in Table II, preincubation with atractyloside prior to butanedione addition fully protected atractyloside binding capacity against butanedione inactivation. Similarly, preincubation with bongkrekic acid prevented the inactivating effect of butanedione on bongkrekic acid binding capacity. Protection by bongkrekic acid was more marked with inside-out submitochondrial particles than with mitochondria, in agreement with the better accessibility of bongkrekic acid to the inverted mitochondrial membrane; in contrast, ADP gave only slight protection. It is noteworthy that preincubation of mitochondria with atractyloside prior to butanedione treatment makes the bongkrekic acid site more susceptible to inactivation by butanedione.

Incubation of mitochondria with phenylglyoxal in borate buffer led to similar differential inactivation as that obtained with butanedione. For example, half-inactivation of atractyloside binding was obtained after 15 min of preincubation with 3 mM phenylglyoxal while after the same time of incubation with 2.5 mM phenylglyoxal virtually no inhibition of bongkrekic acid binding was detectable (Figure 5). In contrast with butanedione inactivation, there was no lag effect in phenylglyoxal inactivation. The kinetics of inactivation were typically pseudofirst order. The plot of the log of the reciprocal of the half-time of inactivation ($t_{1/2}$) against the log of phenylglyoxal concentration yielded straight lines with slopes of 0.96 for atractyloside binding and 1.96 for bongkrekic acid binding (insets, Figure 5). Another assay on phenylglyoxal inactivation gave a slope value of 0.95 for atractyloside binding and 1.80 for bongkrekic acid binding. Similarly to butanedione inactivation, preincubation with atractyloside protected atractyloside binding against inactivation by phenylglyoxal; the same homologous protection held for bongkrekic acid binding.

The rate of the reaction between phenylglyoxal and arginine is greatly affected by the type of buffer used in the reaction mixture (Rogers et al., 1978; Cheung & Fonda, 1979). This also applies for inhibition of atractyloside binding by mitochondria. When borate buffer was replaced by phosphate buffer, the kinetics of inactivation of atractyloside binding were no longer first order (not shown). The inactivation during the

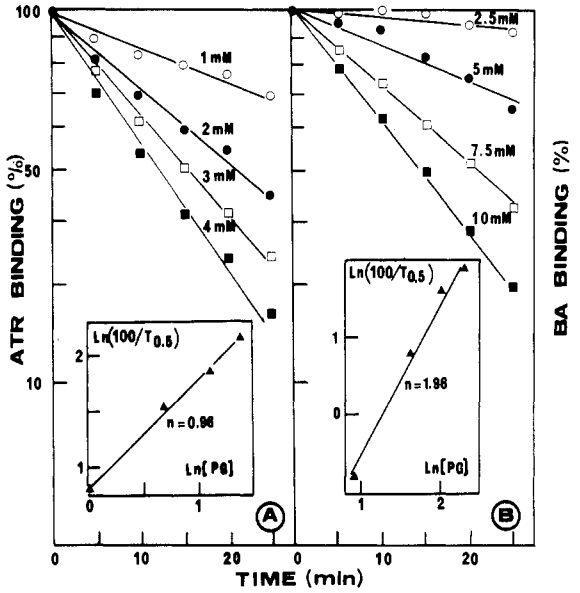

FIGURE 5: Semilogarithmic plot of the inhibition time course of atractyloside and bongkrekic acid binding at different phenylglyoxal concentrations. The incubation medium contained 50 mM borate buffer (pH 7.8), 0.2 M sucrose, 1 mg/mL mitochondrial protein, and various concentrations (mM) of phenylglyoxal as indicated in the figure. After incubation at 25 °C for the times indicated, 1-mL aliquots were withdrawn and diluted with 4 mL of cold 50 mM KCl and 70 mM Mes, pH 6.5, supplemented with 0.6 μM [^{3}H]atractyloside (A) or 0.4 μM [^{3}H]bongkrekic acid (B) as in Figure 4. The percentage of binding was related to a control experiment without phenylglyoxal. The insets show the determinations of the order of the inhibition reaction with respect to phenylglyoxal. The slopes of the plots gave an n value of 0.96 for atractyloside inactivation (A) and 1.96 for bongkrekic acid inactivation (B).

first minute of contact with phenylglyoxal was more rapid in phosphate buffer than in borate buffer; then the inactivation proceeded more slowly. On the other hand, there was no significant change in the rate of inactivation of bongkrekic acid binding, pointing again to the different reactivities of atractyloside and bongkrekic acid sites.

