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REVIEW

SH-Group Reagents as Tools in the Study of Mitochondrial Anion Transport

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SH-group reagents are known to react with a large number of cysteine residues of proteins. Little specificity in their action on individual systems should be expected. In accordance with this, SH-group reagents inhibited a variety of mitochondrial enzymes, including ATPase, components of the electron transport chain, and so on (see Gautheron [1] for review). In 1966 we found that *p*-hydroxymercuribenzoate (PMB)* specifically inhibited the respiration stimulated by either ADP or by Ca^{2+} in mitochondria but not the respiration stimulated by uncouplers [2]. From this we inferred—erroneously as we now know—that PMB was an inhibitor of mitochondrial energy transfer. It soon became apparent in two laboratories at the same time that inhibition of respiration by PMB or by mersalyl was due to their inhibitory action on P_i entry into mitochondria [3–7]. In short time it was reported that other SH-group reagents, NEM, and DTNB were also inhibitors of P_i transport [8] and in the following years several SH-group reagents were added to this series [9–12].

We first thought that SH-group reagents were specific inhibitors of P_i

*Abbreviations: ASPM: *N*-(*N*-acetyl-4-sulfamoylphenyl)maleimide; CPDS: 6,6′-dithionicotinic acid; DTT: dithioerythritol; DTNB: 5,5′-dithiobis(2-nitrobenzoic acid); HEDD: β-hydroxyethyl-2,4-dinitrophenyl disulfide; NEM: *N*-ethylmaleimide; PCMS: *p*-chloromercuriphenylsulfonate; PMB: *p*-hydroxymercuribenzoate.

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transport. It turned out that at very low mercurial concentrations it was mainly P_i transport that was inhibited but by increasing the mercurial concentration more and more transport processes were successively affected [13–16]. Different SH-group reagents inhibited single transport processes in a selective way, affecting one and leaving the other intact [13, 17–19]. It was with their use that the existence of separate transport pathways (carriers) for P_i could be clearly distinguished [18, 19].

In the last few years several SH-group reagents proved to be valuable in situ probes of the carrier proteins embedded into the mitochondrial inner membrane; furthermore, they served as labels for the isolation of these proteins. These novel aspects justify the present review.

Chemistry of SH-Group Reagents

It is surprising that of the enormous list of compounds known to react with SH groups [20–22], only few have been used for studies of mitochondrial functions [23]. Of the reagents hitherto used probably not a single one is absolutely specific for SH groups: They may react under unfavorable conditions with other groups known to be present in proteins. There are good reasons, however, to assume that most of the effects reported with the "SH-group reagents" were really due to their reaction with thiols [20].

In the following the reagents are first classified according to the bond formed with the SH group; aspects of reversibility of the reaction as well as kinetics are covered. Second the reagents are treated according to characteristics of the whole molecule (polarity, lipid solubility) by which selectivity in action of the different reagents is mostly determined.

Types of Bonds Formed between SH Groups and Reagents

Mercaptides. SH groups readily form mercaptides with mercurials in a reversible reaction

$$R_1\text{–}SH + Hg\text{–}R_2 \rightleftharpoons R_1\text{–}S\text{–}Hg\text{–}R_2 \qquad (1)$$

The stability constant for methyl-Hg^+ and some soluble proteins is in the range of 10^{20}–10^{22} [20]. The reaction occurs rapidly with exposed SH groups even at +2°C. The mercaptide formation with proteins is easily reversed on addition of either mono- or dithiols and the equilibrium shown in Equation

(1) can be shifted by excess thiol added to have the original SH group free:

$$R_1\text{–}S\text{–}Hg\text{–}R_2 + R_3\text{–}SH \rightleftarrows R_1\text{–}SH + R_3\text{–}S\text{–}Hg\text{–}R_2 \qquad (2)$$

Of the mercaptide-forming agents the organic mercurial compounds, PMB, PCMS, and mersalyl, were used most extensively. Inorganic Hg^{2+} ions act in a similar way but much less specifically than the organic compounds. It was $HgCl_2$ which was first shown to inhibit P_i transport in bacteria [24], a process probably very close to mitochondrial P_i transport.

Recently ^{14}C-labeled mersalyl became available and its binding to mitochondria was studied (unpublished). A "high" and a "low" affinity binding was clearly distinguished, the apparent K_d of the high affinity binding being about 5–8 μM. The total number of the "high affinity" sites was about 2.5–3 nmol/mg mitochondrial protein in rat liver mitochondria. The apparent K_d depended on the pH of the medium: It was somewhat lower at pH 6.5 and higher at pH 8.0. This corresponds to earlier findings in which less mersalyl was required to inhibit 50% of P_i transport at pH 6.5 than at higher pH values [25].

The mersalyl binding sites overlap—at least partially—with DTNB-reactive sites. Pretreatment of liver mitochondria by DTNB prevented most of the [^{14}C]mersalyl binding. This also corresponds to transport inhibition studies. A difficulty arose when a similar experiment was performed with NEM. Pretreatment of liver mitochondria with NEM did not decrease [^{14}C]mersalyl binding; moreover a slight increase in binding occurred. From transport studies it was unequivocal that NEM prevented the binding of mersalyl to the P_i transport system and conversely mersalyl prevented the subsequent binding of NEM to the same system [26–29]. It has therefore been concluded by us that NEM made some SH groups available for mersalyl which were originally unreactive.