That covalent binding of phenylglyoxal to the ADP/ATP carrier protein in mitochondria is responsible for inhibition of both atractyloside and bongkrekic acid binding was shown by labeling with [^{14}C]phenylglyoxal. Mitochondria were treated with [^{14}C]phenylglyoxal. The ADP/ATP carrier protein was solubilized by Triton X-100, further purified by hydroxylapatite chromatography as described under Experimental Procedures, and exhaustively dialyzed. Analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis (Weber & Osborn, 1969) revealed one peak of radioactivity coinciding with a major protein band of M_r 30000 stained by Coomassie blue, which mostly consists of the ADP/ATP carrier protein. Pretreatment of mitochondria by a saturating concentration of atractyloside (10 μM) prior to labeling with [^{14}C]phenylglyoxal markedly decreased the amount of [^{14}C]phenylglyoxal bound to the 30000 molecular weight protein (blank test). The same observation holds for pretreatment of mitochondria by bongkrekic acid. The difference in bound [^{14}C]phenylglyoxal in the absence and presence of atractyloside or bongkrekic acid was considered as [^{14}C]phenylglyoxal reacting with amino acid(s) (probably arginine) playing a strategic and specific role in the binding of either atractyloside or bongkrekic acid. An attempt was made to establish a correlation between the atractyloside and bongkrekic acid sensitive binding of [^{14}C]-phenylglyoxal to the 30000 molecular weight protein in the purified mitochondrial extract after hydroxylapatite chromatography and the inactivation by phenylglyoxal of atractyloside

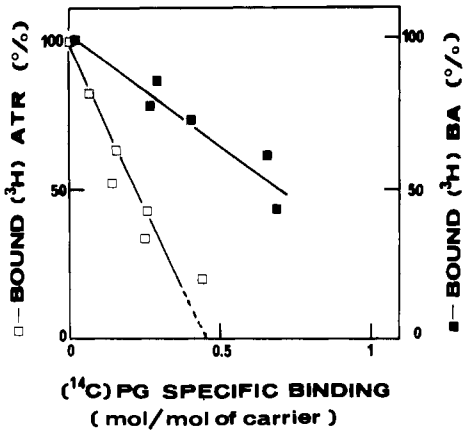

FIGURE 6: Relationship between the atractyloside and bongkrekic acid sensitive binding of [^{14}C]phenylglyoxal and inactivation of binding of [^{3}H]atractyloside and [^{3}H]bongkrekic acid by phenylglyoxal. These assays were performed with beef heart mitochondria (atractyloside binding) and with inside-out submitochondrial particles obtained by sonication of beef heart mitochondria (bongkrekic acid binding). [^{14}C]Phenylglyoxal binding was assayed in the absence and presence of 10 μM atractyloside or 10 μM bongkrekic acid. Decrease in bound [^{14}C]phenylglyoxal resulting from addition of atractyloside or bongkrekic acid was taken as [^{14}C]phenylglyoxal binding sensitive to atractyloside or bongkrekic acid (specific [^{14}C]phenylglyoxal binding). The amount of specific [^{14}C]phenylglyoxal binding was calculated on the basis of a minimal molecular weight of 30 000 for the ADP/ATP carrier and a carrier purity of 80% after hydroxylapatite chromatography and acetone precipitation (for details, see Experimental Procedures). Binding of [^{3}H]atractyloside and [^{3}H]bongkrekic acid was assayed in aliquot fractions, following [^{14}C]phenylglyoxal binding, as described under Experimental Procedures.

and bongkrekic acid binding to mitochondria. The validity of the test rested upon the assumption that the distribution of carrier units, labeled by [^{14}C]phenylglyoxal and nonlabeled, is the same in mitochondria and in the purified mitochondrial extract; this was shown to be the case with the batch of hydroxylapatite used in these experiments (cf. Experimental Procedures). It was also found that the binding of [^{14}C]-phenylglyoxal to the isolated carrier was stable. The correlation data are presented in Figure 6. In the case of the effect of phenylglyoxal on atractyloside binding, the relationship between inactivation of atractyloside binding and binding of phenylglyoxal was strictly linear up to 80% inactivation of atractyloside binding; by extrapolation, it could be calculated that full inhibition of atractyloside binding by phenylglyoxal requires 1 mol of phenylglyoxal per mol of dimer carrier of M_r 60 000, indicating a half-site reactivity for inactivation of the atractyloside site. The dose–effect curve for inactivation of bongkrekic acid binding by [^{14}C]phenylglyoxal was quite different from that obtained with atractyloside binding. It showed a linear relationship up to 40–50% inactivation which can tentatively be extrapolated to about 2 mol of bound phenylglyoxal per carrier dimer of 60 000 dalton for full inactivation; inactivation values higher than 50% were meaningless because the blank test at a high concentration of phenylglyoxal was no valid (no complete protection against phenylglyoxal inactivation by an excess of bongkrekic acid).

Discussion

The ADP/ATP carrier protein in mitochondria is characterized by a binding asymmetry with respect to atractyloside and bongkrekic acid; atractyloside attacks the carrier from the outside of the mitochondrial membrane and bongkrekic acid from the inside. To distinguish between the hypotheses that atractyloside and bongkrekic acid sites are different (double inhibitor site gated carrier) or correspond to a same sequence of amino acids oriented either to the outside or the inside (single-site reorientable carrier) (see introduction), we have analyzed the inhibitory effects of three protein-modifying reagents, HNB, butanedione, and phenylglyoxal, on atractyloside and bongkrekic acid binding. A parallel study of ADP/ATP transport was not possible due to leakage of internal adenine nucleotides, possibly due to alteration of the mitochondrial membrane.

The interaction of chemical modifiers with amino acids at specific sites of proteins (for example, catalytic sites in enzymes) is sometimes difficult to assess. In fact, modification of an amino acid at a distance from the specific site may lead to a change of conformation of this site that consequently looses its reactivity for a specific ligand. However, in such a case, one may expect that the chemical modification alters not only the binding capacity of the protein but also its affinity. HNB, butanedione, and phenylglyoxal were selected on the basis that they do not alter the K_d for atractyloside or bongkrekic acid in partially inactivated mitochondria. This is typical of an all-or-none process of inactivation. Although this behavior cannot be considered as definite evidence for direct modification of the ligand site, it brings strong support in favor of this alternative. Inhibition was also obtained by nitrobenzofurazan (unpublished) and by photooxidation in the presence of Rose Bengal (Vignais et al., 1975); however, in these cases, partial inhibition of atractyloside binding was accompanied by a marked increase in the K_d value for atractyloside, suggesting that the modifications were not restricted to the atractyloside site. Inactivation of atractyloside or bongkrekic acid binding sites by HNB, butanedione, and phenylglyoxal was prevented by pretreatment with the homologous ligands. This result further suggests the idea that the atractyloside or bongkrekic acid sites are distinct.

HNB selectively inhibited atractyloside binding without altering bongkrekic acid binding. Inhibition was accompanied by the binding of HNB to the carrier protein, and protection against HNB inactivation by preincubation with atractyloside was correlated with a decrease in the amount of bound HNB. All these data indicate that HNB interacts with the atractyloside site of the ADP/ATP carrier and does not interfere with bongkrekic acid binding. HNB is known to react readily with tryptophan and more slowly with cysteine; it is much less reactive with other amino acids such as tyrosine (Horton & Koshland, 1965). The carrier protein possesses at least four tryptophanyl residues and four cysteinyl residues (Boulay et al., 1979). The sulfonium salt of HNB was inefficient; since HNB penetrates membranes and its sulfonium salt does not, one may conclude that the HNB-sensitive residue(s) in mitochondria is not directly exposed to the external aqueous medium. Furthermore, since the binding assays with HNB were performed in the presence of β-mercaptoethanol, external cysteinyl residues were not modified. Although the HNB-modified amino acids of the carrier protein were not determined, the most probable candidates for HNB inactivation of atractyloside binding are tryptophanyl or cysteinyl residues in an hydrophobic environment. However, this conclusion must be taken with caution; in fact, there are reports of very rare, but unexpected, reactivities of HNB (cf. Radhakrishnan et al., 1970).