Disulfides. SH groups interact with compounds containing disulfide bonds with the formation of mixed disulfides:

$$R_1\text{–}SH + R_2\text{–}S\text{–}S\text{–}R_2 \rightleftarrows R_1\text{–}S\text{–}S\text{–}R_2 + HS\text{–}R_2 \qquad (3)$$

This reaction is rather slow, taking several seconds or even minutes with exposed SH groups. In the rate of reaction the ionization of both the SH group and the reagents plays a role. With cysteine the rate is accelerated by increasing the pH, i.e., with the ionization of the thiol. This relation does not apply to mitochondria: In these DTNB inhibited P_i transport more readily at pH 6.5 than at pH 7.4 [27].

The reagents in use include DTNB (Ellman's reagent), CPDS, and HEDD [1]. The effect of these inhibitors on transport was not reversed by added monothiols. 2-Mercaptoethanol was in fact used to stop the reaction

of DTNB with mitochondria [27]. The formation of the TNB complex was reversed, however, at least partially by the dithiol DTT. The action of CPDS on transport was not reversed by DTT [30].

Once the disulfide is formed between the SH group and CPDS secondary reactions may occur between the primarily reacting SH groups and some SH groups close to it with the formation of various mixed disulfides [31].

Addition of SH groups on double bonds. The activated double bond in certain maleimide derivatives is easily attacked by SH groups and an addition compound is irreversibly formed:

$$R_1\text{–}SH + R_2\text{–}CH{=}CH\text{–}R_3 \rightarrow R_1\text{–}S\text{–}CH(R_2)(CH_2\text{–}R_3) \qquad (4)$$

The reagent used most is *N*-ethylmaleimide (NEM) but different substituted derivatives such as ASPM [32, 33], *N*-benzyl-, and *N*-cyclohexylmaleimide [34] are available. Compounds of different structure, fuscin [11] and ethacrynic acid [10, 35], also belong to this group: The thiol group attacks the activated double bond. The product is not cleaved by either mono- or dithiols and the bound reagent does not migrate between various SH groups (which effect unfortunately occurs with the previously listed reagents). For this last reason radioactive labels from NEM and ethacrynic acid are easily introduced into membrane proteins (see later and Refs. 28–29, 33, 35–41).

Because the reaction is irreversible, it can be rapidly stopped by large excess of cysteine, 2-mercaptoethanol, or DTT. With this method the rate of radioactive reagent incorporation [33] and of inactivation of transport processes [25, 33] can be determined. The rate of reaction of SH groups with NEM (the latter is not ionized under the conditions used) increased with increasing pH, i.e., with the ionization of the SH group: This was the case with cysteine as well as with the SH groups of the mitochondria. The dependence on pH changed when substituted maleimide derivatives, capable of ionization like ASPM, were used. Cysteine reacted faster with ASPM at higher than at lower pH. In contrast, mitochondrial SH groups reacted faster at lower than at higher pH with ASPM [33]. The reactivity of membrane-bound thiols depends partially on their own ionization and partially also on the ionized state of the reagent.

The inhibitor of pyruvate transport, α-cyano-4-hydroxycinnamate [42, 43], turned out later to be an SH-group reagent [44]. The thiols also react in this case with the double bond of the reagent.

Alkylating agents such as iodoacetic acid and iodoacetamide have long been used for the study of SH groups of various enzymes. They have not yet been used, however, for study of mitochondrial transport.

Oxidizing agents react with SH groups but they have rarely been used as inhibitor of mitochondrial transport. One of them, diamide, was ineffective in inhibiting P_i transport at $+2^\circ C$ [45] but was later found to be partially inhibitory at $20^\circ C$ [46].

Factors Affecting Reactivity: The Role of Reagent Permeability

Low-molecular-weight thiols such as cysteine react with all the SH-group reagents just listed. Enzymologists, however, know that of the total cysteine content of an enzyme some react fast, some sluggishly, and some might be totally unreactive. Reactivity is also very much dependent on the structure of the SH-group reagent. Thus the environment of any cysteine in the polypeptide chain is decisive in reactivity. This is also supported by the recent observations of Halestrap [44]: Using derivatives of α-cyanocinnamate, the hydrophobic portion of the SH-group reagent determined its reactivity with the pyruvate carrier although the inhibition itself was clearly caused by binding the SH groups of the latter. Probably similar phenomena form the basis of the specificity observed for the effect of SH group reagents on transport reactions [23].

Besides this "microenvironmental" factor, i.e., the location of a particular cysteine within the polypeptide chain, the orientation of the protein molecule within the mitochondrion also plays a role. The carrier proteins of the inner mitochondrial membrane are either facing the outer surface or the inner surface of the membrane, or they may be buried within the hydrophobic structure of the membrane. The reactivity of their SH groups depends on whether or not the SH-group reagents are able to penetrate the membrane.

The hydrophobic "core" of the inner mitochondrial membrane is virtually impermeable for charged particles and also to most hydrophilic molecules. In intact mitochondria those SH groups which are not oriented toward the outer surface can react only with reagents which are "penetrants."

SH-group reagents can be classified according to their permeability properties, whether they are able to enter the matrix space of mitochondria [47, 48]. The experimental evidence for the "penetrating character" is their reaction with the intramitochondrial glutathione. On this basis, NEM, fuscin, ethacrynic acid, and 3-chloroethacrynic acid were considered to be penetrants. Other reagents, such as various organic mercurials, CPDS, diamide, and ferricyanide, are clearly nonpenetrants. The permeability of

some reagents may strongly depend on their applied concentration, ionization state (i.e., pH), and also on the time available for penetration. ASPM is, for example, a poor penetrant at low concentration at pH higher than 7, but it will react with glutathione within the mitochondria at higher concentration (300 μM) and below pH 7 [47].

Apparently the penetrant or nonpenetrant character of an SH-group reagent is only one and may be not the most important factor in its selective action on individual transport processes. If permeability were the major factor, then the most permeable reagent, NEM, would have the lower specificity. This is clearly not the case: A number of transport processes are inhibited by the nonpenetrating mercurials but neither by NEM nor by fuscin (see later). Probably the microenvironment of the SH group is more critical in determining its sensitivity and the selectivity of the reagent [44].

The conclusion concerning the penetrant nature of NEM drawn from its reactivity with intramitochondrial glutathione is in marked contradiction with the opinion of Rhodin and Racker [34]. These authors, on the basis of transport studies of submitochondrial particles, consider NEM to be a nonpenetrant SH-group reagent while *N*-benzyl- and *N*-cyclohexylmaleimide were thought to behave as penetrants. The reason why in the experiments quoted the *N*-benzyl- and *N*-cyclohexylmaleimide inhibited P_i transport and the *N*-ethyl derivative did not, is unclear at present. There might be a difference between beef heart and rat liver mitochondria in respect to NEM permeability. On the other hand, the assay system of P_i transport in submitochondrial particles may be responsible for this discrepancy.

Verification and Characterization of Individual Transport Pathways by SH-Group Reagents

P_i *Transport*

The first transport process which was shown to be inhibited by organic mercurials was that of P_i. This inhibition was thought to be specific. It was soon realized that organic mercurials also inhibited the transport of dicarboxylates [16, 17], this latter inhibition requiring higher mercurial concentrations than the P_i carrier [14]. Sill higher mercurial concentrations were needed to inhibit tricarboxylate and 2-oxoglutarate transport and the reaction between the SH groups of these carriers and mersalyl was slow as compared to that of the P_i carrier [14]. Inhibition of the P_i carrier corresponds to mersalyl binding to the high affinity sites and inhibition of the di- and tricarboxylate as well as 2-oxoglutarate transport is probably related to binding of the mercurial to low affinity sites.

TABLE I. The action of SH-group reagents on anion transport in mitochondria

Carrier	SH-group reagent[a]							
	Organic mercurials	DTNB	CPDS	NEM	ASPM	Ethacrynic acid	Fuscin	α-Cyano-cinnamates
Phosphate	+ (low conc)	+	+	+	+	+	+	−
Dicarboxylate	+ (high conc)	?		−				
Tricarboxylate	+ (high conc)			−				
2-Oxoglutarate	+ (high conc)			−				
Pyruvate	+			+				+
Glutamate	−	−		+			+	
Proline	+			−				
ADP/ATP[b]	−	−		+			+	

[a]When neither + nor − is indicated, the inhibition was not investigated.
[b]Sensitive only after pretreatment with ADP or ATP or "energization."

Of the four carriers, NEM exhibits much higher specificity (see Table I). It inhibits P_i transport exclusively through the P_i but not through the dicarboxylate carrier [13, 17–19]; it is without any action on the transport of di- and tricarboxylates and 2-oxoglutarate.

The differential reactivity of the P_i and of the dicarboxylate carriers toward NEM and the organic mercurials is the basic argument for their separate existence as distinct molecular species [18–19]. NEM totally inhibits the net uptake of P_i which occurs in exchange for OH^- ions [17, 49, 50]. NEM, however, does not inhibit the discharge of intramitochondrial P_i by extramitochondrial dicarboxylate or the exchange of intramitochondrial [^{32}P]phosphate for extramitochondrial [^{31}P]phosphate: The inhibition of these latter requires, besides NEM, the addition of 2-*n*-butylmalonate also [13, 17, 18, 51]. Similar conclusions can be drawn from experiments on sulfate transport. Sulfate can be exchanged against phosphate or dicarboxylate anions: This exchange is inhibited by mersalyl or 2-*n*-butylmalonate but not by NEM [52]. Therefore it was concluded that sulfate is transported by the dicarboxylate carrier.

The differential sensitivity of the P_i and the dicarboxylate carriers toward NEM made the separate evaluation of these two carriers in P_i transport possible. P_i transport in the presence of NEM is exclusively due to action of the dicarboxylate carrier whereas P_i transport measured in the presence of 2-*n*-butylmalonate (or similar inhibitors) goes through the P_i carrier. It was found that of the total 224 nmol P_i transported at 0°C/min · mg protein, 205 nmol was transported through the P_i and the rest through the dicarboxylate carrier [29, 51].

The existence of two separate molecular entities involved in P_i transport as just depicted has recently been questioned [53, 54]. 2-*n*-butylmalonate was found to release the NEM inhibition of P_i transport. Other experiments indicated that NEM changed some kinetic parameters of the dicarboxylate–P_i exchange. It was concluded by Lofrumento et al. from these and other experiments that a single carrier catalyzes all transport processes involving P_i. Nevertheless, the fact that complete inhibition of the $^{32}P_i$–$^{31}P_i$ exchange requires both NEM and 2-*n*-butylmalonate simultaneously has never been challenged.