Butanedione in borate buffer is a more specific reagent for arginine than phenylglyoxal in the same buffer. Besides arginyl residues, phenylglyoxal reacts with sulfhydryl groups, leading to the formation of thiohemiacetals (Takahashi, 1968). However, the good parallelism between the differential inactivation of atractyloside and bongkrekic acid binding by both

phenylglyoxal and butanedione suggests that arginyl residues are primarily modified by phenylglyoxal. The order of inactivation with respect to phenylglyoxal was markedly different for atractyloside binding and bongkrekic acid binding: 1 mol of bound phenylglyoxal per mol of active site of the carrier was necessary for inactivation of the atractyloside site while 2 mol of the same reagent per mol of active site was necessary for inactivation of the bongkrekic acid site. Complementary experiments were carried out with [^{14}C]phenylglyoxal to determine the amount of ^{14}C radioactivity incorporated in the ADP/ATP carrier protein after extraction and purification of the carrier protein from the labeled mitochondria. The atractyloside and bongkrekic acid sites specifically inactivated by phenylglyoxal were differentiated as those which are protected against inactivation by preincubation with the homologous ligands. The data showed that the binding of 1 mol of [^{14}C]phenylglyoxal to a mass of carrier protein of 60 000 daltons fully inactivated atractyloside binding. Since the minimal molecular weight of the ADP/ATP carrier protein is 30 000, as determined by $NaDodSO_4$–polyacrylamide gel electrophoresis, the inactivation of atractyloside binding by phenylglyoxal suggests that the ADP/ATP carrier is organized as a dimer of M_r 60 000 characterized by a half-site reactivity; i.e., the binding of 1 molecule of inhibitor to 1 subunit of the dimer fully inhibits the binding activity of the carrier with respect to atractyloside. With the assumption that arginine is the specific target for phenylglyoxal in the ADP/ATP carrier, the stoichiometry of the reaction at the atractyloside site is therefore 1:1, in contrast with the 2:1 stoichiometry reported by Takahashi (1968) for free arginine. It is noteworthy that there are a number of enzymes in which one arginyl residue is modified by a single phenylglyoxal molecule (cf. Mornet et al., 1979). There are 14 or 15 arginyl residues in the purified ADP/ATP carrier protein (Boulay et al., 1979); this implies that phenylglyoxal reacts faster with one particular arginyl residue which is located at or very close to the atractyloside site. Whereas inactivation of atractyloside binding requires the binding of 1 mol of [^{14}C]phenylglyoxal per dimer of M_r 60 000, inactivation or bongkrekic acid binding requires the binding of at least 2 mol of [^{14}C]phenylglyoxal to the same mass of carrier. The difference in stoichiometry is compatible with the observed values of inactivation order, one for inactivation of atractyloside binding and 2 for inactivation of bongkrekic acid binding.

Recent reports have identified arginyl residues at the active site of carbamoyl-phosphate synthetase (Powers & Riordan, 1975), creatine kinase (Borders & Riordan, 1975), mitochondrial and chloroplastic ATPases (Marcus et al., 1976; Vallejos et al., 1977), 3-phosphoglycerate kinase (Philips et al., 1978), hexokinase (Philips et al., 1979), and myosin (Mornet et al., 1979). All these enzymes share the property of binding nucleotides at their active site. The ADP/ATP carrier protein is another nucleotide specific catalyst probably characterized by strategic arginyl residues.