P_i transport was found to be mersalyl sensitive in intact mitochondria from different sources, such as heart, yeast cells, and plant tissue [30, 55–57]. Under some unusual experimental conditions, the P_i transport was not inhibited by mersalyl. In one special system, when intramitochondrial P_i was generated from added ATP in the presence of an uncoupler, mersalyl was inhibitory when no Mg^{2+} was added, but mersalyl did not inhibit P_i efflux in the presence of added Mg^{2+} [45]. Addition of Mg^{2+} ions did not

affect either the mersalyl or the PMB sensitivity of P_i efflux in other systems [5, 7]. P_i transport also became mersalyl insensitive in mitochondria that previously accumulated calcium phosphate [58]. It appears that a non-specific increase in membrane permeability cannot be excluded in these latter experiments.

A mersalyl-sensitive P_i transport system was found in membrane vesicles of the bacterium *Paracoccus denitrificans* [59]. In view of the postulated bacterial origin of eukaryotic mitochondria this fact was not surprising. A mersalyl-sensitive P_i transport system was also found in the plasma membrane of bovine spermatozoa [60], a fact which indicates a more general occurrence of this carrier system (see also the pyruvate carrier).

A difference between the behavior of the P_i carriers of rat liver and pig heart was recently claimed [9, 30]. CPDS is a powerful inhibitor of the P_i carrier in both kinds of mitochondria. P_i, if present during a preincubation period, "protected" ADP-stimulated respiration and ATP synthesis against CPDS inhibition in liver but not in heart mitochondria. This finding might also be explained by an accumulation of P_i within the matrix of liver mitochondria which were incubated in a medium containing high KCl concentration: Subsequent use of P_i present in the matrix could account for the ATP synthesis observed even in the presence of CPDS [61, 62]. Thus it is not necessary to postulate a "protection" of the P_i carrier by phosphate against CPDS inactivation in liver mitochondria.

The difference between the P_i carriers from liver and heart mitochondria was also suggested because CPDS did not induce swelling (i.e., P_i retention) in the P_i-generating system from ATP *plus* uncoupler of Tyler (see Ref. 7 for the method) in heart mitochondria [9, 30]. In this system the swelling depends on the balance between the rate of intramitochondrial P_i generation, the rate of any P_i efflux, and the rate of Na^+ and K^+ entry into the matrix from the medium, this last fact frequently ignored in similar studies. The balance of these three processes could easily be different in rat liver and pig heart mitochondria with the result that rat liver mitochondria swell in the ATP + uncoupler + P_i transport-inhibitor system but heart mitochondria do not swell under similar conditions.

Transport of Citric Acid Cycle Intermediates (Dicarboxylate, Tricarboxylate, and 2-Oxoglutarate carriers)

Organic mercurials are not as specific a group of inhibitors of mitochondrial P_i transport as it was originally thought. Of the other transport processes, inhibition by mercurials of the dicarboxylate carrier was first reported [13, 16, 17, 63], and later inhibition of the tricarboxylate and 2-

oxoglutarate carriers [14]. Some specificity in mercurial action is nevertheless found: Inhibition of the three last mentioned systems requires far higher concentrations of the inhibitor than the P_i carrier; moreover the mentioned carriers are sequentially inhibited by the mercurials if a careful titration is performed [14]. This procedure was used for measuring tricarboxylate carrier activity without the disturbing interference of the dicarboxylate carrier. The tricarboxylate carrier was 50% inhibited in the presence of 55 μM mersalyl: Inhibition of the citric acid cycle intermediate carriers coincides with the binding of mersalyl to the low affinity binding sites.

There is an important difference in the reactivities of the P_i carrier and the three other systems. The P_i carrier reacts with organic mercurials within 1 sec [49], whereas the other carriers react much more slowly, the reaction taking almost 1 min [14, 64, 65]. The slow reaction indicates that the SH groups of these carriers are buried within the molecule, in contrast with the exposed SH groups of the P_i carrier. Substrates protect the former carriers from the inhibitory action of the mercurials: This protection manifests itself in decreasing the rate of interaction of the SH groups with the mercurial [65].

Mercurials were either competitive or noncompetitive inhibitors of transport in the dicarboxylate carrier, depending on the condition of the experiments [64, 65]. The P_i–P_i exchange was under all conditions competitively inhibited by mercurials. The dicarboxylate exchange reactions were competitively inhibited when the mercurial was added together with the substrate and the inhibition turned to be noncompetitive when the mercurial was added before the substrates. Palmieri et al. concluded that of the two separate substrate binding sites of the dicarboxylate carrier (i.e., the dicarboxylate and the P_i binding site) the mercurials interact directly only with the dicarboxylate binding site and the P_i binding site is only indirectly affected, being remote from the dicarboxylate binding site. Sulfate is also a substrate of the dicarboxylate carrier [52]. Kinetic study of sulfate transport revealed that sulfate and malonate bind to the same locus on the carrier whereas phosphate occupies a different site [66], confirming the existence of two substrate binding sites.

Pyruvate Carrier

The existence of a pyruvate carrier in mitochondria was postulated by Papa et al [67]. Further proof for its existence came when inhibitors of pyruvate transport were found, some of them being very specific. General thiol reagents such as mersalyl and NEM inhibited transport [68, 69]. The highly specific inhibitors of pyruvate transport, the α-cyanocinnamate

derivatives [42, 43] turned out later to react with SH groups [44, 71]. For their inhibitory properties the reactivity with SH groups was an absolute requirement. On the other hand, their rate of reaction with the model thiol compound, 2-mercaptoethanol, did not parallel their inhibitory capacity of pyruvate transport. According to Halestrap [44], the apolar part of the α-cyanocinnamate derivatives plays the most important role in the sensitivity toward the inhibitor. α-Cyano-4-hydroxycinnamate reacts very fast with mitochondria at 6°C at micromolar concentration, although general thiol reagents were not inhibitory on pyruvate transport at this low temperature. The very specific inhibition by this last thiol reagent of pyruvate transport suggests that chemical modification of the available SH-group reagents (organic mercurials, maleimide derivatives, and disulfides) could result in giving specific inhibitors for other carrier systems too.

α-Cyano-4-hydroxycinnamate inhibited pyruvate transport not only in mitochondria but also in the plasma membrane of erythrocytes [42]. The structure and the transporting properties of the mitochondrial and the plasma membranes were hitherto considered to be very different. The occurrence of a similar transport system for P_i in mitochondria and in the plasma membrane of bovine spermatozoa and of a similar transport system for pyruvate in both mitochondria and human erythrocytes indicates that a systematic comparison for transport systems of mitochondria and plasma membranes might be rewarding.

Glutamate Carrier (Glutamate-hydroxyl Antiporter)

Glutamate can enter the mitochondria by using one of the transporting systems available [72]: The relative importance of the different pathways varies in the individual organs [73, 74]. One of these pathways, the glutamate-hydroxyl exchange (which may be in fact a glutamate H^+ uniport) is sensitive toward a selected group of SH-group reagents. The aspartate–glutamate exchange is not inhibited by these latter [75, 76].

The inhibition of glutamate transport by only one class of SH-group reagents, notably NEM [77–80], fuscin [11, 79, 81], and avenaciolide [79, 82], as well as the ineffectiveness of another group of reagents, the organic mercurials and DTNB [78, 79], contributed significantly to our picture of SH-group location within this carrier. The inhibitory compounds are able to penetrate the membrane; all these reagents react with intramitochondrial glutathione, and are all lipid soluble. The reagents that do not inhibit are nonpenetrants. From these it follows that the SH groups of the glutamate carrier are either located within the hydrophobic part of the membrane or are exposed toward the matrix face [79]. Meyer and Vignais [79] pointed out

that all these inhibitors "contain a carbon–carbon double bond activated by a conjugated carbonyl double bond." There is probably an additional factor in reactivity, however, the hydrophobic portion of the molecule conferring some specificity on the SH-group reagent. This latter role is indicated by the action of certain avenaciolide analogs among which the inhibitory properties varied according to the substituents. A future screening of various substituted derivatives of either NEM or fuscin for specific transport-inhibitory properties could reveal some highly specific inhibitors.

Of the inhibitors of glutamate transport avenaciolide does not inhibit the P_i carrier [79]; its specificity as an inhibitor of the glutamate carrier only is, however, less than it was originally supposed [83].

1-Fluoro-2,4-dinitrobenzene inhibits the glutamate carrier [84] but it is not yet known whether it does so by reacting with SH groups or by being reactive with amino groups, phenolic hydroxyls, or imidazole.

Although NEM is a powerful inhibitor of the glutamate carrier, the inhibition of transport by NEM is not quite complete. In transport studies an "inhibitor stop" method can be used by the combined application of bromcresol purple and NEM, but even after combination, some transport remains after the application of the inhibitors [80, 84]. This maybe due to simple leak in the membrane eventually caused by NEM itself.

Proline Transport

Recently it was suggested that another amino acid, proline, is transported in liver mitochondria by way of a specific carrier [85]. Proline transport is inhibited by organic mercurials, mersalyl, and PMB, but not by the hydrophobic reagent NEM. Meyer suggested, therefore, that the SH groups of this carrier are located in a very hydrophilic environment.

SH-Group Reagents as in Situ Probes of Carriers

Asymmetric Orientation and Reactivity of Carriers

At least some of the metabolite carrier systems have an asymmetric position in the membrane. Most of the evidence available for this originates from action of inhibitors of the ADP/ATP carrier (see Refs. 86 and 87 for review). These inhibitors have asymmetric binding sites on the membrane: Atractyloside and its derivatives bind and act exclusively at the outer surface and bongkrekic acid and derivatives at the inner surface. It was partially on the basis of these studies that a "reorienting" model of the ADP/ATP carrier was suggested by Klingenberg et al. [88]: In this model the substrate

binding site of the carrier is cyclically reoriented during each transport cycle. By extension of this model, the P_i carrier also was considered to be both asymmetric and reorienting: It was visualized that its essential SH groups were facing either the outer or the inner surface of the membrane, reorienting with each cycle of P_i transport [89]. This hypothesis was also suggested on the basis of experiments with SH-group reagents which were considered to act exclusively on the outer side of the membrane [32, 89, 90].

SH Groups of the ADP/ATP Carrier

It was thought earlier that the ADP/ATP carrier was not significantly inhibited by SH-group reagents. This simple statement had to be modified later: Preincubation of mitochondria with the specific ligands of the carrier, either ADP or ATP, transformed the carrier to be sensitive toward the SH-group reagents NEM and fuscin [91, 92]. The required nucleotide concentration was very low, in the micromolar range. The specific nature of the nucleotide effect was substantiated by the fact that the specific inhibitor of transport, atractyloside, prevented the "unmasking" of the SH groups if it was present during the preincubation period. Only permeant SH-group reagents were active: Polar, nonpenetrating reagents such as organic mercurials were entirely inactive.