The conclusion which emerges from the present studies is that the atractyloside site is characterized by amino acids which are in part different from those involved in the bongkrekic acid site. It is in agreement with previous data on the differential inactivation of atractyloside and bongkrekic acid sites by ultraviolet light, which were interpreted on the basis of an asymmetrical ADP/ATP carrier spanning the mitochondrial membrane and characterized by separate preexisting binding sites for atractyloside and bongkrekic acid (Block et al., 1979). Another model with a single reorientating site for ADP/ATP, atractyloside, and bongkrekic acid was advocated by Klingenberg (1976). In this model, the site is supposed to change its conformation depending on its orientation either to cytosol (C state) or the mitochondrial matrix (M state); the C state is supposed to bind atractyloside and the M state bongkrekic acid. To explain the effect of chemical modifiers in the reorientating carrier, one would have to assume that the modifiers do not attack directly the binding site but act at some distant amino acid, resulting in the immobilization of the carrier in the M or C state. For example, the inhibition of atractyloside by HNB in the case of the reorientating carrier model could be explained by an indirect effect of the HNB-modified amino acids, resulting in the immobilization of the carrier in the M state. However, this reasoning cannot explain the following observations reported in the present paper. (1) Butanedione and phenylglyoxal inhibit atractyloside and bongkrekic acid binding although to different extents and with different reaction orders. (2) The inhibitory effects of HNB, butanedione, and phenylglyoxal are all-or-none effects; i.e., the affinity of the remaining noninactivated sites for atractyloside (and bongkrekic acid) is not modified. This point is especially important in the case of butanedione and phenylglyoxal since these modifiers interfere with both atractyloside and bongkrekic acid binding. (3) Inhibition of atractyloside and bongkrekic acid binding by the different chemical modifiers is prevented by preincubation with the homologous ligands. (4) Some protection is afforded by ADP and ATP against the inactivating effect of HNB on atractyloside binding and of diketone reagents on both atractyloside and bongkrekic acid binding; this latter observation suggests a partial overlapping of ADP/ATP site(s) and atractyloside and bongkrekic acid sites. (5) The final and most important point is that ADP (or ATP) should favor the M state according to the reorientating model and should therefore increase the inhibition of atractyloside binding by the chemical modifiers. In contrast with this prediction, ADP protects significantly atractyloside binding against the inhibitory effects of modifiers. The one single-site reorientating model, i.e., a model where the substrate and inhibitor sites are strictly the same, with two different conformations depending on the outward and inward orientations of the site (Klingenberg, 1976) cannot accomodate all the above observations taken together.

Our results concerning the kinetics of inactivation of atractyloside and bongkrekic acid binding with different modifiers and the binding stoichiometry of [^{14}C]phenylglyoxal strongly suggest that the atractyloside and bongkrekic acid sites are different. This may mean, but does not prove, that the modified amino acid residues are part of the atractyloside and bongkrekic acid binding sites. In fact, the data could also be explained on the basis that the chemical modifiers act at a distance from the atractyloside and bongkrekic acid sites. For example, assuming different conformations of the ADP/ATP carrier in relation with the nature of the inhibitors (atractyloside or bongkrekic acid), one may imagine that modifications of amino acid residues outside the ligand binding site are able to freeze the carrier protein in a conformational state with a selective masking of the binding site for one of the two inhibitors, resulting in apparent binding inactivation. This conformational hypothesis still assumes two distinct sites for atractyloside and bongkrekic acid and is therefore different from the one-site carrier hypothesis, which assumes the same site for inhibitors and substrates (Klingenberg, 1976).

Our statement that atractyloside and bongkrekic acid bind to distinct sites does not preclude that the ADP/ATP site is not able to reorient [the translocation of the substrate site in a fixed pore is in fact a current view in transport mechanisms

(Singer, 1974)] or that the ADP/ATP site may share part of the atractyloside and bongkrekic acid sites. In keeping with the operational definition of sites in catalytic protein (Vallee & Riordan, 1969), the term "binding site" is used here to designate in a restrictive manner those side-chain groups of the carrier protein involved in the reversible attachment of atractyloside and bongkrekic acid. The side-chain groups which are modified by chemical reagents may be involved in binding, directly or indirectly; in the latter case, their modification may affect, in close vicinity, the topology of essential groups which then become ineffective in binding. The term "active center" (Vallee & Riordan, 1969) is less restrictive than that of binding site; in the case of enzymes, it designates those groups involved not only in binding but also in catalysis. By extension, the term "active center" applied to the ADP/ATP carrier protein may be used to designate not only the ADP/ATP binding site but also those regions of the carrier which control the catalysis of transport, for example, the atractyloside and bongkrekic acid binding sites. In other words, in spite of the fact that the atractyloside and bongkrekic acid sites are probably distinct, they may belong to the same active center.