The "unmasked" sensitivity toward NEM was also reflected by an increased binding of ^{14}C-labeled NEM following ADP addition to mitochondria [38]. The SH groups thus exposed by ADP amounted to about 1–2 mole/mole of cytochrome *a* [86].

Not only transport was inhibited by the combination of NEM and ADP. Binding of atractyloside (used as the ^{35}S-labeled compound), a highly specific ligand of the carrier, was diminished by NEM if a low level of ADP was also present [92, 93].

All these changes, i.e., the ADP-induced inhibition of transport by NEM, the decreased binding of labeled atractyloside by NEM + ADP, finally the specific increase of [^{14}C]-NEM binding by low levels of ADP, strongly indicate that the binding of a specific ligand causes structural change in the carrier molecule or in its close vicinity. This is usually described as a "conformational change."

Binding of its specific ligands is not the only factor which modifies the reactivity of SH groups of the ADP/ATP carrier. "Energization" of mitochondria acts in a similar way. In mitochondria incubated in the presence of succinate as electron donor and oligomycin ("energized state") NEM blocks effectively by itself the binding of ^{3}H-labeled atractyloside [93]. Addition of low levels of ADP under these conditions makes the inhibition of [^{3}H]-

atractyloside binding complete. In mitochondria "deenergized" by addition of an uncoupler, NEM alone is entirely inactive in inhibition of [^{3}H]-atractyloside binding, and even in the presence of ADP the inhibitory effect is much less than it is in "energized" mitochondria. Accordingly, both ligand binding (i.e., ADP addition) and "energization" act in a similar sense on SH groups of the ADP/ATP carrier.

It is still unknown where the SH groups connected to ADP/ATP transport might be located and also what the exact nature of the "unmasking" of these groups by ADP is. These groups are probably not at the cytoplasmic side of the membrane since only permeant SH-group reagents can reach them. They are not superficially located at the matrix side of the membrane since their reaction needs "unmasking" either by ADP/ATP or "energization." It is not even clear whether they belong to the carrier molecule itself or to a protein in close juxtaposition to the carrier. Wherever they are, these SH groups do not change their orientation from one side of the membrane to the other during a cycle of ADP/ATP transport [93].

SH Groups of the P_i *Carrier*

Localization and accessibility. In the "reorienting" model of anion carriers a cyclic reorientation of the binding sites (i.e., active sites) during each transport cycle was postulated. In applying this model to the P_i carrier, the SH groups were considered to undergo this cyclic reorientation. This was based on the early finding that the transport inhibitor ASPM does not penetrate the membrane and can act therefore only on SH groups which are located at the outer surface of the membrane [32, 89]. Later studies, however, revealed that there was no such selectivity in the action of ASPM [33, 47]. Cyclic reorientation of the SH groups during the P_i transport cycle awaits further experimental evidence.

SH-group reactivity with certain reagents is modulated by the presence of P_i. Addition of P_i before the SH-group reagent decreased both the inhibition of transport and the binding of the applied reagent to the mitochondria [33, 37, 39, 40]. Using the slowly acting reagents, NEM and ASPM, P_i decreased only the rate of reaction between the maleimide and the SH groups and not the final extent of the reaction. The effect of added P_i was abolished by any treatment of the mitochondria which caused discharge of P_i from the matrix. It was therefore concluded that SH-group reactivity was modulated by the P_i present at the matrix side of the membrane and that the SH groups of the carrier are very close to the site where the P_i is bound before being transported [33].

Recently some observations casted doubt on this interpretation of

experimental results. Proteins with SH groups labeled with radioactive SH-group reagents were isolated from mitochondria (see later). As expected, P_i and also nigericin (see also Ref. 33) diminished the total labeling of the proteins [37, 39]. The fraction in which the labeling was decreased should correspond to the P_i carrier. In contrast with the prediction, those fractions in which the [^{3}H]-NEM labeling was decreased by either P_i or nigericin were easily solubilized from mitochondria by ultrasonic treatment; these fractions were probably not integral membrane proteins [40]. Those SH groups which more probably belong to the P_i carrier were exclusively located in a fraction that resisted solubilization on sonication. There might be some interaction between the P_i (or nigericin) protected system and the P_i carrier, but the relationship is certainly more complicated than suggested earlier.

With the slowly acting SH-group reagent, ethacrynic acid, it was found that energization of mitochondria resulted in faster inactivation of the carrier as compared to the deenergized state [94]. This again indicates the role of the environment in the reactivity of the SH groups.

Are the SH groups reacting with mersalyl, NEM, and DTNB identical? The reversible type of transport inhibition by some SH-group reagents and the irreversible type caused by others helped to identify the SH groups of the P_i carrier [26]. The basis of the identification was that inhibition by organic mercurials is completely reversed by monothiols (e.g., 2-mercaptoethanol), the inhibition by DTNB is largely reversed by dithiols (e.g., by DTT) but not by 2-mercaptoethanol, and finally the inhibition by NEM is not reversed by thiol compounds at all.

If mersalyl was added to mitochondria, it formed mercaptide with the SH groups of the P_i carrier: NEM added later was not able to react with the SH groups blocked by the mercurial ("protected" SH groups), and therefore 2-mercaptoethanol added after NEM reversed the inhibition caused by mersalyl. The degree of reactivation of P_i transport in this system (i.e., mersalyl–NEM–2-mercaptoethanol) depended on the amount of mersalyl added first: 3–4 nmol mersalyl/mg protein completely prevented the inactivating effect of NEM [25, 27]. Addition of mersalyl also "protected" against the inhibition by DTNB. These facts show that mercurials inhibit P_i transport by binding to the same groups as are bound by either DTNB or NEM: The mercurial, DTNB, and NEM binding SH groups of the P_i carrier are identical.

The relationship between the bound SH-group reagents and the resulting inhibition of P_i *transport.* (How many SH groups correspond to the P_i carrier?) The first attempt to relate the SH groups of the carrier to P_i transport was made by simple titration of the P_i transporting function with mersalyl [95]. At that time the dissociation constant of the mercaptide formed was unknown: It was impossible to calculate how many SH groups were

really bound by the mercurial giving a known degree of inhibition of transport. It was found with this method that addition of about one-third of the completely inhibitory amount of mersalyl could be added without any inhibition of P_i transport.

The relationship between the SH groups bound and transport function was reinvestigated by radioactively labeled [^{14}C]mersalyl (see earlier). The inhibition of P_i transport corresponded to binding of mersalyl to the high affinity sites. Transport was completely inhibited after binding of 2.5–3.0 nmol mersalyl/mg protein; transport was not inhibited at all when only about 1 nmol mersalyl (about one-third of the completely inhibitory level) was bound. There is evidence that at least a part of this 1 nmol mersalyl is bound to the P_i carrier without inhibiting it: The "noninhibitory" bound mersalyl protected the P_i carrier against the irreversible inhibitor NEM (see also Refs. 25–29 and 96). To explain the fact that mersalyl can be bound to the carrier without inhibiting it, we postulated earlier that the "carrier unit" has more than one essential SH group. If a single SH group of the carrier is left intact, the carrier remains functionally active. It becomes inhibited only when all of its essential SH groups become bound [26].

By using ^{14}C-labeled mersalyl it was found that the number of the high affinity sites was also about 3 nmol/mg protein. This fact does not mean at present that all the high affinity sites for mersalyl are identical with the SH groups of the P_i carrier. Other mitochondrial proteins might react with mersalyl with high affinity. Comparison of quantitative data concerning the number of the ADP/ATP carrier units with the proposed number of the P_i carrier units based on mersalyl binding suggests caution. The ADP/ATP carrier has specific, high affinity ligands, and therefore its amount in mitochondria was measured accurately: In liver mitochondria there is about 1.2 mole carrier/mole cytochrome *a* (0.2 nmol/mg protein) [89]. This value is about one-tenth that of the P_i carrier calculated on the basis of high affinity mersalyl binding. There is a close functional relationship between the ADP/ATP and the P_i carriers, and because of this it was suggested that their quantities should more closely correspond to each other [33].

The amount of the P_i carrier was also calculated from the quantity of an isolated polypeptide labeled with radioactive NEM [28, 29, 96] (see later for details). On this basis the number of carrier molecules was suggested to be either 30 or 60 pmol/mg protein, i.e., one-hundredth of the amount calculated by mersalyl binding.

With all these calculations a further point should be considered. P_i transport through the P_i carrier is extremely fast in liver mitochondria, being about 200 nmol/min · mg protein at 0°C [51]. This value is much faster than the rate of adenine nucleotide transport. Comparison of the extremely

high rate of P_i transport with the calculated number of carrier units resulted in an unusually high turnover number for this carrier, being about 3500 min^{-1} at 0°C. This latter value is about 20 times higher than that of the ADP/ATP transport system. This comparison leaves us with the dilemma that either the calculated amount of the P_i carrier was right and then the turnover number is unusually high, or that the actual turnover number of the P_i carrier is closer to that of the ADP/ATP carrier but in this latter case the amount of the P_i carrier had to be underestimated as being either 30 or 60 pmol/mg protein.

The Use of SH-Group Reagents as "Specific" Labels of Isolated Carriers

The metabolite transport systems of mitochondria are proteins of the inner membrane. When this fact was realized attempts were made to isolate these systems [23, 28, 29, 37, 38, 40, 41, 97–104]. It was difficult, however, to find a guideline for their isolation: Highly specific and tightly binding ligands were required a priori. This requirement was relatively easily fulfilled with the ADP/ATP carrier [38, 97–102]. It was much more difficult to find a specific label for the other carrier systems, especially the P_i carrier.

Attempts to Isolate the Protein(s) Involved in P_i *Transport*

The labeling and isolation of the P_i carrier was first attempted via its SH groups [37, 39]. The SH-group reagent used, NEM, is tightly bound but very unspecific. In order to increase the specificity, the allegedly "specific" protection of the SH groups by P_i was performed: A protein fraction was looked for in which P_i pretreatment decreased the radioactive NEM labeling. For reasons reported earlier, this approach was unsuccessful.

Radioactive mercurials are not entirely satisfactory labels since the mercaptide formed dissociates easily [41] and the mercurial migrates from one SH group to the other. Mercurials were, however, used successfully in increasing the specificity of labeling by NEM [28, 29, 41, 96]. The basis of this approach was that organic mercurials bind to the same SH groups of the P_i carrier as does NEM [26, 27]. Treatment of mitochondria with organic mercurials (PMB or mersalyl) prior to treatment with radioactive NEM reduced the radioactivity bound covalently to mitochondria; furthermore, it decreased the number of the radioactively labeled protein fractions which could be detected by SDS-gel electrophoresis [40]. It was suggested that the

missing fraction with a molecular weight of about 32,000 daltons represented the P_i carrier.