Acknowledgments

This investigation was supported by research grants from the Fondation pour la Recherche Médicale and the Délégation Générale à la Recherche Scientifique et Technique.

References

Barman, T. E., & Koshland, D. E., Jr. (1967) *J. Biol. Chem. 242*, 5771–5776.

Beyer, R. E. (1967) *Methods Enzymol. 10*, 186–194.

Block, M. R., Lauquin, G. J. M., & Vignais, P. V. (1979) *FEBS Lett. 104*, 425–430.

Borders, C. L., & Riordan, J. F. (1975) *Biochemistry 14*, 4699–4704.

Boulay, F., Lauquin, G. J. M., & Vignais, P. V. (1979) *FEBS Lett. 108*, 390–394.

Brandolin, G., Meyer, C., Defaye, G., Vignais, P. M., & Vignais, P. V. (1974) *FEBS Lett. 46*, 149–153.

Cheung, S. T., & Fonda, M. L. (1979) *Biochem. Biophys. Res. Commun. 90*, 940–947.

Horton, R. H., & Koshland, D. E., Jr. (1965) *J. Am. Chem. Soc. 87*, 1126–1132.

Horton, R. H., & Tucker, W. P. (1970) *J. Biol. Chem. 245*, 3397–3401.

Klingenberg, M. (1976) *Enzymes Biol. Membr. 3*, 383–438.

Koshland, D. E., Jr., Karkhanis, Y. D., & Latham, H. G. (1964) *J. Am. Chem. Soc. 86*, 1448–1449.

Krämer, R., & Klingenberg, M. (1979) *Biochemistry 18*, 4209–4215.

Lauquin, G. J. M., & Vignais, P. V. (1976) *Biochemistry 15*, 2316–2322.

Lauquin, G. J. M., Villiers, C., Michejda, J. W., Hryniewiecka, L. V., & Vignais, P. V. (1977) *Biochim. Biophys. Acta 460*, 331–345.

Levy, H. M., Leber, P. D., & Ryan, E. M. (1963) *J. Biol. Chem. 238*, 3654–3659.

Marcus, F., Schuster, S. M., & Lardy, H. A. (1976) *J. Biol. Chem. 251*, 1775–1780.

Mornet, O., Pantel, P., Audemard, E., & Kassab, R. (1979) *Eur. J. Biochem. 100*, 421–431.

Naik, V. R., & Horton, R. H. (1973) *J. Biol. Chem. 248*, 6709–6717.

Philips, M., Roustan, C., Fattoum, A., & Pradel, L. A. (1978) *Biochim. Biophys. Acta 523*, 368–376.

Philips, M., Pho, D. B., & Pradel, L. A. (1979) *Biochim. Biophys. Acta 566*, 296–304.

Powers, S. G., & Riordan, J. F. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2616–2620.

Radhakrishnan, T. M., Braskaw, R. A., Deranleau, D. A., & Neurath, H. (1970) *FEBS Lett. 7*, 72–76.

Riordan, J. F. (1973) *Biochemistry 12*, 3915–3923.

Rogers, T. B., Borresin, T., & Foeney, R. E. (1978) *Biochemistry 17*, 1105–1109.

Singer, S. J. (1974) *Annu. Rev. Biochem. 43*, 805–833.

Smith, A. L. (1967) *Methods Enzymol. 10*, 81–86.

Takahashi, K. (1968) *J. Biol. Chem. 243*, 6171–6179.

Vallee, B. L., & Riordan, J. F. (1969) *Annu. Rev. Biochem. 38*, 733–794.

Vallejos, R. H., Viale, A., & Andreo, C. S. (1977) *FEBS Lett. 84*, 304–308.

Vignais, P. V. (1976) *Biochim. Biophys. Acta 456*, 1–38.

Vignais, P. V., Brandolin, G., Lauquin, G. J. M., Morel, F., & Vignais, P. M. (1975) *Biomembr. Lipids, Proteins Recept., Proc. NATO Adv. Study Inst. 10*, 167–189.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.