The actual isolation of the labeled protein fraction was performed in three laboratories [28, 29, 41, 96]. The SH groups of the P_i carrier were first "protected" by mercurials, either PMB [28, 29] or mersalyl [41, 96]. In the experiments of Coty and Pedersen [28, 29] sufficient PMB was added to inhibit the transport completely; in those of Hadvary and Kadenbach [96] the mersalyl added reacted only with about one-half of the SH groups of the carrier. Following the addition of the mercurial, "cold" NEM was added in order to react with all those SH groups that were not bound by the mercurial (i.e., noncarrier SH groups). Next the organic mercurial bound to the SH groups of the carrier was removed by a thiol compound, cysteine or DTT. After removal of the excess thiol, either ^{3}H- or ^{14}C-labeled NEM was added to label those SH groups of the carrier which were "protected" by the mercurial. The membrane proteins with the covalent label were solubilized and separated by SDS-gel electrophoresis.

Coty and Pedersen isolated four minor and one major labeled fraction, the latter having a molecular weight of 30,000 daltons. Hadvary and Kadenbach employed a double labeling, the first with and the second without protection by mersalyl: Using also lower levels of the protecting mercurial, these authors arrived at a single labeled fraction having a molecular weight of 28,500 daltons. All the reported molecular weights (28,000–30,000) are probably identical. Touraille et al. [41] found that the [^{14}C]mersalyl binding protein is probably identical with the mersalyl-protected [^{3}H]-NEM binding protein fraction. The authors also suggested further that there might be a relationship between the labeled proteins involved in P_i and in ADP/ATP transport [41].

Hadvary and Kadenbach protected about half of the SH groups and found 30 pmol labeled protein/mg mitochondrial protein. Coty and Pedersen protected all SH groups and found accordingly 60 pmol/mg of the labeled compound. The results are thus in good agreement.

Isolation of the ADP/ATP Carrier by Labeled NEM

The main strategy for isolation of the ADP/ATP carrier was the use of highly specific radioactive ligands, atractyloside, and carboxyatractyloside [38, 97–102]. In addition, the ADP-induced, specific reaction of the ADP/ATP carrier with NEM was also exploited for the labeling [38]. Those SH groups of the membrane which became exposed and were made reactive by prior treatment of ADP were labeled with radioactive NEM and the labeled polypeptides were separated by SDS-gel electrophoresis. The protein which

was considered to be associated with the carrier had a molecular weight in the range of 30,000 daltons: The polypeptides isolated after carboxyatractyloside or by NEM labeling were very similar or even identical. More experimental evidence is needed to prove that the protein labeled by NEM is really within the molecule of the carrier or only in its immediate vicinity [86].

What Is the Role of SH Groups in Transport?

The fact that almost every metabolite carrier in mitochondria requires free SH groups for function suggests that they have an essential, related role in function: A mere coincidence is unlikely. On the other hand, not in a single case do we have any direct and unequivocal evidence for the function they might have.

Three current models try to explain the events occurring at the molecular level during transport. The first is the "mobile carrier" in its original sense, indicating a molecule capable of diffusion within the membrane with reversible binding properties for its substrate(s). The second is the "reorienting carrier," a molecule which is built into the membrane and is able to undergo reversible conformational changes. It "reorients" its functional groups toward one or the other side of the membrane, the substrate binding site facing alternately one or the other side. The third is the "gated pore," a transmembrane channel with a specific "recognition protein" for binding the substrate and allowing its passage into the channel. All three models require a given conformation of the protein moiety. In all three models the transport process consists of (a) substrate binding, (b) movement through the membrane, and (c) substrate release. Any of these steps might depend on the integrity of the SH groups, but SH groups might be essential merely for maintaining the required conformation of the protein.

The few data available concerning the function of SH groups in transport are related to the dicarboxylate and the P_i carrier. Substrate binding by the dicarboxylate carrier was affected by mercurial reagents [14, 64, 65]. It was suggested that SH groups bind a metal ion involved in the binding of the substrate: The mercurials would interfere primarily with the binding of this metal by the SH group.

Contrary to this hypothesis, Papa et al. [105, 106] proposed that mersalyl and NEM increase the affinity of the P_i carrier for P_i and fixed the transported ion to the carrier by binding the SH groups of the latter.

SH groups of the P_i carrier were suggested to undergo "reorientation" during the transport cycle and face alternately outward and inward [32, 33, 89]. The experimental basis of this hypothesis was that only intramito-

chondrial P_i affected the reactivity of the SH groups of the carrier with maleimide derivatives. The reviewer's opinion is that the facts reported are also compatible with a gate-pore type of mechanism and specific recognition proteins having SH groups.

Future Aspects of Use of SH-Group Reagents

It is the author's view that significant new information on transport is not likely to arise from the use of already known SH-group reagents: The synthesis of new ones is necessary. Emphasis should be put on SH-group reagents with a very narrow specificity: The example of α-cyano-4-hydroxycinnamate as a specific SH-group reagent suggests that this is a real possibility. Specific reagents should be more useful also in the isolation of labeled carriers. Progress could come from use of reagents that act on one side of the membrane only. The role of SH groups in transport could be clarified by use of fluorescent and of spin-labeled SH-group reagents. These examples show that progress is in the hands of organic chemists.

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